Genes, Development and Cancer

The Life and Work of Edward B. Lewis

2nd edition

Edited with commentary by
Howard D. Lipshitz

GENES, DEVELOPMENT, AND CANCER

THE LIFE AND WORK OF EDWARD B. LEWIS

E. B. Lewis, 1996

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This edition is dedicated to Pamela Harrah Lewis: artist, naturalist, opera connoisseur, friend.

The study of the fundamental problems of embryology by experimental methods had almost come to a standstill until two new methods of procedure appeared above the horizon—one the direct application of physico-chemical methods to the developing organism; the other, the application of genetics to problems of development. The combination of these two methods holds for us, at present, I believe the most promising mode of attack on the problems of developmental physiology.

Thomas Hunt Morgan (1926)

It would therefore be strange if occasionally a mutation did not arise which rendered its cell relatively irresponsive to some influence in the surrounding medium that normally exercised an inhibiting action on its proliferation . . . We should then have a cancer cell. It is no mere hypothesis gene mutations occasionally occur spontaneously in somatic cells . . . It is equally well known that irradiation enormously increases the frequency of these somatic mutations . . . it is but a logical step to conclude that the carcinomas, sarcomas and leukemias arising after irradiation represent mutations induced by the latter . . . Moreover the study of the manner and conditions of mutation production by irradiation should . . . be of some value in relation to the problem of cancer production.

Hermann Joseph Muller (1937)

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PREFACE

While Edward B. ('Ed') Lewis is famous for his contributions to genetics and developmental biology, few have read his research papers. One reason for this is availability, many having been published in obscure journals or as book chapters. A second is because his papers in those fields are very difficult to read. The difficulty derives from the fact that Lewis has published infrequently, thus many papers are condensed reviews of many years' work presented largely in summary form rather than in detail. It is not unusual for the reader to have to infer the experimental methods, even the results, from a few sentences. Furthermore, he often presents his results in terms of abstract models; thus it can be difficult to separate the data from the models.

A major goal of this book is to make Lewis' key papers accessible to researchers and students. The papers are grouped into several sections that reflect the changing focus of his research. Each section is preceded by commentary designed to place the papers in historical perspective, with respect to Lewis' own ideas as well as to those of the larger scientific community. The commentaries attempt to highlight the key methods and results—as well as the significance—of each paper by explaining the science in terms that should be understandable to upper-level undergraduates, graduate students and professional researchers.

Even those who have read Lewis' seminal papers on genetics and development are often unaware that he made fundamental contributions to understanding the somatic effects of ionizing radiation on humans. Those studies—several of which are reprinted here—spanned a twenty-year period that began in the mid-1950's and provided

accurate scientific information to policy makers during debates over atmospheric testing of nuclear weapons. The commentary highlights why Lewis undertook the analyses, discusses the response he received from scientists and politicians, and revisits his conclusions on ionizing radiation and cancer in light of almost fifty years of subsequent data collection and analysis.

A final goal is to bring together some of Lewis' thoughts on his scientific predecessors, such as C. B. Bridges and A. H. Sturtevant, as well as on his own career. Those who know him well are aware that he abhors anything that smacks of self-promotion. You will, for example, rarely find the use of the first person in a research paper on which he is the sole author. An autobiographical sketch that was requested to accompany the publication of his Nobel lecture was, characteristically, not completed before the deadline. It reveals many of the factors that led Lewis down the path to becoming a scientist. Here, it is published for the first time, together with the Nobel lecture.

Particular thanks are due to Ed Lewis for agreeing to this project, albeit reluctantly. On a personal basis, Ed has been a remarkable mentor, role model and friend for almost twenty years. He was characteristically generous of his time and resources during my several visits to Caltech during the first half of 2003 to write the commentaries and to assemble the component papers. My heartfelt thanks to Ed and Pam—his wonderful wife—for their kindness and hospitality.

Manuel Buchwald, Director of the Research Institute at the Hospital for Sick Children, granted me a leave from my administrative responsibilities to enable me to carry out this rather unorthodox project. Laura Walsh and Denise Gibson at Kluwer Academic Publishers were invaluable in shepherding the book through the various stages from conception to birth. Mitzi Shpak of Caltech assisted in obtaining permissions to reprint the papers from well over a dozen publishers. Ronit Wilk of the Research Institute at the Hospital for Sick Children provided invaluable assistance in checking the accuracy of the page proofs. Andrew Dowsett of the Claremont Colleges provided input on the commentaries from the perspective of a Drosophila geneticist. Susanna Lewis of the Research Institute at the Hospital for Sick Children—my spouse (and no relative of Ed!)—was the first to understand the imperative that drove me to undertake this project and has supported it unconditionally. She read the commentaries and provided excellent, detailed and insightful advice on how to make them more accessible to geneticists and non-geneticists alike.

Howard Lipshitz
Pasadena and Toronto
January–June 2003

PREFACE TO SECOND EDITION

I first discussed the possibility of this book with Ed Lewis about a decade ago. There were two incentives. One was the desire to make Ed's papers on Drosophila genetics accessible. The need for this was brought home to me when teaching a graduate course on Advanced Genetics. I gave the most motivated and thoughtful student in the class the assignment of presenting Ed's 1978 *Nature* paper on the bithorax complex. To my surprise, the student did not even begin to approach an understanding of either the content or the significance of the paper. On reflection I saw the difficulty: the paper telegraphically summarizes 30 years of data and thought and presents this coalescence in a very dense, model-based manner. As I led the class through the paper, I began to appreciate that it might be a service to researchers in the fields of genetics and development—and a gratification to me—to provide explanatory commentary on Ed's work as a whole.

A second incentive came from my interest in history and politics. Ed's papers on the somatic effects of ionizing radiation had a major impact at the time of their publication but are now largely forgotten. He made seminal contributions to the field of radiation and cancer, particularly with his 1957 paper in *Science* on "Leukemia and Ionizing Radiation." Those who lived through the era of atmospheric testing of nuclear weapons that began during World War II and continued for two decades, appreciate how little was understood about the consequences of exposure to radiation at the time and how significant Ed's contributions were. Today, however, few know of Ed's work in this area—even many of his colleagues at Caltech are surprised to hear

of it. More than that, Ed was for a brief period a very public figure, identified by the US Congress and the media as an authority on the effects of atmospheric nuclear testing. In his later years, Ed still felt disturbed by the politically motivated attacks that were levied against him because he reported findings that the government found inconvenient. With respect to his scientific interests, he closely followed developments regarding radiation and cancer, and regularly revisited his own data. As detailed in my commentary, it is a testament to his scientific skill and integrity that his initial risk estimates—made 50 years ago this year—have withstood the test of time.

Ed initially resisted the idea of this book, in part, I think, because he believed that his Drosophila papers were perfectly clear, but also because, being a fundamentally modest person, the idea of self-promotion suggested by such a project was decidedly uncomfortable. Happily, the second line of argument described above eventually succeeded in winning Ed's endorsement of the proposal and, in mid-2002, he agreed that, if I could find a publisher, we should proceed to compile an annotated collection of his papers. I was not so naive as to overlook the fact that his reluctant acquiescence was founded on his conviction that no publisher would be interested. But, of course, he was wrong, and we signed the contract with Kluwer in the fall of 2002.

Unknown to me, in the fall of 2002 Ed received a diagnosis of metastatic prostate cancer and was given 18–24 months to live. I was blissfully unaware of this when I made my monthly visits to Caltech in early 2003 to draft my commentaries. Only after the book went to press in the late summer of 2003 did Ed reveal his diagnosis, and that he had routinely received treatments in between my trips to Caltech. I think that this shows how much he really did want to see the project through, because he knew that it would have been difficult if not impossible for me to complete the commentaries had I known of his condition.

As it turned out, Ed lived to enjoy the publication of the book in January 2004. Knowing that it might be a final opportunity to celebrate Ed's life and career, David Baltimore (then Caltech's President) and Elliot Meyerowitz (Chair of the Biology Division) arranged to mark the occasion with a talk on Ed's life and work, followed by a book-signing ceremony. The original intent was to invite Ed's friends and colleagues from around the world, but Ed vetoed this because he was not sure that he would be healthy enough to attend. For 6 weeks before the event he underwent transfusions and erythropoietin treatment to raise his blood cell counts. Happily, when the event happened in early February, though weak, he was able to attend the talk, the ceremony, and the banquet in his honor given that evening. The lecture hall was filled to overflowing with standing room only, a testament to the affection and reverence that the Caltech community had for Ed.

I did not see Ed again. About a month after the celebration, he had his fly-pushing microscope moved to his house, where he kept up his experiments until he was too weak to continue. He remained in contact with colleagues and friends by phone and e-mail. He completed a historical article on Demerec for publication in *Genetics* and submitted it in June (the article is reprinted in this edition). In July Ed succumbed to his disease.

Ed was not one for sentimentality. Neither did he support the trend among historians of science to—as he put it—"psychoanalyze scientists and study their sex lives." I was very much aware of this when I wrote my commentaries, and avoided talking about Ed "the person" in order to focus almost exclusively on Ed "the scientist." But a more personal perspective has its place. After his death, an invitation to write a biographical memoir on Ed specifically requested "personal reminiscence." I have included that contribution here, because I believe that it does not violate Ed's wish to provide some context to the commentaries.

In addition to inclusion of the biographical memoir and Ed's historical perspective on Demerec, I have updated my commentaries in a few places. Pam Lewis graciously allowed me to add her painting "for Ed" to the compendium of photographs. Hugh, Keith, and Jon Lewis provided invaluable assistance with aspects of chronology and family history. I am deeply indebted to Professor Markus Noll of the University of Zurich, Switzerland, who reviewed my commentaries and provided valuable scientific (and grammatical!) corrections for this edition.

A final aim in producing this edition is to provide an affordable, paperback version that brings Ed and his life's work to a broad audience. I sincerely hope that it meets this goal.

Howard Lipshitz
Toronto
March 2007

INTRODUCTION

E. B. LEWIS AND HIS SCIENCE*

Three great streams of scientific inquiry—into the mechanism of evolution, the laws of inheritance, and the rules that govern the development of an adult organism from a fertilized egg—had their source in the second half of the nineteenth century. These converged a century later to provide deep insights into the mechanistic basis of animal development, how genes control developmental processes, and the evolutionary origins of the metazoan body plan. This convergence was brought about, in no small part, by Edward B. Lewis.

Lewis began his research on Drosophila in 1938 with a primary focus on the nature of the gene and how genes evolve to give rise to new functions. These two questions drove his analysis of what he later came to call the bithorax gene complex (BX-C). Over a period of several decades, his accumulating data on the bithorax series of mutant phenotypes led to a gradual shift in focus—to how genes control development. The culmination was Lewis' famous 1978 paper, which established the BX-C as a paradigm for the genetic control of development (Lewis, 1978). His collection of mutants was essential in the first positional cloning of a gene, notably of the *Ubx* domain BX-C, and the functional genomic analysis that followed (Bender et al., 1983a). Those molecular analyses were to give concrete reality to the formal models that Lewis had proposed to explain his genetic data. Furthermore, they were to lead to the discovery of the homeobox (McGinnis et al., 1984b; Scott and Weiner, 1984) and the confirmation, 40 years after he had initiated his analyses of the bithorax-series mutants, that the gene complex had indeed evolved by tandem gene duplication. Unexpectedly, homeobox gene complexes were found to exist throughout the metazoa

*In the commentaries, gene names and abbreviations are italicized. However, in the reprinted papers, the convention used in the original publication is retained. Only typographical errors have been corrected.

(McGinnis et al., 1984a), and their fundamental organization has been conserved throughout roughly half a billion years of evolution. Thus, understanding the molecular structure, genetic organization, and evolutionary conservation within the metazoan homeobox complexes, brought together, for the first time, the previously disparate fields of genetics, developmental biology, and evolutionary biology.

The perspective from which Lewis began, and his first and enduring love, was genetics. Genetics is an unusual subdiscipline of biology in that its essence is formalism and abstraction. Mendel's laws were the first and greatest of these in that they describe the behavior of abstract units of inheritance, which obey certain laws that could be derived from the quantitative analysis of the different classes of progeny obtained from controlled crosses. In an era dominated by molecular analyses of entire genomes, it is easy to lose sight of the fact that, for close to a century, the gene was an abstraction: a unit of inheritance.

Genetics is a discipline that is based on operational definitions. Thus, it is technically limited by the operations that are performed. As with all scientific disciplines, advances often derive from the invention of new methods. Perhaps no one made more additions to the lexicon of genetic techniques and tools than Lewis. From his invention of the *cis–trans* test for position effects (Lewis, 1945), to the invention of the chromosomal rearrangement test that led to the discovery of transvection (Lewis, 1954b), to his invention of a method that will be referred to in this book as 'add-back genetics' (Lewis, 1978), Lewis took genetic methods to new heights. He constructed most of the "balancer" chromosomes that have been in use in the Drosophila community for the past half century (Lindsley and Zimm, 1992). He invented simple methods for chemical mutagenesis (Lewis and Bacher, 1968), analyses of mitotic chromosome figures (Lewis and Craymer, 1971), and of the patterns on the exoskeleton of mature embryos (Lewis, 1978). Furthermore, his generosity, openness, and willingness to unconditionally share his materials, reagents, and data, kept alive a tradition begun by T. H. Morgan and his students almost a century ago.

Lewis' contributions to genetics and developmental biology are characterized, not just by technical innovation, but by the formulation of abstract models. Most famous among these is his 1978 model for how the BX-C genes control development (Lewis, 1978), which he also animated into a movie showing the action of the postulated BX-C "substances" in directing formation of the body parts. As readers of this book will see, Lewis always viewed his data in abstract terms, and abstraction lies at the heart of almost all of his science. Not surprisingly, therefore, genetics was Lewis' intellectual home for 70 years. However, starting in the early 1990s, his abilities in statistical analysis and his enjoyment of abstraction led him to use computational methods to uncover possible DNA sequence motifs in the giant, *cis*-regulatory regions of the BX-C (Lewis et al., 1995).

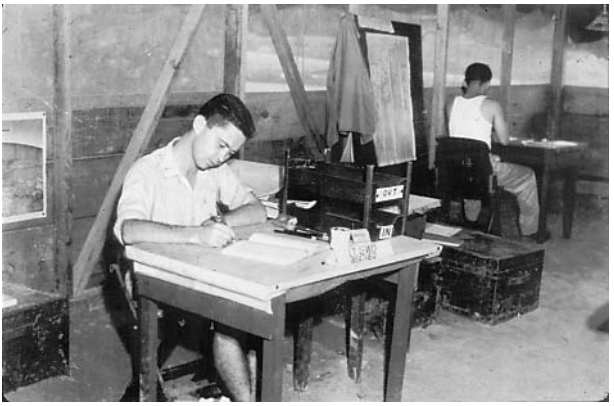
Bertrand Russell's book *The Scientific Outlook* (Russell, 1931) had a profound influence on Lewis as a high school student. This influence was twofold and its importance for understanding Lewis' science cannot be overestimated. First, Russell argued that the goal of all science is to define abstract laws. In particular, he argued that standard language is insufficient to describe reality since it is fraught with misinterpretation,

as well as lacking the exactness of mathematics. Lewis chose to quote from Russell to preface his 1995 Nobel lecture: "The power of using abstractions is the essence of intellect, and with every increase in abstraction the intellectual triumphs of science are enhanced" (Lewis, 1995). Second, Russell described how science is inductive rather than deductive. Science begins with specific examples and then derives general laws from these specific instances. Only then are hypotheses formulated and experiments conducted to test them. Induction and abstraction frame Lewis' science; almost all of his papers start with specific examples, derive general, abstract "rules" and then test the validity of these rules.

Even biologists unaware of Lewis' contributions to genetics and developmental biology will probably have seen his famous four-winged fly figured in their introductory biology textbook. However, many biologists—including radiation biologists and cancer researchers—may not know that Lewis also made fundamental contributions to understanding the somatic effects of ionizing radiation, particularly in causing human cancers. These contributions were not a singularity: they extended for almost 20 years during Lewis' mid-career, from the mid-1950s through the mid-1970s.

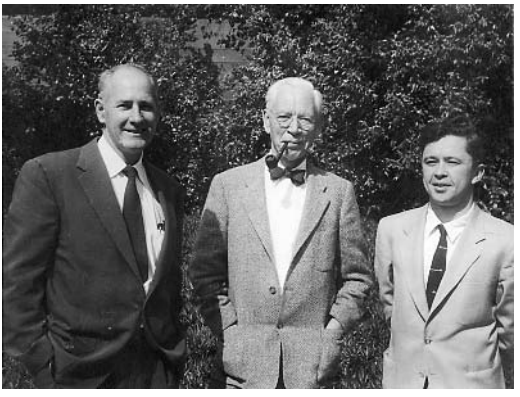
It will be seen that Lewis' detour into radiation effects reflects additional key aspects of his personality: a concern for the individual as opposed to organizations, and the courage to pursue research independent of political expediency. The detour derived from his surprise—in conversation at a faculty lunch table in the Caltech Athenaeum—that physicists should uncritically accept the idea that there is likely to be a threshold, below which ionizing radiation would have no adverse effects on human tissue. Known genetic effects of radiation did not exhibit a threshold, thus Lewis' curiosity was piqued. In an era of atmospheric nuclear weapons testing, and amid assurances from government agencies that radioactive fallout was well below safe exposure levels, Lewis initiated epidemiological studies that revolutionized both scientific and political discussions of radiation effects.

Iconoclasm lies at the root of all science. The larger theme illustrated by Lewis and his research contributions is that science is the product of inquiring minds operating to challenge existing dogma. Lewis' contributions revolutionized views of the nature of the gene, how genes control development, and the effects of ionizing radiation in causing cancer. He accomplished this largely working alone. During nearly 60 years as a faculty member, he had only a handful of postdoctoral fellows and even fewer graduate students. Many of the journals or books in which he chose to publish his research findings score low in citation indices. His publication rate would be considered atrocious by most grant review panels or academic promotions committees. His most famous paper is not a research article, but a review of several decades' worth of experimental results. He did not set out to study the genetic control of development, but that is where—in the circuitous way that science progresses—he ended up. Perhaps the lesson to be learned from Lewis' career is that scientists may be part of a cooperative venture, but it remains the innovative thinking of individuals, rather than a consortium, that drives quantum advances in mankind's understanding of reality. If this book helps explain the work of one such individual, his intellectual quest and curiosity, then it will have accomplished its goal.



Lt. Lewis at his weather officer's desk, G2 section, US Tenth Army, Okinawa, 1945.

Edward, Pamela and Hugh Lewis (3 months old, in high chair) on route to the UK on the Queen Mary, 1948.



G. W. Beadle, A. H. Sturtevant, and Lewis, *circa* 1960. (Courtesy of the California Institute of Technology Archives.)

Lewis in Halloween costume; after 'Le thérapeute (The Therapist)' by René Magritte, *circa* 1985.

Lewis in Halloween costume; after 'Le Mouvement Perpetuel (Perpetual Motion)' by René Magritte, 1988.

Lewis receiving the 1995 Nobel Prize in Physiology or Medicine from King Carl XVI Gustaf of Sweden.

Lewis playing the flute, Toronto, 1996.

D. S. Hogness and Lewis at the EMBO Workshop on "Molecular and Developmental Biology of Drosophila," Kolymbari, Crete, 2002.

A painting by Pam Lewis completed *circa* 1960 "for Ed". Ed's silver flute forms the centerpiece. Those who knew Ed will recognize many of his favorite locations (the cliffs of La Jolla are in the background, the Southern California desert is in the foreground), hobbies (he was an avid chess player), "pets" (crabs, starfish, lizards, spiders), and weaknesses (he was constantly losing his keys). Almost all of Pam's paintings feature insects such as moths and butterflies; this one has more of these than most of her other works. Pam also tells me that she picked up the animal bone seen in the right foreground in the desert, thinking that it was the remains of some exotic wild beast; only after completing the painting did she realize that it was from a pork chop left by a littering visitor! (Courtesy of Pamela H. Lewis.)

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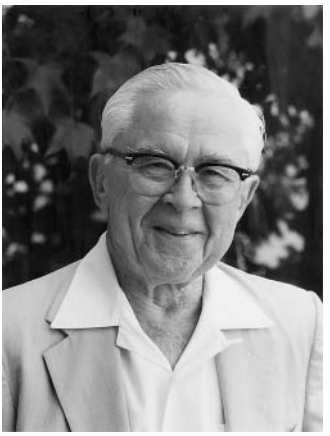
BIOGRAPHICAL MEMOIR

EDWARD B. LEWIS



20 May 1918 · 21 July 2004

I never saw Ed Lewis in an immodest mood. He came closest on a Monday in October 1991 at a Biology faculty lunch table at the Caltech Athenaeum.

By then it was clear that Ed's 45-year-long studies of the *bithorax* gene complex in the fruit fly Drosophila were of fundamental importance for understanding the genetic basis of all animal development (about which, more below). Thus, his colleagues on the Caltech faculty all felt that it was just a matter of time before he was going to receive the Nobel Prize. And it must be admitted that each year we—and he—would become slightly tense as the annual announcement date drew near, wondering whether it was finally his turn.

That day, discussion at the lunch table had turned to the early morning announcement that Erwin Neher and Bert Sakmann had received the Nobel Prize in Physiology or Medicine for their studies of single ion channels in cells. Ed—not one to follow developments in cellular physiology—was at the opposite end of the table from Henry Lester, a faculty member in whose laboratory Neher had spent a sabbatical period in 1989. Henry was expounding at some length on the power of the "patch clamp" technique, and the reasons it deserved the award. Suddenly, from the opposite end of the table came an interjection from Ed: "Pinch clamp? Pinch clamp! Why would

they award the Prize for invention of the pinch clamp?" When the laughter subsided, Henry clarified[1] and Ed was gradually mollified.

Ed Lewis's personality was characterized by modesty, humility, generosity, kindness, and never-ending curiosity. His science was innovative, groundbreaking and, ultimately, revolutionary. His share of the 1995 Nobel Prize in Physiology or Medicine capped a 60-year career in Drosophila genetics that had led the way to the discovery that evolutionarily conserved "master regulatory genes" program the body plan of all animals. Lewis had not begun with an interest in developmental biology; rather, his studies of the nature and evolution of genes had led—in the circuitous fashion that is the hallmark of science—to a gradual shift of focus as he came to realize that the gene cluster he was studying represented an entrée into the rules that govern the assembly of the animal. With the advent of molecular cloning methods and the discovery in the mid-1980s that the gene cluster is conserved in organization and function in all metazoans—thus predating the pre-Cambrian explosion of animal forms more than 500 million years ago—the generality of his studies became apparent and, as mentioned above, prizes began to be awarded on an almost annual basis.

Despite numerous honors, Lewis never lost his humility; neither did he alter his daily routine of many hours at his "fly pushing" microscope. As metastatic prostate cancer weakened him, he set up a microscope at home, where he continued his genetic crosses. He stayed in daily contact with family and close friends by email from home, managed to complete and submit a brief historical article to the journal *Genetics* in mid-June,[2] and finally succumbed to the disease on 21 July 2004.

Edward B. Lewis was born in Wilkes-Barre, Pennsylvania, on 20 May 1918, the second son of Edward Butts Lewis, a watchmaker and jeweler, and Laura Mary Lewis (née Histed). His brother, James ("Jimmy") Histed Lewis, was five and half years older; a sister, Mary Louise Lewis, died of a fever at age two the night before James was born. Edward's full name was supposed to be Edward Butts Lewis, Jr. but his parents forgot to fill his middle name out in full on the birth certificate, so his middle name ended up simply as "B".[3]

Young Edward's parents supported his educational and musical aspirations. This was done despite the hardships of the Great Depression, which led to the closing of the jewelry store in which Edward Sr. worked and a difficult struggle to make ends meet. A great-uncle, Thomas Wyllie, President of the Pittston Stove Company, assisted both Jimmy and Ed financially, enabling them to go to college. After completing high school in 1929, Jimmy worked for a year at Wyllie's company, thus managing to save $1,600 for his college tuition. By winning a scholarship, Jimmy was then able to send

[1]Patch clamp: sophisticated physiological technique that allows recording of electrical currents as small as a picoampere through a single ion channel in a cell membrane. Pinch clamp: metal device to control flow through flexible tubing; used in chemistry laboratories.

[2]E. B. Lewis, Did Demerec discover intragenic recombination in 1928? *Genetics* 168 (2004):1785–1786.

[3]I am greatly indebted to Jon Roderick Lewis, Ed Lewis's nephew, for providing personal details of his father, James H. Lewis, and his grandparents, Edward Butts Lewis and Laura Mary Lewis. He also contributed several interesting anecdotes about Ed's early life of which I was not previously aware, and kindly provided comments on a draft of this article.

some of his savings home to support his parents. However, those years took a terrible toll, probably contributing to Edward Sr.'s untimely death of a stroke at the age of sixty in 1945.

Laura Lewis, Ed's mother, encouraged him to study animals, which he did with a particular focus on toads and snakes. Her patience was, however, tested at least once, when she found one of his rattlesnakes in the closet—he hadn't built the terrarium for it yet. As a teenager Ed used to pay a daily visit to Wilkes-Barre's Osterhout Public Library, whose excellence he praised throughout his life. In the library he read, not only books, but also the scientific journals to which the library subscribed. Thus it was that in late 1934 he spotted an ad for fruit flies in the journal *Science*. Ed was a member of the E. L. Meyers High School biology club, which at that time was chaired by his friend, Ed Novitski, who also went on to become a distinguished *Drosophila* geneticist. For $1 the club obtained the flies, launching both Eds on their future careers.

In his autobiography, Ed emphasized that "by allowing Novitski and me freedom to use the biology laboratory and its supplies to carry out our experiments on *Drosophila*, our biology teacher, who also was the athletic coach, could not have been more helpful in furthering our careers. There was none of the present attitude that one cannot become a scientist without having had the benefit of teachers skilled in the art of keeping their students constantly motivated."[4] In other words, Ed was self-motivated from the outset.

Following high school, Ed spent a year at Bucknell College on a music scholarship. He had begun playing the flute at age 10, when his great-uncle Tom had given him a wooden Haynes flute. A few years later his father gave him a silver flute—undoubtedly at considerable sacrifice. Ed went on to play in the high school orchestra as well as the Wilkes-Barre Symphony, and remained an accomplished and enthusiastic flautist for the rest of his life. For the last forty years of his life, he and his wife, Pam, often spent weekends in La Jolla, during which the high point for Ed was playing chamber music on Sunday morning at the home of the well-known virologist, Marguerite Vogt, of the Salk Institute. Vogt, an accomplished pianist, had spent the years from 1950 to 1963 at Caltech, during which time she became a close friend of the Lewises. In conversation, Ed often praised her earlier work on homeotic mutants of *Drosophila*—conducted under difficult circumstances in Nazi Germany during World War II—as being "many years ahead of its time."[5]

In 1937, Ed transferred to the University of Minnesota to continue his undergraduate education in biostatistics and genetics, although he continued his flute playing as a member of the university orchestra. He was attracted to the University of Minnesota because it had low out-of-state tuition fees (at that time, $25 per year) and because participation in the Reserve Officers' Training Corps (ROTC) was not compulsory.

[4]E. B. Lewis, Autobiographical Sketch. In *Genes, Development and Cancer: The Life and Work of Edward B. Lewis*, ed. H. D. Lipshitz, (Boston: Kluwer Academic, 2004), 497–502.

[5]M. Haas and E. B. Lewis, Cover legend, *Cancer Research* 58.22, (15 November 1998).

During his college years, Ed was assisted financially by his brother, Jimmy, who had by then graduated from George Washington University with a master's degree in international law and had joined the U.S. State Department. Jimmy went on to a distinguished career in the U.S. diplomatic corps, serving as special economic assistant to the ambassador in London (during World War II) and as economic counselor in Copenhagen (after the war). He served as a delegate to the Paris Peace Conference in 1946, minister-counselor for economic affairs in Geneva, and as deputy director-general of the General Agreement on Tariffs and Trade (GATT), ending his career in Helsinki as deputy ambassador to Finland. Ed frequently talked about what an inspiration Jimmy was to him and how he envied Jimmy's ability to read rapidly and broadly. Ed himself was a slow reader—and writer—attributing his low scientific publication rate in part to these handicaps.

A characteristic shared by Ed and Jimmy Lewis—as well as by the author of this memoir—is short stature. In a brief after-dinner speech at the banquet in his honor, following the celebration of publication of his collected papers, Ed could not resist mentioning:

> a letter I received only a few years ago from a student asking what was the hardest thing I had to overcome in my career. I should have written the student and said that the hardest thing was to write up my experiments for publication. . . . But instead of telling the student I suffer from writer's cramp, I wrote him that the hardest thing to overcome was my short stature, which probably seemed rather a flippant reply although I was serious. I was aware that short stature was an even greater problem for my brother, who had a long career in our foreign service and who was a few inches shorter than I. He once said that a Japanese diplomat had told him that he was the only American the diplomat liked because he did not have to look up to him![6]

Jimmy and Ed Lewis also shared a love of opera and of bouillabaisse; both were greatly influenced by the Great Depression, particularly by their parents' struggle; both were self-motivated and successful but kept their success in perspective; both were quiet, modest men with tremendous personal integrity and intellect; and both died of prostate cancer at the age of eighty-six.

At the University of Minnesota, Clarence P. Oliver, professor of genetics, gave Ed a desk in his laboratory along with the freedom to continue the *Drosophila* work that he had begun in high school. By passing examinations in several courses without actually attending the lectures, Ed was able to complete his B.A. degree in biostatistics in two years. He maintained close ties to the university throughout his life and was a generous donor to it; the university awarded him an honorary degree in 1993, the fifty-fourth anniversary of his graduation.

In 1939, Lewis began his graduate research at Caltech under Alfred H. Sturtevant, a renowned *Drosophila* geneticist. His Ph.D. thesis focused on how the position of genes relative to each other in the chromosomes affects their function. Significantly,

[6]At the Ritz-Carlton, Huntington Hotel, Pasadena, California, on 4 February 2004.

Lewis invented a test for gene function known as the *cis–trans* test, which is still taught to undergraduate students in introductory biology courses. It formed the foundation for his later discovery of the rules by which the *bithorax* family of mutants controls the establishment of the body plan.

Completing his Ph.D. in 1942, Ed enrolled as a cadet in the U.S. Army Air Corps training program in meteorology at Caltech and was awarded an M.S. degree in meteorology in 1943. Subsequently, he served at bases in Hawaii and then as a weather officer for the U.S. Tenth Army in Okinawa, a post he assumed shortly after D-Day in the spring of 1945. During this time he was stationed on a command ship in the harbor. He would begin his shift daily at 4 a.m., preparing the weather forecast for relay to the reconnaissance planes that flew over the battle zones on Okinawa. Ed spent many years trying to explain to me—unsuccessfully, I regret to report—why it always rains on the afternoon of the day following a major storm in Southern California, as well as why it rains more heavily in the foothills of the San Gabriel mountains—where our home was located—than down the hill at Caltech itself!

Ed's daily sleep–wake rhythm was unusual, more closely resembling a twelve-hour than a twenty-four hour cycle. He attributed this in part to the rhythm he had been forced to follow as a weather officer during the war. For the decade—1986 to 1995—when my laboratory was located across the hall from his on the third floor of Caltech's Kerckhoff Memorial Laboratories, he would arrive early in the morning to begin work and would follow this with his flute practice, which would echo melodiously through the building. Promptly at eight o'clock, he would disappear to the gym to jog or swim for an hour before returning to continue the day's work. Often he would take a pre-lunch nap on the tattered couch at the rear of his office. Then at noon sharp his door would slam shut and he would head out for lunch at one of the faculty tables at Caltech's Athenaeum, always stopping by my office to invite me to accompany him. After lunch, more laboratory work and some paperwork (which he hated!), then home to an early dinner with Pam, followed by another nap. He usually returned to work at night, enjoying the peace and quiet of that period to carry out the bulk of his *Drosophila* crosses and genetic analyses. In earlier years, he might have napped again in his office before starting the next day's work; but, by the time we became close colleagues, Pam had battled an infection that had led to partial unilateral paralysis, so Ed usually returned home late at night to nap there before returning to the lab shortly after dawn.

In 1946, Ed was appointed an instructor in the biology division at Caltech, having been recruited to that position in 1943 before leaving for military service, by the university president, Robert A. Millikan. He spent his entire independent career at Caltech, was appointed the Thomas Hunt Morgan Professor of Biology in 1966 and attained emeritus status in 1988, though he remained active in research until his death. The trends in U.S. politics in the twenty-first century distressed Ed, who would half-seriously say that it was time to move to Canada. Although I, of course, extolled the virtues of Toronto, I knew that his ties to Caltech were so strong that he would never leave. And he didn't.

Ed met and married Pamela Harrah, a Stanford graduate, in 1946. Their meeting was arranged by George W. Beadle, who had returned to Caltech from Stanford in 1946 to chair the biology division. That same year Ed had taken responsibility for supervising the extensive Caltech *Drosophila* Stock Center and was looking for a stock keeper. While still at Stanford, Beadle called Pam into his office and said, "Hey Pam, how tall are you?" to which Pam replied, "5′3″." Beadle then said, "Your new Boss is 5′4″ tall, he's twenty-eight and maybe you will like him so much, you will fall in love and decide to stay there at Caltech."[7] A few months after meeting, Ed and Pam were married; they remained so until Ed's death more than fifty-seven years later. It was Pam who, working as a technician in the laboratory in 1947, discovered the *Polycomb* gene, which Ed went on to report in his famous 1978 paper in *Nature* as the first "regulator of the regulators." Pam is an accomplished artist; one of Ed's final, albeit unfinished, projects was to self-publish a book of her paintings. Ed and Pam had three sons: Hugh (a lawyer in Bellingham, Washington), Glenn (who died as a teenager in a mountaineering accident on Christmas Eve, 1965) and Keith (a molecular biologist, who lives near Berkeley, California).

Ed's approach to science was strongly influenced by the writings of the British philosopher, Bertrand Russell, who emphasized that abstraction is important and that science is inductive, not deductive. Many of Ed's papers are difficult to read because of the abstract models he formulated to explain his results; however, abstraction framed his science, which can best be understood in those terms. He chose to quote from one of Russell's books—which he had first encountered as a high school student—to begin his Nobel lecture: "The power of using abstraction is the essence of intellect and with every increase in abstraction, the intellectual triumphs of science are enhanced."[8]

Genetics is an abstract discipline; therefore, Ed was at home in genetics, and his first love was always genetics. Almost all of his papers on *Drosophila* present the data in terms of abstract models. Throughout his career he distrusted and avoided overarching theories, instead deriving genetic rules and abstract models directly from the data. It should be noted that Ed not only abstracted models, he always made them current, based on what was happening in other disciplines such as biochemistry and molecular biology.

It is very important when reading Ed Lewis's papers to distinguish between what he called "rules" and what he called "models." Each rule is a description of a particular genetic phenomenon that he had discovered. The models, in contrast, are his abstractions of those phenomena. What is striking—even fifty years after many of Lewis's rules were framed—is that there remains very little understanding of their underlying molecular basis.

Lewis's *Drosophila* research spanned almost seven decades, beginning in the mid-1930s. His laboratory notebooks, begun as a graduate student in 1939 and ending shortly before his death almost sixty-five years later, reveal that he carried out an

[7] Quoted in P. Berg and M. Singer, *George W. Beadle. An Uncommon Farmer: The Emergence of Genetics in the 20th Century.* (Woodbury, N.Y.: Cold Spring Harbor Laboratory Press, 2003), 196.

[8] B. Russell, *The Scientific Outlook* (London: George Allen & Unwin, 1931), 87.

average of thousand genetic crosses a year throughout that period—a rate that is unlikely ever to be equaled.

Lewis's initial Drosophila studies focused on gene function and evolution. His invention of the *cis–trans* test enabled him to determine whether genetic recombination might occur between members of what were then known as "multiple allelic series" (closely linked mutations with similar phenotypes). The *cis–trans* test is simple in concept. In diploid organisms like flies it involves generating offspring that carry the two mutant alleles in *cis* on one chromosome and the two wild-type alleles in *cis* on the homologous chromosome. This can be represented symbolically for alleles *a* and *b* as [*a b*/+ +] where the pluses represent the wild-type alleles. The phenotype of these flies is then compared with that of offspring that carry the alleles in *trans* [*a* +/+ *b*], thus enabling one to ask whether the position of the alleles relative to each other affects the outcome. As can be seen, in an abstract sense the overall genetic constitution of the *cis* and *trans* combinations is the same: both carry two mutant alleles, *a* and *b*, and two wild-type alleles, + and +. They differ, however, in their position relative to each other.

Now, in practice, it can be very difficult to obtain the double-mutant in *cis* [*a b*] since this requires genetic recombination between closely linked alleles, and the recombination frequency is proportional to the distance between them. Furthermore, when Lewis began his studies, it was thought that recombination could not occur between members of a multiple allelic series. Lewis, however, showed that it was, indeed, possible to obtain recombination between the alleles of several such series: first, *Star* and *asteroid* (his Ph.D. work) and, later, *Stubble* and *stubbloid, white* and *apricot*, as well as the *bithorax* mutant series. Since the phenotypes of the *cis* and *trans* combinations differ greatly for all of these series, he was able to conclude that the position of the wild-type and mutant alleles relative to each other is very important for gene function. Furthermore, since *Star* and *asteroid* as well as the *bithorax* series of mutations map to polytene chromosome doublets—which Calvin B. Bridges had hypothesized might represent tandemly duplicated genes that are in the process of evolving to perform new functions—Lewis was led to propose that the separable "pseudoalleles" might indeed represent tandemly duplicated genes that are related both in structure and in function.

Working almost alone, over a thirty-year period from the mid-1940s to the mid-1970s, Lewis invented genetic strategies of unprecedented ingenuity and sophistication. These enabled him to discover that the *bithorax* family of mutants is, in fact, a cluster of genes (which he came to call the *bithorax* homeotic gene complex or BX-C, for short) that function as master regulators of the body plan. The effects of mutations in these genes are striking: they convert flies from two-winged into four-winged or from six-legged into eight-legged versions. This they accomplish by transforming the identity of one body segment into another: so-called "homeotic" transformations.

Already by 1951, Lewis had postulated that the *bithorax* family mutants control the development of particular body segments and that the second thoracic segment is in some sense the developmental "ground state." The function of the *bithorax* genes is,

thus, to convert segments from this ground state to more posterior segmental identity (i.e., from second thoracic to third thoracic as well as abdominal identity). Abrogation of the function of the genes in the *bithorax* complex leads to homeotic transformation of, for example, the third thoracic segment into second thoracic segment, thus creating the second pair of wings.

By the late 1950's, Lewis's focus had shifted from genes, their function and evolution, to how they control development, which he thought should be amenable to the same kind of mechanistic genetic analysis that had been used for biosynthetic pathways in bacteria and their viruses. While the series of rules that Lewis was to discover, rules about how genes control development, could in no way have been derived from the results of those earlier studies on biosynthetic pathways, he was correct in principle. Indeed, it was the genetic approach pioneered by Lewis that, when combined with the molecular methods pioneered by others, led to the deep insights that we now have into the mechanisms by which animals develop.

During the 1960s, Ed Lewis also identified genes—most notably *Polycomb*, the first allele of which Pam had discovered in 1947—that act as "regulators of the regulators," switching the master control gene clusters on or off at different positions along the body axis. He also started to address the spatial and temporal control of development by *bithorax* complex genes through his analyses of genetically chimeric ("mosaic") flies. Using these mosaics he was able to ask whether the *bithorax* complex genes confer the fate of cells autonomously or whether the genes encode diffusible substances, which would be expected to function cell nonautonomously. Strikingly, the genes behaved completely cell autonomously in the epidermis, consistent with their encoding non-diffusible substances that give identity instructions to each cell in which they are expressed. With the recently discovered "*lac* operon" in mind, Lewis suggested that the *bithorax* genes "evidently . . . [produce] a whole set of substances that repress certain systems of cellular differentiation and thereby allow other systems to come into play."[9] Subsequently, he postulated that the *bithorax* substances would function through both activation and repression. Twenty years later, molecular analyses proved this to be correct: the *bithorax* complex encodes proteins that regulate the transcription of mRNAs from their target genes (see below).

Lewis's most famous paper appeared in 1978[10] following a more than ten-year publication drought. Because Lewis summarizes 30 years of research in about six pages and presents almost all of his data in terms of an abstract model, the paper is very difficult to read. However, for those willing to make the effort, it is a revelatory paper; indeed, upon its publication it almost immediately established a new paradigm for the genetic control of development.

The 1978 paper is replete with novel observations and strategies, not least of which is Lewis's analysis of homeotic phenotypes in embryos rather than adults. These

[9]E. B. Lewis, Genetic control and regulation of developmental pathways. In: *The Role of Chromosomes in Development*, ed. M. Locke (New York: Academic Press, 1964), 231–252.

[10]E. B. Lewis, A gene complex controlling segmentation in *Drosophila*. *Nature* 27(1978): 565–570.

analyses proved that the *bithorax* complex genes function throughout development to establish segmental cell fates. Furthermore, they set the stage for ready acceptance by the *Drosophila* community of the large-scale genetic screens for embryonic pattern mutants that were begun in 1978 by Christiane Nüsslein-Volhard and Eric Wieschaus, who shared the 1995 Nobel Prize with Lewis.

To a geneticist, the most remarkable part of the 1978 paper is Lewis's invention of what can be called "add-back genetics."[11] Standard genetics involves mutating or deleting genetic functions and inferring the wild-type role of genes from their mutant phenotypes, a strategy that Lewis had applied very successfully to the *bithorax* complex since the inception of his analyses. In contrast, add-back genetics began by deleting the entire *bithorax* complex and then adding back, bit-by-bit, wild-type pieces of the complex. In this way, Lewis was able to define the location and the wild-type function of genes for which he had not yet obtained mutations. His results led him to propose that there are twelve different genes in the complex, which turn on progressively one at a time from more anterior (fewer genes "on") towards more posterior (more genes "on") segments. Thus the fate of any particular segment would be specified additively by the sum of the "substances" produced by the *bithorax* complex genes turned on in it.

In the early to mid-1970s, David S. Hogness and his colleagues at Stanford University invented recombinant DNA methods for the analysis of whole genomes. In 1978, Hogness and his postdoctoral fellows, Welcome Bender and Pierre Spierer, initiated a collaboration with Lewis that led to the first positional cloning of a gene—part of the *bithorax* complex—and the first functional genomic analyses, which correlated the DNA map, the mRNA transcripts, the genetic mutations, and their phenotypes.[12] This was followed in the mid-1980s by the unexpected discovery by Matthew P. Scott and his colleagues in the U.S. and Walter Gehring's laboratory in Switzerland that genes in the homeotic complexes of *Drosophila* share a closely related DNA sequence (the "homeobox"), which encodes a protein domain that binds to DNA and regulates the production of mRNA transcripts from "target" genes.

The molecular analyses revealed that Lewis, in his earlier "additive control along the body axis by tandemly duplicated genes" hypothesis, had been both right and wrong. Right in that the genes in the complex had indeed evolved by tandem duplication: there are three tandemly duplicated protein-coding genes in the complex, which are characterized by the homeobox. Right, too, in that the spatial expression of these genes is highly regulated along the body axis and, indeed, the genes do become active one after the other, from anterior to posterior, along the body axis as Lewis had postulated. But he was wrong in concluding that there are twelve genes in the *bithorax* complex; there are three. Most of the twelve "genes" that Lewis had identified are in fact *cis*-regulatory regions that control the time, place, and level of expression

[11] H. D. Lipshitz, ed., *Genes, Development and Cancer: The Life and Work of Edward B. Lewis* (Boston: Kluwer Academic, 2004), 165–166.

[12] W. Bender, M. Akam, F. Karch, P. A. Beachy, M. Peifer, P. Spierer, E. B. Lewis, and D. S. Hogness, Molecular genetics of the bithorax complex in *Drosophila melanogaster*. *Science* 221(1983):23–29.

of the homeobox-containing mRNAs. Also, it turned out that the identity of each segment is not a simple additive effect of activating more of the genes (three genes could not additively regulate the identity of that many segments). Lewis could not have predicted these molecular details solely on the basis of his genetic results; the synergism of molecular and genetic methods was required.

One of the most remarkable discoveries made in the mid- to late 1980s was that genes closely related to those studied by Lewis are present in similar clusters in the chromosomes of all animals and that they control the development of these animals in much the same way as in the fly. Furthermore, Lewis's "colinearity" rule—that the order of the homeotic complex genes in the chromosomes corresponds to the order along the body axis of the segments whose development they control—applies all the way from flies to mammals. Thus, a primordial gene complex must have predated the divergence of the ancestors of flies and mammals more then 500 million years ago. It was this extension and generalization of four decades of Lewis's genetic analyses that led to the award, in 1995, of a share of the Nobel Prize for "discoveries concerning the genetic control of early embryonic development." David Hogness summarized it as "one of the best awards that the Nobel committee has made."[13] In typically modest fashion, Ed Lewis's response to the news of the award was, "It's very nice, but actually what is more exciting is the science. . . . It's much more exciting to get the discoveries than to win prizes."[14]

The Nobel Prize did not change Ed's life, his attitude, or his work schedule very much. For the first six months after the award, Caltech provided him with a part-time secretary and a fax machine to assist with the extensive correspondence. Thereafter, the secretary returned to her normal assignment, but Lewis got to keep the fax machine. The celebratory dinner at Caltech in honor of Lewis's award was, for him, more an opportunity to play his flute in a chamber music recital, than to bask in the glow of laudatory speeches. Subsequently, he did attend more public relations functions for Caltech than he had in the past; but this was driven by his dedication to—and love of—that institution rather than by the limelight, to which he never was attracted. He used his prize money to establish a trust with Caltech that would go towards undergraduate scholarships when he died, saying that "Caltech has provided the kind of excellent environment that has allowed me to carry out the research that has led to the award of the Prize. . . . In these days of high tuition costs, scholarships are needed more and more." Undoubtedly, he had in mind the struggle that he and Jimmy had gone through to attend college during the Depression.

More than anything else, however, Ed derived pleasure from returning to his beloved flies, through which he conducted his daily dialogue with the laws of nature. His greatest challenge was not just to make a four-winged *Drosophila*, but to make one that could flap the auxilliary pair of wings and actually fly! This was not just an idle pastime, but a real scientific challenge: the homeotic gene code for the flight muscles

[13] Quoted in the *Los Angeles Times*, 10 October 1995, A18.
[14] ibid.

differs from that for the wings they must flap. For Ed, then, the challenge was to mutate the genes correctly for both the muscles and the wings in order to accomplish his goal. While he never succeeded, for Ed even more than for most scientists it was the journey rather than the destination that was most fascinating.

Ed's journey returned, in the mid-1980s, to gene evolution but now using the newly invented molecular tools and, still later, the completed sequence of the 120 million "letters" in the fly's DNA blueprint. His first love and ongoing tool of choice, however, remained classical genetics, the field to which he had made so many contributions.

Less well known than his studies on the genetic control of development is Lewis's work on the somatic effects of ionizing radiation, which began at the height of the cold war in the mid-1950s. Lewis was drawn into the debate about the effects of low levels of radiation in causing cancer in humans. At that time many scientists and government officials in the U.S. and U.K. argued that there is a threshold dose of radiation below which cancer would not be induced.

In 1954, Admiral Lewis L. Strauss, the chairman of the Atomic Energy Commission in the U.S., had issued a public assurance that the atomic weapons tests would result in an increase in background radiation in some locations within the continental United States that was "far below the levels which could be harmful in any way to human beings." In a landmark study published in the journal *Science* in 1957,[15] Lewis carried out risk estimates for leukemia in survivors of the Hiroshima and Nagasaki atomic bomb attacks, in radiologists, and in other populations exposed to low doses of radiation. His best estimate of the absolute risk of leukemia was one to two cases per million persons per rem per year; low but certainly not negligible. Lewis's analyses also led him to the very important—but at the time highly controversial—conclusion that the threshold hypothesis was not supported.

He also realized that the health effects of radioactive fallout from nuclear weapons tests had been underestimated by federal regulatory agencies. It had been thought that a dose of two thousand rad would be needed to induce cancer and that only bone cancer would occur. This error arose because it had not been understood that radiostrontium would concentrate in bones, thus irradiating the blood-system-producing cells in the bone marrow to cause leukemia. Lewis pointed this out in the 1957 paper, where he calculated that there would be a five to ten percent increase in leukemia incidence in the U.S. from a constantly maintained level of Strontium-90 that was one tenth of the "maximum permissible concentration" (MPC) recommended by the National Commission on Radiation Protection.

Shortly after publication of his 1957 paper, Ed was attacked publicly on NBC's *Meet the Press* television show by Admiral Strauss, who challenged his scientific credentials. Neil Wald of the Atomic Bomb Casualty Commission in Japan and Austin Brues of the Argonne National Laboratories published scientific articles that criticized the accuracy of Lewis's data. The most detailed critique came from Alan W. Kimball, a

[15]E. B. Lewis, Leukemia and ionizing radiation, *Science* 125 (1957): 965–972.

statistician at the Oak Ridge National Laboratory, who challenged Lewis's methods of data analysis. Sewall Wright and James F. Crow, both distinguished geneticists, engaged in an active dialogue with Kimball. The former explained that "Lewis' tests are correct"[16] and the latter pointed out that several of Kimball's theoretical criticisms were "irrelevant"[17] for the type of analysis that Lewis had conducted.

History is on Lewis's side: research over the nearly fifty years since he published his landmark study has supported and confirmed his original conclusions. Current estimates by the National Research Council on the Biological Effects of Ionizing Radiation (BEIR) range from 1.0 to 3.4 cases per million persons per rad (or rem) per year[18], close to Lewis's original estimate of 1.0–2.0 such cases.

Following publication of the *Science* paper, Lewis was called to testify before a U.S. Congressional Joint Committee on Atomic Energy in June 1957. Subsequently, he served on the National Advisory Committee on Radiation of the US Public Health Service as well as on committees of the National Academy of Science concerned with estimating risks of ionizing radiation.

Over the two decades that followed publication of the *Science* paper, Lewis returned repeatedly to questions related to the somatic effects of low doses of ionizing radiation.[19] In one of those studies[20] he reported that drinking cow's milk contaminated with radioactive iodine from fallout or from other sources was likely to affect the thyroid of infants and children far more than the adult organ. Lewis's prediction was highlighted tragically after the meltdown of the Chernobyl nuclear reactor in the Ukraine in 1986, which led to a significant increase in thyroid cancer among children who had consumed cow's milk contaminated with the radioiodine that had been released into the atmosphere over Northern Europe.

Lewis, who preferred the peace and quiet of his laboratory to the public arena, was haunted by the public attention and the politically motivated attacks that accompanied his radiation studies. He always emphasized that he saw himself not as an advocate for or against nuclear weapons and weapons tests, but as a scientist whose responsibility was to provide accurate information to policy-makers, thus positioning them to make educated decisions.

Lewis received many awards and honors. Among these were election as a member of the U.S. National Academy of Sciences (1968), election to the American Philosophical Society (1990), and election as a foreign member of the Royal Society of London (1989). He received (again only a selection is listed) the Gairdner Foundation International Award (Canada, 1987), the Wolf Prize in Medicine (Israel, 1989), the Lewis S. Rosenstiel Award in Basic Medical Research (U.S., 1990), the National Medal of Science (U.S., 1990), the Albert Lasker Basic Medical Research Award

[16] Quoted in Lipshitz (2004), ibid., 399.

[17] Quoted in J. F. Crow and W. Bender, Edward B. Lewis, 1918–2004. *Genetics* 168 (2004):1773–83 (P. 1779).

[18] BEIR V (Washington, DC: National Academy of Sciences, 1990).

[19] For detailed discussion see Lipshitz (2004), ibid., 389–404; J. Caron, Biologists and "the bomb," *Engineering and Science* 67 (2004): 17–27.

[20] E. B. Lewis, Thyroid radiation doses from fallout. *Proc. Natl. Acad. Sci. USA* 45 (1959): 894–97.

(U.S., 1991), the Louisa Gross Horwitz Prize (U.S., 1992), and the Nobel Prize in Physiology or Medicine (Sweden, 1995).

Ed Lewis exhibited a rare combination of intellectual rigor and iconoclasm that was coupled with remarkable personal and scientific integrity and humility. He was kind, gracious and generous in both his personal and his scientific life. In his science he continued the tradition of sharing data and materials that was begun by Thomas Hunt Morgan and his coworkers starting in 1910.

Perhaps one final story captures Ed Lewis the man better than any other. In March 1997, I received a phone call from Ed, who was livid about the contents of an article in the *San Francisco Examiner* entitled "Science student accused of cruelty to fruit flies."[21] The newspaper article reported that a high school sophomore, Ari Hoffman, had won the Marin County science fair but had subsequently been disqualified because thirty-five of the two hundred fruit flies he had used in his experiments had died. Apparently national science fair regulations ban experiments that injure or kill animals of any kind—and fruit flies certainly are animals! Ari's project had been to examine the effects of different doses of radiation on mutation rate and fertility. Herman J. Muller had first shown, in 1927, that ionizing radiation causes mutations roughly in proportion to the dose given to the flies. For this work Muller received the 1946 Nobel Prize in Physiology or Medicine, so young Ari was in good company. Fortunately, the article mentioned that Ari had been able to do the experiments because his father, Dr. William Hoffman, had a laboratory at UCSF and access to a radiation source. Soon Ed was on the phone to Dr. Hoffman, expressing his personal regret that Ari had lost the prize. He was ecstatic to find that others had also challenged the decision, resulting in reinstatement of the award.

But Ed didn't stop there. He obtained Dr. Hoffman's home address and dashed off a letter to Ari, enclosing a cheque "as a token award for your accomplishments from someone who has spent his career studying *Drosophila*. I also started in high school, long before anyone had thought of science fairs . . . [I]f you and your family [are in Los Angeles] and have time we would be pleased to have you visit the lab here." Within days Ari had written back arranging to visit and telling Ed that "the contents of that envelope are my most cherished souvenirs from the fruit fly ordeal. . . . Having a Nobel Laureate support me and show interest in my work is something few can boast about."[22]

Ed will not be remembered only as a great scientist and a fine human being. His friends and colleagues will remember him for his love of life and all things living. He had boundless energy: when not pushing flies he was playing the flute, jogging, or swimming, attending opera performances in Los Angeles, San Diego or San Francisco, playing chamber music, constructing Halloween costumes based on paintings by his favorite artist, René Magritte, jogging on the beach in La Jolla,

[21] *San Francisco Examiner*, 20 March 1997.

[22] I am grateful to Ari Hoffman for permission to quote from his letter to Ed. Ari graduated from Stanford with a degree in biological sciences and commenced studies toward an M.D. degree at UCSF in the fall of 2005.

or scouring its tide-pools for interesting denizens. Unlike all other Caltech faculty, he did not really have an office; his was a mixture of office, laboratory, music room and marine aquarium—always cluttered, always fascinating. Diagrams of his models of "transvection" dating from the 1950s would be propped in the corner; microscope slides with his latest polytene chromosome squashes would be scattered near the microscope; giant sheets of paper bearing the complete DNA sequence of the *bithorax* complex, with different parts shaded in different colors, would be taped to the blackboard that spanned one wall. In one marine aquarium, a pair of clownfish would hover near a sea anemone, excavating the gravel in preparation for consummation of their relationship; in another, each chamber of a multi-chambered box contained a piece of polychaete worm that he had brought back from La Jolla, had cut up, and was in the process of regenerating. At home, a glass tank in the pantry would contain his and Pam's latest batch of baby desert tortoises. Adjacent would be a tank festooned with Pam's annual crop of baby praying mantids; one of his numerous awards—selected because it was weighty enough—would be used to hold the lid on the tank. A tank in the corner of the living room would contain a giant plecostomus or several colorful koi. Even as his illness weakened him, he would proudly escort visitors around his backyard, pointing out his latest crop of ripening tomatoes and the various trees that were coming into fruit.

Ed Lewis was a consummate scientist: always curious, never satiated with knowledge. He is sorely missed as a role model, colleague, and friend to several generations of geneticists worldwide.

Elected 1990

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SECTION I: GENES

LEWIS AND THE NATURE OF THE GENE

BACKGROUND

The first 30 years of research on Drosophila genetics (1910–1940) led to the proof of the chromosome theory of inheritance, invention of genetic mapping by meiotic recombination, discovery of rearranged chromosomes, proof that ionizing radiation causes mutations, discovery and mapping of salivary gland polytene chromosomes, and the cytogenetic mapping of chromosomal rearrangements on these chromosomes (reviewed in Sturtevant, 1965).

The concept of the "gene", the name assigned to Mendel's abstract unit of inheritance by Wilhelm Johannsen, the Danish geneticist (Johannsen, 1909), had evolved further—after discovery of linkage and meiotic recombination—to also be viewed as the unit of recombination. The model of the genes in a chromosome was, thus, very much like beads on a string where the genes were the beads and recombination occurred between the beads, along the string. The length of the string between adjacent beads varied, affecting the genetic map distance between the genes. The idea that genes are indivisible units of recombination led to the concept of multiple allelism: all mutations that map to the same region and show similar phenotypes in *trans*-heterozygous combination are alleles of the same gene (Sturtevant, 1913). With the production of polytene chromosome maps, the bands on the chromosomes came to be regarded as the site of the genes and the interbands as the barren regions in between.

Two discoveries are particularly relevant to understanding Lewis' early papers on genetics. First, Sturtevant discovered what he called a "position effect" at the *Bar* (*B*) locus (Sturtevant, 1925). The original *B* mutation results in a bar-shaped eye, rather than the wild-type round eye, because of absence of anterior eye tissue. Females homozygous for *B* occasionally give rise to offspring with round eyes or with more extremely reduced eyes than *B*. Sturtevant hypothesized that unequal crossing over results in production of these "double-Bar" (*BB*) and normal (round-eyed) progeny. Simultaneous recovery of both products of this unequal crossover in the same fly, and thus proof of Sturtevant's hypothesis, was accomplished by Lillian Morgan, using attached-X chromosomes that carried the *B* mutation (Morgan, 1931). More than a decade after Sturtevant's discovery it was shown that *B* is a small, tandem duplication of seven bands on the X chromosome (Bridges, 1936; Muller et al., 1936).

Sturtevant's key observation was that the phenotype of flies carrying two B mutations is dependent on their position relative to each other on homologous chromosomes: if two *B* mutations are present on the same chromosome but the other chromosome carries the wild-type alleles (*B B*/+) then these flies have somewhat smaller eyes than those that carry one mutant allele on each homolog (*B*/*B*). The implication of this result was that the function of a gene is dependent on its position relative to its neighbors, hence the term "position effect." It should be noted that the situation with regard to *B* is quite special, since *B* is a dominant mutation and is caused by a tandem duplication. Thus the relevance to normal gene function was not altogether obvious.

Second, and of equal importance in Lewis' work, was Bridges' paper on the polytene chromosome maps in Drosophila (Bridges, 1935). It was here that Bridges, in carrying out cytological analysis, called attention to double-banded structures that he called "doublets." He hypothesized that, because each band was thought to represent a gene, such doublets might represent tandemly duplicated genes "offering a method for evolutionary increase in lengths of chromosomes with identical genes which could subsequently mutate separately and diversify their effects."

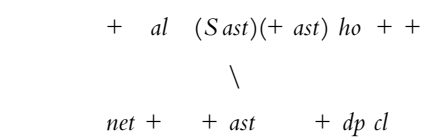
It was Bridges' hypothesis on gene duplication and evolution and the excitement of challenging the concept that crossing-over could not occur between members of a multiple allelic series, that led Lewis to an operational approach to dissect multiple allelic series: the *cis–trans* test.

INVENTION OF THE *CIS–TRANS* TEST FOR POSITION EFFECTS

As an undergraduate, Lewis had worked on a mutation, *Star-recessive* (S^r), which had arisen in a stock of flies carrying the original, dominant *Star* (*S*) mutation. In his first paper (Lewis, 1939), Lewis showed that S^r behaves as a recessive allele of *S*. He in fact, also asked whether he could separate S^r and *S* by recombination, but recovered only a single wild-type fly among 9,294 progeny. Thus, he could not definitively say that this was a wild-type recombinant rather than a reversion of S^r to +, or some other event. Given the "multiple allelism" hypothesis described above, Lewis was driven to conclude that S^r is an allele ("allelomorph" in the term of the day).

Sturtevant gave Lewis the freedom to continue his studies on *S* and S^r when Lewis began his graduate research at Caltech in 1939. By his second paper (Lewis, 1941), he had learned of and applied the trick of increasing crossing over in the chromosome arm containing the gene of interest by using inversions to block crossing over in the other arms (the "interchromosomal effect," Steinberg, 1936). Thus he had now obtained 12 wild-type recombinants among 26,370 progeny, placing the map distance between *S* and S^r at 0.046 cM (centiMorgan, the unit of genetic recombination is defined as percent crossing-over, in this case, 0.046%). However, he had not yet recovered any *S* S^r double-mutants, which would have represented the reciprocal crossover product. Recovery of this product would have been definitive proof of recombination rather than some other genetic event that could explain the wild-type (e.g., reverse mutation associated with crossing over). Importantly, Lewis had recovered a tandem duplication—visualized as four polytene bands—that included the recessive (S^r) allele and that would play a crucial role in his later recovery of the double mutant.

Lewis reported the results of his Ph.D. thesis in a brief abstract in 1942 (Lewis, 1942) and then in detail in 1945 (Lewis, 1945). The 1945 paper is a long and complicated paper in no small part because, in those days, it was customary to actually report the complete results of recombination experiments! To derive the double-mutant Lewis placed the duplication in *trans* to a normal (nonduplicate carrying) S^r chromosome. From this starting configuration he obtained 5 recombinants out of 6,395 progeny. In the paper, Lewis renamed S^r as *asteroid* (*ast*) because his derivation of the double mutant by recombination led him to consider *S* and S^r to be members of a tandem gene duplication. Thus, for example, in presenting mating number 9, the genotype is written: *al (S ast)* (+*ast*) *ho* for the tandem duplication-bearing chromosome and *net* + *ast dp cl* for the unrearranged chromosome. By following the linked markers, Lewis obtained the crossover chromosome carrying both *al* (from the duplication chromosome) and *dp cl* from the unrearranged chromosome. The key question was whether *ast* was indeed present: Was the genotype in fact *S ast* and had *S* been "extracted" from the duplication? This was proved by test crossing to *ast*. Schematically, then, the recombination had occurred between S in the left repeat and ast on the normal chromosome as follows:



to give

+ *al* *S* *ast* + *dp* *cl*.

Lewis went on to also derive the *S* ast^4 double mutant by the same means and with about the same frequency (3/6,667), placing *S* and *ast* between 0.01 and 0.04 cM apart.

Apart from being a technical tour-de-force, Lewis' 1945 paper represents the invention of the *cis-trans* test for position effects that appears in undergraduate genetics textbooks. Specifically in the case of *S* and *ast*, Lewis showed that *S ast*/+ + (the *cis*-configuration) has normal eyes, while *S* +/+ *ast* (the *trans*-configuration) is almost eyeless. Many textbooks present the *cis–trans* test simply as an assay for whether two mutations lie in the same or different genes: Starting with two recessive mutations, *a* and *b*, with similar phenotypes, if the *cis*-configuration *a b*/+ + is phenotypically wild type while the *trans*-configuration *a* +/+ *b* is mutant, then *a* and *b* are in the same gene. If, however, *a* +/+ *b* is phenotypically wild type, then *a* and *b* are in different genes. However, this explanation neglects the subtleties of phenotype between these two extremes. Exploitation of *cis–trans* analysis in the dissection of subtly, but reproducibly, distinct phenotypes was central to Lewis' later analyses of the bithorax series of mutants.

The Discussion section of Lewis' paper is particularly interesting, because it is here that he speculates about the relationship between the *cis–trans* position effect and repeated genes. He points out that *S* and *ast* map to the 21E1-2 doublet, just as *Bar* maps to the 16A1-2 doublet, fully consistent with Bridges' 1935 hypothesis that doublets represent tandemly duplicated genes. He speculates also about how the *cis–trans* position effect might be mediated physically by somatic chromosome pairing, which was known to occur in Diptera, and might be particularly "strong" in the case of tandemly duplicated genes. [That position effects might somehow be related to somatic pairing had been suggested previously by Sturtevant (1925) and Muller (1941).] Lewis proposes that a mutation in one of the genes might alter the pairing of somatic chromosomes, thus altering gene function. This discussion is particularly relevant in hindsight: a decade later Lewis was to return to this model with his discovery and analysis of the phenomenon of "transvection" (Lewis, 1954b).

Lewis ends the landmark 1945 paper with a sentence that sounds prescient: "If doublet structures are repeats . . . then, judging from their widespread occurrence in the salivary gland chromosomes . . . , it is likely that other multiple allelic series may be resolved into duplicate loci which act, by reason of a position effect, as a *developmental unit* [editor's italics]." Almost 20 years later, during his genetic dissection of the bithorax series of mutants, Lewis was to return to the function of what he had by then termed a "gene complex" in the control of development (Lewis, 1967). And, over the following decade, his analyses were to revolutionize understanding of how genes control development.

POSITION PSEUDOALLELES

Subsequent to his 1945 paper, Lewis sought a term other than "allele" to describe closely linked mutations that exhibit similar phenotypes and a position effect in the *cis–trans* test. The need for a new term derived from the ongoing assumption that recombination occurred only between genes. Thus, in his 1951 paper, he coined the term "position pseudoallele" as an operational definition for the outcome of the

cis–trans test in which "the activity of a gene is altered when its relation with respect to a specified allele in a neighboring gene is changed" (Lewis, 1951). For example, *S* and *ast* would now be called position pseudoalleles since they are: (i) closely linked; (ii) exhibit similar phenotypes; and (iii) show the *cis-trans* position effect described in Lewis' 1945 paper. Lewis searched for additional examples of potential tandemly duplicated genes that reside in polytene chromosome doublet bands and that behave as position pseudoalleles. His analyses focused on three of these: *Stubble* (*Sb*) and *stubbloid* (*sbd*) in 89B4-5 (Lewis, 1951); *bithorax* (*bx*), *Bithorax-like* (*Bxl*, aka bx^{D} and later renamed *Ubx*), and *bithoraxoid* (*bxd*) in 89E1-4 (Lewis, 1951); and *white* (*w*) and *apricot* (w^{a} or *apr*) in 3C2-3 (Lewis, 1952).

Before considering Lewis' 1951 paper, it is easier first to review the 1952 paper on *w* and w^{a} (Lewis, 1952). Here Lewis obtains recombinants between w^{a} and *w* (which he calculates to be between 0.005 and 0.02 cM apart) and shows that, in the *cis–trans* test + *w*/ w^{a} + gives a mutant eye color while w^{a} *w*/+ + gives a wild-type, red eye. He makes use of an attached-X chromosome discovered by L. V. Morgan (1931), which enables recovery of both products of a meiotic recombination event in a single female offspring. In normal female flies, only one of the four products of any meiosis is contributed to the offspring. Thus, obviously, it is not possible to recover both products of a reciprocal cross over in the same fly. Morgan's method involves the use of an attached-X chromosome in which two X chromosomes share the same centromere and thus segregate to the same pole (i.e., into the same nucleus) during meiosis. Consequently, a "half-tetrad"—two of the four products of meiosis—could be recovered.

By using attached-X triploid females that also carried a single, unattached X chromosome with appropriate marker mutations, Lewis was able to derive by recombination a starting configuration of the attached-X with the *w* mutation on one arm and the w^{a} allele on the other arm, along with flanking markers that would enable him to detect crossovers. He again used the interchromosomal effect, introducing autosomes carrying inversions to increase recombination in the X chromosome. He thus recovered reciprocal products of a crossover between w^{a} and *w* in the same female. For example:

y	*sc*	+	*w*	*spl*	+		*y*	*sc*	+	+	+	*ec*
		×				to give						
y^{2}	+	w^{a}	+	+	*ec*		y^{2}	+	w^{a}	*w*	*spl*	+

In this way the configuration of w^{a} and *w* changes from *trans* (pinkish yellow eye color) to *cis* (red eyes). Proof that w^{a} and *w* were in fact in *cis* on one chromosome and that the other chromosome carried the + + configuration, was obtained in several ways. The attached-X chromosome can undergo further recombination in subsequent generations to give daughters, still attached-X, which are now homozygous for one or the other arm. Lewis showed that these homozygous daughters are phenotypically yellow scute red-eye echinus (i.e., genotype: *y sc* + + + *ec*) or yellow-2 white-eye and split (i.e., genotype: y^{2} + w^{a} *w spl* +). Another method involved subsequent

spontaneous detachment of the attached-X's. This enabled w^a w double-mutant males to be obtained that could be tested, by back-crossing to either w or w^a females, for the presence of both mutations.

The w mutation was the first to have been discovered in Drosophila (Morgan, 1910) and many alleles had been collected in the 40 years between that discovery and Lewis' experiments. Thus, if any multiple allelic series epitomized the dogma that genes were indivisible by recombination, it was the w series. It thus must have been particularly satisfying for an iconoclastic young scientist like Lewis to succeed in showing that he could obtain recombination between w and w^a!

In his 1951 paper Lewis extended his analyses of position pseudoalleles to the bristle mutants—*Stubble* (*Sb*) and *stubbloid* (*sbd*)—and then to three mutations that affect the morphology of the third thoracic and first abdominal segment of the fly—*bithorax* (*bx*), *Bithorax-like* (*Bxl*), and *bithoraxoid* (*bxd*) (Lewis, 1951). The *sbd* and *Sb* mutations are, respectively, recessive and dominant, and both result in short bristles on the fly. The position effect that Lewis found was particularly spectacular because the strongly dominant *Sb* mutation (*Sb*/+ flies have short and stubby bristles) becomes recessive in the *cis*-configuration, *Sb* sbd^2/+ + (i.e., the flies have long, tapering bristles). In contrast, flies carrying the *trans*-configuration, sbd^2+/+ *Sb*, have extremely short and stubby bristles.

This paper presents for the first time Lewis' analyses of the bithorax position pseudoalleles, which he had begun studying five years earlier. The bithorax pseudoalleles that Lewis used were: (a) mutations in the *bithorax* gene itself (three alleles: *bx*, bx^{34e} and bx^3); (b) *Bxl*, a dominant mutation that lies 0.02 cM more distal (*Bxl* had been called bx^D by Hollander, bxd^D by Lewis and was later renamed *Ultrabithorax* or *Ubx* by Lewis); and (c) a recessive mutation, *bithoraxoid* (*bxd*), which lies 0.01 cM to the right of *Bxl*. The new experimental depth provided by these mutants is apparent in this study. Unlike the situation with *S ast*, w w^a, and *sbd Sb*, the bithorax pseudoalleles showed reproducible qualitative, not just quantitative, phenotypic differences in the *cis–trans* test. As shown in Lewis' Figures 3 and 4, the *trans*-configuration of *Bxl* (= *Ubx*) with either *bx* or *bxd* alleles results in a phenotype very similar respectively to that of *bx*/*bx* and *bxd*/*bxd* (only the combinations involving *bx* and bx^3 are figured in the paper). In contrast, the *cis*-configuration is indistinguishable from the weakly dominant phenotype exhibited by *Bxl*/+ (= *Ubx*/+) flies. For example, in the *trans*-configuration, bx^3+/+ *Bxl*, the anterior portion of each haltere is enlarged and wing-like while, in the *cis*-configuration, bx^3 *Bxl*/+ +, the haltere remains small and only slightly swollen as in the *Bxl*/+ situation.

THE BITHORAX PSEUDOALLELES AND THE CONCEPT OF DEVELOPMENTAL CONTROL

The 1951 paper is a landmark because Lewis introduces two key concepts. First, he introduces the idea that the bithorax series pseudoalleles control the "level" of development of subsegments of the fly. Second, he begins to consider the meaning

of position effects and developmental control in abstract terms, derived in large part from the types of biochemical pathway analyses that were going on in prokaryotes and fungi and that had led to the "one gene one enzyme" hypothesis (Beadle and Tatum, 1941; Horowitz, 1950).

Lewis found that the *bx* mutations convert the anterior region of the third thoracic segment towards the identity of the anterior second thoracic segment. The most obvious change is in the anterior part of the halteres—the small, balancer-like appendages on the third thoracic segment of the fly, which Lewis' studies were to show are homologous to the second pair of wings of other insects. In *bx* mutants, particularly in the case of the strong bx^3 allele, the anterior haltere transforms towards the identity of the anterior wing, as described above. In contrast, the *bxd* mutations convert the posterior third thoracic segment to posterior second thoracic segment identity, exemplified by conversion of posterior haltere to posterior wing. Furthermore, the effects are additive, with *bx bxd* double-mutant flies showing conversion of both the anterior and the posterior third thoracic towards the second thoracic segment in identity.

Lewis points out that *bx bxd* double mutants cause conversion of the first abdominal segment towards the second thoracic segment, contrasting with *bxd* homozygotes in which the first abdominal segment converts to third thoracic segmental identity. Those familiar with Lewis' 1978 paper will recognize at this point the germ of the idea of sequential action of the bithorax complex genes in converting successively more posterior body segments away from the second thoracic "primitive" level (Lewis, 1978). Indeed Lewis spells this out quite explicitly by saying that *bx* mutations cause a change from the third thoracic or metathoracic "level" of development (abbreviated L-mt) to that of the more anterior, mesothoracic "level" (L-ms). Similarly *bxd* mutations convert L-ab to L-mt, and so forth.

Notably, Lewis points out that the segmental transformations caused by *bx* and *bxd* are consistent with the idea that these regions of the fly "behave as though they were embryologically related to the anterior portion of the segment which follows, rather than precedes, them," giving credit to Snodgrass for reaching such a conclusion in more primitive, four-winged insects (Snodgrass, 1935). Almost 35 years later, Martinez-Arias and Lawrence were to define the "parasegmental" nature of Drosophila embryogenesis, an elaboration of this concept that was, by then, supported also by embryological data and molecular studies of the expression patterns of homeotic and other genes (Martinez-Arias and Lawrence, 1985).

Returning to the results of the *cis–trans* tests, Lewis introduces an abstract model for the first time to try to explain the results he had obtained with *S ast*, *sbd Sb*, and *bx*, *Bxl* and *bxd*. As with all of his abstract models, it attempts to formalize the data as well as to integrate it with current, more biochemical, concepts of gene function. He envisages sequential action of the products of the pseudoallelic genes in a biochemical pathway. Note that, at this time it was not known that translation of, as well as most functions carried out by, gene products occur in the cytoplasm, far from their site of transcription in the nucleus. Furthermore, the discovery of transcription

factors that regulate the expression of nuclear genes was many years away. The formal model diagrammed by Lewis thus shows the function of gene products at or near the chromosomes and states explicitly that these products do no diffuse "readily." For example, he postulates the following reaction series:

$$S \xrightarrow{bx^+} A \xrightarrow{Bxl^+} B \xrightarrow{bxd^+} C$$

In words, substrate S is converted to product A by the bx^+ gene product, A is converted to B by the action of the Bxl^+ gene product, and B is then converted to C by the bxd^+ gene product.

In terms of "levels of development," then:

$$\text{L-ms} \xrightarrow{\text{A or B}} \text{L-mt} \xrightarrow{\text{C}} \text{L-ab.}$$

In words, substances A or B are able to convert the level of development of a particular segment from mesothoracic (second thoracic) to metathoracic (third thoracic) in identity, while substance C promotes the next level of development, abdominal identity.

In the *cis* or *trans* mutant situations, Lewis diagrams the consequences. Presented here are a couple of examples, simplified relative to the actual publication.

In *cis* (+ + +/*bx Bxl bxd*):

$$S \xrightarrow{bx^+} A \xrightarrow{Bxl^+} B \xrightarrow{bxd^+} C$$

$$S \xrightarrow[bx^-]{} {<}A \xrightarrow[Bxl^-]{} {<<}B \xrightarrow[bxd^-]{} {<<}C$$

with the outcome that enough A, B, and C are produced by the upper, wild-type chromosome to allow almost normal development of L-mt and L-ab.

In *trans* (*bx* + + /+ *Bxl bxd*):

$$S \xrightarrow{bx^-} {<}A \xrightarrow{Bxl^+} {<}B \xrightarrow{bxd^+} {<}C$$

$$S \xrightarrow[bx^+]{} A \xrightarrow[Bxl^-]{} {<}B \xrightarrow[bxd^-]{} {<}C$$

with the outcome that L-mt is not accomplished because of the reduction in A and B substances that implement this function. To explain why the postulated reduction in C caused by the *bx* mutation on the upper chromosome does not, together with the reduction in C caused by the *Bxl* and *bxd* mutations on the lower chromosome, also produce an effect on L-ab, Lewis is led to suppose that "the somewhat lower production of product, C . . . is evidently not sufficient to lead to a detectable *bithoraxoid* phenotype".

The abstract models are not always easy to follow, neither are they always fully successful in explaining the data, but they are central to understanding Lewis' science as outlined in the Introduction to this book. It is important here to emphasize the

distinction between Lewis' abstract models—of which this was the first—and the abstract "rules" that he later proposed. The models were attempts to explain the data in terms often derived from the biochemical ideas of the day. The rules, in contrast, are abstract summaries of the phenomena that Lewis discovered. Thus, any modern mechanistic explanation must encompass the rules but not necessarily the models. At the time of writing this paragraph, over 50 years later, molecular mechanisms have been defined for only the minority of the phenomena that Lewis observed and formalized into rules.

GENE EVOLUTION BY TANDEM DUPLICATION

In his 1951 paper, Lewis introduces the hypothesis of gene evolution by tandem duplication: "It is the purpose of this paper to consider some of the ways in which 'pseudoalleles' . . . or closely linked genes having similar effects, may provide clues to the mode of origin of new kinds of genes. Our underlying thesis will be that in those instances of pseudoallelism in which there is evidence for close functional similarity among the component genes we may come close to seeing the direct results of a process which produces new genes. In developing this thesis, we hold to the viewpoint that new genes arise from pre-existing genes, and we picture . . . a two-step process; namely, (1) the establishment of a duplication of that gene . . . and (2) the occurrence in one of the two . . . genes thus formed of a 'mutation to a new function' . . . We believe . . . the first step involve[s] gene duplication . . . [because a] gene which mutates to a new function should, in general, lose its ability to produce its former immediate product, or suffer an impairment in that ability. Since it is unlikely that this old function will usually be an entirely dispensable one from the standpoint of the evolutionary survival of the organism, it follows that the new gene will tend to be lost before it can be tried out, unless, as a result of establishment of a duplication, the old gene has been retained to carry out the old function."

He returns to the hypothesis in the last paragraphs of the paper: "This encourages us to believe that, when evidence for close functional similarity among genes of a pseudoallelic series is obtained, we may be dealing with genes which were once (or are still) identical, owing to an origin by a process of duplication . . . The possibility that duplicate genes may often diverge from one another in their functioning in the above way is an attractive one since it gives a conservative, and yet, progressive process such as is required for a general theory of gene evolution."

The above was an elaboration of Bridges' hypothesis of gene evolution through duplication and divergence (Bridges, 1935), and was to form the incentive for much of Lewis' work over the next 30 years. Interestingly, 50 years after publication of the Cold Spring Harbor Symposium paper, Lewis discovered that Bridges had attempted—unsuccessfully—to test the gene duplication hypothesis using bithorax series mutants in 1938, the year of his death (discussed in Lewis, 2003). Two decades after Lewis' exposition, the gene duplication and divergence hypothesis was reintroduced and popularized by Ohno (Ohno, 1970). There, however, Ohno argued against the role

of tandem gene duplications, suggesting that tandem duplications would be unstable owing to unequal crossing over; that gene dosage ratios between functionally related genes would be disrupted; and that such duplications would result in extra copies of "structural" genes that had lost their "regulatory" genes (discussed in Lipshitz, 1996). However, Lewis' original formulation proved to be correct, exemplified perhaps most clearly by the formation of the homeotic gene complexes (also known as homeobox or HOX complexes) at the base of the metazoan evolutionary tree.

FROM CISVECTION TO TRANSVECTION

The position effects that Lewis discovered and studied through 1952, can be lumped together as "cisvection" effects in that the *cis–trans* test was applied to mutations present on otherwise normal, unrearranged chromosomes. Lewis' next advance came from his discovery that rearrangements that would be expected to disrupt pairing of homologous chromosomes, severely affect the interactions among the pseudoallelic bithorax series of mutations (Lewis, 1954b), as he had postulated might occur for tandemly repeated genes (Lewis, 1945). He interpreted the effect to mean that, under normal circumstances, there is pairing-dependent complementation—"transvection"—in the *trans*-configuration of the pseudoalleles. Disruption of pairing thus, in general, reduces complementation and makes the phenotype more severe.

The specific discovery involved the recessive and weak bx^{34e} mutation and the *Ubx* mutation (formerly known as bx^{D} and *Bxl* in his 1951 paper, but now renamed by Lewis). The *trans*-configuration, bx^{34e} +/+ *Ubx*, has flat, oval halteres but almost no metanotal (third thoracic or T3) tissue. Surprisingly, however, when Lewis introduced a rearrangement such as an inversion, into the third chromosome—the genotype of the fly can thus be designated either R(bx^{34e} +)/+ *Ubx* or bx^{34e}+/R(+ *Ubx*) depending on the chromosome in which the rearrangement had been induced—he found that the T3 was much larger (i.e., the mutant phenotype worsened). Furthermore in what Lewis referred to as "rule 1," in all cases in which the phenotype worsened, the rearrangement breakpoint fell within a "critical region" between the third chromosome's centromere and the map position of the *bx* and *Ubx* loci (i.e., between 81F and 89D). In cases where the rearrangement breakpoint in the third chromosome fell outside of the critical region (i.e., was either on 3L (61 to 80) or distal to 89E on 3R, there was no worsening of the phenotype ("rule 2").

Lewis derived rules 1 and 2, and two additional rules (not essential to the present discussion) from this first set of observations, which determined a series of testable predictions. He reasoned that if he began with "unselected" rearrangements (i.e. ones that had not been found on the basis of modification of bithorax phenotypes), the rules could be tested. From existing two-break rearrangements that were unselected in this manner, Lewis derived R(bx^{34e}+) or R(+ *Ubx*) chromosomes as cross-over products from R(+ +)/ bx^{34e} + or R(+ +)/+ *Ubx* females, respectively. He then crossed the derived rearrangements to + *Ubx* or bx^{34e} +, respectively. He tested rule 2 by assaying translocations that had breakpoints outside the critical region, and found that none of these altered the phenotype relative to unrearranged chromosomes. He

tested rule 1 by using rearrangements with breakpoints in the critical region, and found that it held as well; all such breakpoints worsened the phenotype relative to the unrearranged chromosome.

To provide the final proof that the rearrangements were producing their effect by affecting pairing of the bithorax region in homologous chromosomes, Lewis crossed two rearranged chromosomes that have nearly identical rearrangement breakpoints to each other to produce R(bx^{34e} +)/R(+ *Ubx*) flies. (In some instances he could also examine the homozygous rearrangements, when viable.) The prediction was that chromosomal pairing would be restored in the 89E region, and thus the phenotype would return to the less severe form. That this was, in fact, what happened allowed him to derive "rule 5": "that structural homozygosity restores the grade of the *trans*-type to its original grade-0 condition [i.e., weak phenotype]". Reciprocally, Lewis predicted that, if he worsened the structural heterozygosity (read: made somatic pairing even weaker), then the phenotype would become more extreme. This he did by combining rearrangements with very different breakpoints outside of the critical region (e.g., one with breaks in 48C on 2R and 84D combined with another with breaks in 29 on 2L and 87). In this situation the mutant phenotype became more severe.

In the Discussion of his 1954 paper, Lewis contrasts cisvection with this newly discovered phenomenon, transvection. He emphasizes that transvection—pairing-dependent complementation—may only occur in organisms like Drosophila in which the homologous chromosomes are paired in somatic cells. The location of the critical region led him to propose that somatic pairing proceeds proximally to distally (i.e., begins near the centromere and proceeds towards the telomere). Most importantly, he was led to conclude that pairing-dependent complementation implies that a "substance" must be transported between homologs under normal circumstances. Today it seems likely that Lewis had discovered the ability of what are now called "enhancers" (Banerji et al., 1983; Gillies et al., 1983), and their associated binding-proteins, to act in *trans* as well as in *cis* (Mueller-Storm et al., 1989; Morris et al., 1998; Chen et al., 2002).

Lewis' 1954 paper also took up the issue of the dose–response relationship between ionizing radiation and the induced rearrangements. An interesting historical detail here is that some of the mutations were induced by fast neutrons derived from nuclear detonations in Nevada. (George W. Beadle had taken Lewis' flies to the Nevada test site—and reported receiving 10 r from the blast!) In this paper, Lewis demonstrated a near-perfect linear relationship between the dose of fast neutrons and the frequency of induction of transvection suppressing rearrangements. In fact, Lewis could estimate the dose of fast neutrons at different stations positioned near a nuclear detonation simply by scoring the frequency of such rearrangements.

EXTENDING THE BITHORAX PSEUDOALLELEIC SERIES

By 1955, Lewis had identified several additional bithorax series position pseudoalleles, notably the *postbithorax* (*pbx*) and *Contrabithorax* (*Cbx*) mutations (Lewis, 1955). Lewis

had first reported these at the Ninth International Congress of Genetics in Bellagio, Italy, including the remarkable fact that they had been induced simultaneously on the same chromosome (Lewis, 1954a). The *Cbx* and *pbx* mutations cause opposite changes in developmental fate. The recessive *pbx* mutation causes an anteriorly-directed transformation: the posterior part of the third thoracic segment transforms to posterior second (Lewis symbolized this PMT → PMS for posterior metathorax → posterior mesothorax; or T3p → T2p in later symbolism). The dominant *Cbx* mutation causes a posteriorly directed transformation: the posterior second thoracic segment transforms to posterior third (PMS → PMT; or T2p → T3p). Occasionally, the *Cbx* mutation also causes a reduction in the anterior part of the second thoracic segment, transforming it to anterior third (AMS → AMT; or T2a → T3a).

Lewis now had five mutant genes and thus ten possible double-mutant combinations that he could test for cisvection (standard *cis–trans* position effect) and transvection (*trans*-test with chromosomal rearrangement). He presents the results of these tests in Table 2, scoring each combination according to the type of body segment transformation that resulted (I: AMT → AMS; II: PMT → PMS; III: AB_1 → AMT; IV: PMS → PMT). Lewis grouped the comparisons according to whether he was testing recessive versus recessive (group 1), recessive versus dominant *Ubx* (group 2), recessive versus dominant *Cbx* (group 3), or dominant versus dominant (group 4, *Cbx* versus *Ubx*). Most of the cisvection results are similar to what Lewis had obtained previously: both the *cis*- and the *trans*-combinations of different recessive mutations is wild type. An exception is *bxd* +/+ *pbx*, where there is a PMT → PMS transformation. This is Lewis' discovery that *bxd* and *pbx* mutations affect the same genetic function, although the *bxd* mutation is more extensive in effect (since there is also an AB_1 → AMT transformation in *bxd/bxd* that is absent from the *pbx/pbx* or *bxd* +/+ *pbx* phenotype).

Lewis also reports his first analysis of larval phenotype in this study. In all cases the larval phenotypes are consistent with those seen in the adult. He reports that the homozygous *Ubx* mutant is lethal but does not die until the end of the larval stages. The larval phenotype combines transformation types I + II + III such that AMT → AMS, PMT → PMS and AB_1 → AMS (this last because of the additive effects of the transformations). In other words, both the third thoracic and the first abdominal segments are transformed to the second thoracic in identity. Thus *Ubx* behaves as if all the recessive functions defined by *bx*, *bxd*, and *pbx* are eliminated, consistent with the results of the *cis–trans* tests in which *Ubx* fails to complement any of the recessive alleles.

The analyses of *Cbx* in combination with the recessive alleles shows that all combinations in *trans* give the same or almost the same phenotype as *Cbx* alone (e.g., *Cbx* +/+ *bxd* = *Cbx* +/++). Similarly, *Cbx* +/+ *Ubx* gives a phenotype almost the same as *Cbx* +/++ (only a slightly weaker AMS → AMT transformation). But Lewis shows that the *cis*-configuration is striking: *Cbx* in combination with any of the recessive mutants, or even with *Ubx*, results in a severe reduction or even complete suppression of the *Cbx* transformation of AMS → AMT. For example, while

Cbx +/+ *Ubx* causes a strong transformation of AMS → AMT, the *Cbx Ubx*/++ flies have an almost normal second thoracic segment with a very slight defect in the wing structure that causes slight spreading of the wings. Thus Lewis reaches the important conclusion that *Cbx* behaves as if the dominant phenotype is dependent for its production on the presence of the normal bithorax genes in *cis*, particularly on the presence of the wild-type allele of *Ubx*.

The most striking transvection effect in the series is obtained with *Cbx* and *Ubx*: There is no effect in the *trans*-configuration (i.e., *Cbx* +/+ *Ubx* = *Cbx* +/ R(+ *Ubx*) = R(*Cbx* +)/+ *Ubx*). However, when *Cbx Ubx*/++ is compared with *Cbx Ubx*/R(++), the latter are completely wild type, lacking even the slightly spread wings described above. Thus *Cbx* has an effect on *Ubx* in *trans*, which is abolished if pairing of somatic chromosome homologs is prevented. Lewis was later to return to the *Cbx Ubx*/++ combination and to use suppression of the wing spreading as a sensitive screen for rearrangements that break between the centromere and the bithorax complex—the "global rearrangement" screen (Lewis, 1985).

GENETIC VERSUS FUNCTIONAL MODELS

In his 1955 paper Lewis attempts to fit all of his data to the "sequential gene action" model that he first presented in his 1951 paper:

$$\text{A} \xrightarrow{bx^+} \text{B} \xrightarrow{Ubx^+} \text{C-I} \xrightarrow{bxd^+} \text{D-III} \xrightarrow{pbx^+} \text{E-II}$$

He postulates that absence of substance C-I, produced by the Ubx^+ function, leads to the type I transformation (AMT → AMS), absence of D-III, produced by bxd^+, to the type III transformation (AB_1 → AMS), and absence of E-II, produced by pbx^+, to the type II transformation (PMT → PMS). The model is consistent with *Ubx* mutations affecting all three transformations, since Ubx^+ is required for production of the I, II, and III substances. The position of bx^+ is more problematic. Lewis points out that the postulated order of gene action is the same as the order of the genes on the chromosome but that the "order of control with respect to bx^+ and Ubx^+ cannot be deduced from the data and is arbitrarily assumed to be the same as that in the chromosome to simplify discussion." Lewis was later to return to the correspondence of gene order on the chromosome and the spatial control of segment identity along the anterior–posterior body axis with what, by then, had become the "colinearity" rule (Lewis, 1978).

Lewis spends a significant amount of the Discussion attempting to fit the data to the model shown above. Details will not be described here, except to point out that Lewis is unable to fit the *Cbx* mutant into that model. "Since the *Cbx* transformation (type IV) is the inverse of those of type I and type II the possibility of complicated interactions at a physiological level . . . becomes an acute one". *Cbx* is what Lewis later called a gain-of-function mutant (Lewis, 1963a). The type of scheme that Lewis presents in 1955 is derived from loss-of-function phenotypes; a gain-of-function

mutant could only be fit into such a scheme once its true nature was revealed (Lewis, 1963a).

Setting aside the difficulties with *bx* and *Cbx*, Lewis presents the following model for how the different levels of segmental identity are attained:

$$\text{L-mt} \xleftarrow{\text{C-I+E-II}} \text{L-ms} \xrightarrow{\text{D-III}} \text{L-ab}_1$$

What is interesting about this model is that Lewis now explicitly spells out that the "primitive" level of development is the second thoracic segment and that the postulated activities encoded by the bithorax series of genes convert the primitive state of development to that of the third thoracic and first abdominal levels. In words, the substances produced by the action of the *Ubx* (C-I) and *pbx* (E-II) genes promote the level of development from the primitive, second thoracic identity to the third thoracic level, while the substance produced by the action of the *bxd* gene (D-III) promotes the level of development from second thoracic to first abdominal. His recognition of the second thoracic identity as the primitive state was crucial for future analysis.

Of particular historical interest here is that Lewis was fully aware of how biochemists and molecular biologists were viewing the function of genes in pathways and was doing his best to fit his results to their data. That the molecular details of Lewis' models later proved to be incorrect was largely because he was so far ahead of his time with regard to the phenomena he was describing. No contemporary biochemical or molecular model could accommodate his data.

Another point to be made is that Lewis, by presenting his data in the abstract models described above, is here arguing very strongly for what he calls the "genetic" model and equally strongly against the alternative, "functional" model. The genetic model postulates that each member of a pseudoallelic mutant series affects a distinct function. At the time, that was most easily interpreted in the "pathway" type model that Lewis used, since each step represented a different biochemical function and was controlled by a different gene, by analogy to studies in prokaryotes and fungi. The "functional" model was based on the idea advanced, among others by Pontecorvo (1952), that the members of a pseudoallelic series are "alterations at different sites of a single functional unit." This essentially was the multiple allelism hypothesis, which Lewis had countered beginning with his graduate studies on the *S* and *ast* mutants. The simplest interpretation of the functional, or multiple allelism, model was that pseudoalleles represent mutations in a single protein. It was inconceivable to Lewis that a single protein could exhibit such genetic complexity or such a diversity of developmental roles.

As it turned out, the pseudoalleles Lewis was studying all affect a single functional unit, now called the *Ubx* domain. The *Ubx* domain has a single protein-coding transcription unit (Hogness et al., 1985; O'Connor et al., 1988; Kornfeld et al., 1989). The *bx*, *bxd*, and *pbx* mutations are, however, not in the *Ubx* mRNA or protein. Instead, they alter the giant *cis*-regulatory regions that make up 98% of the DNA in the *Ubx* domain. Most of the genetic complexity of the bithorax complex resides in the multiple regulatory regions and not in the proteins whose spatial and temporal

expression they control (White and Wilcox, 1984; Beachy et al., 1985). Thus Lewis' genetic model was wrong in postulating that each genetic function (*bx*, *bxd*, etc.) encodes a distinct substance. However, it was right in that the genetic functions he had identified do not map to the *Ubx* protein. More detailed discussion will be presented in the Molecules and Development section.

FROM GENES TO GENE COMPLEXES

The final paper in this section is Lewis' review on 'Genes and Gene Complexes' (Lewis, 1967). Two papers, published in 1963 and 1964, are discussed in the next section of the book, because Lewis' primary focus in those had switched from an interest in genes and gene evolution, to the genetic control of development (Lewis, 1963a, 1964). All of the preceding papers in this section, as well as the 1967 paper, although they increasingly address the genetic control of development, have genes as their primary focus.

During the 12 years between Lewis' 1955 paper and his 1967 paper, the field of molecular biology had made major strides in understanding how genes encode proteins (mRNA, tRNA, rRNA, triplet genetic code), as well as in how genes are regulated in prokaryotes (specifically, the operon). Lewis had begun to incorporate some of these ideas starting in his 1963 paper, particularly in his interpretation of *cis*-dominant regulatory mutants such as *Cbx*. It should also be noted that, by 1967, the old concept that a gene was indivisible by recombination (i.e., that crossing over occurred only between genes) was long dead. Indeed, that monster had been slain in no small part by Benzer's application of the *cis–trans* test to recombination and function in the rII locus of bacteriophage T4 (Benzer, 1955, 1956). Those analyses had demonstrated that both mutation and recombination occur at the level of single base pairs of DNA, while the cistron—a term invented by Benzer and defined by the *cis–trans* test—is a larger, functional unit within a genetic locus. Ironically, Benzer's remarkably detailed analyses relied upon a clever way to select wild-type recombinants from large numbers of mutant "heterozygotes" for rII locus mutations. His methods precluded the recovery of the double-mutant, and thus the *cis–trans* test for position effect was never carried out in that famous study!

Lewis reviews the literature on gene complexes in bacteria, fungi and humans and then places his work on the bithorax mutants in that context (Lewis, 1967). Of most interest here, is Lewis' extension of his mapping of the *bx*, *Cbx*, *Ubx*, *bxd*, and *pbx* mutants (he calls them *a*, *B*, *C*, *d*, *e* for simplicity) using the half-tetrad method described above for the X-chromosome. The difficulty in applying this method to the mutants in what he was now calling the "bithorax complex" loci, is that they reside on an autosomal arm, 3R. Thus Lewis had to construct attached-3R chromosomes that carried the quintuple mutant listed above on one of its arms and the wild-type alleles on the other arm, together with appropriate flanking markers. Such flies also had to carry attached-3L chromosomes to avoid aneuploidy. [Between 1958 and 1960, Lewis and his colleagues—I. Rasmussen, E. Orias and P. Deal—had constructed attached-L and attached-R second and third chromosomes as well as attached-R

fourth chromosomes in order to permit such *cis–trans* tests for mutations mapping to any autosome (Rasmussen, 1960; Lindsley and Grell, 1968; Lindsley and Zimm, 1992)].

Since Lewis had already defined the phenotypes of all *cis*- and *trans*-types (Lewis, 1955), he was in a position to recognize the simultaneous recovery of reciprocal recombinants in the four regions in which recombination could occur between the five loci within the complex. To give two examples using the genetic symbols rather than Lewis' letters:

- If recombination occurred between *bx* and *Cbx*, then bx^3 + + + +/+ *Cbx Ubx bxd pbx* could be recognized as having an extreme *bx* phenotype (since *Ubx* inactivates the *bx* function on the chromosome carrying the "+" allele of *bx*, resulting in a AMT → AMS transformation; see above). Lewis obtained eight such flies, successfully tested five of these (and verified all ten recombinant chromosome strands using the methods described below).
- If recombination occurred between *bxd* and *pbx*, then bx^3 *Cbx Ubx bxd* +/+ + + + *pbx* could be recognized as having only the *pbx* phenotypes (since *Ubx* inactivates the *pbx* function on the chromosome carrying the "+" allele of *pbx*, resulting in a PMT → PMS transformation; as described above, the *Ubx* mutation in *cis* to *Cbx*, suppresses the *Cbx* transformation of AMS → AMT). Lewis obtained two such flies, successfully tested one of these and verified the two recombinant chromosome strands.

Altogether, Lewis was able to recover and verify 16 recombinants within the bithorax complex. Verification required producing triploids carrying the recombinant attached-3R chromosomes and then isolating normal third chromosomes from their progeny, which now carried one or the other arm of the recombined attached-3R chromosome. Back-crosses to marked multiple-mutant bithorax complex chromosomes verified which mutations were present, as well as that flanking markers had recombined. Lewis' data were fully consistent with the previous gene order that he had established. He also found that in all cases, flanking markers had been exchanged and so no gene conversion had occurred. Furthermore, with his total of 221,000 progeny, he was able to calculate a recombination frequency of 1.5×10^{-4} for three of the four regions (i.e., the loci were 0.015 cM apart). Use of half-tetrad analysis to recover *both* products of a reciprocal crossover event has been rare. Lewis' studies of the *white* locus (Lewis, 1952) and the bithorax complex (Lewis, 1967), and those of others on the *garnet* locus (Hexter, 1958; Chovnick, 1961), are the only such studies in Drosophila to this day.

In the 1967 paper, Lewis reports that the *suppressor of Hairy-wing* mutation suppresses certain alleles in the bithorax complex, including *bx* and *bxd*. Lewis here studies the su^2-*Hw* allele (the original allele had been lost). He had identified su^2-*Hw* in 1948 and had shown then that it suppresses *Hw*, bx^3, *bxd*, *ci*, sc^1, ct^6, y^2 and

more weakly, *f* and *B* (Lewis, 1949). Usually, only one of a number of different alleles tested for a given locus is suppressed. Lewis supposed—incorrectly in retrospect—that the suppressible mutations may be "true point mutations" since they had also been found to revert to wild type. One of the discoveries to be made more than a decade later, when the bithorax complex was molecularly cloned, is that most of the mutations that Lewis had studied are not point mutations, but are caused by insertion of transposable elements (Bender et al., 1983a). The mutations that are suppressed by *su-Hw* all fall into one class, being caused by the gypsy element, a Drosophila homolog of vertebrate retrotransposons (Bender et al., 1983a). Thus, what Lewis had actually discovered in 1967, was a system that prevents expression and mutagenic function of the fly equivalent of a retrovirus (see commentary in Molecules and Development section).

Lewis ends the discussion section of his 1967 paper by emphasizing the suitability of the bithorax complex for studying the genetic control of developmental pathways. He refers to the fact that individual genes of the complex control "the level of development achieved by certain body segments of the fly." He ends: "The bithorax genes appear to control developmental pathways which recapitulate some of the phylogenetic steps that must have occurred during the evolution of the Dipterans from primitive arthropod ancestors." These ideas were to motivate much of his subsequent research on the genetic control of development.

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STAR-RECESSIVE, A SPONTANEOUS MUTATION IN *DROSOPHILA MELANOGASTER**

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In a genetics experiment conducted in November 1937 with *Drosophila melanogaster* and involving the dominant mutant Star (S, in chromosome 2- at locus 1.3; causing an irregularity in the arrangement of eye facets and facet hairs, the homozygote being lethal), a number of flies appeared with small narrow eyes. When these were mated *inter se*, all of the F_1 had abnormal eyes, but these varied in size from that of S/+ (see Table 4) to a narrow slit of red pigment on which only a few facets were scattered. A clue to the manner of inheritance of the type with narrow eyes was found by mating a narrow-eye male to a wild-type female, with the result shown in Table 1.

An F_1 wild-type female was then outcrossed to a Star Curly Dichaete male (genotype S/Cy; D/C3x), with the result given in Table 2.

Since the narrow-eye flies in Table 2 were all non-Curly they must have carried Star; moreover, they must have carried some factor (or factors) which profoundly "enhanced" Star even though that factor had not expressed itself in the mother which was heterozygous for it but was non-Star. For reasons given below the

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Table 1 F_2 Distribution (P_1: wild-type ♀ x narrow-eye ♂).

February 14, 1938	Wild-type	Star
Totals	82	58

Table 2 Result of outcross of F_2 wild-type ♀ x (S/Cy; D/C3x)♂.

28, February 1938	Star Dichaete and Star (C3x)	Curly Dichaete and Curly (C3x)	Narrow-eye Dichaete and Narrow-eye (C3x)
Totals	48	83	22

symbol S^r, meaning Star-recessive, was adopted for this factor at the suggestion of Dr. Bridges.

CHROMOSOME CARRYING STAR-RECESSIVE

In order to determine which chromosome carries S^r, a Star, Star-recessive (C3x) (i.e., narrow-eye) male from Table 2 was crossed to a (S/Cy; D/C3x) female with the result shown in Table 3, where the non-Star offspring have been omitted since it is only in Star flies that S^r can be detected. Distribution with respect to sex was random.

This result agrees with expectation calculated on the basis that S^r is in the second chromosome. We would expect none of the Star Curly nor Star Curly Dichaete offspring to have S^r; while, on the other hand, all of the Star non-Curly flies would carry S^r.

At this point an attempt was made to build up a constant breeding stock of S^r which would not carry Star. From Table 3, a (S/S^r; D/?) male was outcrossed to a Cy/B1 female. F_1 Curly Dichaete (non-Star) males, genotype (S^r/Cy; D/+), were backcrossed to Cy/B1 females. By selecting in the F_2 only the Curly non-Dichaete (non-B1) and mating these *inter se,* a stock was started whose parents had had their I and III pairs of chromosomes entirely replaced by normal ones derived from the Cy/B1 stock. The Cy/S^r x Cy/S^r mating produced approximately one third non-Curly flies which had rough eyes approaching S/+ eye in size. These S^r/S^r when inbred gave a constant rough-eyed stock. Considered apart from its effect with Star in S/S^r, S^r was behaving as a simple recessive. A summary of the phenotypes of the

Table 3 Result of cross (S/Cy; D/C3x) ♀ x (S/S^r; C3x/+) ♂.

March 21, 1938	Star curly	Star Curly Dichaete	Star star-recessive	Star-recessive Star Dichaete
Totals	14	22	11	20

Table 4

	Genotype	Eye	Wing venation	Viability
1.	$S^r/+$	+; occasionally a few facets disarranged	+	+
2.	S /+	Smaller than +; rough	+	Good
3.	S^r/S^r	Smaller and rougher than S/+; may approach + eye in appearance	Sometimes broken at tips of L2–L5	Fair
4.	S /S^r	Smaller than S^r/S^r; few or no facets.	L2–L5 extensively interrupted	Poor

various combinations of S, S^r, and the + allel is given in Table 4; fertility is normal throughout.

LOCALIZATION OF STAR-RECESSIVE

It was noted that in numerous matings in which S/S^r females had been mated to males with normal II chromosomes, e.g., the mating in Table 1, the crossover S S^r/+ +, if phenotypically like S/S^r, never appeared. From this it was now assumed that either S^r is an allel or near the locus of Star, or it is present with an inversion. In order to determine the frequency of crossing over between S and S^r, an al S ho/Cy male was mated to a S^r female and F_1 al S ho/ S^r females were crossed to al ho males (al-aristaless, 0.0; ho-heldout, 4.0) with the result shown in Table 5.

No individuals of the S/S^r type appeared; yet the absence of crossing over between S and S^r was not proven since S S^r/+ + might not have been separable from S/+. Upon testing one al and three ho crossover types to al S ho/Cy, each was found to carry S^r as evidenced by the appearance of Star Star-recessive in the F_1. This indicates that S^r is to the left of ho and corroborates previous findings by a somewhat analogous procedure that S^r is to the left of dumpy (dp. 13.0).

ALLELOMORPHIC TESTS

To determine whether or not S^r is an allelomorph of Star, S^r females were mated to S/Cy males and F_1 S/S^r females were back-crossed to S^r males with the result shown in Table 6.

Table 5 Result of backcross (July 10, 1938) (al S ho/+ S^r + ♀ x al ho ♂).

Parental combinations		Recombinations						
Aristaless Star Heldout	Wild type	Aristaless	Star Heldout	Aristaless Star	Heldout	N	R1 al–S	R2 S–ho
1,339	1,488	25	11	62	42	2,967	1.2%	3.5%

Table 6 Result of backcross (S/S^r ♀ x S^r ♂).

30, August 1938	Star Star-recessive	Star-recessive	Wild-type	N
Totals	1,507	1,727	1	3,235

Table 7 Result of backcross (al S ho/+ S^r + ♀ x S^r ♂).

24, October 1938	Star Star-recessive	Star-recessive	N
Totals	1,711	1,871	3,582

If crossing over between S and S^r occurred, the class S^r/+ would be wild-type and could be separated from S/S^r; and S^r/S^r; even though the reciprocal crossover S S^r/S^r might be inseparable from S/S^r. The exceptional wild-type female which appeared was shown to be of genotype S^r/+, which would be one of the crossover types sought. Yet, since only one occurred in 3,235 flies it could not be certain that it was a crossover; for, a reversion of the S^r gene to + or the appearance of an inhibitor of S^r might have produced the same result.

The experiment was repeated using al S ho/S^r females by S^r males, with the result given in Table 7.

A final test made use of the mating: al S ho/S^r females by males homozygous for the three recessives—al, S^r and ho—with the result shown in Table 8.

With the exception of a possible crossover type in Table 6, there is strong evidence that Star-recessive is an allelomorph of Star.

Table 8 Result of backcross (al S ho/+ S^r + ♀ x al S^r ho ♂).

Parental combinations		Recombinations						
Aristaless Star Star-Recessive Heldout	Star-recessive	Aristaless Star-recessive	Star Star-Recessive Heldout	Aristaless Star Star-Recessive	Star-Recessive Heldout	N	Region 1 al–S	Region 2 S–ho
394	1,948	19	9	36	71	2,477	1.1%	4.3%

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ANOTHER CASE OF UNEQUAL CROSSING OVER IN *DROSOPHILA MELANOGASTER*

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Females homozygous for the sex-linked dominant, Bar, occasionally give rise to wild-type reversions and to forms with more extreme eye reduction than Bar. This behavior was shown by Sturtevant[1] to result from unequal crossing over. The Bar-reverted type was considered to be a deficiency for the Bar gene; while the extreme form, called Ultra-Bar or Double-Bar, was interpreted as a duplication for that gene. Later, Wright[2] suggested that Bar itself had something additional present which when lost by unequal crossing over would give back a normal chromosome (Bar-reverted).

The cytological nature of Bar was cleared up independently by Bridges[3] and Muller, et al.,[4] who investigated the salivary gland chromosomes. They found Bar to be a tandem duplication, in normal order, for an X chromosome section composed, according to Bridges' detailed analysis, of six bands. Bridges further demonstrated that Bar-reverted had the identical banding of a normal chromosome, whereas Double-Bar had a serial triplication for the region present twice in Bar and once in Bar-reverted.

[1]Sturtevant, A. H., *Genetics*, **10,** 117–147 (1925).
[2]Wright, S. *Amer. Nat.,* **63,** 479–480 (1929).
[3]Bridges, C. B., *Science,* **83,** 210–211 (1936).
[4]Muller, et al., *C. R. Acad. Sc. USSR,* 1(X), 87–88 (1936).

The second case of a tandem duplication being responsible for a dominant "mutation" is that of the sex-linked Hairy wing, which Demerec[5] has shown is a repetition for a single heavy band near the tip of the X chromosome. However, its location in a region of extremely low crossing over prevented a study of unequal crossing over.

This paper is a preliminary report on an autosomal tandem repeat which was detected as a suppressor of the dominant mutant, Star (*S*, 2-1.3).

An analysis of the salivary gland chromosomes of this suppressing factor, when homozygous, when closely paired with a normal chromosome and when present as an unpaired haploid strand, consistently showed the presence of a tandem duplication in direct order near the left end of the second chromosome. The section present twice appears to include the two faint bands, 21 *D* 4—5, and the heavy, frequently capsulated doublet, 21 *E* 1—2; i.e., a section of at least four bands.

Dp(2)*S* appeared in a study of changes at the Star locus, a consideration of which is essential before discussing the properties of this repeat. *S*/+ has roughened, slightly reduced eyes; *S*/*S* is always lethal. Star-recessive (S^r) is the name tentatively given to a recessive mutant near if not at the Star locus. S^r/S^r has smaller, rougher eyes than *S*/+, and may have gaps at the tips of the wing veins. The compound, S/S^r, is much more extreme, having a narrow diamond-shaped eye and extensively interrupted venation.

Bridges has reported that the salivary gland chromosomes of Star are apparently normal. The same appears to be true for Star-recessive and also for the dominant Suppressor of Star (*Su*—*S*, 2—1.3±) found by Curry.

The allelic relation between *S* and S^r is, as yet, ambiguous. From *al S ho*/S^r females (*al* = aristaless, 2—0.0; *ho* = heldout, 2—4.0), wild-type "reversions," which are always heldout crossovers and which are cytologically normal, occur with a frequency of 0.01% (4 : 31,106); by using females heterozygous for inversions in all of the other chromosome arms, their frequency has been stepped up to 0.046% (12 : 26,370). Yet, a crossover complementary to the reversions has not been detected. The situation may be similar to a case, recently reported by Oliver,[6] of reversions, associated with crossing over in one direction, arising from females carrying two alleles of the lozenge eye mutation. For the sake of simplicity, *S* and S^r are considered as alleles in this paper.

Dp(2)*S* arose spontaneously as a single individual among approximately 49,000 offspring of *al* S^r *ho*/S^r females individually mated to *al S ho*/*Cy, E*—*S* males. The fly had normal aristae, nearly wild-type (Star suppressed) eyes and heldout wings. Tests showed that the mother had contributed al^+, *ho* and *Dp*(2)*S*, whose origin was therefore associated with crossing over. *Dp*(2)*S*/+ and *Dp*(2)*S*/S^r look wild type.

[5]Demerec, M., *Genetics*, **24,** 271–277 (1939).
[6]Oliver, C. P., *Proc. Nat. Acad. Sci.*, **26,** 452–454 (1940).

Dp(2)*S*/*Dp*(2)*S* is also normal except for an occasional slight extra vein near the fifth longitudinal vein. This wild-type action is in striking contrast to the pronounced phenotypic effects of Bar and Hairy wing.

A study of unequal crossing over in the heterozygous duplication has shown that the Star locus is included in the repeated sections; i.e., it has been possible to recover from *al Dp*(2)*S ho*/*S* females unequal crossover products which have *S* inserted into the left (distal) section of the repeat, and others with *S* introduced into the right (proximal) region. The latter occur with a frequency of 0.3% (6 : 1,948) or roughly 30 times as frequently as the former (0.01% or 2: *ca* 23,000). In terms of genetic length this indicates that the *S* locus is included in the extreme right portion of each of the two regions present in duplicate. The original duplication, since it arose from homozygous S^r, might be expected to have a S^r gene in each of these positions. Using parentheses to bound the repeated regions, its composition may be written: *Dp*(2)*S* = (. . . S^r.)(. . . S^r.). That S^r is present in the proximal section has been demonstrated by its recovery from

$$\frac{al\,(\ldots S^r.)(\ldots S^r.)\ ho}{(\ldots S.)}$$

females as S^r *ho* crossovers, whose cytological picture is normal; their frequency is 0.35% (57:16,568) or approximately that of the complementary *al* (. . . S^r.)(. . . *S*.) class mentioned above. In the two cases where *S* was inserted into the left section, the product may be written: (. . . *S*.)(. . . S^r.) *ho*, and its origin visualized as the result of the following pairing:

$$\frac{al(\ldots S^r.)(\ldots S^r.)\ ho}{(\ldots S.)},$$

accompanied by a crossover between the *S* locus and the breakpoint of the duplication. The complementary crossover is expected in this case to be *al* S^r, or the removal of S^r from the left section. Yet, although a total of 243 S^r *ho* types have been detected, no cases of *al* S^r have occurred. This may mean that S^r is slightly to the right of *S*, as was suggested, in part, by evidence given above. On this basis, either S^r is just outside the duplication or it is so close to the breakpoint that crossing over has failed so far to remove it from the left section.

Although the phenotypic effects of the original duplication are consistent with the assumption that one S^r and a normal allele of S^r are acting, the origin of *Dp*(2)*S* from homozygous S^r would seem to indicate that this action is more likely a position effect. A preferable notation, for the present, would be *Dp*(2)*S* = (. . . S^r?.)(. . . S^r.).

From *al* (. . . S^r?.)(. . . S^r.) *ho*/+ females, normal S^r *ho* chromosomes and *al* (. . . S^r?.)(. . . S^{r+}.) occur with approximately equal frequencies as expected.

There is genetic evidence, not of a crucial character, that the locus of net (2—0.3±) is also included in *Dp*(2)*S* at the extreme left end of each section. If this is

the case then the total frequency of crossing over between the loci of net and Star in heterozygous *Dp*(2)*S* is greater when the distal section is involved (1.4%) than when the proximal one takes part (0.7%). As in the experiments previously given, these data are obtained from females heterozygous for inversions in some of the other chromosome arms with the result that the normal *net–S* distance of 1% is increased to 2% or more.

From females homozygous for the original duplication whose repeated sections may be supposed occasionally to pair in an unequal manner, diagrammatically,

$$\frac{al(\ldots S^r?.)(\ldots S^r.)\ ho}{(\ldots S^r?.)(\ldots S^r.)},$$

two types of unequal crossover products have been obtained which, apart from phenotype, are analogous to the derivatives produced by homozygous Bar females. The normal chromosome products, corresponding to Bar-reverted, are detected on the basis that they carry S^r; their frequency is 0.25% (9 *al* S^r + 5 S^r *ho*:5594). New chromosomes with three sections in tandem repetition, as is the case with Double-Bar, occur with approximately the same frequency as the normal types, namely, 0.18% (8 *al* + 3 *ho*, triplications:6000); their action is to suppress, completely, *S E–S* (*E–S* = Enhancer of Star, 2—6.±), whereas *Dp*(2)*S*(. . . S^r?.)(. . . S^r.) only partially suppresses the small rough eye effect seen in *S E—S*/+. The unequal crossover types have been examined in the salivary gland chromosomes and the analogy with the Bar derivatives has been found to hold. The homozygous triplication, symbol, *Tr*(2)*S*, has slightly bulging eyes with large facets; in addition to occasional slight extra veins, described for the homozygous *Dp*(2)*S*, there is often a branching of the second longitudinal vein. The wing effects are perhaps to be ascribed to the locus of net.

Homozygous *Tr*(2)*S* females have produced S^r chromosomes with only one section present, and also a new "dominant" unequal crossover product, which over S^r (or S^{r+}) has eyes resembling those of homozygous *Tr*(2)*S*. A cytological analysis supports the conclusion that the "dominant" is a repeat of five sections. When homozygous this quintuplication, symbol, *Qn*(2)*S*, is still quite viable and fertile, and has the same effects, but much more intensified, as homozygous *Tr*(2)*S*. *Qn*(2)*S* would correspond to Quadruple-Bar obtained by Rapoport[7] from attached-X females homozygous for Double-Bar.

SUMMARY

1. An autosomal tandem duplication is described, whose origin was associated with crossing over.

[7]Rapoport, J. A., *Bull. Biol. Méd. Exp.*, **2,** 242–244 (1936).

2. Genetic evidence indicates that the locus of Star and possibly the net locus are included in each of the duplicate sections.
3. The homozygous duplication gives unequal crossover products analogous to Bar-reverted and Double-Bar. A repeat of five sections has been derived from the homozygous triplication.

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THE RELATION OF REPEATS TO POSITION EFFECT IN *DROSOPHILA MELANOGASTER*

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INTRODUCTION

In *Drosophila melanogaster* it has been established that the action of at least some genes is affected by their position in the chromosomes. Critical evidence for this phenomenon of position effect has been given by Sturtevant (1925), Dubinin and Sidorow (1935), and Panshin (1935). Position effects have been detected only in cases in which the relative position of a gene with respect to its neighbors has been altered by a chromosomal rearrangement. It is conceivable that the effective position of a gene may also be changed as a result of a substitution in an adjacent gene of one allele by another. Such a possibility was put to experimental test by Sturtevant (1928) for the dominant mutants, Delta (*Dl*) and Hairless (*H*), three units apart in the third chromosome; however, no difference could be demonstrated between *Dl H*/++ and *Dl*+/+*H*.

On the other hand when a similar type of comparison is made with mutants at the Star and asteroid loci, a position effect can be detected, as will be shown below. The purpose of this paper is to present the evidence for an asteroid locus as distinct from that of Star, to examine the conditions which may make possible the detection of a position effect in this instance, and to study "changes" at the Star and asteroid loci associated with certain chromosomal rearrangements.

THE STAR AND ASTEROID MUTANTS

Star (*S*) is a spontaneous dominant mutant at 1.3 in the second chromosome (Bridges and Morgan, 1919; Stern and Bridges, 1926). *S*/+ flies have slightly reduced eyes with irregularly shaped facets and disarranged facet hairs. The *S* homozygote is lethal. In a line of *S*/+ flies which had been inbred for several generations in mass cultures, several individuals appeared which had smaller eyes than those of *S*/+. An analysis of one of these showed that it carried *S* and, in the opposite chromosome, a dominant intensifier of *S*. By itself this factor behaved as a recessive, rough-eyed mutant in the left end of the second chromosome. This mutant, formerly called Star-recessive (S^r), is known from evidence presented below to be close to the right of *S* and has been renamed asteroid (*ast*).

The compound *ast*/+ occasionally has a very slightly roughened eye, but it is usually inseparable from wild type. The eyes of flies homozygous for *ast* are smaller than those of *S*/+ and the longitudinal wing veins, L_2, L_3, L_4, and L_5, occasionally have terminal gaps. The compound, *S*+/+ *ast*, has an extremely small, diamond-shaped eye with many of the facets fused and the rest of irregular size and distribution. The longitudinal veins, which are sometimes interrupted in *ast*/*ast*, usually have large terminal gaps in *S*+/+ *ast*.

Asteriod^2 (ast^2) is probably an allele of *ast*, although the possibility that it is a recessive allele of *S* has not been excluded. It appeared spontaneously in a single F_1 male from a mating of *ast* females to *al S ho*/*Cy* males (*al*, aristaless at 0.0; *ho* heldout at 4.0). The fly had Curly (*Cy*) wings and rough eyes and was therefore readily distinguishable from the *Cy*/*ast* class. From an identical type of mating, conducted at a later time, another allele of *ast*, ast^3, arose in the first or second generation and was detected in the same way as was ast^2. In the case of both ast^2 and ast^3 it was shown that each had arisen in a Curly inversion chromosome. Although these mutants have not been separated from the inversion in the left arm of the second chromosome, they have been freed of the Curly mutant. Homozygous ast^2 and homozygous ast^3 types have good viability but the former is sterile in the female; in each case the eye effect is similar to that of homozygous *ast*, but the wing venation is normal. The compound, S/ast^2 is lethal. The S/ast^3 types are late-hatching, but those which emerge have good viability and fertility; they have normal venation and extremely small eyes which are similar to, but slightly larger than, those of *S*/*ast*.

In a search for spontaneous reverse mutations of *ast* to ast^+ from homozygous *ast* females, a slight allele of *ast*, ast^4, appeared in a single F_1 male of composition, *al S ho*/ast^4*ho*, from a cross of *al ast ho*/*ast* females to *al S ho*/*Cy* males. Here the calculated number of offspring in which ast^4 could be detected was approximately 15,700, and the observed recombination value for the *al–ho* interval was 5.5%. The possibility that the observed association of mutation with crossing over in the *al–ho* region was due to chance is perhaps strengthened by the fact that an allele, ast^5, which closely resembled ast^4, arose in a noncrossover individual of composition, *al ast ho*/*al* ast^5 *ho*, from a mating (conducted at 30°C) of *al ast ho*/*ast* females to

Table 1 A rough classification of eye types of various combinations of *S* and *ast* alleles, including those with Enhancer of Star, *E-S*. The extent of variability is indicated, where necessary, in parenthesis. Asterisk (*) indicates that individuals of type in question may show gaps in the longitudinal wing veins.

	S^+ ast^+	S^+ ast^4	S^+ ast^3	S^+ ast	S^+ ast^2	S ast^+	S^+ast^+E–S
S^+ ast^+	1						
S^+ ast^4	1	1 (1–5)*					
S^+ ast^3	1	2 (2–3)	5				
S^+ ast	1 (1–2)	3 (1–5)*	4 (4–6)	5* (1–7)			
S^+ ast^2	1	4 (3–5)	5	6 (4–8)*	5 (♀, sterile)		
S ast^+	3 (2–3)	5* (4–6)*	7 (late hatching)	8 (6–8)*	lethal	lethal	
S^+ast^+E-S	1	2	2	4	3	5 (5–6)	2

al ast ho males. $Ast^4/+$ and ast^4/ast^4 are wild type, although the latter may become as extreme as *ast/ast* under unfavorable culture conditions. The compound, S/ast^4, has a smaller eye than that of $S/+$ and resembles rather closely *ast/ast* not only in having a similar eye effect but also in occasionally producing a similar pattern of gaps at the tips of the longitudinal veins.

The phenotypic effects of all possible combinations of +, *S, ast,* ast^2, ast^3, and ast^4 are indicated in Table 1 by means of a rough, arbitrary grading of the eye effect, based on inspection rather than measurement. Wild type is taken as Grade 1. Grade 2 has a very slight facet irregularity. Higher numbers indicate decreasing size and increasing roughness of the eye. The possibility that modifiers at other loci are a complicating factor in the phenotypic expressions of the various *ast* alleles has been excluded rather rigorously in the case of *ast*, and to some extent for ast^2, ast^3, and ast^4.

In summary, the alleles, *ast* and ast^4, are more nearly similar to each other than to ast^2 and ast^3 in having good viability when opposite *S*, a variable expression, and a

tendency to interrupt the wing venation; whereas, *ast*^{2} and *ast*^{3} have peculiar lethal effects with *S* and, by themselves, a relatively constant expression and no perceptible effect on the wing venation.

In Table 1 the interactions of *S* and the *ast* alleles with the dominant, Enhancer of Star (*E–S*, at 6± in the second chromosome) are also shown. The relative specificity of *E-S* in strongly enhancing *ast*/+ and *ast*^{2}/+ is shown by the fact that it had, when heterozygous, little, if any, such effect on other recessive rough-eyed mutants which were tested in the heterozygous condition. The mutants thus tested were: echinus, facet, split, rough^{ex2}, uneven, morula, roughish, rolled, rubroad, scabrous, rough, and roughoid. Another interaction, not shown in Table 1, is that with either homozygous net or homozygous plexus, each of which is partially suppressed by *S*/+ and tends to be completely suppressed by *ast*/*ast* and *S*/*ast*.

Bridges (1936) has reported that *S* is apparently normal in the salivary gland chromosomes. Since the *S* and *ast* loci are now known to lie in the region of the 21E1–2 doublet, particular attention has been paid to this region in a cytological study of *S*, *S*^{2}, and *S*^{R} (two alleles closely resembling *S*^{1}), *ast*, *ast*^{2}, *ast*^{3}, and *ast*^{4}. Each of these mutants has been found to be apparently normal in the salivary gland chromosomes.

THE EVIDENCE FOR AN ASTEROID LOCUS

Data summarized in Table 2 represent part of the evidence that *ast* and *ast*^{4} are located close to the right of *S*, and they suggest that *ast*^{3} is located close to the right of *S*^{2}. The first indication that *ast* was not an allele of *S* was the appearance of a single +/*ast* fly among 3,235 offspring of a cross of *S*/*ast* females to *ast* males (Mating 1). Additional data were then collected from *al S ho*/*ast* females which were individually mated in separate experiments to *ast, al ast ho*, and *al S ho*/*Cy* males with the results shown in Matings 2a, 2b, and 2c, respectively. Mating 2a produced a normal fly which was shown by a cross to *al S ho*/*Cy* to have had the probable composition, *ast*/*ho*; seven other apparently normal offspring from Mating 2a were shown by the same test to have been instances in which homozygous *ast* had overlapped wild type. Mating 2b yielded no normal-eyed offspring. From Mating 2c, two non-*Cy* females with *ho* wings and eyes resembling those of *S*/+ were recovered in separate cultures. These females, which were non-virgin, were separately mated to *al S ho*/*Cy* males; in each case, some of the F_1 flies were phenotypically identical with the parental female. In one case a pair mating of these F_1 flies was made, and it produced an F_2 which had "*S*/+" eyes and normal eyes in the ratio, 2:1. Thus the females produced in Mating 2c had the probable composition, *al S ho*/*ho*.

In Mating 2 the "+" types, which may be interpreted as wild type crossovers between *S* and *ast*, occurred with a frequency of 0.009%. Here the total number of offspring in which the "+" types could be detected is calculated, as shown in Column 3, from that class which had approximately the same viability as those types—namely, the "*ast*" offspring in Matings 2a and 2b, and the "*Cy*" class in 2c.

Table 2 The frequency of "+" crossovers between the *S* and *ast* loci. The inversions for which the parental females were homozygous are shown in column 2. Calculated total progenies (column 3) are based on total counts of the class shown in parenthesis. The number and composition of the tested crossover chromosomes are indicated in parenthesis in column 4.

	Mating	Inversions	Total	Wild type crossovers	Percentage
1	$\frac{S\ +}{+\ ast}$ ♀ × *ast* ♂♂	Unknown	2 × 1,728 ("*ast*")	1(?)	
2a	$\frac{al\ S\ +ho}{+\ +\ ast\ +}$ ♀ ± *ast* ♂♂	Unknown	2 × 6,933 ("*ast*")	1(*ho*)	
b	(") ♀ × *al ast ho* ♂♂	Unknown	2 × 2,038 ("*ast*")	0	0.009
c	(") × $\frac{al\ S\ ho}{Cy}$ ♂♂	Unknown	15,753 (*Cy*)	2 (*2, ho*)	
3	$\frac{net\ +\ S\ \ +\ +\ dp\ cl}{+\ al\ +\ ast\ ho\ +\ +}$ ♀ × *In(2L)Cy, ast*^{2} ♂♂	*In(1)dl-49, cm*^{2}; *In(2R)Cy, cn*^{2}; and in about $\frac{1}{2}$ ♀♀, *In(3L + 3R)P*.	2 × 16,068 (*ast*/*ast*^{2})	15 (12, *al dp cl*)	0.047
4	$\frac{net\ S\ \ +\ +\ dp\ cl}{+\ +\ ast^4\ ho\ +\ +}$ ♀ × *In(2L)Cy, ast*^{2} ♂♂	*In(1)dl-49*, cm^2; and in some, *In(3L + 3R)P, ra.*	2 × 7,656 (*ast*^{4}/*ast*^{2})	2 (*2, dp cl*)	0.01
5	$\frac{+S^2\ \ +In(2L)t}{al^2\ +\ ast^3 In(2L)Cy}$ ♀ × $\frac{al\ s\ ho}{Cy,\ E–S}$ ♂♂	*In(1)dl-49, cm*^{2}; *In(2R)Cy, cn*^{2}; and in about $\frac{1}{2}$ ♀♀ *In(3LR) sep, ri p*^{p}.	17,334 (*Cy*)	7 (4, *al*^{2} *In–t*)	0.04

A more satisfactory method of recovering the "+" crossovers was employed in Mating 3, Table 2. Here the parental *S/ast* females were heterozygous for the mutants, *net* (net, at 0.0–), *dp* (dumpy, at 13.0), and *cl* (clot, at 16.5), as well as for *al* and *ho*. These mutants were arranged to give the approximately alternated composition, *net S dp cl/al ast ho*. In addition, the parental females were made heterozygous for inversions in chromosome arms other than 2*L*, since this procedure was known to increase the frequency of crossing over between *al* and *ho*. In order to obtain females of the required composition, *al ast ho In(2R)Cy/Cy, E–S* females were mated to *In(1) dl-49, cm*^{2}*/ Y; net S dp cl/ Cy(2L) dp*^{2} *b pr*; *In(3L + 3R) P/+* males. The F_1 *In (1) dl-49, cm*^{2}*/+*; *net S dp cl/al ast ho In (2R) Cy* females, of which one half are expected to be heterozygous for the Payne inversions in both arms of chromosome 3, constituted the parental females employed in Mating 3. The parental males

in this mating were homozygous for *In (2L) Cy, ast*^{2}. Since *S/ast*^{2} is lethal, the use of *ast*^{2} rather than *ast* served to reduce by one-half the total number of offspring to be examined for the "+" crossovers. At the same time, *ast*^{2} made easier the detection of such crossovers, since *ast*^{2}*/ast*, in contrast to *ast/ast*, had not been observed to overlap wild type.

In addition to 16,068 "*ast/ast*^{2}" offspring, Mating 3 yielded 15 flies with normal eyes. Of the latter, one was sterile and another (with Minute bristles) was lost. The remaining 13, composed of eight males and five females, were individually outcrossed to an *al S ho/Cy, E–S* stock. In each case, the F_1 non-*Cy* flies, except for those expected on the basis that the tested individual was a nonvirgin female, were aristaless and had the "*S*/+" type of eye. Two types of *Cy* flies, those with normal eyes and those of the expected "*ast*^{2}*/E–S*" type were obtained in the F_1 of each of these matings. An F_2 was raised from each of 12 of the 13 progeny tests by mating the *Cy*, normal-eyed flies *inter se*. The F_2 non-*Cy* offspring had in each case normal eyes and were phenotypically *al, dp* and *cl*. Thus, at least 13 "+" crossovers between *S* and *ast* were recovered in Mating 3, of which 12 were shown to carry the marker *dp* and *cl*, as well as *al*. It will be noted that the composition of the crossover types is expected to remain intact in the above progeny tests since those types were constantly kept against the Curly inversion in the left arm of chromosome 2. Stocks of two separate occurrences of the "+" crossover types from Mating 3 were kept for further tests. These included matings to *ast, ast*^{3}, and *ast*^{4} and to the *S* deficiencies of *Df-S*1 and *Df-S*2, which will be described later. The results of each of these matings were consistent with the assumption that the "+" crossovers carried the normal alleles of *S* and *ast*. A cytological analysis of these two stocks showed no departure from normal in the critical *S* region.

The results of Mating 3 are consistent with those of Mating 2 in indicating that *ast* is close to the right of *S*. The frequency of normal-eyed flies in Mating 3 was 0.08%. Since the *S/ast*^{2} class is lethal, the calculated frequency of "+" crossover types is 0.04%. Inspection of Table 5 shows that significant increases in crossing over in the *al–ho* region are obtained by the method adopted in Mating 3 of introducing inversions into other chromosome arms. The increase in frequency of "+" crossovers in Mating 3, compared to Mating 2 can be best attributed to this procedure.

It was further found that *S+/+ ast*^{4} females produced occasional "+" crossover types (Mating 4). The original *ast*^{4} *ho* strain, kept by mass inbreeding was used as the source of *ast*^{4}. The "+" types were detected and tested by methods analogous to those used in Mating 3, although a less "efficient" inversion setup was employed. Only two normal-eyed flies were recovered in this experiment. Each was found to be a "+" crossover type similar to those obtained from *S+/+ ast* females. The lower frequency—namely, 0.01%—of "+" crossovers from *S+/+ ast*^{4} females as compared with 0.04% from *S+/+ ast* females is at least consistent with the use of fewer inversions in Mating 4 as compared to Mating 3. In their interactions with

ast, ast^2, ast^3, *S* and *E–S*, the "+" crossovers from Mating 4 behaved as though they carried the normal alleles of *S* and *ast*. Likewise their cytological picture was apparently normal for the *S* region of the salivary gland chromosome.

Technical difficulties arising from the association of S^2 and $asteriod^3$ with *In(2L)t* and *In(2L)Cy*, respectively, prevented a study of their crossing over relationships with respect to either *ast*, ast^4, or *S*. However, since the sequences in these two inversions are nearly identical to each other (Bridges and Li, 1936), the linkage relations of S^2 and ast^3 could be determined directly. For this purpose, *In(2L)Cy*, al^2 ast^3 *b pr* (homozygous) females were crossed to *In(1)dl-49*, cm^2/Y; *In(2L)t*, S^2 *In(2R) Cy/Cy(2L)*dp^2*b pr; In(3LR)sep, ri* p^p *sep*/+ males. The F_1 "S^2/ast^3" females, one half of which are expected to carry the third chromosome "separated" inversion of Muller were individually mated to *al S ho/Cy, E–S* males with the results shown in Mating 5. Since S^2/S is lethal, only three general types of offspring were recovered. In addition, a total of seven non-Curly, aristaless flies resembling "*S*/+" were detected in the F_1.

It will be noted by reference to Fig. 1 that the left break points of *In(2L)t* and *In(2L)Cy* each lie in the region to the left of *ho* and to the right of *S*. Therefore, although no marker mutants close to the right of *ast* are employed in the parental females, that region is strategically marked by the inversions themselves. Of the seven "+" types, four were successfully studied cytologically. The procedure here was separately to cross the aristaless "*S*/+" flies to an *al S ho/Cy, E–S* stock. The F_1 Curly males were backcrossed to *al S ho/Cy, E–S* females. In each of the four tests some of the F_2 larvae carried the overlapping inversion complex, *In(2L)t/In(2L)Cy*. At the same time it was determined that each of the "+" crossover types was apparently normal in the critical 21E region. The "+" crossovers were mated to *ast* and ast^3 (as well as to *S* and *E–S*), and their behavior was that expected on the basis that they carried the normal alleles of *S* and *ast*. Thus the probable composition of each of the four crossover chromosomes was al^2 S^+ ast^+ *In(2L)t*. The frequency of the "+" crossovers can best be computed on the basis of the Curly offspring which totaled 17,334. This gives an estimated frequency of 0.04% (7/17,334). These results, when compared with those of Matings 2 and 3, indicate that S^2 is probably an allele of *S* and not a dominant allele of *ast*; similarly, ast^3 probably is an allele of *ast* rather than a recessive allele of *S*.

At first attempts to recover crossovers having *S* and *ast* or *S* and ast^4 in the same chromosome failed. These experiments, shown in Table 3, Matings 6, 7, and 8, were conducted on the assumption that *S ast*/++ or *S* ast^4/++ would resemble phenotypically *S*+/+ *ast* or *S*+/+ ast^4, respectively, or that they would be at least more extreme than *S*+/++. In Mating 7b, use was made of a suppressor of *S, Dp-S*, in the parental males, on the basis that it might permit the survival and detection of *S ast*. However, no offspring with eyes more extreme than "*S*/+" (or wild type in the case of Mating 7b) were detected in any of these experiments among a total of 15,108 offspring.

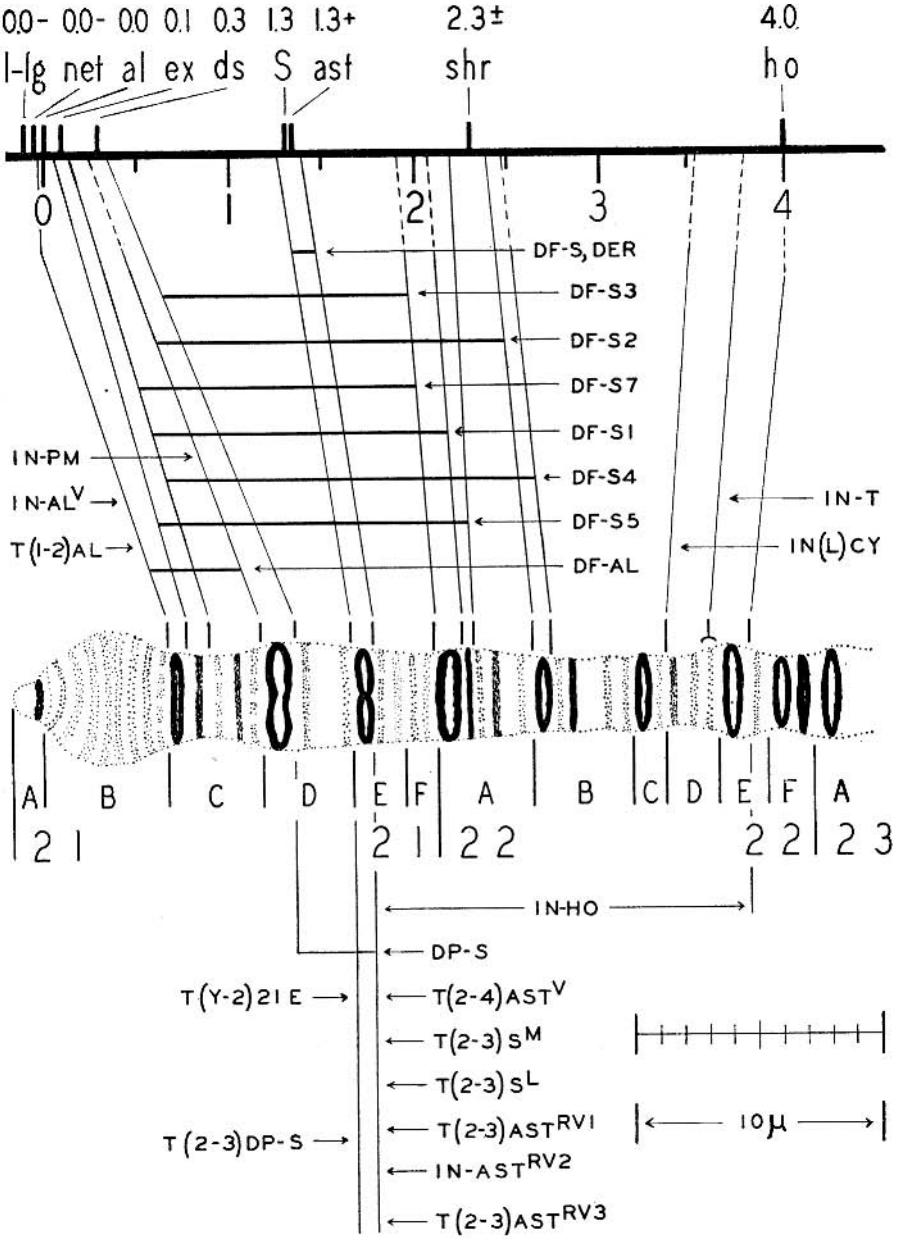

Figure 1. A correlation of the linkage map of the extreme left end of the second chromosome with the salivary gland chromosome structure.

Table 3 The frequency of "*S ast*" and "*S ast*^{4}" crossovers. (See text and Table 2 for description.)

	Mating	Inversions	Total progeny	"*S as*" or "*S ast*^{4}" crossovers	Percentage
6	$\frac{al\ S\ +ho}{+\ +\ ast\ +}$ ♀×*al ho* ♂♂	Unknown	2,967	0	—
7a	$\frac{net\ +\ S\ +\ +\ dp\ cl}{+\ al\ +\ ast\ ho\ +\ +}$ ♀×*al ho* ♂♂	*In(1)dl-49, cm*^{2}; *In(2R)Cy, cn*^{2}; *In(3L)P, Me* *In(3R)C, Sb e*	3,005	0	—
b	(")♀×*al Dp–S ho* ♂♂		6,222	0	—
8	$\frac{net\ S\ +\ +dp\ cl}{+\ +\ ast^4\ ho\ +\ +}$ ♀×*al ho* ♂♂	*In(1)dl-49, cm*^{2}; and in some, *In(3L + 3R)P, ra*	2,914	0	—
9	$\frac{+\ al\ \ (S\ ast)\ \ (+\ ast)\ ho\ +\ +}{net\ +\ \ +\ ast\ \ \ \ \ \ +\ dp\ cl}$ ♀×*al go* ♂♂	*In(1)dl-49, cm*^{2}; *T(2;3)Me, Me*	4,864	3 (2, *al S ast dp cl*)	0.06
		In(1)dl-49, cm^2; *T(2;3)Me, Me/ In(3LR)CxD, D*	1,531	2 (1, *al S ast dp cl*)	0.1
10	$\frac{al\ \ (S\ ast)\ \ (+\ ast)\ \ ho\ \ +\ +}{+\ \ +\ ast^4\ \ \ \ \ \ +\ dp\ cl}$ ♀ ×*net* ♂♂; *net ho* ♂♂; and *al ho* ♂♂	*In(1)do-49, cm*^{2}; *T(2;3)Me, Me/ In(3LR)sep, p*^{p}.	6,667	*3*(*3, al S ast*^{4} *dp cl*)	0.04

An indirect way of obtaining the *S ast* and *S ast*^{4} combinations suggested itself as a result of independent studies of the tandem Star Duplication, *Dp-S* (Lewis, 1941). To understand the method employed, some of the properties of this duplication require review.

A combination of genetic and cytological studies now indicates that this duplication includes two known loci, *S* and *ast*. Cytologically, this aberration is a tandem repeat in direct order for the four bands: 21D3,4 and the 21E1–2 doublet structure or "capsule" (see Fig. 1). As originally obtained, this duplication, which arose from an *ast* female, had the probable composition, (+*ast*)(+*ast*), formerly written (S^r)(S^r). This notation is to be taken as indicating that the regions in parentheses are in adjacent, direct order and that each carries the normal allele of *S* and the recessive *ast*. Many variations in the genetic composition of *Dp-S* have been obtained, but for present purposes only the derivative, (*S ast*)(+*ast*), formerly written (*S*)(S^r), need be considered. This derivative was obtained among the offspring of a mating in which (+*ast*)(+*ast*) *ho*/*net S dp cl* females were individually mated to *In*(2*L*)*Cy, ast*^{2} *b pr* males. The great majority of the F_1 had normal eyes—i.e., the (+*ast*)(+*ast*)/*ast*^{2} class; in addition, there were 206 offspring with eyes resembling

"*ast*/*ast*^{2}." Four possibilities seemed likely for the origin of these types. Thus, by unequal crossing over, *ast* could have been extracted from the duplication in two ways, either as (1) *ast dp cl*, or as (2) *net ast ho. S* could likewise be inserted into the left section of the duplication in two ways, either as (3) *net* (*S ast*)(+ *ast*) *ho*, or as (4) *net* (*S* +)(+ *ast*)*ho*. Two other duplication types possible here—namely, (+ *ast*)(+ +) and (+ *ast*)(*S* +)—were known to be wild type opposite *ast*^{2} and therefore could not be detected in this experiment. Of the 206 cases, 192 were successfully tested to a *net ast ho* stock. In each of these progeny tests some of the F_1 flies were phenotypically *net ast ho* (except for two which were *ast ho*). Thus the first possibility, the extraction of *ast* from the left section of the duplication, was not realized. A salivary gland chromosome analysis was then made of 83 of the "*ast*" crossover types. Only two of these proved to carry *Dp-S*. These were indistinguishable phenotypically from the 81 *net ast ho* types which did not carry the duplication. Since type (4) is complementary to type (1), which did not occur in this or related experiments, it can be assumed that the composition of these two duplication types was (*S ast*)(+ *ast*). This genetic structure, at least with respect to the *S* locus, was confirmed by extracting from the left (distal) section of the repeat the newly introduced *S*. This was done in three ways. The results of two of these methods are shown in Matings 9 and 10 of Table 3. Females of composition, *al* (*S ast*)(+ *ast*) *ho*/*net ast dp cl* (Mating 9) or *al* (*S ast*)(+ *ast*) *ho*/*ast*^{4} *dp cl* (Mating 10) when mated to *al ho* males produced for the most part only normal-eyed flies. However, each type of female produced several aristaless offspring with eyes resembling those of "*S*/+" flies. These rare types were also produced when *al* (*S ast*)(+ *ast*)/*dp cl* females were mated to *al ho* males. No types with eyes more extreme than "*S*/+" were detected in any of these experiments.

In the next step, three separate occurrences of what may be called the "*S*" crossovers from Mating 9, and two such cases from Mating 10 were tested for the presence of *ast* or *ast*^{4}. The results of these five tests are grouped and summarized in Matings 11, 12, 13, and 14 of Table 4. Matings 11 and 12 were tests of "*S*" crossovers derived from Mating 9 and in each of these three tests one (*net*) *ast dp cl* crossover was recovered. Likewise, at least one *net ast*^{4}*dp cl* crossover was detected from each of the two tests of "*S*" crossovers from Mating 10.

These linkage studies indicated that the probable composition of the "*S*" crossovers from Matings 9 and 10 were *al S ast dp cl* and *al S ast*^{4}*dp cl*, respectively. In these studies the *ast* and *ast*^{4} crossovers were derived from *S ast*/+ + and *S ast*^{4}/+ + females, respectively. Still another *S ast* crossover type from Mating 9 was tested in Mating 14 by a different procedure, but one analogous to that by which the *S ast* type was first recovered. In this case *net S ast dp cl*/*al* (+ *ast*)(+ *ast*) *ho* females were mated to *In*(2*L*)*Cy, al*^{2} *ast*^{3} *b pr* males. Here, too, it was possible to recover an individual whose composition was shown to be *al ast dp cl*/*In*(2*L*)*Cy, al*^{2} *ast*^{3} *b pr*. The evidence that the rare crossover types obtained in the matings shown in Table 3 carried *ast* or *ast*^{4} was based on phenotypic studies which included obtaining homozygotes for the crossover chromosome as well as testing to *S* +, *S ast*, and *S ast*^{4}.

Table 4 The frequency with which *ast* and ast^4 were recovered from *S ast*/++ and *S* ast^4/++ females, respectively. (See text and Table 2 for description.)

	Mating	Inversions	Total	"*ast*" or "ast^4" crossovers	Percentage
11	$\frac{al\ S\ ast\ dp\ cl}{+\ +\ +\ +\ +}$ ♀ × *In(2L)Cy*, $al^2 ast^3$ ♂♂	*T(2;3)Me, Me*	2 × 2,304	1 (*ast dp cl*)	0.02
12	$\frac{+\ al\ S\ ast\ +\ dp\ cl}{net\ +\ +\ +\ ho\ +\ +}$ ♀ × *al ast ho* ♂♂ and *In(2L)Cy*, $al^2 ast^3$ ♂♂	*In(1)dl-40*, cm^2; *Dp(2)T(2;3)Me, Me*	2 × 1,594	2(2, *net ast dp cl*)	0.06
13	$\frac{+\ al\ S\ ast^4\ +\ dp\ cl}{net\ +\ +\ +\ ho\ +\ +}$ ♀ × *In(2L)Cy*, $al^2 ast^3$ ♂♂	*In(1)dl-49*, cm^2; *Dp(2)T(2;3)Me, Me*	2 × 2,754	4(3, *net* ast^4 *dp cl*; 1, *net* ast^4)	0.07
14	$\frac{net\ +\ S\ ast\ +\ dp\ cl}{+\ al\ (+\ ast)(+\ ast)\ ho\ +\ +}$ ♀ × *In(2L)Cy*, $al^2 ast^3$ ♂♂	*In(1)dl-49*, cm^2/ *In(1)AM*, pig^4; *T(2;3)Me, Me*	2 × 996	1(*al ast dp cl*)	0.05

A cytological study was made of five of the *S ast* types from Mating 9, two *S* ast^4 types from Mating 10, and several of the *ast* and ast^4 crossovers from Matings 11–13. None of these carried *Dp-S*, and with the exception of one *S ast* type, all were apparently normal in the critical *S* region of the salivary gland chromosomes. The exception here showed a slight disturbance near the 21E1–2 doublet. The exact nature of this irregularity was not determined but it appeared to involve a duplication or inversion of a single faint band. This aberrant *S ast* type was not included in the crossover studies shown in Table 4; however, a cytologically normal *ast* crossover was extracted from it by the same procedure as that used in the other cases.

In their phenotypic reactions, *S ast* and *S* ast^4 were indistinguishable from *S* ast^+ in tests to +, *E–S*, ast^2, and ast^3. All combinations in which *S* was homozygous were lethal regardless of the *ast* composition. However, opposite *ast*, and similarly opposite ast^4, *S* ast^4 produced a larger eye than *S ast* which in turn produced a larger eye than *S* ast^+. Thus, the following sequence, arranged in order of decreasing eye size, could be established: *S* ast^4/+ ast^4, *S ast*/+ ast^4, *S* +/+ ast^4, *S* ast^4/+ *ast*, *S ast*/+ *ast*, and *S* +/+ *ast*. The largest eye produced here is still somewhat smaller than that typical of *S*/+.

It was felt that the difference in eye size between *S ast*/+ *ast* and *S* +/+ *ast* (or between *S ast*/+ ast^4 and *S* +/+ ast^4) was not sufficiently distinct owing to the inherent variability of these types to make practicable the detection of an *S ast* crossover from a mating of *S* +/+ *ast* females to *ast* (or ast^4) males. However, it was

Table 5 A comparison of the standard recombination values (together with their standard deviations) for the *al-ho* region, with those obtained when the parental females were heterozygous for various inversion (or translocation) complexes.

Number of mating	Sequence in major chromosome arms of parental ♀♀					Percentage recombination	
	X	2L	2R	3L	3R	*al-S*	*al-ho*
6	(standard)					1.2 ± 0.2 (36/2,967)	4.7 ± 0.4 (140/2,967)
7a,b	$\frac{dl\text{-}49}{+}$	$\frac{+}{+}$	$\frac{Cy}{+}$	$\frac{P}{+}$	$\frac{C}{+}$	4.3 ± 0.4 (128/3,005)	10.9 ± 0.3 (1,011/9,227)
12, 13	$\frac{dl\text{-}49}{+}$	$\frac{+}{+}$	$\frac{+}{+}$	$\frac{Dp(2)T(2;3)Me}{+}$		4.2 ± 0.3 (167/4,008)	12.1 ± 2.7 (18/149)
—	$\frac{+}{+}$	$\frac{Dp\text{-}S}{+}$	$\frac{+}{+}$	$\frac{+}{+}$	$\frac{+}{+}$	—	4.3 ± 0.2 (343/8,000)
9	$\frac{dl\text{-}49}{+}$	$\frac{Dp\text{-}S}{+}$		$\frac{T(2;3)Me}{+;+}$		—	13.8 ± 0.6 (455/3,297)
9	$\frac{dl\text{-}49}{+}$	$\frac{Dp\text{-}S}{+}$		$\frac{T(2;3)Me}{+;In(3LR)CxD}$		—	17.3 ± 1.7 (85/490)

possible to recover *S ast*^{4} from *S* +/+ *ast*^{4} females on the basis of the more distinct difference existing between *S ast*^{4}/+ *ast* and *S* +/+ *ast*. In this experiment, Mating 4a, not shown in the tables, *In(1)dl-49, cm*^{2}/+; *al S ho/ast*^{4}*dp cl; In(3LR)sep*/+ females were mated to *al ast ho* males. Total counts were not made but among a large number of offspring several aristaless flies were obtained which had eyes intermediate between *S* +/+ *ast* and *ast/ast*^{4}. Three of these upon further testing were found to carry the desired *S ast*^{4} crossover, on the basis that it behaved phenotypically exactly like the *S ast*^{4} types obtained from Mating 10. The remaining cases were found to be instances in which *S/ast* had overlapped wild type.

In addition, a stock of one of the complementary *ho* crossovers which occurred in Mating 4a was established. It was then possible to make up flies having the composition *al S ast*^{4} *dp cl/ho* whose second chromosomes were the two complementary crossovers derived from mating 4a. The phenotype in this case was again indistinguishable from *S ast*$^+$/+ +.

In summary, some 39 crossovers were obtained between the Star and asteroid loci. Twenty-two of these were "+" crossovers derived from *S* +/+ *ast, S* +/+ *ast*^{4}, or *S*^{2} +/+ *ast*^{3} females. Their frequency varied between 0.009% in the absence of

inversions in the parental females to 0.047% when the parental females were heterozygous for *In(1)dl-49, In(2R)Cy*, and, in some, *In(3L + 3R)P*. Six *S ast* or *S ast*^{4} crossovers were detected in the progeny of (*S ast*)(+ *ast*)/*ast* and (*S ast*)(+ *ast*)/*ast*^{4} females, respectively. The frequency varied between 0.04% and 0.1%, depending on the particular inversions present.

A SURVEY OF THE EXTREME LEFT END OF CHROMOSOME 2

Early in the genetic analysis of *S* and *ast*, an attempt was made to correlate those loci with the salivary gland chromosome structure in the left end of chromosome 2. This correlation was particularly necessary as a basis for a cytological analysis of the *S* and *ast* mutants. Genetic and cytological correspondence were obtained not only for *S* and *ast* but also for most of the other mutant loci in their immediate vicinity. A genetic map of this region, which includes approximately the first four map units of the left end of the chromosome 2, is shown as the top line in Fig. 1. This section includes at least ten known loci. Descriptions and linkage data for the mutants, aristaless (*al*), expanded (*ex*), and dachsous (*ds*), as well as for Star, have been given by Stern and Bridges (1926). The mutant, telegraph (*tg*, at 0.0±), also described by these authors, has been lost and is omitted from the linkage map shown here. An account of net (*net*), shrunken (*shr*), heldout (*ho*), and lethal giant larvae (*l-gl*), together with Bridges' revised location of *l-gl* at 0.0±, is given by Bridges and Brehme (1944).

The drawing, in Fig. 1, of the first two sections of chromosome 2L is semi-diagrammatic and composite, based on moderately stretched chromosomes which were stained with aceto-orcein according to the method of LaCour. The chromosome is labeled to correspond with Bridges' (1935) map. Reference to the latter map shows that the section extending from 21A to just after 23A1–2 corresponds, as calculated by Bridges, to about 4.4 map units on the genetic map. In Fig. 1, this value has been used as the basis for the correlation of the genetic and cytological maps.

Much of the material which has made possible the genetic and cytological correspondences shown in Fig. 1 was obtained from an experiment in which adult wild type (Canton-S) males were X-rayed (3,000 r units) and mated to *al ast ho* females. The results of this experiment are summarized in Table 6. Total counts were not made; however, a random sample of 16 out of 196 cultures, in which the parental males were allowed to remain for the first 5 days, averaged 82 flies per culture. A total of 103 transfer cultures produced an additional 3,398 flies. Therefore the combined total number of offspring was roughly 19,500. F_1 aberrant individuals, in which the eyes were smaller and rougher than in *S*/+ flies, or whose phenotypes resembled *al* or *ho*, were separately crossed to an *al S ho*/*Cy, E–S* stock. A preliminary examination was made of the salivary gland chromosomes of F_1 larvae from each of the fertile progeny tests. This was followed up in most cases by a more detailed cytological analysis after a balanced culture had been established.

Table 6 Results from X-rayed (3,000 r units) wild-type ♂ × *al ast ho* ♀. Total offspring: approximately 19,000. (*Df* = Deficiency, *In* = Inversion, *T* = Translocation.)

Changes resembling:	Number not analyzed	Number analyzed				Total
		Associated with aberrations			"Point mutations"	
		Df	*In*	*T*		
aristaless	5	1	1	0	0	7
Star	5	5	0	1	1?	12
asteroid	4	0	0	1	1?	6
heldout	9	0	1	0	0	10
Notch	13	—	—	—	—	13

No substantial case of "point mutation" to either *al* or *ho* was detected in this experiment. A possible change of this type to a *S* and to an *ast* allele, simultaneously, will be discussed later. The following is a description of those analyzed changes shown in Table 6 which were found to be accompanied by a chromosomal aberration; an additional *S* deficiency (*Df-S7*) obtained from a related X-radiation experiment is also included:

Df-al—Deficiency (2) aristaless—Lewis 1940. *Df/+* is an extreme Minute with rough eyes and slight *ex*- and *ds*-like effects. Deficient for *al, ex*, and *ds:* but not for *l-gl, net* or *S*. *Df-al/net* has a weak net effect. The loss extends from just before the 21C1–2 doublet to just before the 21D1–2 doublet.

Df-S1—Deficiency (2) Star—Lewis 1940. *Df/+* has a slightly smaller and rougher eye than "+" and is less extreme and more variable than "*S/+*"; viability and fertility are good. *Df/ast* is somewhat less extreme than *S+/+ ast*, and more like *S ast/+ ast*; otherwise the *Df* acts exactly like *S*. Deficient for *ds, S,* and *ast;* but not for *l-gl, net, al, ex, shr,* or *ho*. The breaks follow the medium 21C3 band and just precede the heavy 22A3 band.

Df-S2—Deficiency (2) Star-2—Lewis 1940. Phenotypic effects are like those of *Df-S1*. Deficient for *ds, S, ast,* and *shr;* but not for *l-gl*, *net*, *al*, *ex*, or *ho*. The loss appears to extend from just to the left of 21D1–2 doublet to just preceding the 22B1–2 doublet. It is also possible that the breaks occur in the middle of these two doublets.

Df-S3—Deficiency (2) Star-3—Lewis 1940. Phenotypic effects are like those of *Df-S1*. Deficient for *S* and *ast;* but not for *l-gl*, *net*, *al*, *ex*, *ds*, *shr*, or *ho*. The loss extends from just to the right of 21D1–2 doublet to just before the 22A1–2 doublet.

Df-S4—Deficiency (2) Star-4—Lewis 1940. Phenotypic effects are like those of *Df-S1*. Deficient for *ds*, *S*, *ast*, and *shr*; but not for *l-gl*, *net*, *al*, *ex*, or *ho.* The breaks follow the medium 21C3 band and the 22B1–2 doublet.

Df-S5—Deficiency (2) Star-5—Lewis 1940. Phenotypic effects are like those of *Df-S1* except that *Df-S5/+* has a slight *ex*-like effect. Deficient for *ex*, *ds*, *S*, and

ast; but not for *l-gl*, *net*, *al*, *shr*, or *ho*. The breaks occur just after the 21C1–2 doublet and after the heavy 22A3 band.

In(2LR)al^V—Inversion (2LR) aristaless-variegated— Lewis 1940. *al^V/al* is similar to homozygous *al;* however, in the presence of an extra Y chromosome, *al^V/al* is wild type. The homozygote is lethal. *Df-al/al^V* is viable and variegated in that sometimes only one instead of both of the aristae is missing. The euchromatic break appears just to precede the 21C1–2 doublet; the right break is in the heterochromatin of chromosome 2R.

T(2;3)S^L—Translocation (2;3) Star—Lewis 1940. *S^L/+* resembles *S/+*; *S^L* also resembles *S ast$^+$* opposite *ast, ast^2, ast^3, ast^4*, and *E–S*. This is a complex rearrangement involving at least three breaks: one follows the 21E1–2 doublet of chromosome 2L; chromosome 3R has a break which apparently just precedes 88D8 and another in the basal heterochromatin. The tip of chromosome 2L up to and including 21E1–2 is translocated to heterochromatin of chromosome 3, but it has not been possible to determine from a salivary gland chromosome analysis whether the section from the base of 3R to 88D is inverted or whether there is an insertion of heterochromatin (without inversion) between 88D and 21E. The tip of 3R to 88D is exchanged for the tip of 2L.

In-ho—Inversion (2) heldout—Lewis 1940. *In/ho* is phenotypically like homozygous *ho*. Opposite *Df-S3, S*, or *ast*, the inversion acts as though it carries the normal alleles of *S* and *ast*. The homozygote has reduced eyes with anterior indentation; the wings are reduced to tiny stubs (*In/vg* is wild type); the female is fertile, but the male lacks genitalia and the anal apparatus. The break points of this short inversion occur just to the left of the 21E1–2 doublet and just to the right of the 22E1–2 doublet.

T(2;4) ast^V—Translocation (2;4) asteroid-variegated—Lewis 1940. *ast^V/+* is wild type; *ast^V/ast* and *ast^V/S* resemble but are more variable than *ast/ast* and *S+/+ ast,* respectively. *ast^V/Df-S3* is lethal. *ast^V/ci* gives a cubitus interruptus (*ci*) effect. The variegated *ast*-like effect is completely suppressed in the XXY female. Duplication and deficiency derivatives are viable. *Df-ast^V/+* resembles *Df-al/+* except that the former has a much more roughened eye; the venation of *Df-ast^V/net* resembles homozygous net; *Df-ast^V/l-gl* is lethal. *Dp-ast^V/l-gl/l-gl* is viable. One break just follows the 21E1–2 doublet and the other is in the heterochromatin of chromosome 4.

Df-S7—Deficiency (2) Star-7—Lewis 1940. From X-rayed *net ho/net Dp-S sp cl* male. Associated with *net* and *ho*. Loss extends from just after the medium 21C3 band to just preceding the heavy 22A1–2 doublet. Lost.

With this material it was possible to locate with varying degrees of precision most of the known mutants at the extreme left end of the second chromosome. The results are diagrammed in Fig. 1. The locations of *al*, *ex*, and *shr* are based directly on tests of these mutants to the *S* deficiencies and *Df-al*. It is likely that the locus of *ho* is in the neighborhood of section 22E on the basis of genetic and cytological studies of *In(2)ho*. The location of *l-gl* follows from studies of the duplication and deficiency derivatives of *T(2;4) ast^V* which "cover" this locus in contrast to the *S* deficiencies and *Df-al*.

Net (0.0–)

The process of locating the *net* locus was complicated by the enhancement of *net* by Minute deficiencies of the *Df-al* and *Df(2)ast*^{V} type, and by the possible complete suppression of the *net* phenotype by the *S* effect of the *S* deficiencies. The latter possibility was excluded by the fact that *net Df-S7/net* flies had only a very slightly suppressed *net* venation. Critical evidence for the location of *net* was obtained by the use of a duplication for the left end of chromosome 2 derived from *T(2;4)b* of Dobzhansky. Females of composition *T(2;4)b, net/net* were crossed in separate experiments to *Df-al/Cy* and *Df-S5/Cy* males. The non-Curly duplication offspring having the composition *Dp(2)b*, *net/net/Df-al* and *Dp(2)b*, *net/net/Df-S5*, respectively, had normal venation. On the other hand, when females of the above type were mated to *T(2;4)ast*^{V}*/Cy* males, some of the duplication offspring had typical *net* venation and therefore presumably had the genetic make-up *Dp*(2)*b, net/net/Df*(2)*ast*^{V}. These results are consistent with the fact that *Df*(2)*ast*^{V}*/net* flies show a more extreme *net* venation than *Df-al/net* flies. Hence the locus of *net* must lie to the left of the 21C1–2 doublet.

The locus of *net* (originally 0.3± on the genetic map) was re-examined, but the results were negative with respect to whether *net* is to the left or to the right of *al*. Thus, on the basis that it is to the right of *al*, no crossovers were obtained between the locus of *al* and *net* among 90 tested crossovers between *al* and *S*. Again, on the basis that it is to the left of *al*, there were no crossovers between *net* and *al* among ten tested crossovers between *net* and *S*. From the cytological evidence, however, it is likely that the *net* locus is to the left of *al*. It should be noted here that the map order of *net* (0.0–) and *l-gl* (0.0–) is unknown.

As has already been mentioned, (+ *ast*)(+ *ast*) *ho/net S dp cl* females produced a very rare type having the composition, *ast ho*. A total of all matings in which females of this type were used produced three *ast ho* to 247 *net ast ho* crossovers, the latter occurring with a frequency of 0.35%. The *ast ho* types, which were normal cytologically, were first interpreted as indicating that the locus of *net* was included in the region duplicated in *Dp-S* (Lewis 1941). This tentative conclusion is now no longer consistent with the deficiency evidence for the location of *net*. The *ast ho* types may have resulted from double crossing over, which is very improbable for a region only one to two map units in length, or from unequal crossing over involving sister chromatids of the duplication chromosome.

Dachsous (0.3)

The deficiencies, *Df-al*, *Df-S2*, and *Df-S6* (identical with *Df-S2*, but independent in origin) appear to be deficient for the *ds* locus as determined by tests of these deficiencies not only to the mutant *ds*^{1}, but also to *ds*^{W}, *ds*^{33k}, and *ds*^{38k}. Yet,

cytologically the above *S* deficiencies do not appear to overlap *Df-al* (Fig. 1). In the case of both *Df-S2* and *Df-S6* it is possible that the loss involves only the right half of the 21D1–2 doublet; in any case, they are separated from *Df-al* by at least one disc of the chromosome. Perhaps the simplest explanation of these ambiguous results is that either *Df-al* or *Df-S2* (and *Df-S6*) is a true deficiency for the *ds* locus while the other has a position effect at, or an interaction with, the *ds* locus resembling deficiency for that locus.

Star (1.3)

Purely genetic evidence has shown that the *S* locus is included in the repeated section of *Dp-S*. That evidence has included not only the "extraction"of the normal allele of *S* from each section of (+ *ast*)(+ *ast*) but also the "insertion" of *S* itself into each section. Hence, the *S* locus must lie in the region from 21D3 to 21E1–2, inclusive—that is, the region present twice in *Dp-S*. This shows that the deficiencies, *Df-S1* to *Df-S7*, inclusive, which have in common a loss of that section and which closely resemble *S*, phenotypically, are indeed deletions for the *S* locus.

A more precise location of *S* can be made with the use of $T(2;4)ast^V$ and *T(Y;2)21E*. The latter translocation was kindly supplied by Dr. Schultz, who found (unpublished) that the second chromosome was broken just to the left of the 21E1–2 doublet and translocated with the Y-chromosome. *T(Y;2)21E* over *S* or *ast*, acts as though it carries the normal alleles of *S* and *ast*. The deficiency for the tip of chromosome 2 derived from this translocation closely resembles *Df-al* in its effects. Whereas $Df(2)ast^V/S$ is lethal, *Df(2)21E/S* is very similar to $Df(2)ast^V/+$. This suggested that the loss of the 21E1–2 doublet is associated with an *S* effect. To eliminate as far as possible the effects of deficiencies for euchromatic regions other than the 21E1–2 doublet, these two translocations were combined to produce a derivative having the tip of chromosome 2L up to and including 21D4, derived from *T(Y;2)21E*, and the remainder of the second chromosome, from 21E3 on derived from $T(2;4)ast^V$. For this purpose *y/Y* (attached-X): *T(Y;2)2IE/Cy, E-S* females were mated to $T(2;4)ast^V/Cy$, $(2L)\ dp^2\ b\ pr$ males. F_1 Curly flies with eyes resembling those of *S/E-S* were mated *inter se* to establish a balanced stock of this newly derived deficiency. An examination of the salivary gland chromosomes of this stock showed that the expected combination of the two translocations was realized. This synthesized deficiency has been called Deficiency (2) Star-derived, *Df-S-der*. As kept in stock, *Df-S-der* has a deficiency for the 21E1–2 doublet, a duplication for part of the Y chromosome, and a deficiency in the heterochromatin of chromosome 4. Phenotypically, *Df-S-der* was indistinguishable from the other *S* deficiencies in its effects opposite +, *S*, *ast*, ast^3, and ast^4. Thus, the *S* locus is closely confined to the region of the 21E1–2 doublet structure of the salivary gland chromosomes.

Asteroid (1.3+)

It is well recognized that the determination of genetic and cytological correspondences by deficiency evidence alone may sometimes be misleading, owing to the possibility of position effects accompanying these deficiencies. As has already been described, such difficulties were encountered in attempting to ascribe the *ds* locus to some precise section of the chromosome. In view of the position effect known to exist at the *S* and *ast* loci, it is apparent that these loci present a special problem. In the case of *S*, its location by means of deficiencies could be independently checked by the use of a tandem duplication for that locus—namely, *Dp-S*. Similar methods have been used in the location of *ast*.

It can be safely assumed that *Df-S4*, which involves a loss of the shrunken locus, is a deficiency for the *ast* as well as the *S* locus. Moreover, it more closely resembles *S ast* than *S* ast^{+} in its effects opposite *ast*. Inasmuch as *Df-S-der* is phenotypically indistinguishable from *Df-S4*, it is likely that it too involves a loss of both the *S* and *ast* loci. It is important to note here explicitly that *Df-S-der* is derived from *T(Y;2)21E*, which has no detectable departure from normal when opposite *S* or *ast*; and from *T(2;4)ast*^{V} which likewise behaves like wild type when the variegation is suppressed by an extra Y chromosome. Now it has been determined that the presence of an extra Y chromosome does not change the phenotypic effects of *Df-S-der*. It may therefore be assumed that those effects are attributable mainly to the deficiency for the 21E1–2 doublet.

Considerable evidence is available for supposing that the *ast* locus, like the *S* locus, is included in *Dp-S*. This evidence will be described in a later section and comes from a further study of the position effect at the *S* and *ast* loci by means of chromosomal aberrations and by means of various derivatives of *Dp-S*. Formal genetic proof that the *ast* locus is included in *Dp-S* is lacking, however, since the occurrence of the crossover between an asteroid locus in the left section of the duplication and the break point of the duplication has not been definitely detected.

Suppressor of Star

The dominant Suppressor of Star, *Su-S*, found by Curry, is mentioned here since it has been reported to be close to, if not an allele of *S*. A reinvestigation of *Su-S* has shown that it is probably not the result of a point mutation but rather that its effect is attributable to the double deficiency involved in *In(2L)Cyt*, with which it was associated at the time its effect was first detected. This was shown by deriving, a new *In(2L)Cyt*, from a mating of *In(2L)Cγ*, $dp^{2}/In(2L)t$ females to *S dp/Cγ* males. *In(2L)Cyt* was detected in the progeny of this cross by virtue of its suppressing effect on *S*. The complementary or double duplication derivative was also detected in this experiment by virtue of its slight enhancement of *S*. A cytological analysis of *In(2L)Cyt*, made by Bridges and Li (1936), showed the presence of a deficiency for 22D3 and all of section 34A. There is some evidence of a preliminary nature for supposing that it is the deficiency in 22D which is responsible for the suppression of

S by *In(2L)Cyt*. Whether or not the location of Enhancer of Star, *E–S*, (locus, 2–6±) coincides with that of *Su-S* is not known. Existing stocks of *E–S* that have been examined cytologically show the presence of the Curly inversion and not the double duplication derivative complementary to *In(2L)Cyt*. The enhancement of *S* by this duplication type is by no means as extreme as that by *E–S*.

THE PHENOTYPES OF DP-S DERIVATIVES

Unequal crossing over provides a mechanism whereby different *S* and *ast* alleles can be introduced into *Dp-S*. It seemed likely that a study of the derivatives, so obtained, should shed further light on the nature of position effect at these loci. Using only the following six combinations of *S* and *ast* alleles: + +, + ast^4, + *ast*, *S* +, *S* ast^4, and *S ast*; there are 36 possible ways of varying the composition of *Dp-S*. Actually, it was not feasible to vary the composition of the *ast* locus in the left section of the duplication. This leaves 12 combinations, of which all but two, (+ *ast*)(+ ast^4) and (*S ast*)(+ ast^4), have been synthesized. The derivatives may be divided into the following two groups:

Group I	*Group II*
1. (+ *ast*)(+ +)	6. (*S ast*)(+ +)
2. (+ *ast*)(+ *ast*)	7. (*S ast*)(+ *ast*)
3. (+ *ast*)(*S* +)	8. (*S ast*)(*S* +)
4. (+ *ast*)(*S ast*)	9. (*S ast*)(*S ast*)
5. (+ *ast*)(*S* ast^4)	10. (*S ast*)(*S* ast^4)

In each of these ten types the presence of the duplication was confirmed by an examination of the salivary gland chromosomes. Only a brief description will be given of the methods by which these types were originally obtained.

All members of Group I were obtained from (+ *ast*)(+ *ast*). The unequal crossover, *net* (+ *ast*)(+ +) *ho*, was detected among the progeny of *net* (+ *ast*)(+ *ast*) *dp cl/al ho* females as a complete suppressor of S^2 *E–S* (*Cy*); whereas, (+ *ast*)(+ *ast*) only partially suppresses S^2 *E–S*. The derivatives 3, 4, and 5 could be obtained with relative ease from (+ *ast*)(+ *ast*)/*S*, (+ *ast*)(+ *ast*)/*S ast*, (+ *ast*)(+ *ast*)/(*S* ast^4) females, respectively, when mated to *S*/*Cy* males. The marker genes are omitted here for simplicity. The new combinations, 3, 4, and 5, when opposite *S*, are found to be inseparable in appearance from *S*/+; while (+ *ast*)(+ *ast*)/*S* has a normal or only very slightly roughened eye.

A brief description has already been given of the method of deriving (*S ast*)(+ *ast*) from (+ *ast*)(+ *ast*). Because of the difficulty of introducing *S* into the left section of *Dp-S*, and of detecting the product when it is produced (*S ast*)(+ *ast*) was used as the source of the remaining members of Group II. This, then, was accomplished by methods similar to those by which members of Group I were derived from (+ *ast*)(+ *ast*) except that the detection of the Group II derivatives was on a different basis. One example will be given to illustrate this difference. Females of composition

Table 7 Phenotypic effects of certain *Dp-S* heterozygotes. The composition of the duplication chromosome is shown at the left while that of the normal chromosome is shown at top. The entries in the table indicate standard diploid types to which the appropriate *Dp-S* heterozygotes are very closely comparable.

		+	*ast*	*S ast*^{4}	*S ast*	*S*	*Df-S3*	*Df-S-der*
1	(+ *ast*)(+ +)	"+"	"+"	"+"	"+"	"+"	"+"	"+"
2	(+ *ast*)(+ *ast*)	"+"	"+"	"*ast*/+"	"*ast*/+"	"*ast*/+"	"*ast*/+"	"*ast*/+"
3	(+ *ast*)(*S ast*^{4})	"+"	"*ast*/+"	"*S*/+"	"*S*/+"	"*S*/+"	"*S*/+"	"*S*/+"
4	(+ *ast*)(*S ast*)	"+"	"*ast*/+"	"*S*/+"	"*S*/+"	"*S*/+"	"*S*/+"	"*S*/+"
5	(+ *ast*)(*S* +)	"+"	"*ast*/+"	"*S*/+"	"*S*/+"	"*S*/+"	"*S*/+"	"*S*/+"
6	(*S ast*)(+ +)	"+"	"*ast*/+"	"*S*/+"	"*S*/+"	"*S*/+"	"*S*/+"	"*S*/+"
7	(*S ast*)(+ *ast*)	"*ast*/+"	"*ast*"	"*S ast*^{4}/*ast*"	"*S ast*/*ast*"	"*S*/*ast*"	"*S ast*/*ast*"	"*S ast*/*ast*"
8	(*S ast*)(*S ast*^{4})	"*S*/+"	"*S ast*^{4}/*ast*"	lethal	lethal	lethal	lethal	lethal
9	(*S ast*)(*S ast*)	"*S*/+"	"*S ast*/*ast*"	lethal	lethal	lethal	lethal	lethal
10	(*S ast*)(*S* +)	"*S*/+"	"*S*/*ast*"	lethal	lethal	lethal	lethal	lethal

al (*S ast*)(+ *ast*)/*net S dp cl*, were mated to *al ho* males. F_1 *al* "*S*/+" offspring were then outcrossed to an *al ho* stock, and the F_1 larvae from this latter mating were then analyzed cytologically for the presence of *Dp-S*. It was found in this way that roughly one-half of the *al* "*S*/+" crossovers had carried the duplication; in other words, they were of the expected type, *al* (*S ast*)(*S* +). Similar methods had to be used for each of the remaining members of Group II.

The phenotypic effects of ten *Dp-S* derivatives when opposite +, *ast*, *S ast*^{4}, *S ast*, *S*, *Df-S3*, or *Df-S-der*, are shown in Table 7. Those types which have a normal eye or occasionally a very slightly roughened one are compared with *ast*/+, to which they are perhaps most similar. Although a deficiency for, and only for, the region present twice in *Dp-S* is not available, an approximation can be realized by comparing the effects opposite *Df-S-der*, and *Df-S3*, whose deficiencies are respectively smaller and larger than that region. From Table 7, it is apparent that these deficiencies act alike in all combinations shown. Two important combinations of these deficiencies are those with (+ *ast*)(*S* +) and (*S ast*)(+ +). In each case the effect is more nearly similar to that of *S*/+ than to *Df-S*/+. The effects of these two deficiencies when opposite (*S ast*)(+ *ast*) are compared with those of *S ast*/*ast*; they might equally well be compared with *ast*/*Df-S*.

It has already been reported that types analogous to double-Bar and quadruple-Bar have been obtained from *Dp-S* (Lewis, 1941). The triple form, since it arose from (+ *ast*)(+ *ast*) females by unequal crossing over, has the composition (+ *ast*)$_3$. The subscript, 3, here is used to indicate that the section present in parenthesis occurs three times in tandem repetition. Using a similar notation, the quintuplication, which was derived from homozygous (+ *ast*)$_3$ females, has the composition (+ *ast*)$_5$. A comparison of (+ *ast*)$_2$/(+ *ast*)$_2$ and (+ *ast*)$_3$/*ast* shows no detectable difference. That is, the eye looks normal in each case, and slight extra veins are occasionally present in each. No (detectable) position effect was found in a comparison of (+ *ast*)$_3$/(+ *ast*)$_3$ with (+ *ast*)$_5$/*ast*. Each of these latter types has an eye slightly larger than normal with bulging and somewhat disrupted facets; extra veins are also present in each to apparently the same extent.

Experimental evidence for the nature of the position effect in (+ *ast*)(+ *ast*) has been derived from a study of a translocation, *T(2;3) Dp-S*, obtained from X-radiation of (+ *ast*)(+ *ast*) males. A salivary gland chromosome analysis showed the presence of a reciprocal translocation, having one of its breaks within the duplication, just to the left of the 21E1–2 doublet of the right section of *Dp-S*, and the other break in heterochromatin of 3R. In the presence of a normal heterochromatin balance, the phenotypic effects of *T(2;3)Dp-S* are such as might be expected if it contained two doses of *ast*, just as the notation (+ *ast*)(+ *ast*), would indicate; thus, *T(2;3) Dp-S/S* is similar to, although more variable than, homozygous *ast*. However, when studied in the XXY female, this latter combination has a normal eye, or occasionally a slightly roughened one, typical of that of (+ *ast*)(+ *ast*)/*S*. In other words, the effects of (+ *ast*)(+ *ast*) appear to be the same whether the *S ast* regions are relatively close, as in *Dp-S*, or are widely separated, as in *T(2;3)Dp-S*. It is important to note here explicitly that the phenotypic effects of (+ *ast*)(+ *ast*), itself, are not changed by the addition of a Y chromosome.

X-Ray Induced Changes at the *S* and *ast* Loci

An analysis of X-ray induced changes at the *S* and *ast* loci offers another line of attack on the nature of the position effect at these loci. Such a study must be limited by the practical difficulty of obtaining a sample of such changes without employing their phenotypic effects as a basis for their detection. In the experiment shown in Table 6 it was possible to detect changes from wild type which closely resembled either *S* or *ast*. Neglecting deficiencies, a change of each type was detected—namely, $T(2;3)S^L$ and $T(2;4)ast^V$. To these aberrations should be added the *S* translocation of Muller (Muller and Painter, 1929), $T(2;3)S^M$, which likewise arose from wild type. The latter *S*, however, was detected by its dominant *S*-like effect opposite wild type. In spite of the differences in the bases for detection of S^L and S^M, the phenotypic effects of these changes, as far as could be determined by tests to various *S* and *ast* alleles, were identical with that of *S* itself. The addition of an extra Y chromosome did not alter the phenotype of either S^L or S^M. The recessive change, ast^V, was more similar to ast^1 than the other *ast* mutants in its effects on the venation and eyes.

Cytologically, S^M, S^L, and ast^V have in common a break immediately following the 21E1–2 doublet, as indicated in Fig. 1. In this connection, one other aberration obtained from wild type, *In(2)ho*, also has a break at this point. As already noted, *In(2)ho* does not appear to be associated with a change at the *S* or *ast* loci. Similarly, *T(Y; 2)21E* has no detectable effect at these loci. Its break in the second chromosome, however, is immediately to the left of he 21E1–2 doublet.

Only one change at the *S* and *ast* loci not associated with a demonstrable rearrangement in the salivary gland chromosomes occurred in the experiment shown in Table 6. This change has been given the tentative symbol, $S^X ast^X$, since, as shown below, it behaves genetically as though it involves a simultaneous change at each locus. $S^X ast^X$/+ flies show an occasional gap near the tip of the second longitudinal vein and have slightly smaller eyes than those of *S*/+ flies. It was found that if these slight

differences from *S* are due to modifiers, those modifiers must be very closely linked to $S^X\,ast^X$. Opposite the *ast* alleles, *ast,* ast^2, ast^3, and ast^4, $S^X\,ast^X$ closely resembled $S\,ast^+$.

On the assumption that $S^X\,ast^X$ represented merely a new dominant allel of *S* a search was made for "wild type" crossovers from $S^X\,ast^X/ast^4$ females similar to those obtained from S/ast^4 females. In this experiment females of composition *In(1)dl-49,* cm^2/+; $S^X\,ast^X/ast^4$ *ho*; *In*(3*LR*)*sep*/+, were individually mated to *al S ho/Cy, E–S* males. This mating produced, among 3,505 non-Curly offspring, a single female resembling the expected wild type class. The latter fly had normal (non-*ho*) wings, but the eyes were slightly smaller than those of *S*/+. Upon further testing, the crossover chromosome appeared to carry a very slight *ast* allele, ast^X. It is of course possible that ast^X arose in the above experiment as a mutation of ast^4. The following properties of ast^X were determined: ast^X/+ and ast^X/ast^X are wild type; S/ast^X closely resembles $S^X\,ast^X$/+ except that the former frequently, if not always, has normal venation; $S^X\,ast^X/ast^X$ has a somewhat smaller eye size than that of $S^X\,ast^X$/+. A salivary gland chromosome analysis of ast^X, as well as of $S^X\,ast^X$, failed to show any disturbance in the left end of the second chromosome. The possible significance of the $S^X\,ast^X$ change will be taken up in the discussion of this paper.

It will be recalled that the spontaneous *S* duplication, *Dp-S*, has a break immediately to the right of the 21E1–2 doublet (of the left section of the duplication), similar to that found in S^L, S^M, and ast^V. Phenotypic studies of *Dp-S* suggested that the *ast* mutant originally present in the left section of the repeat acts as though it had reverted to wild type. With this result particularly in mind, *al ast ho/net ast dp cl* males were X-radiated (6,500 r units) and mated to *S/Cy, E-S* females. "Reversions" of *ast* were looked for among the F_1 flies. The following three changes closely resembling reversions of *ast* to ast^+ were found, all of which were fertile:

T(2;3)ast^{rv1}*—Translocation (2;3) asteroid reverted-1*—Lewis 1942. The translocation carries the mutants *al* and *ho*. Phenotypically, ast^{rv1} is inseparable from wild type opposite +, *ast,* ast^3, ast^4, $S\,ast^+$, *S ast,* $S\,ast^4$, *E–S*, and ast^V. The homozygote is lethal. However, ast^{rv1}/Df-*S*4 is viable and resembles *S*/+ rather than *Df-S4*/+. The eye is slightly less rough in either ast^{rv1}/S^M or ast^{rv1}/S^L than it is in S^M/+ or S^L/+. At least three breaks are involved in this complex rearrangement. Chromosome 2L is broken just to the right of 21E1–2; chromosome 3L has a break just after the heavy dotted 68C2 band and 3R has a break which probably just precedes 88D9 (Bridges' 1935 map). The new rearrangement is in the form of cyclical exchange of tips. Thus, 2L tip replaces the tip of 3R, which in turn replaces the 3L tip, which in turn replaces the tip of 2L.

In(2L)ast^{rv2}*—Inversion (2L) asteriod-reverted-2*—Lewis 1942. The inverted chromosome also carries the mutants, *al* and *ho*. Phenotypically, ast^{rv2} is identical with ast^{rv1}, including its effects opposite *Df-S4* and S^L. S^M/ast^{rv2} overlaps wild type. ast^{rv1}/ast^{rv2} is wild type. A preliminary salivary gland chromosome analysis showed a break immediately following the 21E1–2 doublet and one in section 31, the section between then being inverted.

T(2;3)ast^{rv3}—Translocation (2;3) ast^{rv3}—Lewis 1942. The translocation was associated at the time of its origin with the mutants, *net*, *dp*, and *cl*. The homozygote and *ast^{rv3}/Df-S4* are lethal. Incomplete phenotypic studies indicate that opposite *S*, *S*, *ast*, *S ast*^{4}, and *S^L*, *ast^{rv3}* produces a slightly rougher eye than that characteristic of *S/+*. *ast^{rv3}/ast*^{3} and *ast^{rv3}/E–S* are wild type. The rearrangement involves a reciprocal exchange of the extreme tips of chromosomes 2L and 3L, with breaks just to the right of 21E1–2 and to the left or right of 61C1. In its new location this latter region of chromosome 3L assumes a heterochromatic character.

Again, as in *Dp-S*, apparent reverse mutation of *ast* was associated with a chromosomal rearrangement in which the 21E1–2 doublet was transferred to a new position. As yet, no spontaneous or induced reverse change of *ast* to *ast*$^+$ unaccompanied by a chromosomal rearrangement has been detected.

The above results further support the conclusion that *ast* as well as *S* is included within the confines of the 21E1–2 doublet, for it is this doublet and not the material to the right of it which has undergone rearrangement in both *Dp-S* and the X-ray induced asteroid-reverted changes. That is, in *Dp-S* the material to the right of 21E1–2, since it not present twice, is in its normal position following the 21E1–2 doublet of the right section of the repeat.

From the same X-radiation experiment which produced the asteroid-reverted changes, a complete suppressor of *S* was detected as a single (sterile) male with normal eyes and wings. Several changes resembling partial reversion of *ast* were also detected. Among those of the latter which were analyzed, two were found to be due to permanent changes. They were complex rearrangements in which the 21E region was normal, but each had in common a break in or close to section 22D and had that section translocated to heterochromatin of chromosome 3. It is possible that the partial suppressing effect on *S/ast* of these aberrations is related to that shown by the double deficiency *In(2L)Cyt* or *Su-S*, which has one of tits deficiencies in 22D.

DISCUSSION

A plausible interpretation of the *S* and *ast* loci can be developed by assuming that they have resulted from duplication of an ancestral locus, that duplication now being established in the species. This notion is chiefly based on the finding that *S* and, very probably, *ast* are included in the 21E1–2 doublet structure of the salivary gland chromosomes. The possibility that such structures might represent instances of duplication of a single band, or short section, was first pointed out by Bridges (1936). It is interesting to note that Bridges chose the 21E1–2 doublet as a characteristic example of this type of duplication.

That the doublets involve two discrete bands was shown experimentally by Bridges et al. (1936) in an analysis of the Notopleural deficiency. In the case the break points of the deficiency occurred between the halves of two doublets. Bridges (Drosophila Information Service **9**) has reported other instances, chiefly spontaneous, where

breaks have separated the halves of a doublet. Similarly, Metz (1937) has found that some of the small deficiencies which occur in stocks of *Sciara ocellaris* involve the loss of only one band of a doublet structure. Metz (1938) also holds to the view that doublets have arisen by a process of duplication and, on this basis, that these "deficiencies" are either the original unduplicated condition or are secondary losses from duplications already established in the species.

Small, intrachromosomal duplications have been given the convenient name "repeat" by Bridges. The doublet structures fall in the category of "tandem repeats" in which the duplicated section immediately follows (or precedes) the original section. The tandem repeat may be either "direct" or "reverse." Thus, if the section present twice in the repeat is represented by the letters, CD, then a direct tandem repeat can be written a CDCD, and a reverse tandem repeat as CDDC or DCCD. The direct repeats such as Bar, or the Star Duplication, are unstable in the sense that they can give rise by unequal crossing over to the unduplicated condition, CD, or to a complementary triplicated form, CDCDCD. Reverse repeats, however, are expected to be stable in the sense that unequal crossing over (if it occurs at all) should result only in a dicentric chromatid, which will be lost to the polar bodies, and an acentric fragment.

The problem arises as to which of these two categories of repeats the 21E1–2 doublet may be assumed to belong. This cannot be decided from cytological evidence alone, since only the duplication of one disc is involved. *A priori*, only the stable or reverse type would be expected to become established in the species. This is supported by the fact that all of the tandem repeats, which have been described for the normal salivary gland chromosomes of *D. melanogaster* and in which the sequence can be determined, are of the reverse type. Moreover, if the 21E1–2 doublet were a direct repeat, then it might be anticipated that some of the changes observed at either the *S* or *ast* loci would represent either the unduplicated or the triplicated forms. However, no cytological changes whatsoever in the 21E1–2 doublet were apparent in any of the mutants at these loci or in any of the analyzed crossovers between these loci.

Assuming from the above considerations that the 21E1–2 doublet is a tandem reverse repeat, we can draw the genetic analogy that *S* and *ast* are reversely repeated loci. There are at least two methods by which reverse repeats may offer a basis for producing changes which resemble mutation but which are due to crossing over in the repeat. Firstly, genetic differences in the duplicated section of a naturally occurring repeat may arise within a stock or between different stocks. Such differences need not be detectable, phenotypically, since the repeated section is present in four doses. Applied to the *S* and *ast* loci, this mechanism suggests that such differences as are observed between $S+/+\ ast$ and $S\ ast/++$, or between $S+/+\ ast^4$ and $S\ ast^4/++$, may be due, not to position effect, but to unknown genetic differences in what were thought to be normal alleles. However, this is very unlikely in that the "wild type" crossovers derived from either $S+/+\ ast$ or $S+/+\ ast^4$ females are identical not only with each other but also with normal chromosomes from unrelated stocks, even in the critical test opposite $S\ ast$ or $S\ ast^4$. In particular, the critical test held in an

experiment in which the "wild type" crossover and $S\ ast^4$ were each recovered in the same experiment from $S+/+\ ast^4$ females.

Secondly, consider a tandem repeat in which the two halves are no longer exactly identical, genetically, and assume that an inversion of the repeat has been produced. The original repeat may be written CDD′C′, where the primes indicate genetic differences between the repeated regions. For simplicity, the inversion may be assumed to involve only the repeat itself and may be represented as C′D′DC. It is apparent that this simple case has a highly important consequence—namely, that the inversion cannot be detected cytologically. Crossing over in CDD′C′/C′D′DC heterozygotes will give rise to genetically different products. For example, if crossing over occurs between D and D′, then these products are CDDC and C′D′D′C′. This mechanism is suggested as a possible basis for the behavior of the X-ray induced *S*-like change, $S^X\ ast^X$, which from linkage studies appeared to involve a change at the *ast* locus as well as the *S* locus. Here it is necessary to assume that the X-rays induced an inversion of just the repeated region, since $S^X\ ast^X$ was normal, cytologically. The new sequence may be written ast^+S^+, and the *S*-like effect may then be attributed to a position effect rather than to a point mutation. On this basis, the "mutant" ast^X, recovered from $S^X\ ast^X/++$ females may be interpreted as a duplication for the *S* locus and a deficiency for the *ast* locus—i.e., its composition may be written S^+S^+. This mechanism does not seem applicable to the spontaneous *S* and *ast* mutants, since the crossover corresponding to S^+S^+ does not differ detectably from $S^+\ ast^+$; furthermore, S^+ could not be here assumed to be identical with ast^+, for then the inversion itself would accomplish nothing new.

The concept of *S* and *ast* being repeated loci is also useful in that it relates, by analogy, the position effect observed at these loci to that occurring at a known repeat region—namely, the Bar Duplication. As a result of the cytological finding of Bridges (1936) and Muller, et al. (1936) that Bar is a duplication, the position effect demonstrated by Sturtevant (1925) may now be stated as depending on the way in which four, rather than two, homologous sections are distributed between the two homologs, thus, equal distribution of the four doses of the Bar region results in a larger eye than when three doses are present in one homolog and the fourth in the other. The position effect, here, would appear to extend over a distance at least as great as the length of the duplication, or perhaps as great as the length of triplicated region. This is remarkable in view of the fact that other types of rearrangements which involve only euchromatic portions of the chromosomes and which seem to be accompanied by position effects do not show this spreading effect.

It is suggested that this spreading effect is due not alone to the closeness of the two elements in Bar, or the three elements in *BB*, but likewise to the homology of those elements. This possible role of repeats in producing pronounced position effects will be discussed more fully in connection with position effects at the *S* and *ast* loci.

Several methods of attack were used in studying position effect at these loci. Firstly, their effective position could be altered by allelic substitutions alone. Thus, no demonstrable chromosomal aberrations appeared to accompany *S*, *ast*, ast^4, $S\ ast^4$, or

S ast; yet, a striking difference exists (1) between *S/ast* and *S ast*/+, and (2) between *S/ast*^{4} and *S ast*^{4}/+. Similarly, *S ast/ast*^{4} is genetically equivalent to *S ast*^{4}*/ast;* yet the former has a larger eye and less interrupted wing venation than has the latter. It may be emphasized that in comparisons (1) and (2) there is an important qualitative difference in that neither *S ast*/+ nor *S ast*^{4}/+ has ever been observed to overlap the small, roughened eye types characteristic of either *S/ast* or *S/ast*^{4}; whereas, *S ast/ast*^{4} and *S ast*^{4}*/ast* may occasionally resemble one another.

This position effect at the *S* and *ast* loci may be stated more analytically. The viable, diploid combinations of *S*, *ast*^{4}, *ast*, *S ast*^{4}, *S ast*, and wild type can be arranged in the following series (the symbol, >, means "has a larger and less roughened eye than," while that symbol, =, means "is phenotypically approximately equivalent to"): +/+ = *ast*^{4}/+ > *ast*/+ > *ast*^{4}*/ast*^{4} > *S ast*^{4}/+ = *S ast*/+ = *S*/+ > *ast/ast*^{4} > *S ast*^{4}*/ast*^{4} > *S ast/ast*^{4} > *S/ast*^{4} = *ast/ast* > *S ast*^{4}*/ast* > *S ast/ast* > *S/ast*. From this seriation it may seen that if (n) represents any one of the six combinations of these alleles, then the order may consistently be written: $+/n > ast^4/n > ast/n > S\ ast^4/n \geqq S\ ast/n \geqq S/n$. Here, the order, *S ast*^{4}*/n* > *S ast/n* > *S/n*, is determinate only when (n) is *ast* or *ast*^{4}.

The above analysis shows that the order of effectiveness of the *ast* alleles is different, depending on whether they are adjacent to S^+ on the one hand or to *S* on the other. Thus, when these alleles are adjacent to S^+, their effective order may be written: + > *ast*^{4} > *ast*; while adjacent to *S*, it becomes: *ast*^{4} > *ast* > +. The difference between *S ast*^{4}*/ast* and *S ast/ast*^{4} leads to the conclusion that more than just a change in the order of effectiveness of the *ast* alleles is involved; however, the above change of order is another way of stating the pronounced position effects detected in comparisons (1) and (2). The problem arises as to which, if any, of these six haploid combinations of *S* and *ast* alleles does not exhibit a (detectable) position effect. (In this discussion the term, position effect, when applied to the haploid chromosome, is meant to imply an interaction between two, or more, neighboring genes or their products, which leads to a different phenotype than would arise if the genes were widely separated from one another.) As yet, no solution to this fundamental problem is available, since it has not been possible to study the effects of one locus independently of the other. Although none of the available aberrations which have breaks in the vicinity of *S* and *ast* appear to separate these loci, or to involve a loss of one locus and not the other, the effects of these aberrations do have a bearing on the above problem. The study of these aberrations constitutes a second attack on position effect at these loci.

Exclusive of the *S* deficiencies, the available rearrangements involving the *S* region exhibit two significant features. First, only two of these, *T(Y;2)21E* and *T(2;3)Dp-S*, have a break just preceding the 21E1–2 doublet. It is likely that in the latter as well as the former translocation, there is no detectable effect on the immediately adjacent *S* and *ast* alleles. On the other hand, the remaining aberrations have a break immediately following the 21E1–2 doublet, and all of these, except for *In(2)ho*, are associated with a pronounced effect at these loci. This result parallels that found with certain other loci. Thus, among those rearrangements, which have breaks on either side of the scute

locus and which do not involve heterochromatin, only those having a break just to the right of the locus are accompanied by a scute-like change. (For a discussion of this case and its bearing on the evidence for position effect accompanying euchromatic exchanges, see Muller, 1941.) The Bar "locus" is another example. Although little material is as yet available, the wholly euchromatic exchanges having a "Bar" effect, including Bar itself, consistently appear to have a break just preceding the 16A1–2 doublet, which structure is known, particularly from studies of Griffen (1941) and Sutton (1943) to be associated with the Bar effect.

The second important feature is revealed by a study of the non-variegated rearrangements involving the *S* and *ast* regions—namely, all of the available aberrations except for *T(2;4)ast*^{V} and *T(2;3)Dp-S*, whose effects are changed by the addition of a Y chromosome. The former types are associated either with no change whatsoever at the *S* and *ast* loci, as in the case of *In(2)ho* and *T(Y;2)21E,* or they are accompanied by an identical type of change, the type itself depending on whether the rearrangement occurred in wild type or in *ast*. Thus, the dominant *S*-like changes, *S*^{M} and *S*^{L}, are the only non-variegated aberrations, originating from wild type, associated with a change at the *S* and *ast* loci. In spite of their completely independent origins, and the different bases on which they were detected, these two changes are indistinguishable from each other in all combinations studied. Similarly, the rearrangements which arose from *ast* and which are accompanied by a change at this locus act like complete reversions of *ast* to wild type. That is, partial reversions, although detectable in this case, were not recovered. In the above category belong the X-ray induced changes, *ast*^{rv1}, *ast*^{rv2}, and *ast*^{rv3}. To these may be added the spontaneous *Dp-S*, which appears to have a change to wild type in the action of the *ast* allele in the left section of the repeat.

The above *S*-like and reverted-like *ast* changes can be related on the basis of a possible identical association of the 21E1–2 doublet region in *S*^{L} and *ast*^{rv1}. Thus, these translocations exhibit a remarkable coincidence in that they apparently have identical breaks in 21E and 88D. Whether the 21E1–2 doublet is intimately associated with 88D in *S*^{L}, as well as in *ast*^{rv1}, is not determinate, owing to the complication of an additional break in heterochromatin in the *S*^{L} rearrangement. However, it is likely that there is a causal rather than a chance basis for this coincidence of breaks.

From these considerations, it may be surmized that the same rearrangement which gave rise to an *S* change from wild type, would, if the original constitution had been *ast*, have given rise to a reversion to wild type. The behavior, if the original composition had been *S*, is conjectural. In this connection, however, extensive experiments on the X-radiation of *S* (unpublished) have failed to give reversions of *S* to wild type. Unfortunately, *Dp-S*, which promised to be the most suitable material for attacking this problem, has so far failed to give crossing over between the *ast* locus of the left section and the break point of the duplication. The effects of *Dp-S* combinations in which the left section carries *S ast*, instead of *ast*, tend to indicate that the effects of that section are similar to those of *S ast* (or *S*).

The third method of attack on the *S-ast* position effect makes use of *Dp-S* to obtain combinations equal on a quantitative genetic basis, but involving differences in the distribution of the *S* and *ast* alleles between the two homologs or between the two sections of the repeat. Inspection of Table 7 shows that a great many combinations of this type are lethal; such as, (*S ast*)(*S ast*)/*S* and (*S ast*)(*S* +)/*S ast*. In many cases, no difference can be detected between the members—that is, (+ *ast*)(*S* +) cannot distinguished from (*S ast*)(+ +) in a great many combinations studied. In certain instances, very sharp differences exist, as in each of the seven comparisons involving the equivalent types, (+ *ast*)(*S ast*) and (*S ast*)(+ *ast*). Superficially, such differences appear to indicate an interaction between the two sections of the repeat—that is, a position effect extending across the repeated regions. A much more likely and consistent assumption is that in those duplication derivatives having S^+ *ast* in their left section, the rearrangement has induced a position effect on these loci causing them to act like S^+ ast^+. There are three reasons for making this assumption. Firstly, it satisfactorily explains the effects observed for each of the *Dp-S* derivatives which carry *ast* in their left section, without introducing the additional assumption of position effects extending from the *S* and *ast* loci in one section to their duplicates in the other section. Secondly, three other aberrations, ast^{rv1}, ast^{rv2}, and ast^{rv3}, have just such a reverse type of change in the originally present *ast* mutant, and, as in *Dp-S*, this change appears to be a position effect arising from the new association of the 21E1–2 doublet. Finally, the study of *T(2;3)Dp-S* indicated that, at least in the case of $(+\ ast)_2$, the effects of these two sections are the same whether in close juxtaposition, as in the duplication itself, or are widely separated, as in the translocation.

These conclusions do not necessarily imply that the behavior of *Dp-S* and the *B* duplication are at variance. For it now seems clear that the *B* effect is induced primarily by the new association of the 16A1–2 doublet of the right section of the repeat, rather than to mere duplication of that region, or solely to a position effect between the two sections of the duplication. The difference between *B*/*B* and *B B*/+ indicates that the latter type of position effect does occur; however, it is not clear as to which combination, *B* or *B B*, has the stronger effect in bringing about that difference. Unfortunately, the comparison of $(+\ ast)_2/(+\ ast)_2$ with $(+\ ast)_3/ast$, in which no difference was detected, is not critical, for in neither of these cases does the increase in dosage of the *S-ast* region result in an appreciable change of the eye from normal.

Sturtevant (1925) and Muller (1941) have suggested that position effect may be related to the phenomenon of somatic pairing found in the Diptera. This suggestion appears particularly plausible when applied to tandem repeats, since the forces of somatic pairing bring about a very intimate pairing of the duplicated sections within a homolog, and, by so doing, should increase the possibility of the genes in those regions, or the genic products, interacting with one another or competing in a gene reaction. From these considerations, the pronounced position effect existing between the *S* and *ast* loci has been interpreted as a function of their repeat nature and the very short distance between them, both genetically and cytologically speaking. A mutant at one of these loci is viewed as a change in the gene which alters the forces of

somatic pairing normally existing between those loci. This possibility is of interest in that it is not necessary to assume that the immediate gene product is altered. The rearrangements which are associated with changes at these loci are likewise considered as upsetting the normal somatic pairing relations between them. It appears that certain relatively nonspecific changes in the material just to the right of these loci can cause a considerable change in the position effect normally existing between them.

In general, it may well be found that rearrangements, at least of the wholly euchromatic type, which are associated with position effects, either have a naturally occurring repeat at the basis of those effects or are themselves repeats. *Dp-S* and *B* may be considered as complex types, since they involve a duplication of a doublet structure. The doublets, 21E1–2 and 16A1–2, appear to be responsible for the strong position effects associated with *Dp-S* and *B*, respectively. Superimposed on this position effect is another one which presumably arises from the close juxtaposition of additional doublet regions—a phenomenon as yet detectable only in the case of *B* and/or *B B*.

A repeat interpretation was suggested by Oliver (1940) for the behavior of two "alleles" of lozenge, lz^s and lz^g, in *D. melanogaster*. This case appears at present to be in many ways similar to that reported here at the *S* and *ast* loci. The alleles, yellow and reddish-alpha, in *D. virilis*, analyzed by Demerec (1928), may also be interpreted as duplicate loci, but it is also clear that this alone will not account for the remarkable behavior of the reddish-alpha character. If doublet structures are repeats, as the evidence thus far indicates, then, judging from their widespread occurrence in the salivary gland chromosomes of Drosophila, it is likely that other multiple allelic series may be resolved into duplicate loci which act, by reason of a position effect, as a developmental unit.

SUMMARY

Two loci, Star and asteroid, in the second chromosome of *D. melanogaster* were found to be extremely closely linked, the normal distance between them being estimated as 0.02 map unit.

These loci exhibit a position effect which can be detected solely by varying their genetic composition.

A correlation of genetic and cytological analyses gave evidence that *S* and *ast* are included in the 21E1–2 doublet structure of the salivary gland chromosomes. It also gave pertinent data on the location of other mutants in the vicinity of these loci.

Position effect at the *S* and *ast* loci was also studied by means of chromosomal rearrangements which had breaks in the neighborhood of these loci. A special case was the study of a spontaneous tandem duplication of the Bar type—namely, the Star Duplication, *Dp-S*.

From certain cytological considerations, *S* and *ast* may be interpreted as repeated loci which have become established in the species. The possibility is discussed that repeats have special potentialities for showing pronounced position effects.

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THE PSEUDOALLELISM OF WHITE AND APRICOT IN *DROSOPHILA MELANOGASTER**

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The classical example of multiple allelism is the series of eye-color mutants at the white (*w*) locus in *Drosophila melanogaster*. The alternative interpretation of this series, namely, that it is made up of "pseudoalleles," or closely linked genes with similar effects, has usually been considered ruled out by two kinds of evidence. In the first place early attempts to resolve the series by crossing over failed in spite of numerous tests involving most of the mutants available at the time.[1–4] Secondly, a heterozygote for two different mutant genes of the series does not have the phenotype expected for nonallelic genes, namely, wild type (or red) eye color, but instead has a mutant eye color which is usually intermediate between the colors of the two respective homozygotes. In recent years, however, several cases have been found in which nonallelic genes give a positive phenotypic test for allelism by virtue of a position effect.[5–7] In such cases, which have been

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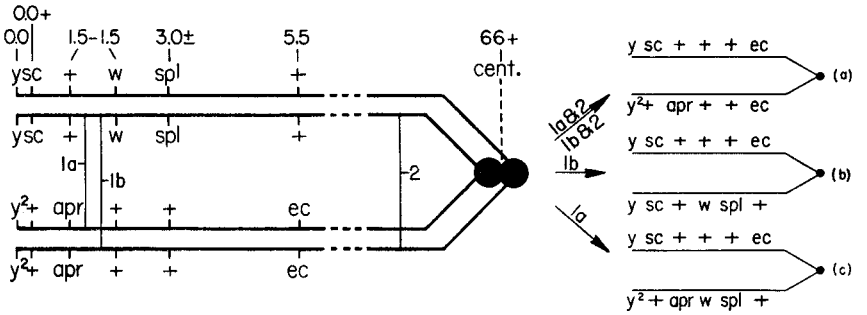

Figure 1. The diagram on the left is the attached-X constitution of Type A from which the majority of crossovers between *apr* and *w* were detected. The types of exchange which give rise to such crossovers are labeled: 1*a* (reciprocal) and 1*b* (nonreciprocal). On the right are shown the resultant three classes of crossover-containing attached-*X*'s. An additional exchange (exchange 2) between the locus of *ec* and the centromere (cent.) is required for the production of class (*a*).

termed "position pseudoalleles,"[7] mutant genes at the different loci (say, *a* and *b*) give a mutant phenotype in the $a+/+b$ heterozygote, but a wild type, or more nearly wild type, phenotype in the $ab/++$ heterozygote.

With the above considerations in mind and with the aid of more adequate techniques for studying crossing over than were available in the early studies, the white gene and its "allele," apricot, have been reinvestigated. This paper presents the evidence that these two genes occupy separate loci and that they constitute another example of position pseudoallelism. In what follows, the apricot gene, formerly symbolized as w^a will be designated by a new symbol, namely, *apr*.

In order to investigate the possibility of crossing over between *w* and *apr*, females with attached-X chromosomes were employed so that the two complementary products from any such crossing over would sometimes be recoverable simultaneously in a single individual. The first step was the construction of attached-X's heterozygous for *w*, *apr*, and suitable marker genes on either side of the *w* locus (at 1.5 in the X chromosome). The markers chosen were yellow (*y*, at 0.0; body and bristle color); yellow-2 (y^2; allelic and dominant to *y*; with black instead of yellow bristles); scute (*sc*, at 0.0+; missing bristles); split (*spl*, at 3.0±; abnormal bristles and rough eyes); and echinus (*ec*, at 5.5; enlarged facets).[8,9] By the standard method[10] of using attached-X triploid females, an attached-X diploid female of the following or "Type-A" constitution was obtained (see Fig. 1): y^2 *apr ec*/*y sc w spl*. Such a female is phenotypically yellow-2 and "dilute-apricot"; the latter is a pinkish yellow eye color which is often distinguishable from the "apricot" or yellowish pink color of homozygous *apr*. A Type-A female produces predominantly three phenotypic classes

[8]Bridges, C. B. and Brehme, K. S., *Publ. Carnegie Inst. Wash.*, **552**, 1–257 (1944).
[9]Further descriptions and references to the original literature may be found in the work of Bridges and Brehme.[8]
[10]Emerson, S. and Beadle, G. W., *Z. indukt. Abstam. u-Vererb-Lehre*, **65**, 129–140 (1933).

of attached-X progeny; namely, (1) yellow-2 dilute-apricot, (2) yellow-2 apricot echinus, and (3) yellow scute white split. Females belonging to Class 1 prove to be primarily of Type-A constitution like the mother. Classes 2 and 3 are the diagnostic ones for a Type-A constitution since they correspond to the two kinds of homozygotes with respect to the original linked sets of mutant genes in the mother, namely, y^2 *apr ec* and *y sc w spl*, respectively. Such homozygous classes result from certain types of exchange(s) in the region between the *ec* locus and the centromere (see, e.g., exchange numbered 2 in Fig. 1). Since the latter region is over 50 map units in length, a single Type-A attached-X female usually produces numerous daughters belonging to each of the above homozygous classes; conversely, the constitution of an individual female with respect to the above group of genes is usually readily determined from inspection of the two principal classes of homozygous daughters. Although certain types of exchange within the *y – ec* region lead to constitutions and phenotypic classes other than those discussed above, the relatively short genetic length of this region makes such exceptions to the above rules either infrequent or absent in the progeny of a single female.

Type-A attached-X females were next made heterozygous for chromosomal rearrangements in the second and third chromosomes, since such an autosomal constitution is known[11] to be very effective in causing an increase in crossing over in the X chromosome. For the second chromosome the two Curly (*Cy*) inversions[9] were chosen (marked by the dominant wing-mutant, *Cy*).[12] For the third chromosome a new complex rearrangement of X-ray origin was chosen. This rearrangement involves five breakage points[13] distributed throughout the third chromosome and is inseparably associated with a dominant bithorax-like change, termed Ultrabithorax-130 (Ubx^{130}). The Ubx^{130} heterozygote is readily classified on the basis of an enlarged distal segment of the haltere (while the homozygote is lethal).

The first indication of crossing over between *w* and *apr* came in the offspring of a preliminary mating, Mating 1, in which the parental yellow-2 dilute-apricot females were heterozygous for only the Ubx^{130} rearrangement. All of such females were descendants of the original Type-A female described above. The parental males in Mating 1 were heterozygous for a *Cy* chromosome which was known to carry both of the *Cy* inversions. In 19 cultures of this mating the individually mated parental females proved to have had a Type-A constitution. One of these cultures produced a single yellow-2 red echinus female. This female in turn produced 23 daughters, of which 12 were phenotypically like the mother, while 6 were yellow scute red echinus and 5 were yellow-2 apricot echinus. This is the result to be expected if the original red-eyed fly had the following constitution: *y sc ec*/y^2 *apr ec*. As the result of detachment of the attached-X's in one of the above six homozygous *y sc ec* females, *y sc*

[11] Steinberg, A. G., *Genetics*, **21**, 615–624 (1936).

[12] The presence of the *Cy* inversion in the right arm of this chromosome was not always insured in these experiments since it carried no dominant marker.

[13] The new rearrangement in the third chromosome is as follows: 3L tip to 61A-C/96A to 93B/89D-E to centromere to 74/61A-C to 74/89D-E to 93B/96A to tip of 3R.

ec males were obtained and these also proved to have red eyes. The *y sc ec* chromosome was then tested against a known deficiency for the white gene (Notch-8)[9] and the resultant females also had the wild-type eye color. Thus, the *y sc ec* chromosome appears to be completely wild type with respect to the white region. The simplest interpretation of the origin of this chromosome is that it represents a wild-type crossover chromatid which resulted from an exchange between the *w* locus and an *apr* locus lying to the left of *w* (see exchange la or lb of Fig. 1). The presence of the y^2 *apr ec* chromosome in the original red-eyed female is readily accounted for by assuming a nonreciprocal exchange (see exchange 2 of Fig. 1) between the *ec* locus and the centromere.

A search for additional red-eyed flies was continued among the progeny of the second and final type of mating, Mating 2, in which the parental yellow-2 dilute-apricot females were heterozygous for *Cy* as well as Ubx^{130}. There were four groups of such parental females. The first group was selected from among the daughters of the 19 Type-A cultures of Mating 1, described above; the second group was selected from among the daughters of only those females belonging to the first group which proved to be of Type-A constitution; while the third and fourth groups were similarly selected from the daughters of the second and third groups, respectively. The parental females of Mating 2 were in every case individually mated to males carrying the sex-linked mutant genes, y^{3ld}, sc^8, *apr*, *B* (Bar) and lz^3 (lozenge-spectacled).[9] The *B* mutant served here to identify any free-X daughters, which result rarely from detachment of the attached-X's in the mother. The nearly white (*apr* lz^3) eye color of this type of male facilitated the search for red-eyed flies among the progeny as a whole. (The yellow body color of this type of male served a similar purpose with respect to any nonyellow progeny, which might result from crossing over between *y* and y^2, but none of these was found.)

Among 897 fertile cultures[14] of Mating 2, a total of 794 cultures proved to have had parental females which were either of Type-A constitution or were heterozygous for at least *y*, *sc* and *spl*, as well as *apr* and *w*; while the remaining 103 cultures had to be discarded either because of too few progeny or because of an insufficiently marked maternal constitution. From the above 794 adequately constituted cultures, there was a total of 12 independent occurrences of red-eyed females among an estimated 40,100 attached-X offspring. Ten of these 12 red-eyed females came from Type-A mothers. As shown in Table 1, these ten were distributed among three phenotypic classes with respect to the sex-linked marker genes. Each of the two yellow-2 red echinus flies of Class (a) proved on progeny testing to have had attached-X's made up of a *y sc ec* or wild-type crossover chromosome and a y^2 *apr ec* chromosome, as in the first case of a red-eyed fly from Mating 1, above. Of the four yellow scute red females of Class (b), one died and each of the others proved to have had a *y sc ec* chromosome associated with a *y sc w spl* chromosome. Class (b) individuals are those expected following a single nonreciprocal exchange (see exchange lb of Fig. 1) between *apr* and *w*. The four

[14]The author is indebted for technical assistance in the preparation of these matings to W. Gencarella.

Table 1 The number and classes of red-eyed daughters of y^2 *apr ec*/*y sc w spl*, or "Type-A," attached-X females (heterozygous for autosomal rearrangements) from Mating 2. The classes correspond as lettered to those shown in Figure 1.

Class	Phenotype	Corresponding Genotype	Number of Flies
(a)	yellow-2 echinus	$\frac{y\ sc\ +\ +\ +\ ec}{y^2\ +\ apr\ +\ +\ ec}$	2
(b)	yellow scute	$\frac{y\ sc\ +\ +\ +\ ec}{y\ sc\ +\ w\ spl\ +}$	4
(c)	yellow-2	$\frac{y\ sc\ +\ +\ +\ ec}{y^2\ +\ apr\ w\ spl\ +}$	4

yellow-2 females of Class (c) each produced the following principal classes of homozygous attached-X daughters: yellow scute red echinus and yellow-2 white split. Each of the four females of Class (c) must therefore have represented a case of simultaneous recovery of a *y sc ec* wild-type crossover chromosome and the complementary double mutant, or y^2 *apr w spl*, crossover chromosome. Of the two remaining red-eyed flies among the above group of 12, one was yellow-2 in phenotype and arose from a y^2 *apr*/*y sc w spl* mother (identical in constitution with Type A except for the loss of *ec*). The principal two classes of homozygous daughters of this red-eyed female were phenotypically yellow scute red and yellow-2 white split. The other red-eyed fly, also of yellow-2 phenotype, came from a y^2 *w spl*/*y sc apr ec* mother (identical with Type A except for an interchanging of the markers distal to *apr*). The principal two classes of homozygous daughters of this latter red-eyed female were yellow-2 red echinus and yellow scute white split in phenotype. Thus, each of the above two red-eyed flies must have represented a case in which both crossover chromatids from an exchange between *apr* and *w* had been recovered simultaneously.

The above analysis of the 12 red-eyed attached-X females from Mating 2 actually provided a total of 18 crossovers between *apr* and *w*; that is, each of the 12 carried a wild-type crossover while six of them carried the complementary double mutant crossover, as well. The observed amount of crossing over was 0.03% (12/40,100 ±). This value probably overestimates the standard map distance between *apr* and *w*, since the observed total amount of crossing over in the whole region from *y* to *spl* in these experiments was calculated to be 11.5% (based on a complete phenotypic analysis of 11,985 attached-X progeny from a large sample of Type-A cultures of Mating 2), or nearly four times the standard value of 3.0 ±%.[15] This increase, however, was not distributed uniformly over the *y* − *spl* region. Thus, the *y* − *apr* region was increased from 1.5% to 8.9% or nearly sixfold;[16] while the *w* − *spl* region increased from 1.5± to 2.6% or about 1.7 times. By assuming that crossing over in the *apr* − *w* region was increased by a factor lying within these limits, the standard map distance between *apr*

[15]No definite case of a double crossover in the *y* − *spl* region was observed.
[16]Two verified crossovers between *y* and *sc* were detected as y^2 *sc w spl* phenotypes.

and *w* can be calculated as being within the range of 1/6–1/1.7 of the observed value of 0.03 unit, or roughly 0.005–0.02.

Indirect proof was obtained above for the occurrence of six double mutant, or *apr w*, crossovers from Mating 2. In none of these cases, however, has it been possible to distinguish, phenotypically, *apr w* from *w*. Thus, the heterozygote, *apr w*/+ +, invariably has red eyes in striking contrast to the pinkish yellow eye color of *apr* +/+ *w* heterozygotes; while the *apr w* homozygote is like that of *w* in having white eyes. In addition, two *apr w* detachment males were obtained from one of the females belonging to Class (c) of Table 1. Such males also have white eyes. Similarly, *apr w*/+ *w* females have white eyes, and *apr w*/*apr* + females have pinkish yellow eyes like those of + *w*/*apr* +.

An attempt was next made to obtain direct proof for the presence of *apr* in the double mutant combination by searching among the progeny of *apr w*/+ + females for a dilute-apricot phenotype. For this purpose a third type of mating, Mating 3, was employed. The parental females in this case were phenotypically yellow-2 red (and were in some cases heterozygous for *Cy* and/or Ubx^{130}). Each was derived as either a second or third generation daughter of one of the original four yellow-2 red females from Mating 2 (the same one, in fact, from which the above *apr w* detachments were ultimately derived). The parental females of Mating 3 were individually mated to males which carried wild-type X chromosomes (and were heterozygous for Ubx^{130}). To forestall, in so far as possible, any contamination of this mating with dilute-apricot attached-X females, all cultures containing such females had been purposely destroyed before Mating 3 was initiated, except for one Type-A culture in which, however, all of the flies were homozygous for the Gowen gene *(c3G)*.[9] Since this gene results in complete suppression of crossing over in the female, Type-A females from this latter type of culture are readily recognizable because they breed true for the Type-A constitution and are nearly sterile. From one culture of Mating 3 in which the parental female proved to have had the constitution, *y sc ec*/y^2 *apr w spl*, a single dilute-apricot (yellow-2) female (which also happened to carry *Cy* and Ubx^{130}) arose among the otherwise red- or white-eyed progeny. The principal classes of homozygous daughters of this latter female were yellow-2 apricot echinus and yellow scute white split in phenotype; thus, the maternal constitution in this case must have been y^2 *apr ec*/*y sc w spl*, or Type A. In this case there must have been simultaneous recovery of the *w* + and *apr* + crossover chromatids that are to be expected following an exchange between the *apr* and *w* loci in the parental *apr w*/+ + female.

DISCUSSION

The above crossing-over studies have shown that at least two loci are at the basis of the "multiple allelic" series of white mutants. Only brief consideration can be given here to the relation of this finding to results obtained from several other types of studies that have already been made of this series. Firstly, phenotypic studies have shown that

this series is comprised of two qualitatively distinct groups of mutants. Thus, the eye color of mutants belonging to the "apricot" group is darker in the male than in the female; while the converse is true for mutants of the "eosin" group. Similarly, Bridges' specific modifier, Pale, acts to darken the eye color of mutants of the apricot group, and to lighten it for those of the eosin group.[17] Moreover, it has been pointed out[17] that "w" (presumably the same w as used in the present studies) belongs to this latter group since the associated "white" phenotype is made even lighter in color in the presence of Pale.

The above difference in properties between the apricot and eosin group of mutants will more than likely turn out to reflect a functional difference between the apr^+ and w^+ genes. Superimposed on such a difference, however, would be the position effect associated with these genes; namely, the striking phenotypic difference that exists between $apr + / + w$ (with pinkish yellow eye color) and $apr\ w / + +$ (with red eye color). This result suggests that a mutant gene at one of the loci blocks, or impairs the functioning of, the normal allele of the gene at the other locus, when both are present in the same chromosome, as in the $apr + / + w$ heterozygote; while no impairment of the functioning of the two different wild-type alleles is phenotypically detectable when both of these are present in the same chromosome. The simplest assumption is that the effect is one way; that is, that the mutant gene *apr* impairs the functioning of w^+, or that w impairs that of apr^+. This leads to a simple model in which one of the genes controls a step A $\rightarrow$ B, and the other a step, B $\rightarrow$ C, in a biochemical reaction chain of the type: A $\rightarrow$ B $\rightarrow$ C. The position effect can then be assumed to result from a failure of substance B to diffuse readily from one chromosome to the other so that the chain of reactions in one of the chromosomes of the heterozygote is carried out more or less independently of the chain in the homologous chromosome. Further details of the application of this type of model to position pseudoallelism have been given elsewhere.[7]

Cytological studies of Panshin[18] and of others[9] have shown that the w^+ gene is located within the confines of the 3C2-3 doublet, or two-banded, structure of the salivary gland chromosomes (see Bridges' revised map[19]). Moreover, the evidence of Panshin was based on the critical method of synthesizing a deficiency for the gene in question by combining parts of two appropriate rearrangements, neither of which acts like a deficiency for that gene. In the case, the rearrangements were the white-mottled-5 (w^{m^5}) and roughest-3 (rst^3) inversions in the X chromosome. The right break-points of these two inversions are essentially similar and in the heterochromatic region. The left breakpoint of (w^{m^5}) lies between 3C1 and 3C2 (Sutton[9]);[20] while that of rst^3 lies between 3C3 and 3C4 (Emmens and others[9]). Panshin combined, as the result of crossing over, the left end of (w^{m^5}) with the right end of rst^3. The

[17] Morgan, T. H., Bridges, C. B., and Schultz, J., *Yearbook Carnegie Inst.*, **30**, 408–415 (1931).

[18] Panshin, I. B., *Compt. rend. acad. sci. U. R. S. S.*, **30**, 57–60 (1941).

[19] Bridges, C. B., *J. Heredity*, **29**, 11–13 (1938).

[20] Other studies cited by Bridges and Brehme,[8] indicating that this break lies between 3C2 and 3C3 would not alter the present argument.

resultant chromosome is a deficiency for the 3C2-3 doublet and it proved to act like a deficiency for the w^+ gene; on the other hand, other synthesized deficiencies either for the 3C1 band, or for bands to the right of the 3C2-3 doublet, did not act as though they were deficiencies for this gene. From these results, it is probable that apr^+ also lies within the 3C2-3 doublet.

The doublet structure has been implicated before in certain cases of position pseudoallelism. As has been discussed more fully in connection with these other cases,[5,7] the probable significance of this cytological finding is that the pseudoallelic genes associated with a doublet represent an established duplication of a single ancestral gene. To explain the finding that the two members of the duplication now appear to differ in function, it has been presumed that one of the genes has, by mutation, diverged in function from the other, and, in such a way, that it now controls a reaction successive to (or, on Horowitz' hypothesis for the evolution of biochemical synthesis,[21] antecedent to) that of the original gene.

SUMMARY

The white (*w*) and apricot (*apr*, formerly w^a) genes of the "multiple allelic" white-series in *Drosophila melanogaster* are found to be pseudoallelic genes whose order in the chromosome is *apr* – *w*. This result was based on a total of 21 crossovers between the *apr* and *w* loci. The total percentage of crossing over between them was 0.03%, under conditions giving 2–6 times the normal amount of crossing over in the surrounding regions. The standard map distance between *apr* and *w* is inferred to be in the range of 0.005–0.02 unit. The *apr* and *w* genes are a typical example of position pseudoallelism. Thus, a position effect is present, whereby *apr* +/ + *w* females have a pinkish yellow eye color, while *apr w*/ + + females have a wild-type or red eye color. The use of attached-X chromosomes enabled the *apr w*, or double mutant, combination to be derived simultaneously with the complementary wild-type crossover in a total of six instances. The results are discussed in relation to phenotypic and cytological studies of the white-series, and also in relation to other studies of position pseudoallelism.

[21] Horowitz, N. H., these Proceedings, **31**, 153–157 (1945).

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PSEUDOALLELISM AND GENE EVOLUTION

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It is the purpose of this paper to consider some of the ways in which "pseudoalleles" (McClintock, 1944), or closely linked genes having similar effects, may provide clues to the mode of origin of new kinds of genes. Our underlying thesis will be that in those instances of pseudoallelism in which there is evidence for close functional similarity among the component genes we may come close to seeing the direct results of a process which produces new genes.

In developing this thesis, we hold to the view point that new genes arise from preexisting genes, and we picture the origin of a new gene as being generally accomplished by a two-step process; namely, (1) the establishment of a duplication of that gene (or a higher repetition), and (2) the occurrence in one of the two (or more) genes thus formed of a "mutation to a new function," the result of which will be termed the "new gene." Such a mutation will be defined as one which permits the gene to produce an immediate product which is different, chemically speaking, from the one it originally produced—the production of the latter product will be termed the "old function of the gene."

We believe that it will generally be necessary to postulate the first step involving gene duplication, because of the following *a priori* considerations regarding the immediate fate of a new gene. A gene which mutates to a new function should, in general, lose its ability to produce its former immediate product, or suffer an impairment in that ability. Since it is unlikely that this old function will usually be an entirely dispensable one from the standpoint of the evolutionary survival of the

organism, it follows that the new gene will tend to be lost before it can be tried out, unless, as a result of establishment of a duplication, the old gene has been retained to carry out the old function.

That the establishment of chromosomal duplications would offer a reservoir of extra genes from which new ones might arise, is an old speculation, which gained prominence when Bridges (1935) invoked it again as the result of his discovery that the salivary gland chromosomes of *D. melanogaster* show many small intrachromosomal duplications or "repeats," in the homozygous, and hence, established condition. Metz (1937, 1947) found similar evidence in a study of such chromosomes of *Sciara*, and also evidence that duplications were not yet established, in the sense that strains of *Sciara ocellaris* collected from the wild were, in some instances, heterozygous for minute rearrangements of the duplication type. By far the most frequent type of structure resembling duplication is the "doublet" or "capsule"; such structures (see, e.g., the bands labeled 21 El-2 in Fig. 6) appear to result from the pairing of two like discs or "bands" along their edges to give a heavy staining shell inclosing a non-staining center. We shall return to this type of case later. Meanwhile, we may conclude that there is direct cytological evidence for the establishment of duplications in nature and that the most frequent type may possibly be adjacent gene repetitions.

It becomes much more difficult to obtain evidence for the all-important second step; namely, mutation to a new function. There is, of course, abundant evidence which points to the ability of certain genes, at least, to mutate to alleles which differ from one another in their action qualitatively, rather than simply quantitatively. We might mention here such examples as: self sterility alleles in plants (particularly the results obtained from a direct experimental attack on these cases by D. Lewis, 1947, 1949); multiple allelic series affecting the control of cellular antigens (e.g., Stormont, Owen and Irwin, 1951); and cases of complementary allelism (one of many examples being the "*R*" locus of maize, whose mutation properties have been studied in detail by Stadler (1946, 1951) and coworkers. Although we may ascribe such qualitative differences between alleles to qualitative differences in the immediate gene products of those alleles (i.e., to mutations to new functions), there are other possible interpretations. Models in which the gene has two independently varying attributes, namely, a combining power for its substrate, and an efficiency with which it produces a product, are satisfactory in some cases as shown by Stern and Schaeffer (1943); still other models, such as those suggested by Wright (1941), may be involved. We will stress here, however, another type of interpretation; namely, that the qualitatively different changes, which appear to be multiple alleles of a single gene, are in many cases changes within pseudoallelic genes. We stress this because in the case of several series of apparent multiple alleles, which have been subjected to very close scrutiny from the standpoint of genetic divisibility by crossing over, a pseudoallelic basis has resulted (such cases will be considered in some detail below). Finally, it may well be inherently difficult to recognize the occurrence of a mutation to a new function, if

such a mutation usually leads to a loss of the old function; for a mutant type would then result which would be difficult to distinguish from an inactivation of the gene.

It is evident from the above discussion that the problem of determining in the laboratory whether mutations to new functions do arise is a very elusive one. We propose that, in many cases, the qualitatively different types of "alleles" which have been experimentally observed, merely reflect functional differences which have already become established in the species among a series of pseudoallelic genes. As a working hypothesis, we may view a pseudoallelic series as a close succession of "old" and "new" genes which have originated and become established by the two-step process outlined above for the origin of new genes. We turn the problem around and inquire to what extent the qualitatively different types of "alleles," at hand, can be used to differentiate the functions of the component genes of the pseudoallelic series. We wish of course to determine whether such genes are functionally closely related at the level of the immediate gene products, for if they are not so related we have no reason to suppose that proximity of the pseudoallelic genes reflects a common origin of such genes; instead other forces, such as purely fortuitous ones, may have brought them together.

We shall then be largely concerned with a limited number of instances of pseudoallelism; specifically, those examples of the phenomenon in which the evidence points to a fundamental similarity in the functioning of the component genes, and, in a few very favorable cases, to a cytological homology of such genes as well. Consideration of pseudoallelic cases in which the possibility of functional similarity is, as yet, suggested only by a similarity in the gross phenotypic effects produced by the component genes will be omitted here; examples of such are: (1) the achaete and scute bristle mutants in *D. melanogaster* (see e.g., Serebrovsky, 1930, 1938; Muller and Prokofyeva, 1935; Sutton, 1943); (2) the dominant and recessive short-tailed mutants in the house mouse (Dunn and Caspari, 1942, 1945); (3) the pale yellow and pale green seedling color mutants in maize (McClintock, 1944); and (4) the miniature and dusky wing mutants in *D. virilis* (Komai and Takaku, 1949).

THE BIOCHEMICAL APPROACH

One type of evidence which would point to closely related functions existing among members of a pseudoallelic series would be the control by such members of closely related steps in a series of biochemically defined reactions. Such steps, if defined only in terms of the end products of the reactions rather than the direct gene products controlling the reactions, must be interpreted with great caution from the standpoint of our purposes. We consider next a few examples of the biochemical approach to the study of pseudoallelism.

Stephens (1948, 1949) has obtained evidence that in *Gossypium* two pseudoallelic genes, which affect pigmentation of the flower and other parts of the plant, are closely related with respect to the end products which they control. The two genes

concerned, namely, ghost spot, *G*, and spotless, *S*, evidently control the following two chemical steps:

$$\text{Anthoxanthin} \xrightarrow{(G)} \text{Leuco-substance} \xrightarrow{(S)} \text{Anthocyanin}$$

The evidence suggested possible reversibility of the first reaction, in the sense that the anthoxanthin apparently came from a precursor identical with the leuco-substance in the above sequence.

The genetic evidence for pseudoallelism in the above case comes from the work of Yu and Chang (1948). They found that *G* and *S*, and still a third gene affecting the above types of pigments in the plant, are most readily interpretable on the basis of their genetic data as three closely linked loci rather than multiple alleles of a single gene (R_2) as previously thought. Although the evidence is not conclusive (marker genes to demonstrate any association with crossing over, not being available), the hypothesis of three closely linked genes is strengthened over any other (such as one postulating unequal crossing over in minute duplications) by their finding that the recombination types which appeared in the offspring of given crosses did not permit more than one possible order for the three postulated loci.

Studies of biochemical mutants in *Neurospora* have provided little evidence, as yet, for close linkage between any of the genes known to control different but related steps in the synthesis of essential metabolites (Houlahan, et al., 1949; Horowitz, 1950). There are, of course, two possible explanations of this; namely, (1) the genes concerned are not fundamentally related in their function with respect to such steps, or (2) they are so related but either were not in close proximity at the time of their origin or were close together but became separated as the result of chromosomal rearrangements. Bonner (1950) has recorded the first possible exception in the case of three mutants affecting nicotinic acid synthesis; these show recombination relationships suggestive of close linkage between three genes.

A specific search for pseudoallelism in *Aspergillus* was initiated by Pontecorvo (1950) and suggestive evidence that three biotin-deficient mutants are pseudoallelic rather than multiple allelic has been reported by Roper (1950).

It is noteworthy that the biochemical examples, discussed above, were for the most part found as the outgrowth of studies of apparent multiple allelic series. Another promising example of this has been the study of the *A* "locus" in maize. Here, Laughnan (1949) has obtained genetic evidence, as yet inconclusive, of divisibility of the series into two closely linked genes. Biochemical studies by Sando and Bartlett (1922) and Laughnan (1950) are incomplete and somewhat conflicting. It may be of interest to note, however, that there are at least superficial resemblances, both chemically and phenotypically speaking, between the *A* "locus" in maize and the R_2 "locus," containing the *G* and *S* genes discussed above, in cotton. A comparative evolutionary study of pseudoallelism, which the latter case would permit if it is valid, is one of the most promising methods of learning more about the mode of gene evolution; such a study may soon be feasible among the (sexual) microorganisms,

where mutants affecting a specified type of biochemical synthesis can readily be selected for.

THE POSITION EFFECT APPROACH

It might have been supposed that valid evidence for a close relationship in basic functioning of two or more genes would have to be obtained with the aid of biochemical methods. This might have been true were it not for the phenomenon of position effect (Sturtevant, 1925): i.e., the change in a gene's activity which may accompany a change in its position with respect to neighboring genes. The clear demonstration of the presence of this phenomenon in four instances of pseudoallelism in *D. melanogaster* will be considered next.

In the pseudoallelic series which are discussed below, the normal alleles of the component genes are functionally so closely related that, to act normally, they must lie adjacent to one another in the same chromosome; as opposed to one lying in one chromosome and the other(s) in the homologous chromosome. Thus, if we let, a^+ and b^+, represent the normal alleles of two adjoining pseudoallelic genes, then the following general relations hold for the known examples. When the phenotype of $+\,+/a\ b$ is wild type, then the corresponding $a\ +/+\ b$ heterozygote has a mutant phenotype. When the phenotype of $+\,+/a\ b$ is a mutant one, then it is like that expected for incomplete dominance of the normal alleles in that this phenotype resembles that of a heterozygous deficiency (*Df*) for the two genes (namely, $+\,+/Df$); in such cases, the $a+/+\ b$ heterozygote always has a more extreme mutant phenotype than that of $+\,+/a\ b$. These types of relationships are, by definition, one form of the position effect phenomenon; namely, a form in which the activity of a gene is altered when its relation with respect to a specified allele in a neighboring gene is changed. It will be convenient to refer to genes so related as "position pseudoalleles." The concept involved here is not a new one. Thus, with the discovery of the position effect phenomenon, it was realized that comparisons of the above two types of corresponding heterozygotes would, if they gave different phenotypes, provide additional examples of the phenomenon. A quantitative study, however, of the dominant mutants, Delta (*Dl*) and Hairless (*H*), which are rather closely linked (3.7 units apart) and which show strong phenotypic interactions, did not reveal a significant phenotypic difference as between $Dl\ H/+\,+$ and $Dl+/+\ H$ (Sturtevant, 1928). Offerman (1935) also discussed the concept in some detail, particularly as a basis for understanding how rearrangements may result in position effects.

Since in the position pseudoalleles thus far analyzed, the type of position effect and the phenotypic relationships appear to be basically similar in each case, we will discuss at the outset a few of many possible models which could be used to interpret the observed results. We assume first of all that the mutant alleles of the pseudoallelic genes are acting like partial or complete inactivations of those genes; i.e., we shall not discuss the elaborate possibilities which arise when the mutant alleles are assumed to represent mutations which result in the formation of new gene products. We also

assume that the component genes are not identical; i.e., that they do not represent duplicate genes which have yet to diverge in function. For, in the event of such identity, assumptions that the observed mutant alleles represent mutations to new functions and that different types of such mutations occur at the different loci, together with other elaborations, seem to be required before the observed results are adequately interpreted.

We might consider a model in which the genes would act together as a true physiological unit with respect to their immediate gene products; that is, such products would be coupled in some way into one complex functioning product so that a change in either component gene would destroy or impair the activity of the complex. We regard this model, however, as less satisfactory, from the standpoint of being a working hypothesis in the present examples, than either of the following models:

Among schemes which would postulate that the pseudoallelic genes control separate but related chemical steps, there are two general types; namely, (1) a competitive type of scheme in which the relation between the two genes may be diagrammed as follows (where the symbol, S, represents the common substrate of the system; and "A" and "B" signify the immediate gene products which result from the action of the genes, a^+ and b^+, respectively, on S):

$$\mathrm{A} \xleftarrow{a^+} \mathrm{S} \xrightarrow{b^+} \mathrm{B}$$

and (2) a sequential type of reaction series which may be diagrammed as follows:

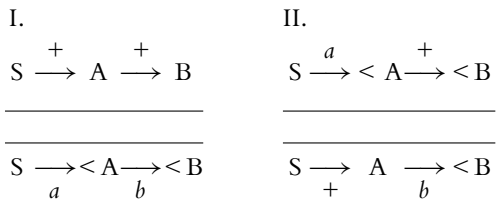
$$\mathrm{S} \xrightarrow{a^+} \mathrm{A} \xrightarrow{b^+} \mathrm{B}$$

We will prefer this latter model since the former one does not seem to adapt itself so readily to the type of position pseudoallelism which will be under consideration here. The second model involving sequential reactions provides a useful working hypothesis; it becomes relatively simple to apply it to the cases at hand; and it represents a progressive process such as is needed for any general theory for the origin of new gene functions.

The question immediately arises as to how such progressive divergence as that represented by the sequential type of reaction model can evolve. It has been pointed out (Lewis, 1950) that one way of picturing the process is to assume that the reaction under the control of the old gene is a reversible one, say, $\mathrm{K} \rightleftharpoons \mathrm{L}$. Under these conditions, the old gene would be expected to share a specificity for both the substrate, K, and the product, L, of the reaction (by analogy with reversible enzymatic reactions). If a mutation to a new function occurs, then the substance which would act as the substrate of the new gene could be either K or L. If K is utilized by the new gene to produce a new product, the competitive type of relationship between the old and new genes would result; but in the event that the new gene utilizes L to form some new product, M, then a sequential reaction of the type, $\mathrm{K} \rightarrow \mathrm{L} \rightarrow \mathrm{M}$, would result. Indeed, if the postulated substances are highly complex molecules, as is likely since

they represent immediate gene products, such progressive steps may only be possible when the genes concerned had a common origin; for unrelated genes would not be expected to be structurally similar enough to share a specificity for the same complex molecule.

We picture the above sequentially related reactions as occurring at the site of the genes in the chromosomes. We will diagram the application of the model to the case of the $+\ +\ /a\ b$ and $a\ +/+\ b$ heterozygotes as follows (where the symbol, <, is used to express a reduced amount of the corresponding product, and where the parallel lines represent the two homologous chromosomes):



We assume that the product, A, is produced here in such small amounts or is so limited in its ability to diffuse, that the reactions in one chromosome take place more or less independently of those in the other chromosome. The outcome in the two cases is most easily seen if we choose the extreme case in which (1) the mutants, *a* and *b*, result in complete blocks of their respective reactions, and (2) the product, A, does not diffuse from one chromosome to its homolog. Under these conditions, heterozygote II produces no B at all (and therefore has a mutant phenotype), while heterozygote I produces an amount of B which is normal for one dose of a^+b^+ (and therefore is wild type in phenotype whenever there is complete dominance). At the other extreme, if the mutants, *a* and *b*, are only slightly less active than their respective normal alleles, or if product, A, readily diffuses between the two chromosomes, then the two heterozygotes will tend to approach the same phenotype (in general, the wild-type one), so that a detection of a position effect would become increasingly difficult.

Although we assume to begin with, in the sequential reaction model, that a rather radical qualitative difference exists between the two pseudoallelic genes with respect to their primary reactions, it is evident from an application of the model to position pseudoallelism that such a difference may often not be directly reflected in the phenotypes of heterozygotes, I and II, above. Obviously, qualitative phenotypic differences can only be estimated after all possible genotypes, in a given case, have been constructed and analyzed phenotypically. If such differences can be found then we adopt the working hypothesis that they result from a qualitative difference in activity between products, A and B. In most of the examples of position pseudoallelism, discussed below, we can do little more than estimate from phenotypic considerations whether there are or are not qualitative differences between the component genes in each case.

Parenthetically, it may be noted that another type of hypothesis for the mechanism of the position effect has been elaborated by Ephrussi and Sutton (1944); namely, the "structural hypothesis," as opposed to the type which has been employed above involving localized interactions of immediate gene products. They have termed the latter a "kinetic hypothesis." The latter, as these authors have discussed, did not seem too promising as a working hypothesis for the variegated-type of position effect in which there was evidence for long range effects (i.e., effects extending over 50 gene diameters). There appears, however, to be no valid reason for supposing that position pseudoallelism is in any way analogous to the phenomenon of variegation. We have preferred here to develop a kinetic hypothesis, since it provides a more useful working hypothesis and the distances involved in the known cases of pseudoallelic genes are so extremely restricted (limited so far to two or three gene diameters) that diffusion of chemical substances may be considered an important variable.

Green and Green (1949) have established the existence of three pseudoallelic genes as the basis of a series of sex-linked lozenge (*lz*) mutants, which act phenotypically exactly like multiple alleles of a single gene. Thus, if the letters, *a*, *b*, and *c*, be used to represent the three genes (they would correspond to lz^{BS}, lz^{46}, and lz^{g}, in their notation), then *a b*/++, *a c*/++, and *b c*/++ are wild type in phenotype, while the alternative genotypes (*a* +/+ *b*, etc.) are in every case lozenge-like in phenotype (i.e., have a specific type of roughened eye effect). Since the results for any pair of these three mutant types are like those expected on the above sequential reaction type of model, a consistent result will be given by extending the sequence one more step, B → C, under the control of the third gene. (It is to be noted that the order in which the genes control the steps need not be the same as the order of the genes in the chromosome, and, in the present case, the former order appears, as yet, to be indeterminate). A mutant allele of any one of the three genes would then be expected to reduce the amount of the final product of the sequence, C, provided that the reactions in the two homologous chromosomes are more or less independent of one another. If the major phenotypic effect results mainly from a reduction in the amount of C, then a lozenge phenotype would be expected to result, whenever each chromosome carries at least one mutant allele, whether the same one (the homozygous mutant) or two nonallelic ones (the *a* +/+ *b* type of heterozygote). One of the most important implications of the lozenge case is that it provides an example in which there is very close mimicry of a multiple allelic series in a phenotypic sense. In the light of this example there is some question as to whether valid phenotypic criteria for allelism can be developed.

The extent to which there may exist qualitative differences among the three lozenge genes has not been determined; this must await detailed phenotypic studies of the many possible genotypes which the series now permits. It can now be inferred from the work of Oliver (1941), which pointed to the occurrence of wild type crossovers, or "reversions," as they were called, between lz^{g} and lz^{s}, that lz^{s} is another mutant lying to the left of lz^{g}. The conclusion by Oliver and Green (1944) that lz^{s} + /+ lz^{g} females may be intrinsically more fertile than the homozygote for

Figure 1. A position effect comparison involving the Star (*S*) and asteroid (*ast*) mutants. The female on the left has the genotype, + + /*S ast*; that on the right has the genotype, *S* +/+ *ast*. (Photographs by Dr. W. Hovanitz; unpublished).

either mutant can now be stated as implying that there may be qualitative differences between the activities of at least two of the individual gene products.

The phenomenon of position pseudoallelism was first discovered in the case of the Star (*S*) and asteroid (*ast*) mutants, which act phenotypically as multiple alleles of a single gene, but which represent separate loci, about 0.02 map unit part, near the left end of the second chromosome (Lewis, 1942; 1945). In this case, *S ast*/+ + is exactly like *S* +/+ + in that it invariably produces a slightly rough, and slightly smaller than normal, eye; while the alternative form of the genotype, *S* +/+ *ast*, invariably has a very small and rough eye (and abnormal veins) as shown in Fig. 1.

It was possible to show that the double mutant combination, *S ast*, was acting like a known joint deficiency (*Df*) for the two genes in several sensitive tests, and specifically, that *Df*/+ + is phenotypically indistinguishable from *S ast*/+ + (or *S* +/+ +). Thus, as far as could be detected, the joint activity of the normal alleles of each gene is not impaired when they are together in the same chromosome. A sequential type of reactions series may be invoked for the relationship between the Star and asteroid genes with respect to their immediate gene products in order to explain the phenotype of *S* +/+ *ast*. However, it is not possible to decide, as yet, the order in which the genes would have to control the postulated successive steps.

Distinct qualitative differences which would differentiate the Star and asteroid genes have not been found from a phenotypic analysis of the many possible

genotypes which were constructed. The rough-eyed phenotype, however, is clearly an undesirable one for such an attempt. There is only an indirect indication of a real difference in action between the two genes, in the sense that the various possible combinations of alleles of each gene (employing two recessive alleles, *ast* and ast^4, at the asteroid locus) do not arrange themselves in a linear series. Thus, when tested opposite either + *ast* or + ast^4, the seriation of the available combinations is as follows (where the symbol, >, means "has a more nearly normal eye than"):

$$++ > +\,ast^4 > +\,ast > S\ ast^4 > S\ ast > S+ .$$

Thus, a curious type of reversal in the order of the effectiveness of the asteroid alleles in promoting eye development occurs depending upon the allele of Star to which they are adjacent.

The next two examples of position pseudoallelism (which have been reported only briefly, Lewis, 1948; 1949) were the outgrowth of an attempt to find additional examples of the Star-asteroid type among other apparent multiple allelic series in *D. melanogaster*. The first example involves certain short-bristle mutant types; namely, the dominant mutant, Stubble (*Sb*), and recessive mutants which will be termed stubbloid (*sbd*) alleles. The latter are represented by two alleles, *sbd* and sbd^2 (formerly symbolized as Sb^r and $Sb^{r\,2}$, respectively, by Bridges and Brehme, 1944). The stubbloid locus has been found to lie an estimated 0.01–0.03 unit to the left of the Stubble locus at 58.3 in the third chromosome (see Fig. 6).

The results of the position effect comparison (which has thus far been made only with sbd^2 and *Sb*) are very striking. Flies of genotype, sbd^2 *Sb*/++ invariably have long, tapering bristles like those of wild-type flies; while sbd^2 +/+ *Sb* flies always have extremely short and stubby bristles. The difference is particularly striking since *Sb*/+, as well as homozygous sbd^2, flies have bristles roughly intermediate between the two extremes just described.

A joint deficiency for the stubbloid and Stubble genes has been obtained and its action is like that of the double mutant combination, sbd^2 *Sb*. Thus, *Df*/++ flies have normal bristles as in sbd^2 *Sb*/++ flies. The application of our sequential reaction type of model becomes useful not only in interpreting the phenotype of sbd^2 +/+ *Sb*, but also in suggesting a basis for the curious dominant action of the Stubble mutant when it is next to the normal allele of stubbloid (that is, Stubble acts as an "antimorph" in the terminology of Muller, 1932). If we assume that the sequential steps involving immediate gene products are controlled as follows:

$$\mathrm{S} \xrightarrow{sbd^+} \mathrm{A} \xrightarrow{Sb^+} \mathrm{B},$$

then we may assume that a block in the second step, such as would be expected to occur when the mutant allele of Stubble is substituted for the normal allele, would lead to an accumulation of the product, A. Such an accumulation could then act as a dominant effect if we suppose that it leads to abnormal bristle development; in the

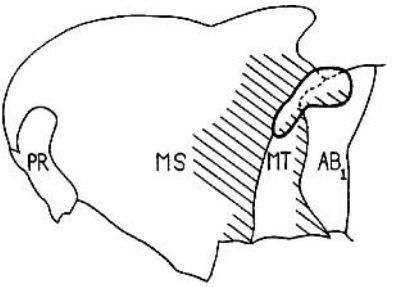


Figure 2. A drawing of the thorax and first-abdominal (AB_1) segment of *Drosophila melanogaster*. Only the outlines of the prothorax (PR), mesothorax (MS), and metathorax (MT) are shown. The haltere or dorsal appendage of the metathorax, is drawn in heavy outline. The shaded areas represent the regions defined in the text as the posterior mesothorax and posterior metathorax. (Greatly modified from Zalokar, 1947.)

combination, sbd^2 *Sb*, an accumulation of A would be prohibited by a block imposed on the first reaction by the mutant stubbloid allele.

From a detailed phenotypic study of the Stubble and stubbloid mutants, Dobzhansky (1930) was able to demonstrate distinct but slight qualitative differences between the two. It would be premature, however, to attempt to relate these differences in any definite way to a difference in developmental activity of the hypothetical immediate gene products, A and B, in the above reaction sequence.

In our final example of position pseudoallelism which involves the so-called "bithorax" and "bithoraxoid" mutant types, three separate loci have been established as the result of crossing over analyses. At the first locus (58.8 in the third chromosome) there are three recessive mutant alleles available; namely, *bx*, bx^{34e}, and bx^3. At the second locus, about 0.02 unit to the right of *bx*, there is a dominant mutant allele, *Bxl*, which formerly was symbolized as bx^D (Hollander, cited by Bridges and Brehme, 1944) and also as bxd^D (Lewis, 1949). Finally, one recessive mutant allele, *bxd*, is known at the third locus (about 0.01 unit to the right of *Bxl*).

The analysis of the present example is considerably facilitated by the existence of at least two qualitatively distinct phenotypes among the mutant effects. In one, which will be called the "bithorax phenotype," the anterior portion of the metathorax (see Fig. 2) comes to resemble the anterior portion of the mesothorax; while the posterior metathorax remains unchanged (see Fig. 3 for an extreme bithorax-type of haltere). This phenotype is typical of the *bx* mutants when homozygous or in combination with each other, and also of heterozygotes between *Bxl* and a *bx* mutant, provided the two mutants are in opposite chromosomes (see Fig. 4 for examples of the latter heterozygotes).

Distinctly different developmental effects which make up the "bithoraxoid phenotype," can be observed in the *bxd* homozygote or in the *Bxl* +/+ *bxd* heterozygote. In such types, the posterior portion of the metathorax comes to resemble the posterior

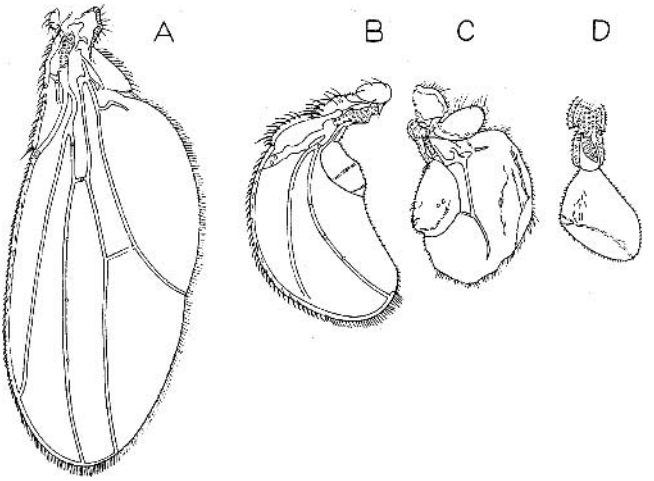


Figure 3. Camera lucida drawings of: (A) the normal (mesothoracic) wing; (B) the appendage which arises on the dorsal metathorax of an extreme bithorax-type of individual; (C) the appendage, corresponding to (B), which arises in an extreme bithoraxoid-type; (D) the haltene of the wild-type fly. The position of sense organs is shown by small circles. (Only B and C are drawn to the same scale).

portion of the mesothorax, the anterior metathorax remaining unchanged (see Fig. 3 for an extreme bithoraxoid-type of haltere). At the same time there is always a thoracic-like modification of the first abdominal segment, one of the most striking results of which is the occasional production of a pair of first abdominal legs, which arise in addition to the normal three pairs of thoracic legs; on rare occasions in *bxd*/*bxd*^{121} flies (where *bxd*^{121} is an allele of X-ray origin) abdominal wing-like halteres of the bithoraxoid type arise in addition to the abdominal legs.

At first sight the two mutants, *bx* and *bxd*, appear to affect different portions of the body segments in a more or less mutually exclusive way typical of complementary alleles. However, examination of double mutant combinations, such as the *bx bxd* homozygote reveals an additional feature regarding the nature of the bithorax effect. Such double mutants have a combined bithorax and bithoraxoid phenotype; moreover, the effect of the *bx* mutant now becomes recognizable not only in the anterior portion of the metathorax, which becomes mesothoracic-like as in the single *bx* mutant type, but also in the anterior portion of the first abdominal segment, as well. That is, the latter region is metathoracic-like in *bxd* flies and becomes mesothoracic-like in the double mutant flies. (These effects are recognizable thus far only in the ventral portion of the first abdominal segment—a mesothoracic-like modification of the dorsal part not having been found.)

The effect of the *bx* mutant may be summarized as a change from a metathoracic level of development (L - mt) to a mesothoracic level (L - ms), whether it occurs in the anterior portion of the metathorax, itself, or in the corresponding region of

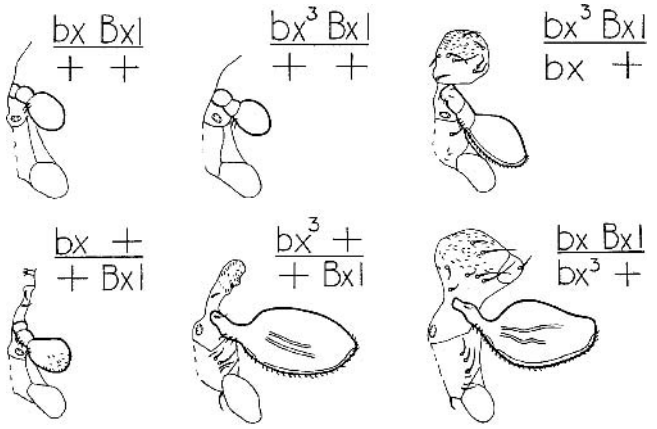


Figure 4. Position effect comparisons involving the bithorax (*bx*) and Bithorax-like (*Bxl*) mutants, illustrated by camera lucida drawings of the metathoracic regions only (see Fig. 2 for orientation) of flies belonging to six genotypes with respect to these mutants; the pair of genotypes in each vertical column are identical except for the way in which the alleles are distributed between homologous chromosomes.

the first abdominal segment, provided that region has been made metathoracic-like by the presence of the *bxd* mutant. The effect of the *bxd* mutant may be defined as a change in the latter region from a first abdominal level of development (L - ab) to a level, L - mt. (The posterior portions of the mesothoracic segments and metathoracic segments, shown in Fig. 2, can also be brought into this scheme if it is assumed that these regions are at levels, L - mt and L - ab, respectively; i.e., such regions appear, in the presence of the mutants, to behave as though they were embryologically related to the anterior portion of the segment which follows, rather than precedes, them—a conclusion which Snodgrass (1935) has drawn for the thorax of four-winged insects.)

The role of the *Bxl* mutant in the above phenotypic picture is difficult to evaluate, especially since it is lethal in the adult stage when homozygous. The + *Bxl* +/+ + + heterozygote differs from wild type only in having the distal segment of the haltere enlarged to about twice its normal volume (as Hollander, cited by Bridges and Brehme, 1944, has shown). In many respects, the *Bxl* mutant acts as though it were a double mutant combination of the *bx bxd* type. Firstly, such double mutant combinations exhibit in the larval stage pairs of metathoracic and first-abdominal spiracles (in addition to the single, or mesothoracic pair characterizing normal larvae); this mesothoracic-like modification of the first-abdominal and metathoracic segments is also found in larvae of the + *Bxl* + homozygote. Secondly, *Bxl* acts as if it were an allele of the *bx* mutants, as already noted, in the phenotypic sense that bx^k +/+ *Bxl* flies (where bx^k refers to *bx*, bx^{34e}, or bx^3) have a bithorax phenotype; and, thirdly, it acts as an allele of the *bxd* mutant in that *Bxl* +/+ *bxd* flies have a bithoraxoid phenotype; while, finally, + *Bxl* +/ bx^k+

bxd heterozygotes have combined bithorax and bithoraxoid phenotypic effects. Each of these phenotypes is the result of a position effect type of interaction in that the corresponding heterozygotes, bx^k *Bxl*/+ +, *Bxl bxd*/+ +, and bx^{34e} *Bxl bxd*/+ + + (which carries the only triple mutant combination available), are phenotypically indistinguishable from the single mutant heterozygote, *Bxl*/+, described above (see Fig. 4 for examples of the position effect in the case of the *bx* and bx^3 alleles).

A model which is consistent with the above facts, and with most of the other known relationships within this pseudoallelic series, can be most readily made by assuming that the three genes control successively related reaction steps according to either of the following two schemes:

1. $\text{S} \xrightarrow{bx^+} \text{A} \xrightarrow{Bxl^+} \text{B} \xrightarrow{bxd^+} \text{C}$, or

2. $\text{S} \xrightarrow{Bxl^+} \text{A} \xrightarrow{bx^+} \text{B} \xrightarrow{bxd^+} \text{C}$,

where a reduction in amount of substance, C, is assumed to lead towards a bithoraxoid phenotype, and a reduction in the amount of B or A towards a bithorax phenotype. Stated in another way, the postulated substances will be assumed to control levels of development, already defined above, according to the following scheme:

$$\text{L - ms} \xrightarrow{\text{A or B}} \text{L - mt} \xrightarrow{\text{C}} \text{L - ab}$$

Three lines of evidence directly support the sequential type of model in this case. Firstly, X-ray induced bithoraxoid-like changes have been obtained (from treatment of wild-type males) which show no detectable change in the action of the bx^+ and Bxl^+ genes. In one of the three changes of this kind, called bxd^{100}, it has been possible to show genetically by duplication and deficiency studies, that a rearrangement has occurred which separates the *bx* and *Bxl* loci from that of *bxd* (cytologically, a transposition of the section from 89 B6 to 89 E 1-2, inclusive (see Fig. 6), to the left arm of the third chromosome (66C) has occurred. Two other of the induced changes of this type have been analyzed cytologically and found to have breaks in common with that of bxd^{100}; namely, separating the doublet, 89 E1-2 from the adjoining 89 E3-4 doublet. Thus, the normal functioning of the bx^+ and Bxl^+ genes does not appear to depend upon their being in proximity to bxd^+. Moreover, if these rearrangements owe their phenotypic effect (which is a more extreme bithoraxoid effect than that of the spontaneous mutant, *bxd*) to a position effect of the bxd^+ gene (rather than to mutation of that gene, accompanying the rearrangement), then it may also be concluded that the normal functioning of the bxd^+ gene is directly dependent upon its being in proximity to the bx^+ and/or Bxl^+ genes.

The second line of evidence supporting the sequential type of scheme is that the addition of a duplication containing the normal alleles of the *bx* and *Bxl* (but not the *bxd*) genes (readily derivable from the bxd^{100} rearrangement) to the homozygous *Bxl* mutant overcomes the recessive lethal action of this mutant and gives an adult with an extreme bithoraxoid phenotype; a haltere from a fly of this duplication genotype

is shown in Fig. 3, as the example of an extreme bithoraxoid effect. Moreover, when two doses of this duplication are added to homozygous *Bxl*, the extreme bithoraxoid phenotype remains unchanged; and the only effect of the second addition is to diminish the anterior portion of the haltere, which is slightly enlarged in the above case with only one dose of the duplication, to its normal size. As a kind of control for these experiments, a duplication containing the normal alleles of all three genes was added to + *Bxl* +/+ + + and also to homozygous + *Bxl* +; the duplication phenotypes, here, were found to be wild type in the former case and like that of the single mutant heterozygote, *Bxl*/+, in the latter; i.e., with no bithoraxoid phenotype detectable. We conclude that the *Bxl* mutant is not directly concerned with the production of the bithoraxoid phenotype, but that it is a change which leads to a reduction in, if not complete loss of, a product (B, in the above scheme) which acts as the substrate for the bxd^+ gene.

The third line of evidence supporting the above sequential reaction series is derived from a consideration of the effect of a *bx* mutation on its adjoining bxd^+ allele. According to the postulated sequence, a *bx* mutant would be expected to result in a weakening in the activity of the adjoining bxd^+ allele in direct proportion to the degree to which that mutant represents an inactive state of the *bx* gene. X-ray induced changes which have been found to act as *bx* mutants have (in six cases analyzed) always acted as though they were more extreme mutant alleles of the *bx* gene than the spontaneous alleles. Further tests of each of the induced *bx* alleles show that they act as though they were also extreme mutant alleles of *bxd* (and *Bxl*). All such cases have been found to be associated with chromosomal rearrangements (of a type other than deficiency) in which one break had occurred apparently just to the left of the three loci (that is, just to the left of the first doublet of region 89 E). Since the effects associated with each of these rearrangements resemble those of simultaneous inactivation of all three of the pseudoallelic genes, a position effect basis for those effects seems probable and can be readily interpreted on our sequential reaction series by assuming that the first gene of the series, bx^+, has, for one reason or another, been inactivated as the result of the rearrangement. The spontaneous mutant alleles of *bx* do not show a detectable change in the action of the bxd^+ allele (as measured by the production of a bithoraxoid phenotype) when homozygous nor when opposite a chromosome bearing the spontaneous *bxd* mutant allele. Thus, in the latter case, bx^k + +/ + + *bxd* flies (where bx^k represents, as before, either the *bx*, bx^{34e} or bx^3, allele) are wild type as are also each of the corresponding bx^k + *bxd*/+ + + genotypes. However, the most extreme of the spontaneous mutants, bx^3, does show a detectable reduction in the activity of its adjoining bxd^+ gene in the sense that this mutant when opposite a chromosome carrying an extreme bithoraxoid type of change of X-ray origin (such as, bxd^{100}, described above) results in a very slight bithoraxoid phenotype (detectable as a wing-like modification of the posterior region of the haltere).

We have already discussed the way in which sequentially related reactions may be used to make a general model for explaining the known cases of position pseudoallelism. Having obtained three lines of evidence pointing to the validity of the sequential type of reaction series in the case of the bithorax pseudoalleles, we

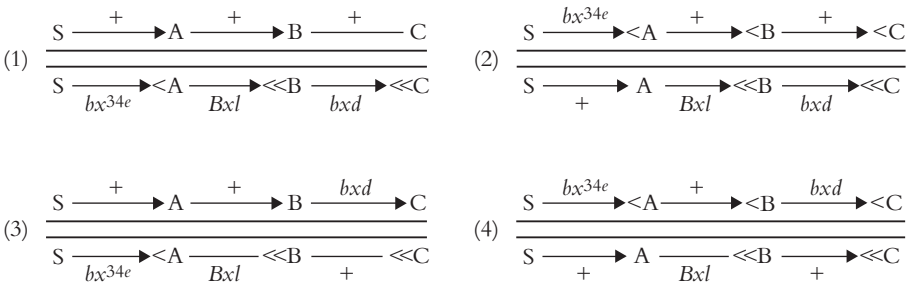


Figure 5. The postulated gene-controlled reactions at the site of the chromosomes (represented as two parallel lines) in the four possible genotypic arrangements of three mutant and three normal alleles of the bithorax pseudoallelic series. The symbols, < and ≪, are used to indicate slightly reduced, and greatly reduced, amounts, respectively, of the appropriate immediate gene product, A, B, or C.

will consider the application of the general model to a few of the position effects which become detectable in the present case when the distribution of mutant alleles between homologous chromosomes is varied. An instructive example is available in the case of the mutant alleles, bx^{34e}, *Bxl*, and *bxd*, since the triple mutant combination, as well as all possible double mutant combinations, has been derived. With this material the four possible heterozygotes carrying one dose of each mutant allele and one dose of the corresponding normal alleles have been constructed and analyzed phenotypically. The four genotypes and the postulated relationships with respect to immediate gene products are diagrammed in Fig. 5. For the sake of simplicity we arbitrarily choose the case in which the first two steps in the postulated reaction series are controlled in the order: *bx-Bxl*; the reverse of this order is equally applicable since it is sufficient in the examples shown to describe the results solely in terms of products, B and C.

We assume, as before, that the immediate gene products (A and B, at least) do not diffuse readily, for one reason or another, from the site of the genes in one chromosome to the corresponding site in the homologous chromosome. Actually, there is some evidence which will be presented elsewhere that a slight amount of interaction, presumably involving diffusion of these products, does occur at this level.

We assume that bx^{34e}, since it behaves as a slight *bx* allele, leads to a relatively slight reduction in the amounts of products, B and C (through first reducing the amount of A); while more extreme reduction in the amount of these two substances is assumed to follow a blocking of the second step by the *Bxl* mutant, and a similar reduction in the amount of C is assumed to follow a blocking of the third step by the *bxd* mutant. The heterozygote, $+\,+\,+/bx^{34e}$ *Bxl bxd*, designated as (1) in Fig. 5, departs from wild type only in having an enlarged haltere like that of the single mutant heterozygote, *Bxl*/+. It is also known that $+\,+\,+/Df,\ bxd^{100}$ (where *Df,* bxd^{100} stands for a deficiency for the *bx* and *Bxl* genes, obtained from the bxd^{100} rearrangement, described above) is

phenotypically like *Bxl*/+. Thus, one dose of the normal alleles of all three genes is sufficient to result in a nearly normal phenotype, which is like that expected for simple incomplete dominance of those alleles. Heterozygote (2), bx^{34e} + +/+ *Bxl bxd*, on the other hand consistently has a moderate bithorax phenotype, which would be expected because of the lowered production of product, B, relative to that produced in heterozygote (1); the somewhat lower production of product, C, in (2) as compared to (1), is evidently not sufficient to lead to a detectable bithoraxoid phenotype. The heterozygote (3), + + *bxd*/bx^{34e} *Bxl* +, is found to have a constant extreme bithoraxoid phenotype as expected on the basis of a strong reduction in the amount of the product, C in both chromosomes; no reduction in the amount of B is expected over that occurring in heterozygote (1), and it is not surprising, therefore, that the typical bithorax phenotype is lacking, here. Finally, (4), bx^{34e} + *bxd*/+ *Bxl* +, consistently has a combination of the bithorax and bithoraxoid phenotypic effects (and is nearly lethal). This is not surprising when it is noticed that this heterozygote is expected to produce a reduced amount of product, B, comparable to that of (2), and a reduced amount of C, comparable to that of (3).

THE CYTOLOGICAL APPROACH

Cytogenetic studies have been made of the Star-asteroid, Stubble-stubbloid, and bithorax-bithoraxoid examples of position pseudoallelism. The results are summarized in Fig. 6. At the time that each of these series was subjected to a crossing over analysis, the salivary gland chromosome location of these genes was unknown. Each case was,

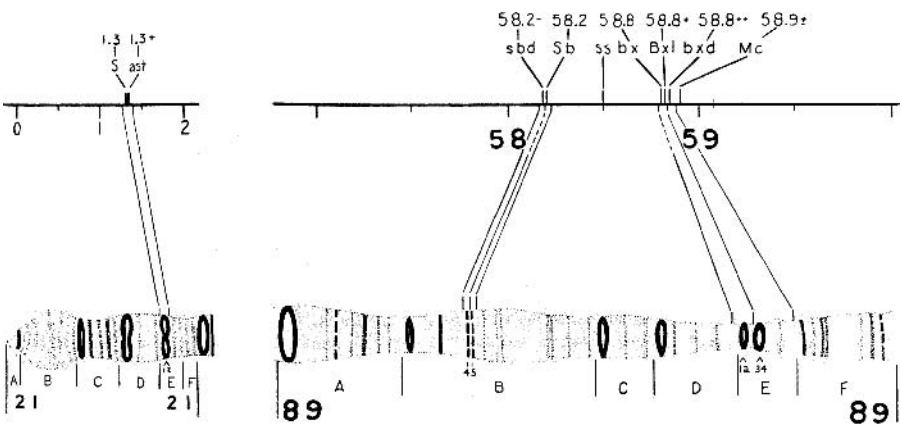


Figure 6. A correlation of the genetic and the salivary gland chromosome locations of three sets of pseudoallelic genes which exhibit position pseudoallelism. At the left are the correspondences found near the extreme left end of the second chromosome (modified from Lewis, 1945); at the right is shown a section from the middle of the right arm of the third chromosome of *Drosophila melanogaster.* (The symbols, *ss* and *Mc*, refer to the loci of spineless and Microcephala, respectively; other symbols are described in the text.)

therefore, an unselected one with respect to the outcome of the cytological analysis. The Star and asteroid loci were found to be included within the doublet, or double banded structure, 21 E1-2, near the extreme left end of the second chromosome (Lewis, 1945). A location in the immediate vicinity of, if not within, a doublet structure, 89 E1-2, near the middle of the right arm of the third chromosome, has been established for the bithorax and Bithorax-like loci. The locus of bithoraxoid is most probably in the adjoining 89 E3-4 doublet, although the possibility that it lies one or two bands to the right of this structure is not excluded. The two doublets of subdivision 89E are possibly partially homologous to one another, in the sense that they frequently appear as one coalesced structure, as figured by Bridges (1935); proof that two doublets are present has been obtained by finding several rearrangements in which they are separated from one another. Finally, the stubbloid and Stubble loci lie within the region of the two separate bands, 89 B4 and 5, on the basis of studies of rearrangements having breaks just to the left, between, and to the right of these bands. Whether the two bands in this case are homologous or not, we cannot say; they appear similar to one another, morphologically, and in staining properties, although the second band, 89 B5, is somewhat thinner than the first.

It should be noted that there is no evidence for minute rearrangements (or larger ones) accompanying any of the spontaneous mutants belonging to the above three pseudoallelic series. That is, all such mutants, which include all those employed in the position effect comparisons discussed above, appear normal in the salivary gland chromosomes. Similarly, the wild-type crossovers and the complementary double mutant combinations, which have been obtained for all possible combinations of the mutants in each series, show no detectable rearrangement in these chromosomes for all cases analyzed (which include all except a few combinations in the *bx* pseudoallelic series).

Although there is no proof that the doublet structures are adjacent gene duplications, as Bridges (1935) postulated them to be, we regard the finding that two of the pseudoallelic series are associated with doublets as suggestive evidence that the component genes, in such cases, owe their origin to a duplication process. It is evident, however, that a great deal of work remains to be done on the nature of the doublet structures and many more pseudoallelic series need to be found and analyzed before general conclusions can be drawn.

CONCLUSIONS

Those cases of pseudoallelism in which there is evidence for a position effect between the component genes, together with cytological evidence suggesting that such genes are associated with established chromosomal duplications, provide experimental material for the study of gene evolution. The position effect evidence, is most readily interpretable on the basis of a functional relationship of the neighboring genes at the level of the immediate gene products. Such evidence for a basic functional similarity

of the component genes is reinforced by the cytological evidence that the genes concerned may be structurally similar to one another, as well.

From cytological considerations (of the salivary gland chromosomes of the Diptera) it would appear that established duplications are common and that they are often in the form of adjacent repetitions of chromosomal material. This encourages us to believe that, when evidence for close functional similarity among genes of a pseudoallelic series is obtained, we may be dealing with genes which were once (or are still) identical, owing to an origin by a process of duplication. Such evidence may also be obtained from biochemical studies of pseudoallelic series and preliminary work in this field has been briefly discussed. For the purposes at hand, however, the biochemical approach will be of value only in so far as the analysis will permit valid deductions at the level of the immediate gene products.

The known pseudoallelic series which exhibit position effect evidence for functional similarity may be viewed as examples in which the component genes were once identical, but have diverged from one another with respect to function, and now control a series of sequentially related biochemical reactions of the type: $A \rightarrow B \rightarrow C$. In the case of the bithorax pseudoallelic series, position effect evidence in direct support of this type of model has been obtained. The remaining three cases of pseudoallelism of the position effect type appear to be simply interpretable on the basis of this model.

The possibility that duplicate genes may often diverge from one another in their functioning in the above way is an attractive one since it gives a conservative, and yet, progressive process such as is required for a general theory of gene evolution. Indeed, as has been discussed, considerations of the complex structural specificities which are expected to obtain at the level of the gene and its immediate products suggest that the development of sequential steps at this level may only be possible in the case of genes which were once identical.

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DISCUSSION

ALTENBURG: The bithorax and certain other cases were used by Dr. Lewis to emphasize the importance of repeats in explanation of the evolution of loci at which there is a high degree of pseudoallelism. Such cases were also described earlier in the present symposium by Dr. Stephens, involving the highly complicated series of pattern alleles in cotton. For duplication of a locus might be repeated a number of times, followed by later differentiation of the duplicated segments in the evolution of the normal phenotype. Mutations occurring in these differentiated segments could then result in different phenotypic effects.

The first definitely established case of pseudoallelism was the achaete-scute case in Drosophila, explained by Muller as a repeat. This case, however, unlike those described by Dr. Lewis, does not admit the possibility, early realized by Muller, and incorporated in an article by Offerman in 1935, of being explained alternatively by supposing that the neighboring loci may have been of an entirely different nature from one another from the start, and that they merely influence one another's expression through position effects. The evidence required to settle this point has in this case been supplied by the breakage test.

LEWIS: Although achaete and scute show morphological similarity this does not necessarily imply a fundamental similarity between the two genes at the level of the immediate gene products. The writer's contention is that in those cases which have been referred to as "position pseudoalleles" the position effect evidence (lacking in the achaete-scute case) strongly implies that the immediate gene products are similar to one another. The chief reason for believing that these position pseudoallelic series bear on the problem of gene evolution is that, in the three examples cytologically analyzed, there is evidence of cytological homology of the component genes in each series. Cytological evidence for homology of the achaete and scute genes is as yet lacking (see Sutton, 1943).

HADORN: It is an extremely interesting fact that the two pseudoalleles, *bx* and *bxd*, seem to affect independently different regions of the metathoracic discs. There are in these primordia apparently two independent fields, one concerned with the anterior, the other with the posterior half of the adult segment. Each mutant would change the field properties in such a way that mesothoracic-like structures are now found.

LEWIS: The metathoracic discs of bithorax and bithoraxoid mutant larvae are larger than the corresponding discs of wild-type larvae and show an expected resemblance to the mesothoracic discs. Since these discs do not show an obvious morphological division into the two expected fields, it would be of great interest to transplant portions of the discs as Dr. Hadorn and others have done for the imaginal discs of other organs.

PAPAZIAN: The absence or low incidence of crossing over between pseudoalleles is to be expected from an evolutionary point of view. Since crossing over is almost certainly under hereditary control it will be subject to natural selection. Imagine, following the views expressed by Lewis, that a gene duplicates and that each new part changes functionally. Now if these newly changed parts or pseudoalleles have an advantageous function and if they must be adjacent in order to function, evolution will proceed in one of two directions, either crossing over between them will be suppressed, or each part will become functionally autonomous and no longer a pseudoallele in the above sense.

LEWIS: Since crossing over does not alter the physical distance between genes, its occurrence does not constitute a pressure operating to separate pseudoallelic genes. The occurrence of spontaneous rearrangements will constitute such a pressure, but the magnitude of that pressure will be low, owing to the unlikelihood that a break will occur between the pseudoallelic genes. Actually, a finite amount of crossing over between such genes would be desirable from an evolutionary standpoint, since it would insure the possibility of obtaining favorable combinations of alleles at the different pseudoallelic loci.

WHITING: The speaker has defined the gene as the unit within which there is no crossing over. Three years ago I questioned the existence of genes (P. W. Whiting, Biol. Bull. *95*: 257) as units of hereditary transmission, pointing out that ideologically the gene is the lineal descendent of the subcellular units of past philosophies—micellae, pangenes, etc., and that we have no evidence that crossing over does not occur in the homozygote within that portion of the chromosome acting as a unit in the heterozygote. The 13 sex alleles found by Goldschmidt in the Z-chromosome of *Lymantria* and the 8 in the W-chromosome act as units in the hybrids but there may well be intragenic crossing over in the pure races. Similarly in Drosophila, the sex gene is diffuse, scattered throughout the active portion of the X. This acts as a unit segregating from the Y or the lack of X in the sex heterozygote, the male, but it is not a unit in the sex homozygote, the female, as shown by the localized sex-linked genes for which the female may be heterozygous. The germ plasm is genic material but it does not consist of genes. It produces genes by such structural reorganizations as may subsequently mendelize with the original condition.

LEWIS: The speaker's remark about the definition of the term, gene, was given as a very brief aside to the main paper. Since there are conflicting viewpoints on the matter of this definition, we will amplify here our reasons for employing the crossing-over process as an essential part of a working definition of the gene. The definition of any particulate unit must be in terms of its indestructibility by some breakage or splitting process. The crossing-over process and any which leads to chromosomal rearrangements are the only yet known processes of this kind which can be used in defining the unit of heredity. The point we wish to emphasize is that the discovery of what we have called position pseudoallelism relegates the rearrangement process to second place. Thus, if a chromosomal rearrangement separates units which must lie close together in order to function normally, then the effect of that rearrangement

will be to cause an apparent destruction of one or more of the units which are acting physiologically as one. In practice, we would conclude nothing in such a case until we knew the behavior of the physiological unit following an exact reversal of the rearrangement to the original condition, or following a recovery of that unit in its original arrangement as the result of crossing over. Actually, it has never been possible to effect either of these reversals (except in the case of position effects involving the heterochromatic regions, where the issue has been one of deciding whether the gene has changed by virtue of its being next to heterochromatin as opposed to euchromatin). Crossing over, on the other hand, is the unique process which results in recombinations of chromosomal parts without altering the physical distance between genes. It is therefore capable of leading, and has led in the examples cited in this paper, to the recognition of smaller units within a portion of the chromosome acting as a physiological unit. These smaller units we still call genes.

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THE THEORY AND APPLICATION OF A NEW METHOD OF DETECTING CHROMOSOMAL REARRANGEMENTS IN *DROSOPHILA MELANOGASTER*[1]

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A new method of detecting chromosomal rearrangements in *Drosophila melanogaster* has been applied to the problem of measuring the biological effects of ionizing radiations from nuclear detonations. The method, itself, is an outgrowth of studies of the bithorax pseudoallelic genes near the middle of the right arm of the third chromosome (Lewis, 1951). It will be called the "bithorax" method. Results of applying it to the detection of X-ray induced rearrangements will be considered first.

MATERIALS AND METHODS

The bithorax method employs two mutant genes of the bithorax pseudoallelic series; namely, the recessive, bithorax-34e (bx^{34e}, locus 58.8) found by Schultz (Bridges-Brehme, 1944) and the dominant, Ultrabithorax (*Ubx*, locus 0.02 unit to the right of *bx*) found by Hollander (loc. cit.) who named it bithorax-Dominant (bx^{D}). The name Ultrabithorax is intended to supersede the original name and also the name Bithorax-like (*Bxl*) by which it has also been known (Lewis, 1951).

The bx^{34e} homozygote has a slight but highly constant and symmetrical mesothoracic-like modification of the metathoracic segment. The dorsal metathorax

[1]This study was aided by a contract with the Atomic Energy Commission operating through the Office of Naval Research, Department of the Navy, and the California Institute of Technology (NR 164010).

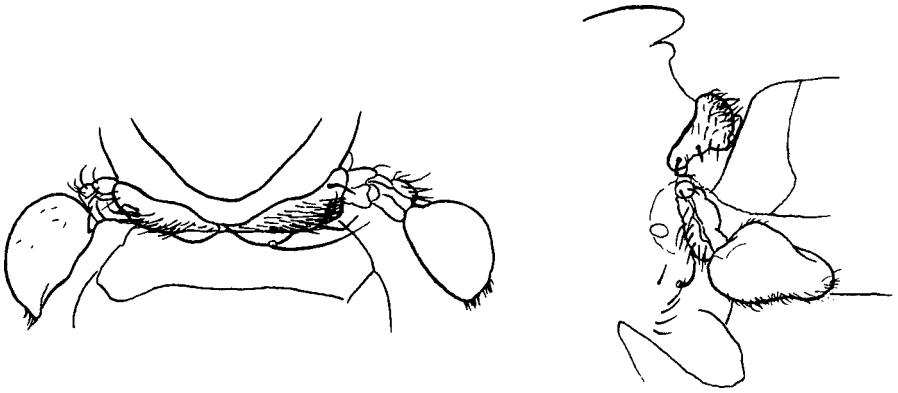

Figure 1. Dorsal (*left*) and lateral (*right*) views of the metathoracic region of the bx^{34e} homozygote. (Drawing by E. M. Wallace.)

or "metanotum" which in wild-type flies is little more than a "line" separating the mesothorax from the abdomen (see Zalokar, 1947), is transformed in the bx^{34e} mutant flies into a narrow band of hairy and bristled tissue (Fig. 1). The halteres in the mutant flies are somewhat enlarged and slightly wing-like. The *Ubx* gene is lethal when homozygous while the heterozygote, *Ubx*/+, differs from wild-type chiefly in having the distal segment of the haltere enlarged to about twice its normal volume. Since bx^{34e} and *Ubx* are pseudoallelic genes there are two forms of the double heterozygote to be considered; namely, the *cis*- and *trans*-forms—a useful terminology proposed for such cases by Pontecorvo (1950). The *cis*-form, or bx^{34e} *Ubx*/ + +, cannot be distinguished phenotypically from the + *Ubx*/ + + heterozygote just described. On the other hand the *trans*-form or bx^{34e}+/+ *Ubx* has oval, flat halteres which are consistently larger and much more wing-like than those of the bx^{34e} homozygote. Paradoxically, however, the *trans*-heterozygote (or "*trans*-type" as it will be referred to hereafter) has virtually no metanotal tissue such as is seen in the bx^{34e} homozygote. In other words, the *trans*-type is at once more extreme and less extreme in the degree of its bithorax effects than the bx^{34e} homozygote, depending upon whether the haltere or the metanotum is considered.

As will appear, the extent of development of the metanotum in the *trans*-type is extraordinarily sensitive to certain structural rearrangements of the third chromosome. The following rough, arbitrary system has been developed for measuring this phenotype: grade 0 indicates no appreciable development of the metanotum; grade 1 indicates tufts of hairy metanotal tissue which do not, however, form a continuous band across the dorsal side of the thorax; grades 2, 3, and 4 indicate progressively wider, continuous bands of such tissue—grade 3 having approximately the width seen in the bx^{34e} homozygote (Fig. 1) and grade 4 having a width about that of the first abdominal segment, to use a convenient reference point. The procedure is to score the

grade of the *trans*-types which arise from a mating of bx^{34e} homozygotes with *Ubx*/+ heterozygotes (the chromosome carrying the normal alleles of these genes should also be marked and in the experiments reported below carried the "balancer" Payne, *Dfd;* that is, carried the dominant Deformed (eye) mutant and the Payne inversions in the left and right arms of the third chromosome). Either of these genotypes may be used as the treated or male parent but the bx^{34e} homozygote is the one more usually used. In the fast neutron, gamma ray, and nuclear detonation studies, bx^{34e} males were treated and mated to *Ubx*/+ females, which carried an attached-X type of X chromosome—the so-called double-X of Muller; in these cases only the F_1 male *trans*-types were scored. Standard culture bottles were used; they were incubated throughout at 25°C, and crowding was avoided (e.g., the controls had only one pair of parents). These environmental conditions are essential for maximum sensitivity of the method.

In order to minimize subjective errors in grading the *trans*-types, the control and treated culture bottles were given randomly drawn numbers by another person who also removed all of the parental flies before emergence of the F_1 generation. This coding procedure was followed in experiments designed to calibrate the method for different types of radiations. Finally, the scoring and grading of all flies was done throughout by one person (the author).

Cytological analyses were conducted by examining the salivary gland chromosomes of individuals heterozygous for the treated chromosome (containing either bx^{34e} or *Ubx*, as the case might be) and an untreated chromosome of normal sequence carrying a mutant at another of the bithorax pseudoallelic loci, namely, bithoraxoid (*bxd*). The latter mutant permitted the bx^{34e} and *Ubx* containing third chromosomes to be readily distinguished from one another in the larval stage; thus, bx^{34e} +/+ *Ubx* males are outcrossed to bithoraxoid females to produce bx^{34e} +/+ *bxd* larvae which are wild-type in phenotype, and *Ubx* +/+ *bxd* larvae which lack setae on the first abdominal segment. Salivary gland chromosome map designations were made from Bridges' 1935 map.

The source of X-rays was a Westinghouse 150-KV Industrial Unit employing a Machlett radiographic tube which was operated in all cases at 120 KV, 8 ma., with a 1 mm Al filter. Exposure of flies to gamma rays (from Cobalt-60) and to fast (pile) neutrons were carried out at the Argonne National Laboratory. For the X-ray studies the males were put into small gelatin capsules; while for all of the other types of treatments males were transported (by air) and treated in cotton-plugged polystyrene plastic tubes (2½" long; ½" diameter; and 1/16" wall thickness). In all of these latter cases, the males were received at the laboratory and mated within 24 h after the treatments. Such males were removed and discarded on the seventh day after treatment.

X-RAY EXPERIMENTS

The results of grading the metanotum of trans-types in the F_1 generation following treatment of adult parental males are shown in Table 1. It is apparent that a significant number of the *trans*-types from each X-ray treatment fall into grades 2–4, inclusive; while none of the controls fall into these grades. In this latter connection it is

Table 1 The grading of *trans*-heterozygotes.

Treatment	Dosage	Phenotypic grades 0	1	2	3	4	Total[a]	Percent grades 2–4, inclusive	Standard deviation (%)
	Control	2,559	4	0	0	0	2,563	0.00	....
X-rays	3,000 r[b]	2,917	33	45	16	9	3,020	2.32	±0.26
(120KV)	4,500 r	2,667	48	97	25	6	2,845	4.50	±0.39
Gamma rays	Control	1,303	9	0	0	0	1,312	0.00	....
(from Co^{60})	3,000 r	2,274	28	30	5	4	2,341	1.67	±0.26
Fast neutrons	200 rep	1,823	35	20	4	0	1,882	1.28	±0.26
(from Argonne	400 rep	2,117	40	24	5	1	2,187	1.37	±0.25
pile)	800 rep	1,325	44	37	13	3	1,422	3.73	±0.50
	1600 rep	299	37	19	8	4	367	8.45	±1.45
	Control	398	0	0	0	0	398	0.00	....
Nuclear detonation	(70 rep)	869	5	3	0	0	877	0.34	....
(mostly fast	(150 rep)	1,159	11	8	1	0	1,179	0.76	±0.25
neutrons)	(190 rep)	1,114	26	8	3	0	1,151	0.96	±0.29
	(330 rep)	1,202	23	18	2	1	1,246	1.69	±0.36
	(940 rep)	646	29	28	6	0	709	4.80	±0.80
	(1100 rep)	177	26	8	3	1	215	5.58	±1.57

[a] In the X-ray series and its control, males and females are included in the totals. In all of the remaining experiments the totals refer only to males.
[b] In this experiment the treated male parent was *Ubx*/+; in all other cases the male parent was homozygous for bx^{34e}.

noteworthy that all of the control and 3,000 r cultures in these experiments were pooled and coded by the method already described. Among 278 individuals falling into grades 1–4, inclusive, in these experiments, the ratio of females to males was 124:154. It is probable that the excess of males is an expression of a slight sexual dimorphism in the phenotype.

The vast majority of *trans*-types which belong to grades 2–4, inclusive, transmit an average grade significantly greater than the approximately grade-0 average of the control *trans*-types. On the other hand many *trans*-types belonging to grade 1 fail to show inheritance of the effect upon appropriate testing; e.g., in the 3,000 r experiment, 19 grade-1 males were individually progeny tested by mating to bx^{34e} females; of these tests, 13 were fertile and only six transmitted the effect (usually averaging about grade 1) to the F_1 *trans*-types. In general, the average grade of the *trans*-types produced in the F_1 of a progeny test is similar to, or within one grade unit of, that of the parental *trans*-type. A small percentage of asymmetrical individuals arise. These are usually cases in which one half of the body is of grade 0. Such cases are scored as grade 0 and have rarely transmitted the higher grade. The remaining asymmetrical types are classified according to the lower grade present since that grade is the one usually transmitted in such cases. In general, a population of a given *trans*-type shows little tendency towards asymmetrical metanotal development.

The next step was to determine what proportion of the *trans*-types belonging to the different graded phenotypes carried chromosomal rearrangements. A total

Table 2 Correlation of grade of male *trans*-heterozygote with X-ray induced rearrangement (R) involving the third chromosome.

Original grade of male	R absent	Critical region	R involving non-critical region	Total
0	33	0	4	37
1	5	9	0	14
2–4	3	74	0	77
				128

of 128 treated third chromosomes (obtained from progeny-tested male *trans*-types) were analyzed by the salivary gland chromosome method. (This total includes 74 cases from preliminary 4,000 r X-ray experiments, not shown in Table, in which the cultures were scored by another person.) The results are shown in Table 2. Among 37 *trans*-types belonging to grade 0, only four (10%) carried rearrangements involving the treated third chromosome. On the other hand, among 91 *trans*-types belonging to grades 1–4, inclusive, 83 (91%) carried rearrangements involving that chromosome. Moreover, a striking difference in the distribution of breakages in the third chromosome is evident. Thus, every rearrangement associated with a *trans*-type of transmitted grade of 1 or higher had at least one breakage point in the region extending from the centromere to immediately to the left of the section including the *bx* and *Ubx* loci (89E; Lewis, 1951); that is, in the region from section 81F to 89D, inclusive. This relation will be referred to here as Rule (1). The region will be called the "critical region," and comprises 506 discs or bands as measured on the revised map of Bridges (1941). On the other hand, Rule (2), in each of four cases in which the rearrangement was associated with a grade of 0 the breakage point in the third chromosome fell outside of the critical region (the sections being, 64F, 76D or E, 80, and 94D). The eight instances of transmission of the altered grade which were not associated with visible rearrangement require comment. Five of these cases were of grade 1 to begin with and were transmitted as grade-1 types. These may have been due to changes in modifier genes or to rearrangements not readily detectable by the salivary gland chromosome method (such as, wholly heterochromatic exchanges). The three cases of this kind among the higher grades were all of grade 2 and were transmitted as grade-2 types. One proved to be caused by, or associated with, a second chromosome Minute (bristle) change (but four other known Minute types which were tested did not act as modifiers *per se* of the *trans*-type). The other two cases without rearrangements have not been analyzed further but one has been preserved in stock.

Two-, three-, four-, and five-break rearrangements occurred in the ratio of 53 : 14 : 13 : 3, respectively, among the 83 rearrangements discussed above (Table 2). The multiple-break, as well as the two-break, cases were distributed throughout all grades but the former were proportionately more often associated with the higher grades of 3 and 4 than were the latter.

A number of features stand out clearly when a plot is made of the distribution of breakages among the two-break rearrangements detected by the bithorax method. A total of 81 such cases were available from the X-ray experiments. (This total includes 53 cases from Table 2 and 28 cases from progeny tests of female *trans*-types.) The result is shown in Table 3.

Two more general rules may now be formulated from the pattern of breakages seen in this table. Thus, Rule (3), when one breakage point occurs in the proximal half of the critical region (from section 81 to 85 or 86, inclusive), the other breakage point tends to occur in the distal half of one of the other major euchromatic chromosome arms (X, 2L, 2R, or 3L). On the other hand, Rule (4), when one breakage occurs in the remaining or distal part of the critical region (from 86 or 87 to 89D, inclusive), the other breakage point may occur apparently anywhere in the entire chromosome complement (including Y, 4 and 3R). The phenotypic grades seem to be correlated roughly as follows: for the same breakage position in the critical region, the more distal the break in the other arms the more extreme the grades; while for the same breakage position relative to the centromere in other arms, roughly speaking, the more distal the break in the critical region the more extreme the grade (although there is no definite trend in the proximal one half of the critical region in this respect).

The bithorax method is unique among the known position effect methods in that it detects wholly euchromatic rearrangements with considerable efficiency. Thus, from Table 3, among the 81 two-break rearrangements 29 (36%) were of this type (not involving breakages in the heterochromatic sections: 20, 40, 41, 80, 81, nor 101). In spite of this result, the method is seen to be very inefficient at detecting inversions in the right arm of the third chromosome. Thus, only two of these were detected; yet from the data of Bauer (1939) it is known that significantly more inversions involving 3R occur than translocations between 3R and any one of the other autosomal arms when rearrangements are detected by the salivary gland chromosome method.

Finally, aside from the broad relationship laid down by rule (3) above, there is no significant evidence that breaks in the critical region tend to be preferentially associated with specific regions of other chromosomes, when allowance is made for the fact, well established by Bauer (loc. cit.), and Kaufmann (1946), that certain regions normally show a relatively higher breakage frequency than their salivary gland chromosome physical distance would indicate (such as section 3 in X and any heterochromatic section such as 41).

The validity of the above defined rules can best be tested by employing unselected rearrangements; i.e., those selected by other methods of detection. For this purpose the available two-break rearrangements were surveyed and the required R(bx^{34e} +) or R(+ *Ubx*) chromosome rearrangement (R) types were derived as crossover products from R(+ +)/bx^{34e} + and R(+ +)/*Ubx* +, females, respectively. The breakage points in such rearrangements were either already known (Bridges-Brehme, loc. cit.) or in some cases given below were determined for this study.

Three translocations (T), not involving the critical region, were used to test the validity of rule (2); namely, *T(1;3)v* (10/93B), (the latter set of numbers in parenthesis

Table 3 Position of breakage in X-ray induced two-break rearrangements detected by the bithorax method. Each entry is the number of the salivary gland chromosome breakage section in the noncritical region; wherever two or more virtually identical rearrangements occur, the number of such cases is shown in parenthesis.

Distance from tip of second break[a]	Number of the breakage section in critical region									Total (by arm)						
	81F	82	83	84	85	86	87	88	89	X	Y	2L	2R	3L	3R	4
1	60(2)		60	61		60	21; 61					1	4	2		
2	22(3)											3				
3	3; 58(2); 63(3)	63	3					63	98	2			2	5	1	
4	57(2); 64(2)	64											2	3		
5	5; 25; 56(3); 65									1		1	3	1		
6	55(2)												2			
7	67(2)		60										1	2		
8	68	28; 53				8		8		2		1	1	1		
9	52												1			
10	30(2); 70(2)			70±[b]	30			30				4		3		
11	91							11		1					1	
12							62			1						
13				48		33						1	1			
14																
15					75	75		35				1		2		
16																
17						37±		37±				2				
18																
19						39±	39					2				
20						Y[c]; 80±	20; Y; 41; 101	Y; 40(2); 41 ±(2); 101	Y(2); 41(3) 80; 80±	1	5	2	5	5		2
Total	32	4	3	3	2	8	8	13	8	7	5	18	22	27		2

[a] These distances are in terms of the number of salivary gland chromosome sections from the tip for any given major chromosomal arm.
[b] The sign ± indicates that the breakage location was only roughly made to the nearest section; 80± signifies that the breakage is in either section 80 of 3L or, less likely, in 81 of 3R.
[c] Y symbolizes a breakage position in the heterochromatic Y chromosome, which corresponds roughly to section 20 of the X chromosome.

indicating the breakage points in terms of salivary gland chromosome regions), *T(2;3)S*^{M} (21E/79D), and *T(2;3)bw*^{VDe4} (59D/80). None of these rearrangements enhanced the bithorax phenotype of the *trans*-type; that is, R(*bx*^{34e}+)/+ *Ubx* for each of the three rearrangements is phenotypically like the *trans*-types which carry no rearrangements involving the third chromosome. Secondly, every rearrangement tested whose breakage points agree in type with those described by Rule (1) enhanced the grade of the *trans*-type; of these, the examples obeying Rule (3) were: *T(2;3)bw*^{VDe3} (59E/81F), *T(2;3)Dp-S* (21E/81F; Lewis, 1945); and the inversion, In (3LR)*sep* of Muller (65E/85E); while an example obeying Rule (3) or (4) was *T(3;4)*c (86B-C/101F). Finally, two unselected rearrangements having breaks in the proximal part of the critical region but the other breaks not in conformity with Rule (3) are of interest; namely, *T(2;3)A* (39B or C/83B), and *T(2;3)B* (33/81F). The former is barely detectable as a modifying rearrangement of the *trans*-type, while the latter is just detectable by its modification of the *trans*-type to an average grade of about 1.

Preliminary (4,000 r) X-ray experiments had shown that the frequency of phenotypically modified *trans*-types was roughly the same whether the treated male parent was *bx*^{34e} or *Ubx*/+. This indirect evidence suggested that R(*bx*^{34e}+)/+ *Ubx* was equivalent to R(+ *Ubx*)/*bx*^{34e}+ for the identical type of R. This rule has been verified repeatedly in this work using both the selected and unselected rearrangements available. The necessary R(*bx*^{34e}+) and R(+ *Ubx*) crossovers were, for example, both obtained in the case of *T(2;3)bw*^{VDe4} and *T(3;4)c*. In the case of the former rearrangement the two kinds of *trans*-types are mostly in the range of grade 3 and 4 and are not phenotypically distinguishable from one another. In the latter case of *T(3;4)c*, the two *trans*-types are again indistinguishable from one another phenotypically and range from grade 0 to grade 2.

Thus far, only heterozygosity for structural changes has been considered. A number of rearrangements selected by the bithorax method have proved viable when homozygous so that it becomes possible to compare structural homozygotes of the general genotype, R(*bx*^{34e}+)/R(+ *Ubx*), with the structurally heterozygous *trans*-types. More often, however, it has been necessary to cross two nearly identical rearrangements, one associated with *bx*^{34e} and the other with *Ubx* in order to approach structural homozygosity for the rearrangement sequence. The results of such studies have indicated, almost without exception, that structural homozygosity restores the grade of the *trans*-type to its original grade-0 condition—rule (5). Thus, the selected rearrangements, *T(2;3)*39–86, *bx*^{34e} (39/86C-D) and *T(2;3)*39–87, *Ubx* (39A-C/87A-C) produce when combined a *trans*-type of the original grade, although each by itself gives a grade average of about 2. Exceptions to Rule (5) are very instructive and are illustrated by the following two examples: *In(3LR)*64-82 (64C/82A) and *T(2;3)*57–81 (57D-E/81F). Each is viable when homozygous and each has been used to make a structurally homozygous *trans*-type, which proves to be predominantly of grade 1 or 2 in its phenotype instead of grade 0. All of such exceptions thus far have been cases in which the bithorax region has been shifted to a much more distal location than it normally occupies. Thus, in the case of the homozygote for the pericentric inversion and the

homozygote for the translocation which have just been discussed, the bithorax region (89E) now is located more than 1,220 discs and more than 1,450 discs, respectively, from the centromere compared to its normal location of 506 bands.

At the other extreme, when the intensity of structural heterozygosity in the *trans*-type is increased by combining two quite different kinds of two-break rearrangements, the phenotype tends to become very extreme. For example, the translocation, $T(2;3)48–84$, bx^{34e} (48C/84D) has been combined with $T(3;4)c$, *Ubx* and with $T(2;3)29–87$, *Ubx*, already described. In each case the phenotypic grades of the double structural heterozygote are in the range of 3 and 4, whereas the three heterozygotes by themselves each have grades of mostly 1 and 2.

One rare type of rearrangement must be considered in applying the bithorax method. Rearrangements associated with breakages immediately adjoining the 89E1-2 doublet act like very extreme *bx* and *Ubx* mutations or position effects. A number have been obtained from X-raying wild-type males, or bx^{34e} males in other kinds of experiments. When bx^{34e} males are treated and mated to the *Ubx*/+ female, as in the bithorax method, such changes are expected to occur but to be lethal when opposite the *Ubx* chromosome from the female. On the other hand when the reciprocal cross is made a *trans*-type carrying a rearrangement of this kind is expected to result on rare occasion. Two such cases were detected in the 3,000 r experiment shown in Table 2 as very extreme grade-4 individuals. The effect of such cases on the per cent of individuals falling into grades 2–4, inclusive, is however negligible.

The addition or subtraction of a Y chromosome does not noticeably alter the grade of the *trans*-type either in the presence or absence of structural heterozygosity for rearrangements involving the critical region. An example was the translocation, $T(2;3)bw^{VDe3}$ which was studied in the XXY and XO male in the form, $R(bx^{34e}+)/+$ *Ubx*, in each case the phenotype remains of grade 3 or 4, even though the variegation for the normal allele of the brown gene (bw^{+}) in this case shows the expected modification due to changes in numbers of Y chromosome.

An important feature of the bithorax method is the fact that its rearrangements only modify the phenotype of the *trans*-type. Thus, e.g., $R(+\,+)/bx^{34e}+$; $R(+\,+)/+$ *Ubx*; $R(bx^{34e}+)/bx^{34e}+$; and $R(+\,+)/bx^{34e}$ *Ubx* have in each case proved phenotypically indistinguishable from the corresponding genotype without the rearrangement. This feature holds in particular for such rearrangements as $T(2;3)bw^{VDe3}$, which, as already noted, is an extreme modifier of the grade of the *trans*-type.

FAST NEUTRON AND GAMMA-RAY EXPERIMENT

It was anticipated that the flies to be exposed to the nuclear detonation would receive chiefly fast neutron radiation; i.e., they would be shielded by inclosure in lead chambers. For this purpose it was obvious that the bithorax method of detecting rearrangements had to be calibrated in terms of known dosages of fast neutrons. The results of exposures of adult males (bx^{34e} homozygotes) to a series of known dosages of Argonne pile neutrons and to a 3,000 r dosage of gamma rays (from a Co^{60} source)

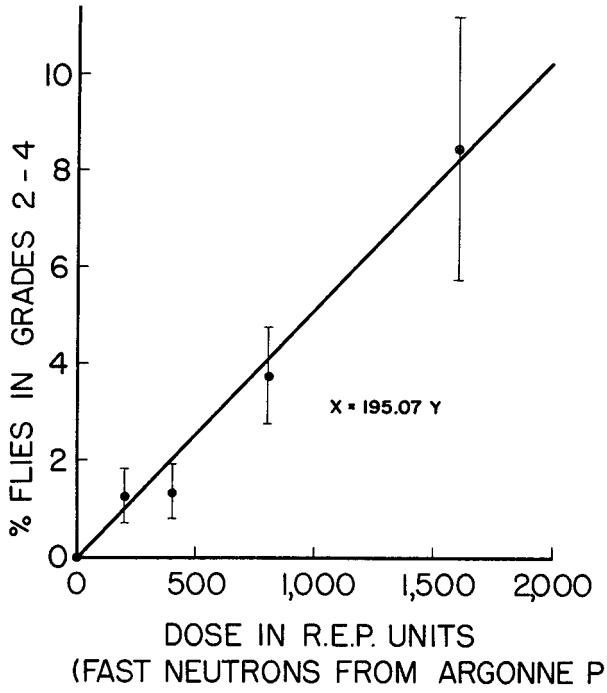

Figure 2. The relation of rearrangement production as measured by the percentage flies in grades 2–4, inclusive, with dosage of fast neutrons in roentgen-equivalent-physical (r.e.p.) units. For each experimental point the 95% confidence interval is shown.

are included in Table 1. All of the fast neutron, gamma ray and control cultures in this experiment were pooled and coded by the method already described. Only the F_1 male trans-type were scored.

The fast neutron results show a reasonable fit to a linear relationship with dosage in rep (roentgen-equivalent-physical) units (Fig. 2). The data have been fitted by the method of least squares to a linear curve of the form $x = ay$ (that is, to a curve which, on *a priori* grounds is expected to pass through the origin) where x is the dosage in rep units and y is the percent of individuals falling into grades 2–4, inclusive. The value of the constant, a, fitted in this way is 195.07. This curve served as the best available prediction curve for the unknown dosages received at the nuclear detonation sites.

NUCLEAR DETONATION EXPERIMENT

The results of applying the bithorax method to the detection of rearrangements produced by the fast neutrons (contaminated to some extent by gamma rays) resulting from one of the nuclear detonations are also included in Table 1.

The dosage that the flies received at various stations has been estimated simply by substituting the values of the percentage of flies falling into grades 2–4, inclusive, into the equation, x = 195.07y, which was the curve obtained above when the bithorax method was calibrated with known dosages of fast neutrons. The values of x thus obtained (and rounded to two significant figures) are shown in parenthesis in the column headed "Dosage" of Table 1. The validity of this procedure rests of course on a number of assumptions not too easily tested; namely, (1) that fast neutrons from the nuclear detonation will be identical with those from the pile in their rearrangement inducing capacities for Drosophila, and (2) that the percentage of gamma-ray contamination in the two cases is also the same. Nevertheless, these biological estimates derived from the bithorax method of the dosages received at the various test stations are found to agree quite well in each case with physical measurements.

DISCUSSION

The new type of position effect which underlies the bithorax method of detecting chromosomal rearrangements has important implications for the theory of the position effect phenomenon (review by Lewis, 1950). Some of these implications will be briefly discussed here.

Essentially, the new type of position effect is closely related to that revealed when *cis*- and *trans*-heterozygotes for certain pairs of pseudoallelic genes are compared. Thus, in the latter type of position effect (Green and Green, 1949; Lewis, 1945, 1951, 1952) the normal alleles of two such genes function better when both are together in the same chromosome. A model for this type has already been discussed in some detail (Lewis, 1950, 1951; see also similar speculations by Pontecorvo, 1950). It postulates that (1) a product A produced by gene, a^+, is utilized in some way by gene, b^+, to make a product B; and (2) A is effectively transported only along the chromosome (see Fig. 3). This type of position effect will be referred to here as the "cisvection effect."

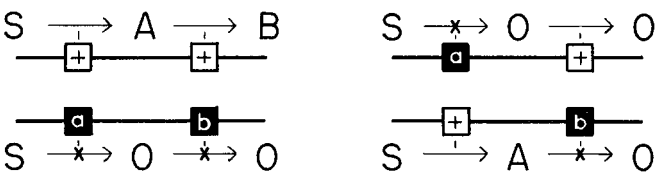

Figure 3. Diagram illustrating the possible mechanism of the position effect associated with the *cis*-(*left*) and *trans*-heterozygote (*right*) for certain pairs of pseudoallelic genes. For simplicity, the mutant genes, a and b, shown as black squares, are assumed to result in complete blockages of the reactions under their control, and the substance A is assumed to show no diffusion from one homologous chromosome to the other. The normal alleles of the mutant genes are signified by squares containing "+."

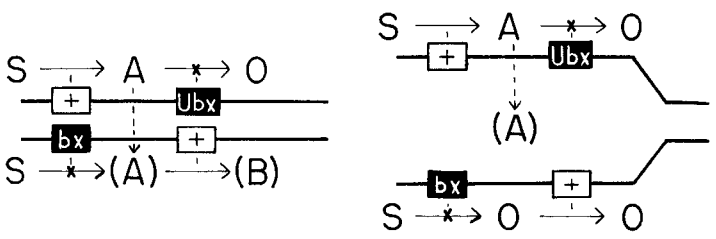

Figure 4. Diagrams illustrating the possible mechanism underlying the new type of position effect seen in the bithorax method of detecting rearrangements. The diagram on the left shows the postulated significance of somatic pairing in promoting the transport of substance A from one homologue to the other of a trans-heterozygote between bithorax (*bx*) and Ultrabithorax (*Ubx*) mutant genes. In the diagram on the right a reduction in somatic pairing, such as might be caused by a heterozygous rearrangement proximal to these genes, is assumed to prevent the "leakage" of A from one chromosome to the other.

On the above model the intensity of the cisvection effect, measured by the degree of phenotypic difference between the *cis*- and *trans*-heterozygotes, might be expected to be a function of the distance separating homologous chromosomes. Specifically, in an organism with somatic pairing, such as Drosophila (Metz, 1916), there may be a greater possibility of transport of A from one homologue to the other than in an organism without somatic pairing. This consideration led to the experiment of testing whether rearrangements which might upset the pairing of the chromosomes in the bithorax region would alter the phenotype of the *trans*-heterozygote. This prediction has been fully verified by the above rearrangement studies employing the bx^{34e} and *Ubx* genes. The position effect that is revealed by modifying the *trans*-heterozygote by means of chromosomal rearrangements will be referred to as the "transvection effect." A diagram of a model for this effect is shown in Fig. 4.

The principal rules governing the transvection effect have been seen to hold for both unselected and selected rearrangements and have reasonable interpretations on a somatic pairing basis. Thus, Rules (1) and (2), that a rearrangement to be effective in significantly modifying the phenotype of the *trans*-type must have one breakage point in the critical region, suggest that somatic pairing is initiated at the centromere, or in the chromocentral regions, and then proceeds distally. Rule (3), that a breakage in the proximal part of the critical region tends to occur with another breakage in the distal half of an autosomal arm, suggests that owing to the formation of a chromo-center, or proximal association of nonhomologous arms, rearrangements with both breakages in the proximal regions of different arms give relatively little failure of pairing (just as wholly heterochromatic translocations are virtually undetectable in the salivary gland chromosomes). Rule (4), that a breakage in the distal part of the critical region may occur with a breakage apparently anywhere else in the complement, suggests merely that the remaining distance between the position of the former breakage and that of *bx* and *Ubx* genes is so short that the probability of somatic pairing occurring in this interval is made relatively small. Finally, Rule (5) that structural homozygosity does not in general modify the phenotype of the *trans*-type is obviously expected on a

somatic pairing basis. Exceptions to this later rule involved rearrangements in which the bithorax region is now at an extreme distal location. These exceptions suggest that, again, somatic pairing begins proximally but that it may not reach completion in abnormally long chromosome arms relative to the timing of the transvection effect.

Somatic pairing as a causative or modifying factor in the position effect phenomenon has been often considered, as reviewed elsewhere (Lewis, 1950). Its detailed consideration by Ephrussi and Sutton (1944) warrants attention at this point. Thus, on their interpretation the forces of somatic pairing are assumed to cause a (reversible) deformation of the genes in the case of the structural heterozygote, rather than an alteration in the interaction of localized gene products. Their hypothesis, however, was designed for the general case in which there is an effect of a rearrangement on the wild-type allele of gene, *a*, such that R(+)/*a* differs phenotypically from +/*a*. The rearrangements with which the transvection effect deals do not show any effect on such heterozygotes in the case of either the bx^{34e} gene and its normal allele or the *Ubx* gene and its normal allele; nor is there a difference when bx^{34e} *Ubx*/+ + and bx^{34e} *Ubx*/R(+ +) are compared. Thus, the available evidence in the case of the transvection effect strongly implies that transport of gene products rather than deformation of the genes is involved.

Much more detailed studies will be needed before speculations are warranted regarding the manner in which the developmental changes seen in the transvection effect are mediated. But it should be noted that this type of position effect provides a possible new approach to studying gene action, since it specifies the conditions under which experimental variation in the strength of somatic pairing may *per se* cause a change in gene action.

The results of the application of the bithorax method to the measurement of rearrangement production will be only briefly discussed. It may be inferred from the data of Bauer and others (Bauer, 1939) that the present method detects roughly 10% of the rearrangements that would be detectable by the salivary gland chromosome method (for example, at a dose of 3,000 r of X-rays, the present method recovered 2.3% flies in grades 2–4, inclusive, while the cytological method showed that 18.8% of the treated sperm carried rearrangements). This must be considered a very high yield of rearrangements for a position effect method and it is in all probability dependent upon the great length of the critical region. Thus, the present method has roughly ten times the efficiency of perhaps the next most efficient position effect method available; namely, that employing the Dubinin-effect, or position effect of the cubitus interruptus gene (inferred from the data of Khwostova and Gavrilova, 1938).

There is an indication from the present data that gamma rays may be slightly less effective than X-rays in producing chromosomal rearrangements in Drosophila but the point is by no means established (cf. similar findings with Tradescantia by Kirby-Smith and Daniels, 1953). It is safe to conclude, however, from the present data that fast (pile) neutrons are many more times effective than gamma rays or X-rays in producing rearrangements in this organism.

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SUMMARY

A new type of position effect called the "transvection effect" permits rapid and highly efficient detection of chromosomal rearrangements in the first generation following an induction treatment. Several unique features are involved: (1) the position effect extends over vastly greater distances than heretofore demonstrated (over 500 discs of the salivary gland chromosomes); (2) wholly euchromatic as well as euchromatic-heterochromatic rearrangements are efficiently detected; and (3) the position effect is detectable only in a double heterozygote between pseudoallelic mutant genes, the arrangement of which must be of the *trans*-type (a +/+ b). Interference in somatic pairing exerted by structural heterozygosity is postulated to reduce the transport of an essential gene product from one chromosome of this heterozygote to the other. By the use of this new method fast (pile) neutrons have been found to be more effective than X-rays or gamma rays in producing rearrangements in Drosophila, and estimates of the dose of fast neutrons at different stations during a nuclear detonation have been derived.

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SOME ASPECTS OF POSITION PSEUDOALLELISM*

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INTRODUCTION

The phenomenon of pseudoallelism promises to contribute much to our understanding of the gene—how it functions, how it mutates, how it evolves. The functional aspect will be the central theme of this paper. Attention will be focused chiefly on examples of "position pseudoallelism"; namely, those cases in which there is a position effect or phenotypic difference between the "*cis*-type" ($a\ b/+\ +$) and "*trans*-type" ($a+/+b$) of double mutant heterozygote. In most of these examples a close functional relationship between the adjoining loci is indicated.

There are currently two contrasting interpretations of position pseudoallelism. On the first or functional interpretation the mutants at the different pseudoallelic loci are alterations at different sites of a single functional unit which is called the gene. On the second or genetic interpretation the mutants at the different loci are alterations in different units each of which is called a gene, whether it be a single functional unit or not.

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The functional interpretation is currently advocated by Pontecorvo (1952) on the basis of studies (Roper, 1950; and Roper in Pontecorvo et al., 1953) of three biotin mutants in *Aspergillus nidulans*, which are presumptive position pseudoalleles although recovery of *cis*-types has not been reported; and by MacKendrick and Pontecorvo (1952) on the basis of studies of certain pairs of white "alleles," which, by analogy with the case of apricot and white (Lewis, 1952), may be assumed to be position pseudoalleles. The chief difficulty with this type of interpretation is that adequate criteria for recognizing a functional unit are not available. Mere appearance of functional identity, as in the case of the above biotin mutants, or certain inositolless mutants in Neurospora which are also presumptive position pseudoalleles (Giles, 1951), is obviously not sufficient. Thus, it is easy to see how two or more units which control different reactions in a sequential series can mimic the action of a single unit. Another criterion has been the phenotypic test of allelism; thus, two recessive mutants, each arising independently from a standard or wild type, have been considered alleles of a single gene if the heterozygote between them has a mutant phenotype. This criterion is inadequate since it does not take into account the possibility of position effects; thus, the finding that the trans-type in the case of position pseudoallelism is mutant in phenotype is readily explained by a position effect involving the products of two different functional units (Lewis, 1951).

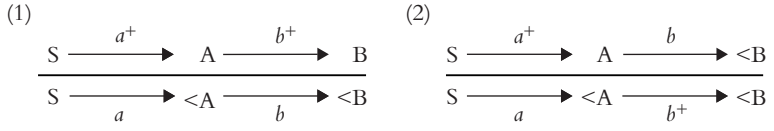
Paradoxically, the application of the phenotypic test of allelism has largely been responsible for endowing the gene with complex functional attributes rather than a unity of function. Thus, most of the well-studied cases of multiple allelism (or pseudoallelism, as the case may be) that have been identified largely by means of this test show evidence for at least two, more or less independently varying, functional components; for example, the dumpy mutants (Muller, 1922) and the scute and achaete series of mutants (beginning with the work of Serebrovsky, 1930) in Drosophila, and the "*R*" mutants (Emerson, 1921; Scadler, 1946, 1953, 1954) and the "*A*" mutants (see Laughnan in this Symposium) in maize. The difficulties in setting up criteria for functional unity become more aggravated in the case of allelic or pseudoallelic series of dominant mutants and/or mutants of obscure origin. Such cases tend to show even greater degrees of complexity at the functional level; e.g., self-sterility alleles in Oenothera (Lewis, 1949), the "E" mutants in Bombyx (reviewed by Tanaka, 1953) and the genes controlling cellular antigens in cattle (Stormont et al. 1951).

The evidence from established cases of position pseudoallelism in Drosophila suggests not a functional unity but as many functional components as there are different loci. This has been discussed in detail before (Lewis, 1951) for the cases of Star-asteroid (Lewis, 1945), Stubble-stubbloid, three loci of the bithorax series, and the three loci of the lozenge series investigated by Green and Green (1949). In the lozenge case the evidence is incomplete but already points to at least two functional components (fertility and eye effects). The more recent example of apricot-white pseudoallelism, referred to above, well illustrates the same point, since the series of white "alleles" has long been known (Morgan et al., 1931) to be separable into two

qualitatively distinct groups with respect to reaction to Bridges' specific modifier gene, Pale, or to sexual dimorphism. Thus, the eye-color of mutants of the "apricot" group (including apricot, blood, coral, and honey) is darker in the male than the female, while it is lighter in the male than in the female in the case of the "eosin" group (including white, as well as eosin and most of the other mutants of the series). The data of MacKendrick and Pontecorvo (1952) can be interpreted to mean that coral and blood each lie to the left of white, parallelling the finding that apricot lies to the left of white. To this limited extent, at least, the separation of the white series by the crossing-over test coincides with the separation by the test of qualitatively different function. In the recent case of two vermilion position pseudoalleles, Green (1954) has used a more or less specific modifier gene to distinguish the two; but the evidence for functional differentiation is not quite so convincing in this case. Finally, there is the probable case of two singed position pseudoalleles discovered by Ives and Noyes (1951), which awaits report of the *cis*-type; here, too, the evidence from existing mutants (see Bridges and Brehme, 1945) suggests two, more or less independently varying attributes (fertility and bristle effects).

By contrast the genetic interpretation refers the complexity of functioning which typifies pseudoallelism to interactions at the level of different gene products. The standard operational criteria for defining the gene—indivisibility by the crossing-over or rearrangement test—are thus still preserved; and there remains a one-to-one correspondence between gene and locus.

Specifically, a model of gene action in terms of sequential biochemical reactions has proved a fruitful working hypothesis. This kind of model was formerly advocated by Pontecorvo (1950) on the basis of theoretical considerations of millimicromolar reactions (McIlwain, 1946), and was independently put forward (Lewis, 1949) and elaborated in some detail (Lewis, 1950, 1951) to explain the position effect which characterizes position pseudoallelism. This model assumes that (1) the normal allele of one of the pseudoallelic genes, a^+, controls a reaction: S → A, while the normal allele of a second gene of the series, b^+, controls a reaction: A → B; (2) the mutant alleles block or impair these reactions; and (3) the substance A, at least, is produced at, or very close to, the site of the gene in the chromosome and is effectively transported along the chromosome more readily than it is transported to the homologous chromosome. As may be seen from the diagrams below, the *cis*-arrangement of the wild-type alleles (1) is expected on the above assumptions to give a more nearly normal action (production of substance B) than the *trans*-arrangement (2):



A new kind of supporting evidence for this model has come from the discovery that, in the case of the bithorax pseudoallelic series, structural heterozygosity for

certain chromosomal rearrangements (*R*) significantly alters the phenotype of particular *trans*-types towards a more extreme departure from wild-type, yet, in general, does not alter the phenotype of the *cis*-type. This new type of position effect, detected by comparing $R(a+)/+b$ or $a+/R(+b)$, on the one hand, with $a+/+b$ on the other, will be referred to as the "transvection effect." As recently described in some detail (Lewis, 1954b), the majority of X-ray-induced rearrangements which have at least one breakage point between the centromere and the locus of *bx* (a distance of over 500 "bands" of the salivary gland chromosomes) evoke the transvection effect; moreover, the results of the analysis of these strongly supports the hypothesis that a reduction in somatic pairing is the causative factor in modifying the phenotype of the *trans*-type. The transvection effect phenomenon is readily understandable on the above model by assuming that a reduction in somatic pairing effectively blocks the residual transport of substance A (diagram 2, above) from its site of production in one chromosome to the corresponding site in the homologous chromosome.

The position effect which is detected by comparing the *cis*- and *trans*-type and which is the basis for defining position pseudoallelism now needs a term to distinguish it from the transvection effect, and will henceforth be referred to as the "cisvection effect" (Lewis, 1954b). A variety of cisvection and transvection effects are known in the bithorax case, and the remainder of this paper will be devoted to a systematic treatment of these phenomena.

CISVECTION EFFECTS

Evidence for five loci in the bithorax pseudoallelic series has recently been presented (Lewis, 1954a). In order from left to right starting at locus 58.8 in the third chromosome, the loci are: bithorax (*bx*), Contrabithorax (*Cbx*), Ultrabithorax (*Ubx*), bithoraxoid (*bxd*), and postbithorax (*pbx*). The evidence was, however, incomplete in that between two pairs of genes, *Cbx* and *Ubx*, and *bxd* and *pbx*, only the wild-type crossovers had been recovered from females of the *trans*-type. Since then the complementary crossovers, *Cbx Ubx* and *bxd pbx*, respectively, have been obtained from females of the appropriate *trans*-type. Cisvection effects have been studied by comparing all ten possible double mutant combinations in the *cis*- and *trans*-types for the mutants, bx^3, *Cbx*, *Ubx*, *bxd*, and *pbx*. Before considering them, however, the individual mutant phenotypes will be briefly reviewed.

Three of the mutants, bx^3, *Ubx*, and *bxd*, are spontaneous in origin and have been described in some detail before (see Bridges and Brehme, 1944; Lewis, 1951; *Ubx* was formerly designated as bx^D, in the former reference; as *Bxl*, in the latter; and as bxd^D by Lewis, 1949). The *Cbx* and *pbx* mutants are of X-ray origin, and, as recently reported (Lewis, 1954a) are remarkable in that they appear to have been induced simultaneously. Each of these five mutant genes appears to be normal in the salivary gland chromosomes. These genes effect well-defined transformations of certain body segments or parts of body segments. At least four sharply distinct and more or less independently varying transformations of this kind have been recognized. On the

Table 1 Individual mutant phenotypes of the bithorax pseudoallelic series.

Name of locus	Genotype	Type of body segment transformation			
		I	II	III	IV
bithorax	bx^3/bx^3	++++	0	0	0
postbithorax	pbx/pbx	0	++++	0	0
bithoraxoid	bxd/bxd	0	+++	+++	0
Contrabithorax	Cbx/+ and Cbx/Cbx	0	0	0	++++
Ultrabithorax	Ubx/+	+	0	0	0

0 = little or no transformation; hence wild-type, or nearly so; +, ++, +++, ++++ = very slight, slight, moderate, extreme degrees, respectively, of the indicated transformation

basis of these, each mutant can be rather precisely described and readily distinguished from any one of the others (see Table 1). Thus, the bx^3 homozygote has the anterior portion of the metathorax (AMT) transformed into a structure which very closely resembles the anterior portion of the mesothorax (AMS)—a transformation (Type I) which will be symbolized: AMT → AMS. The *pbx* homozygote has a second type of transformation (Type II); namely, a conversion of the posterior portion of the metathorax (PMT) into a structure very closely resembling the posterior portion of the mesothorax (PMS), or symbolically: PMT → PMS. The *bxd* homozygote also has this Type II transformation, but not quite so well developed, and, in addition, always has a thoracic-like modification of the first abdominal segment (AB_1). The latter transformation (Type III) is primarily towards a structure resembling AMT, and will be symbolized: AB_1 → AMT; however, the presence of posteriorly wing-like halteres on AB_1 (thus far found only in bxd/bxd^{121}, where bxd^{121} is of X-ray origin) implies that posteriorly AB_1 changes towards PMS. The *Ubx*/+ genotype has an extremely slight Type-I transformation (recognizable only in the haltere, whose distal segment is enlarged and more hairy on the anterior margin than the wild-type haltere). The *Ubx* homozygote is lethal in the adult stage but the larval phenotype and interactions with other mutants of the series indicate that it is phenotypically like double-mutant homozygotes between *bxd* and a *bx* mutant; i.e., combines Type-I, -II, and -III transformations (the combination of Type I and Type III giving a transformation of AB_1 towards AMS). Whether *Ubx* differs qualitatively from such double mutant combinations is not clear. The *Cbx* homozygote, as well as the virtually identical *Cbx*/+ genotype, has a fourth transformation (Type IV); namely, a reduction in the development of PMS so that it partially resembles, especially in the case of the wing, PMT—or a change in the mesothorax which may be written: PMS → PMT. Occasionally, the *Cbx* phenotype has also a reduction in AMS so that the latter begins to resemble AMT, especially in the case of the wing which becomes almost completely haltere-like.

Since the first known mutant of the above series, bx^1 of Bridges (see Bridges and Brehme, 1944), is highly variable and may occasionally overlap wild type, the false impression may have arisen in some quarters that these so-called homeotic mutants are

Table 2 Cisvection and transvection effects involving the bithorax pseudoallelic series (Legend as in Table 1).

Group	Mutants in heterozygote	Type of heterozygote	Type of body segment transformation			
			I	II	III	IV
		cis	0	0	0	0
	a. bx^3 and bxd	*trans*	0	0	0	0
		R-*trans*	0	0	0	0
		cis	0	0	0	0
1.	b. bx^3 and pbx	*trans*	0	0	0	0
		R(*cis*)	0	0	0	0
		R(*trans*)	0	0 to +	0	0
		cis	0	0	0	0
	c. bxd and pbx	*trans*	0	+++	0	0
		R(*trans*)	0	+++	0	0
		cis	+	0	0	0
	a. bx^3 and Ubx	*trans*	+++	+	0	0
		R(*trans*)	++++	+	0	0
		cis	+	0	0	0
2.	b. Ubx and bxd	*trans*	+	+++	+++	0
		R(*trans*)	+	+++	+++	0
		cis	+	0	0	0
	c. Ubx and pbx	*trans*	+	++++	0	0
		R(*trans*)	+	++++	0	0
		cis	0	0	0	++
	a. bx^3 and Cbx	*trans*	0 to +	0	0	++++
		R(*trans*)	0 to +	0	0	++++
		cis	0	0	0	++++
3.	b. Cbx and bxd	*trans*	0	0	0	++++
		R(*trans*)	0	0	0	++++
		cis	0	0	0	++++
	c. Cbx and pbx	*trans*	0	0 to +	0	++++
		R(*trans*)	0	0 to +	0	++++
		cis	+	0	0	+
4.	a. Cbx and Ubx	*trans*	++	+	0	+++
		R(*cis*)	+	0	0	0
		R(*trans*)	++	+	0	+++

intrinsically highly variable. On the contrary, all of the above described mutant effects are surprisingly uniformly expressed, with the exception of the variability noted for the *Cbx* mutant. In no case have any of these phenotypic effects, including those of the *Cbx* mutant and the slight dominant effect of *Ubx*/+, been observed to overlap the wild-type phenotype.

With the above five mutant genes and their ten possible double mutant combinations, there are ten possible pairs of *cis*- and *trans*-types to be compared for cisvection effects. All ten pairs have been constructed and their phenotypes are summarized in Table 2 in terms of four transformation types described above. For the sake of systematic presentation, the results will be discussed in terms of the

four groups of comparison that can be made on the basis of dominant and recessive relationships.

Group-1 comparisons involve only the recessive mutants. The *cis*- and *trans*-types for bx^3 and *bxd* are both wild-type in phenotype; thus, no cisvection effect is in evidence. The *cis*- and *trans*-types for bx^3 and *pbx* are also wild type; in contrast with the previous comparison, however, these genotypes can be shown to differ phenotypically by making each a structural heterozygote for chromosomal rearrangements which evoke moderate to extreme transvection effects. As described elsewhere (Lewis, 1954b), the translocation, $T(2;3)bw^{VDe3}$, which has the major portion of the right arm of the third chromosome reciprocally translocated to the distal portion of the right arm of the second chromosome, is such a rearrangement, and is useful to employ since it has an inseparable, dominant, variegated-brown effect. By the use of this rearrangement, it is found that the *cis*-type, $R(++)/bx^3$ *pbx* remains wild-type; while each of the *trans*-types, $R(bx^3+)/+$ *pbx* and $bx^3+/R(+$ *pbx*), occasionally has a very slight wing-like development of the posterior region of the haltere; i.e., a slight Type-II transformation. Finally, the *cis*-type, *bxd pbx*/++, is wild-type; while the *trans*-type, *bxd* +/+ *pbx*, has a moderate Type-II transformation, thus indicating a strong cisvection effect.

Group-2 comparisons involve each of the three recessive mutants with the dominant *Ubx* mutant. Each of the three comparisons of this kind shows pronounced cisvection effects. On the one hand, the three *cis*-types, bx^3 *Ubx*/++, *Ubx bxd*/++, and *Ubx pbx*/++ are phenotypically indistinguishable from each other and from the single dominant mutant heterozygote, *Ubx*/+, which, as already noted, differs from wild type only by a very slight Type-I transformation. On the other hand, bx^3+/+ *Ubx* combines a moderate Type-I (figured by Lewis, 1951) with a very slight Type-II transformation; *Ubx* +/+ *bxd* combines the above very slight Type-I with moderate Type-II and moderate Type-III transformations (since no haltere-like structure has been observed on AB_1 of this genotype, it is not possible to observe whether the very slight transformation of Type I combines in that segment with the Type III one to produce a mesothoracic-like modification of this segment); while the remaining *trans*-type, *Ubx* +/+ *pbx* combines the very slight Type-I with a very extreme Type-II transformation.

Group-3 comparisons involve each of the three recessive mutants with the dominant *Cbx* mutant. The comparison of bx^3 *Cbx*/++ with bx^3+/+*Cbx* reveals another type of cisvection effect. Thus, the *trans*-type is like the single mutant heterozygote, *Cbx*/+; it has a well-developed Type-IV transformation. The *cis*-type, on the other hand, has only a slight transformation of this type. The *trans*-type sometimes also has a very slight Type-I transformation which the *cis*-type lacks. The *trans*-types, *Cbx* +/+ *bxd* and *Cbx* +/+ *pbx*, show no striking differences from *Cbx*/+, nor from their respective *cis*-types; however, *Cbx* +/+ *pbx* appears to have the beginning of a Type-II transformation in the region of the haltere, while its *cis*-type is wild type in this respect. At the same time, there are striking differences between the *cis*- and *trans*-types in each of these two latter comparisons when they are studied in

the presence of a recessive, sex-linked, partial suppressor of *Cbx* (symbol, *su-Cbx; locus*, 30±; spontaneous in a stock of *v* Bx^r; bxd^{51j}, kindly supplied to the author by M. M. Green). Thus, males of the *cis*-types, *su-Cbx*; *Cbx bxd*/+ +, and *su-Cbx*; *Cbx pbx*/+ + (as well as the genotypes, *su-Cbx*; *Cbx*/+ and *su-Cbx*; bx^3 *Cbx*/+ +) differ from wild type only in having a very slight Type-IV transformation; that is, they have an almost complete suppression of the dominant effect of *Cbx*. By contrast, in addition to this latter very slight transformation of Type IV, males of the *trans*-type, *su-Cbx*; *Cbx* +/+ *bxd*, have a moderate Type-III, and males of the *trans*-type, *su-Cbx*; *Cbx* +/+ *pbx*, have a slight Type-II transformation. Furthermore, males of the *trans*-type, *su-Cbx*; bx^3+/*Cbx* +, have a slight Type-I transformation as well as the very slight Type-IV one.

Finally, group-4 comparisons involve the two dominant mutants. The *cis*-type, *Cbx Ubx*/+ +, is remarkable in that there is scarcely any detectable Type-IV transformation; the alula of the wing is reduced and the wings are often slightly spread, but the phenotype otherwise is wild type except for the typical very slight Type-I transformation characteristic of *Ubx*/+. On the other hand, the *trans*-type, *Cbx* +/+ *Ubx* has a moderate transformation of Type IV (not quite so extreme as that of *Cbx*/+), a slight one of Type I (the haltere being larger and more hairy than that of *Ubx*/+), and a very slight one of Type II.

It is a general rule that cisvection effects are very striking position effects, in that the *cis*- and *trans*-types not only differ strikingly in phenotype, but they show little or no tendency to overlap one another in phenotype over a wide range of environmental conditions. This rule applies to all of the above cisvections shown in Table 2, except that the phenotype of *R*(bx^3+)/+ *pbx* may overlap wild type and hence overlap that of *R*(+ +)/bx^3*pbx*; however if a more complex rearrangement (with respect to structural heterozygosity of the *bx* region) than $bw^{V\,De3}$ is employed then there is virtually no overlapping of these two phenotypes.

To summarize, a broad spectrum of cisvection effects have been met with in the case of the *bx* pseudoallelic series. Both *cis*- and *trans*-types for a given pair may prove to be wild type under perhaps all conditions (bx^3 and *bxd*). This case is analogous to that of miniature (*m*) and dusky (*dy*) mutants studied by Slatis and Willermet (1953) who find that *m* +/+ *dy* and *m dy*/+ + are each virtually wild type, although the latter may possibly have significantly shorter wings than the former. The *cis*- and *trans*-types may be wild type under normal conditions but differ phenotypically when both are made into identical structural heterozygotes (bx^3 and *pbx*). It becomes evident from this case that there may be no sharp line between pseudoallelism with and without the position effect phenomenon: thus, the possibility of position effect is obviously to be kept in mind in the numerous cases of pseudoallelism where both *cis*- and *trans*-types are wild type or otherwise identical in phenotype; e.g., cases of pseudoallelism in mice (Dunn and Caspari, 1942, 1945) or cotton (see reviews of this and other cases by Stephens, 1951; Komai, 1950). A striking cisvection effect occurs between two recessive mutants (*bxd* and *pbx*). The latter case is analogous to the cases of lozenge, apricot-white, and vermilion pseudoalleles, already referred to.

Striking differences occur in every comparison involving any one of the recessive mutants with *Ubx*; these effects are analogous to those observed with the Star-asteroid series. In comparisons involving *Cbx* unusual relations arise. Thus, the *trans*-type may be mutant but not strikingly different from *Cbx*/+; yet position pseudoallelism is indicated by the strikingly different and much more nearly normal *cis*-type (*Cbx* and bx^3). Or both *cis*- and *trans*-types may be mutant and nearly, if not quite, identical; yet cisvection effects can be revealed in the presence of a sensitizing modifier gene; *su-Cbx*, (*Cbx* and *bxd*, or *Cbx* and *pbx*). *Ubx* acts as a virtually complete suppressor of the dominant effect of *Cbx* in the case of the *cis*-type, but has only feeble interactions with *Cbx* in the case of the *trans*-type. This comparison and that involving *Cbx* and bx^3 parallel somewhat the Stubble-stubbloid case (Lewis, 1951); thus stubbloid acts as a complete suppressor of the dominant Stubble phenotype in the case of the *cis*-type but gives an extreme mutant phenotype in the case of the *trans*-type.

TRANSVECTION EFFECTS

To study transvection effects, the translocation, bw^{VDe3}, has been used to produce structural heterozygosity. This rearrangement has been combined by crossing over with all of the single mutant types except *Cbx* and most of the double mutant types (the exceptions being *Cbx Ubx, Cbx bxd* and *Cbx pbx*). In no case has heterozygosity for this translocation modified the phenotype of a heterozygote for a single mutant gene, and in only one case (discussed below) is the phenotype of the *cis*-type between two mutants modified. The manner in which certain of the *trans*-types are altered by heterozygosity for this translocation will be discussed by systematically considering the four groups of double mutant types already adopted. The results are also shown in Table 2. In the case of Groups 1 and 2, comparisons have been made in every case between the *trans*-type without structural heterozygosity, on the one hand, and the two forms of the structurally heterozygous *trans*-type, or "*R*(*trans*)-type" as it is designated in Table 2, on the other hand. In no case has there been any obvious difference between the two forms of an *R*(*trans*)-type; that is, between $R(a+)/+b$ and $a+/R(+b)$. Both forms of the "R(cis)-type," as the structurally heterozygous *cis*-type is designated, have also been constructed in all of the Group-1 and -2 cases. Again, in no case involving Groups 1 and 2 has there been an obvious phenotypic difference between such pairs; moreover, since in such cases the *R*(*cis*)-type does not differ from the *cis*-type, the *R*(*cis*)-type has been omitted from Table 2 except where explicitly needed. In the case of Groups 3 and 4 of Table 2, only one form of the *R*(*trans*)-type or *R*(*cis*)-type has been constructed; and only where the phenotype of the *R*(*cis*)-type was different from that of the *cis*-type is it included in the table.

Among the Group-1 comparisons of *trans*-types with and without structural heterozygosity, only the one involving bx^3 and *pbx* shows a transvection effect. In this case, $R(bx^3+)/+$ *pbx* occasionally has a very slight Type-II transformation in contrast to the wild-type phenotype of $bx^3+/+$ *pbx*.

Among the Group-2 comparisons, the only transvection effect yet detected involves bx^3 and *Ubx*. In this case, *R*(bx^3 +)/+ *Ubx* has an extreme type-I transformation compared to a moderate type-I found in bx^3 +/+ *Ubx*.

Among the Group-3 comparisons, no obvious transvection effects are present. However, preliminary studies utilizing the above-described modifier gene, *su-Cbx*, have shown that *su-Cbx*; *R*(bx^3 +)/+ *Cbx* males have a significantly more extreme Type-I transformation than su-Cbx; bx^3 +/+ *Cbx* males.

Among the Group-4 comparisons, there is one transvection effect and it is of a new type. Whereas, in all other examples of this phenomenon, it is only the *trans*-type that is modified by the appropriate kind of structural heterozygosity, in this case it is only the *cis*-type which is modified. Thus, the comparison of the *trans*-type and the R(*trans*)-type for *Cbx* and *Ubx* reveals no obvious phenotypic difference. On the other hand, the *R*(*cis*)-type is more nearly wild-type than the *cis*-type; that is, *R*(+ +)/*Cbx Ubx* lacks the very slight type-IV transformation characteristic of + +/*Cbx Ubx*. The latter result has, moreover, been verified for a number of different *R*'s besides $bw^{V\,De3}$.

It is a general rule that the phenotypic differences involved in the recognition of the transvection effects are relatively slight ones. Maximum sensitivity for the detection of such differences requires attention to environmental conditions: constant temperature (25°C) and sufficient food. Combinations of two different rearrangements or single rearrangements more complex than $bw^{V\,De3}$, for example, have been used effectively to increase such differences, as in the case of bx^3 and *pbx*, noted above. Such techniques, as well as the use of sensitizing modifier genes, may help to reveal transvection effects in the cases where none have yet been detected. Although isogenic stocks have not been employed, an approach to complete control over the genetic background has been made in most of the observed transvection effects by utilizing a variety of stocks of each mutant type (containing different substitutions with respect to closely linked marker genes), and by utilizing different chromosomal rearrangements of independent origin. Such techniques have actually failed to reveal differences traceable to modifier genes; that is, the results are reproducible under a wide variety of genetic backgrounds and with different rearrangements.

To summarize, transvection effects have been detected between bx^3 and *Ubx*, bx^3 and *pbx*, bx^3 and *Cbx* (if sensitized by the presence of *su-Cbx*), and *Cbx* and *Ubx*, as well as between bx^{34e} and *Ubx* (as reported in a previous study—Lewis, 1954b). Thus, such effects are not dependent upon the presence of some particular mutant type. The mutants, *Cbx* and *Ubx*, give a unique result in that a phenotypic difference arises only between the *R*(*cis*)-type and the *cis*-type; while in all of the other cases, it arises only between the *R*(*trans*)-type and the *trans*-type.

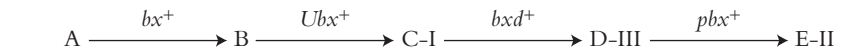
DISCUSSION

An attempt will be made below to construct a model of gene-controlled reactions in the case of the bithorax pseudoallelic series which will (1) take account of the principal

mutant transformations, (2) explain as simply as possible the observed cisvection and transvection effects, and (3) give some picture of how the genes control the wild-type segmentation pattern.

In a previous report (Lewis, 1951) it was pointed out that the position effects between bx^3 and *Ubx*, and *Ubx* and *bxd* (cisvection effects, in the newer terminology), can be simply interpreted on the basis of three successive, gene-controlled reactions occurring at the chromosomal level. The order of control could be postulated to be either: $bx^+ - Ubx^+ - bxd^+$ or $Ubx^+ - bx^+ - bxd^+$. In addition to the finding that this scheme satisfactorily accounted for the phenotype of a great many genotypes, it received independent support from studies of chromosomal rearrangements having one breakage point within the pseudoallelic series itself. Thus, rearrangements which apparently separate bx^+ and Ubx^+ on the one hand from bxd^+, on the other, were found to have no detectable change in the action of the bx^+ and Ubx^+ genes but were found to act like extreme mutant changes in the action of bxd^+. For example, the homozygote for the transposition, bxd^{100} (which has bx^+ and probably Cbx^+ and Ubx^+ transposed to the left arm of the third chromosome) has extreme transformations of the *bxd* type (Type-II and Type-III), but not of the *bx* type (Type-I). On the above *trans*-type between bx^3 and *bxd* might also be expected to give the *bxd* types of transformations; while the *cis*-type would be expected to be wild type. Although the *trans*-type as well as the *cis*-type in the case of bx^3 and *bxd* is wild type, this was not regarded as necessarily inconsistent with the above scheme since both bx^3 and *bxd* could be regarded as intermediate alleles of their respective gene-controlled reactions. The latter interpretation was especially plausible since (1) *bx*-like mutants of X-ray origin, which are more extreme than bx^3, give strong Type-II and Type-III transformations in the *trans*-types with *bxd*; and (2) extreme *bxd*-like mutants of X-ray origin, such as bxd^{100}, give a very slight Type-II transformation in the *trans*-type with bx^3. In neither of these cases could the assumption of a cisvection be tested, since the complication of chromosomal rearrangements associated with the X-ray induced changes prevented the recovery of *cis*-types.

The new findings reported here concerning the *pbx* mutant help to clarify the above findings regarding the Type-II transformation, and permit an extension of the above scheme to include the pbx^+ gene. Thus, the Type-II transformations which have just been discussed, as well as those which characterize the trans-types between *pbx* on the one hand, and bx^3, *Ubx*, or *bxd*, on the other, are all readily accounted for if it is assumed that (1) pbx^+ controls a reaction subsequent to that of bxd^+, and (2) a reduction in the concentration of the gene product of pbx^+ leads to the Type-II transformation. This gives the scheme "Scheme 1" for the sequence of gene-controlled reactions. Although the order of control of reactions by bxd^+, pbx^+, and either bx^+ or Ubx^+ is the same as the gene order in the chromosome, the order of control with respect to bx^+ and Ubx^+ cannot be deduced from the data and is arbitrarily assumed to be the same as that in the chromosome to simplify the discussion of this scheme.



Scheme 1

The omission of Cbx^+, whose locus in the chromosome is between those of *bx* and *Ubx*, is intentional, since as discussed below its role in such a scheme is not clear. In the above notation the roman numeral after the symbol of the gene product identifies the type of transformation controlled by that product. More exactly, a reduction in the relative concentration of substance C-I is postulated to lead to a transformation of Type-I; of D-III to one of Type III; and of E-II to one of Type-II. The substances, C-I and D-III, at least, are assumed to be produced in sufficient concentration to act not only as substrates for the appropriate successive step in the chain, but also as agents which somehow ultimately direct the control of specific physiological processes. The intermediate substances may be thought of as enzymes, as enzyme percursors, or more likely as products of enzymatic activity, that activity in the latter case coming either directly from the gene or from an enzyme closely held to the gene. The essential requirement for the interpretation of the observed cisvection and transvection effects is that these substances be produced at, or very close to, the site of the genes in the chromosome, rather than at other points in the cell.

Specifically, the Type-II transformations discussed above are accountable for on Scheme 1 in the following way. In the case of the trans-type, $bx^3 +/+ bxd^{100}$, the bx^3 mutant is assumed to reduce relatively slightly the concentration of B, which then leads indirectly to a relatively slight reduction in E-II; in the other chromosome, the separation of bx^+ and pbx^+ is expected to result in a relatively extreme reduction in the concentration of E-II; the observed very slight Type-II transformation is thus accounted for. In the case of $bx^3 +/+ pbx$, bx^3 is assumed to act as in the preceding case, while a relatively extreme reduction in E-II in the other chromosome is postulated to result from a blocking of the final reaction by the *pbx* mutant; the observed result, that a very slight Type-II transformation arises in this case only if there is at the same time structural heterozygosity, will be discussed below. In the case of $Ubx +/+ pbx$, the *Ubx* mutant is assumed to cause a relatively extreme reduction in C-I and in turn of E-II, while the *pbx* would act as in the previous case; the observed extreme type-II transformation is thus accounted for. Finally, in the case of $bxd +/+ pbx$, the *bxd* mutant is assumed to cause a moderate reduction of D-III and therefore in turn of E-II; while the *pbx* mutant would act as before; the observed moderate Type-II transformation is thus accounted for. In the case of the three respective *cis*-types, $bx^3 pbx/++$, $Ubx\ pbx/++$, and $bxd\ pbx/++$, the chromosome carrying the wild-type alleles of the pseudoallelic genes is assumed to produce its normal quota of substance E-II and this quota is assumed to be sufficient to prevent the Type-II transformation, even though the other chromosome might fail to produce any E-II; the observed failure of the *cis*-types to show a Type-II transformation is thus accounted for. The remaining cisvection effect comparisons involving the four loci

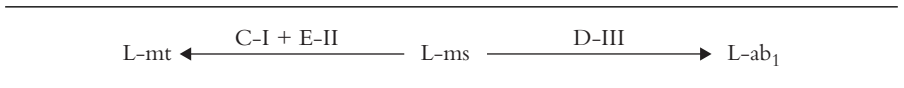
shown in Scheme 1 are those involving the following pairs: bx^3 and *Ubx*, bx^3 and *bxd*, and *Ubx* and *bxd*. As already noted, these cases have been discussed elsewhere (Lewis, *loc. cit.*) in detail in terms of a model including the first three steps of Scheme 1. The only change that Scheme 1 introduces is to interpret Type-II transformations as being controlled not by the product of the bxd^+ gene, but by that of pbx^+. In the case of bx^3 +/+ *bxd*, it remains necessary to postulate a threshold effect to explain its wild-type phenotype; in other words, the reduction in amount of substances D-III and E-II which is expected in this genotype on Scheme 1 is postulated to be below the threshold required to give a detectable mutant phenotype.

The application of the sequential reaction type of interpretation to transvection effects has been discussed elsewhere (Lewis, 1954b) and will only be briefly considered here. In the case of bx^3 +/+ *Ubx*, for example, the Ubx^+ gene is assumed on Scheme 1 to receive some of its substrate B from the action of the bx^3 gene and some from diffusion of B from its site of production in the homologous chromosome; while in the presence of structural heterozygosity, the Ubx^+ gene is assumed to be deprived (relatively speaking) of this latter source of B as the result of reduced somatic pairing of the homologous chromosomes at that site. A similar argument would apply to the remaining transvection effect observed in Groups 1 and 2 of Table 2; namely, that involving bx^3 and *pbx*; here, however, the substance(s) involved might be any of the intermediates: B, C-II and/or D-III.

It is important to consider how, conversely, the production of substances C-I, D-III and E-II would be expected to control the development of the wild-type segmentation pattern. This should provide a test of the utility of Scheme 1 and an aid to the understanding of the mutant phenotypes. Thus, the production of substances C-I and E-II is postulated to cause the wild-type metathoracic segment, to advance from a primitive level of developmental determination, which will be designated as a mesothoracic-like level, "L-ms," to a metathoracic- like level, or "L-mt." On evolutionary grounds, L-ms is more primitive than L-mt, since the Diptera almost certainly evolved from four-winged ancestors. A reduction in concentration of C-I or E-II would be expected on this basis to cause AMT or PMT, respectively, to tend to remain at the level, L-ms, which agrees with the respective definitions of the Type-I and Type-III transformations.

The production of substance D-III is postulated to cause the first abdominal segment of the wild-type organism to change from a primitive level, which again will be designated L-ms, to a first-abdominal level, or "L-ab_1." On evolutionary grounds, L-ms is probably more primitive than L-ab, since the ancestors of the insects certainly had legs on the abdominal segments, and the immature stages of many insects bear ventral abdominal appendages. In an earlier discussion of these levels of developmental determination (Lewis, 1951) it was assumed that this product of the bxd^+ gene (D-III) causes the first abdominal segment to change in a way that would be the exact reverse of the Type-III transformation ($AB_1 \rightarrow MT$); that is, causes change: L-mt $\rightarrow$ L-ab_1. This earlier assumption is unsatisfactory on evolutionary grounds and does not give a clear picture of the way in which development of this segment

would take place in certain genotypes; it will therefore be discarded in favor of the above assumption that the production of D-III leads to the change: L-ms → L-ab$_1$. In other words, the first abdominal segment is postulated to develop according to two alternate pathways, rather than according to two successive pathways (namely, L-ms → L-mt → L-ab$_1$), as formerly assumed. The alternate pathways and their specific controlling substances will be designated Scheme 2, and may be represented as follows:



Scheme 2

This scheme gives for the first time an adequate explanation of the development of AB$_1$ in the principal mutant types as well as in the wild-type organism. The following examples illustrate the way in which Scheme 2 is assumed to apply in such cases (see Table 1 for the observed effects). Thus, the *bx*^{3} homozygote possesses a sufficient quantity of substance D-III to direct AB$_1$ along the pathway to L-ab$_1$. The *bxd* homozygote lacks a sufficient quantity of D-III to direct the segment along this latter pathway, and lacks sufficient E-II to direct the posterior portion towards PMT, so that this portion should remain at level, L-ms; however, it has sufficient C-I to direct the anterior portion towards the level, L-mt. Finally, homozygotes for double mutants between *bx* and *bxd* mutants, or for *Ubx*, lack sufficient quantities of all three substances, so that AB$_1$ should remain at the level, L-ms.

The remaining segment of the wild-type organism to be considered in connection with Schemes 1 and 2 is the mesothorax. It is presumed that Scheme 1 does not effectively come into play during the development of this segment, perhaps because of an anterior–posterior gradient in the distribution of the initial substrate A. Thus, lack of sufficient quantities of substances C-I, D-III and E-II would leave MS at its primitive level of development, L-ms.

Scheme 1 has been found to account adequately for all of the genotypes constructed to date involving the *bx*^{3}, *Ubx*, *bxd* and *pbx* mutants, except for one case: *bx*^{3} + +/+ *Ubx bxd*. (Some 56 such genotypes among a possible total of 136 have been constructed, the remaining ones involving chiefly triple and quadruple mutant combinations, which have not yet been synthesized.) This exceptional case, omitted above since it is a triple mutant heterozygote, has a more extreme Type-I, and a less extreme Type-II transformation than does the genotype, *bx*^{3} + +/+ *Ubx* +. On Scheme 1 it would be expected that the former genotype would, if it did so at all, differ from the latter by having a less extreme Type-I transformation, as a result of the possibility of an accumulation of substance C-I, and a more extreme Type-II transformation, as a result of two blocks along the pathway to E-II in the + *Ubx bxd* chromosome, compared to only one in the + *Ubx* + chromosome. For in other cases, just such predictions are verified; for example, *bx*^{3} + *bxd*/*bx*^{34} + + has a less extreme Type-I transformation

than bx^3 + +/bx^{34e} + + (where bx^{34e} is an intermediate *bx* allele); and *Ubx bxd*/+ *bxd* has a more extreme Type-II transformation than does *Ubx* +/+ *bxd*.

The above difficulty in interpreting one exceptional genotype on Scheme 1 has not been resolved and indicates that at least one more variable is needed, if it is to remain a valid working hypothesis. There is such a variable in the case of substance B, since it has not been assigned a specific function (other than as substrate for the Ubx^+ gene). There is also the question of the role of Cbx^+ in Scheme 1, since this gene and its product constitute an additional variable, yet to be considered. But just what role Cbx^+ may play is not clear. Many of the interactions of the *Cbx* mutant with other mutants of the series indicate that Cbx^+ may control an additional step near the beginning of the reaction sequence; while its dominant effect may be attributed to an accumulation of some product such as B or C-I which would begin to cause MS to develop towards MT. However, since the *Cbx* transformation (Type-IV) is the inverse of those of Type-I and Type-II, the possibility of complicated interactions at a physiological level between the numerous hypothetical gene products involved in such transformations becomes an acute one.

The unique finding of a transvection effect by comparing *cis*- and *R*(*cis*)-types involving *Cbx* and *Ubx* can be formally reconciled with the other transvection effects, even though the role of *Cbx* in Scheme 1 is not clear. Thus, it may be that in *Cbx Ubx*/+ + a higher concentration of substance B reaches the *Ubx* gene than does so in *R*(+ +)/*Cbx Ubx*, where the homologous chromosomes are relatively farther apart; this would mean more of substance C-I, or a more nearly normal Type-I transformation, in the *cis*-type than in the *R*(*cis*)-type; in turn this would lead, paradoxically, to the observed result that the *cis*-type has a more extreme Type-IV transformation than the *R*(*cis*)-type, since the available evidence suggests that the more extreme the Type-I change the less extreme the Type-IV change.

CONCLUSIONS

It is concluded that there is an ordered complexity to the variety of cisvection and transvection effects, as well as individual mutant effects, in the case of the bithorax pseudoallelic series. The "functional" interpretation has to explain such results in terms of a concept of a single functional unit of probably considerable complexity—analogous to the old concept of the gene as deduced from the behavior of complex "multiple allelic" series. The "genetic" interpretation explains such results in terms of a working hypothesis in which each of the component genes of the series acts as a functional unit in the modern sense—namely, as an agent controlling a single, specific reaction.

SUMMARY

Contrasting interpretations of position pseudoallelism are discussed, and the types of position effect which characterize this phenomenon are illustrated, with special

reference to the case of the bithorax series of five pseudoallelic loci in *Drosophila melanogaster*. Most of the results in this latter case can be simply interpreted on the basis of a chain of gene-controlled reactions in which each intermediate substance is postulated to act in a dual capacity: as substrate for the succeeding reaction and as a determiner of a specific physiological process. The model also gives a consistent picture of the way in which different levels of developmental determination may be controlled by the different genes of the series.

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GENES AND GENE COMPLEXES

E. B. LEWIS

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Mendel was ahead of his time, but he was not so far ahead that he could not arrive at the first great generalizations of genetics—his first and second laws. How fortunate that Mendel did not discover linkage, for he might then have failed to deduce the second law. How fortunate, too, that Mendel, by having chosen to work on peas, failed to discover "gene conversion," not to mention "meiotic drive," for he might then have failed to deduce even the first law.

Mendel left it for others to study the role played by the hereditary factors in development, but he did make an observation in this area that turns out to be another sound generalization not without exception. It will be recalled that five of his seven pairs of factors were functionally interrelated, in the sense of affecting the color or form of the seeds or seed pods; nevertheless, these five pairs, as well as the other two, are known to assort independently. This observation that the hereditary factors or genes are distributed throughout the genome in a more or less random fashion with respect to their function in development remains valid for many of the known genes of higher organisms. It might well be called Mendel's third law. How fortunate for Mendel that his five functionally related genes did not happen to reside in an operon!

I would like to turn now to certain exceptions to this "third law"—namely, cases in which two or more functionally related genes do lie close together in the chromosome, forming a pseudoallelic series; or, to introduce a simpler term, a "gene

complex" (Brink 1932).[1] The term "gene cluster" is also in common use for such cases.

Gene complexes seem to be scattered here and there throughout the genome of higher organisms, and in bacteria and phages the genes may often be arranged in an almost continuous succession of such complexes. It now seems likely that the study of gene complexes will have much to tell us not only about the way in which genes control and regulate biosynthetic and developmental pathways but also about the way in which new genes arise from old ones. The existence of gene complexes, however, has added to the difficulty of developing operational criteria for defining the gene. Therefore, it may first be useful to review the present status of the gene concept.

THE GENE CONCEPT

The first operational definition of a hereditary unit was provided by Mendel's concept of a factor which assorts independently of other factors. For many years it was a satisfactory approximation to treat the gene as if it were at once a unit of mutation, a unit within which crossing over or other forms of chromosomal breakage and reunion did not occur, and a unit of function determining the production of a specific protein. The work of Benzer (1955) and of others (especially Yanofsky, 1963) has shown that certain kinds of mutational and recombinational tests can resolve the gene into smaller units—the "muton" and "recon" (Benzer, 1957), respectively. These smaller units not only coincide with one another but almost certainly correspond to the individual base pairs of the DNA molecule.

It still seems best to reserve "gene" for the larger functional unit. That unit is now thought of as the portion of the genetic code that carries the information needed to specify the production of a single polypeptide. This unit is sometimes called the "code-message," while "code-word" or "codon" denotes the triplet of base pairs coding for a single amino acid.

In most cases the code-message unit is thought to "transcribe," or code for the production of, an intermediate ribonucleic acid (RNA) template which is then "translated" into a polypeptide. This is the concept of the "structural gene" (Jacob and Monod, 1961). Evidently, some code-message units code for RNA molecules that do not function as templates, such as the transfer RNA's. Such units have quite properly also been considered genes. In the Jacob-Monod terminology, they would constitute one type of "regulatory gene."

The relationship among some of the terms currently in use for the genetic units is summarized in Fig. 1. The gene, or code-message unit, occupies a "locus," consists of a linear array of mutons, and exists in a number of alternative forms or "alleles." A gene complex, sometimes called a "pseudoallelic series," occupies a "region," and consists of a linear array of genes or "pseudoalleles." At least some of the gene complexes of

[1]The "gene complexes" of *Oenothera* have now come to be called "Renner complexes" or simply "complexes"; therefore, no confusion between this usage of the term and the present one should arise.

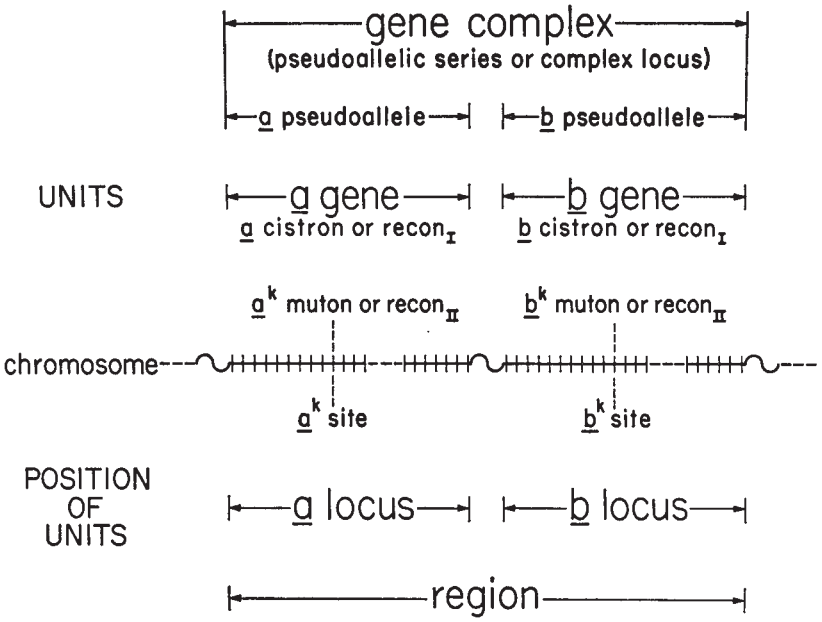

Figure 1. Diagram of some of the terms that have been proposed for the genetic units and for their location in the chromosome.

microorganisms form "operons" (Jacob and Monod, 1961); that is, in addition to a cluster of linked structural genes, the gene complex contains a region, or "operator," which controls the coordinate repression or derepression of all the genes of the operon. The operator, which may be a separate region or a part of one of the structural genes, seems to be the point at which transcription of the genetic message of the operon is initiated.

We need operational methods of defining a unit that is approximately equivalent to the structural gene. Functional, recombinational, mutational, and rearrangement tests have all been used to help define such a unit. In the discussion which follows, these four tests are examined separately; however, it should be kept in mind that they are closely interrelated and no one test is likely to prove adequate for distinguishing between a gene, on the one hand, and a gene complex, on the other.

Functional Test

The *cis–trans* test provides a purely genetic method of defining a unit of function—the "cistron" (Benzer, 1957). The *cis–trans* test involves comparing the phenotypes of the two possible forms of the heterozygote for two linked mutants, *a* and *b*. If the *cis* (*a b*/+ +) and the *trans* (*a* +/+ *b*) forms are both wild type in phenotype, then *a* and *b* fall in different cistrons, and *a* and *b* are said to "complement" each other; conversely,

if the *cis* and *trans* forms differ (the *trans* being mutant and the *cis*, wild type), then *a* and *b* fall in the same cistron, or are said to fail to complement.

In many situations, the *cis–trans* test works well. At one extreme if *a* and *b* tend to be far apart on the genetic map and to complement, they probably are mutant alleles of two different structural genes. Or, at the other extreme, if *a* and *b* are so close that no genetic recombination is observed in many trials and if they fail to complement as well, they probably are mutant alleles of the same structural gene. A logical difficulty arises in the latter case, however, since there might in fact be two structural genes so closely linked and functionally interrelated that they behave like one. In practice such cases of gene complexes are known to occur. For an understanding of the ramifications of the *cis–trans* test it becomes necessary to consider the four possible relationships which can arise for the *trans*-heterozygote, depending upon whether there are two closely linked structural genes or only one structural gene and depending upon whether the mutants complement or not. Actually, examples of all four possible relationships have been found, and a biochemical basis for each relationship can be inferred from the known examples of its type (Fig. 2). For simplicity, it is assumed

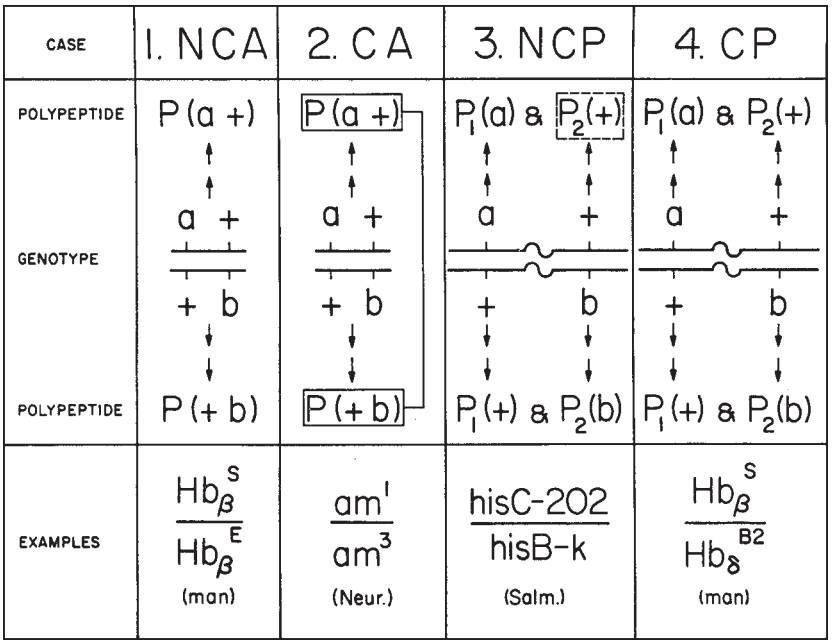

Figure 2. Diagram of four possible kinds of *trans*-heterozygotes for two closely linked genes, *a* and *b*, depending upon whether the genes are: (1) noncomplementing alleles (*NCA*); (2) complementing alleles (*CA*); (3) noncomplementing pseudoalleles (NCP); or (4) complementing pseudoalleles (CP); The arrows signify the steps of transcription and translation which intervene between the gene and its polypeptide (*P*).

that in all four cases the mutants *a* and *b* complement in the *cis* arrangement. Often, as in all of the examples shown in Fig. 2, the double mutant type has not yet been identified. In such cases, the *cis–trans* test is made without the *cis*-heterozygote, which is then assumed to have a wild-type phenotype.

The first type of *trans*-heterozygote shown in Fig. 2 involves noncomplementing alleles (NCA). This is the classical case of multiple allelism. An example, well understood at the polypeptide level, is the *trans*-heterozygote for the mutant genes which determine the abnormal human hemoglobins, S and E. Blood samples from two such heterozygotes were shown by Acksoy and Lehmann (1957) to lack the normal beta chain of hemoglobin A and to contain only the S and E types of beta chains. The latter chains are known to differ from the normal beta chains in having single amino acid substitutions in the sixth and 26th positions, respectively (Hunt and Ingram, 1959). Pedigree data support, but are not sufficient to establish, allelism of the mutant genes determining the S and E beta chains (Acksoy and Lehmann, 1957). The requisite genetic information to construct a normal beta chain is available in the *trans*-heterozygote in the form of the wild-type, or "+," sites corresponding to the S and E mutant sites; yet in human somatic cells such + sites seem to be unable to cooperate at the DNA, RNA, or protein levels to produce normal beta chains. In this case the unit defined by the *cis–trans* test probably coincides with the structural gene.

The two mutants of a *trans*-heterozygote for complementing pseudoalleles (CP) by definition occupy separate but closely linked and functionally related loci (case 4 of Fig. 2). Again, an example taken from the genetics of human hemoglobins well illustrates the biochemical basis of this case. A number of *trans*-heterozygotes have been found which have a mutant allele, usually the Hb_{β^S} allele, of the gene determining the beta chain of the normal major hemoglobin component A_1, and a mutant allele, $Hb_{\delta^{B2}}$, of the gene determining the delta chain of the normal minor component A_2 (Ceppellini, 1959; Horton and Huisman, 1963; Boyer et al., 1963). The *trans*-heterozygote in this case produces four kinds of hemoglobin (A_1, A_2, S, and B_2) corresponding to the four polypeptides derived from the mutant and wild-type alleles of the two genes. In 41 opportunities no recombinants between the two loci were observed (Boyer et al., 1963). This result is consistent with the close linkage that would be expected between pseudoallelic genes. That the genes for the beta and delta chains form a gene complex (i.e., are functionally closely related to one another, as well as being closely linked) is made clear from amino acid sequence determinations of the corresponding polypeptides (Ingram and Stretton, 1961; Baglioni, 1963). Thus, the beta and delta chains are each composed of 146 amino acid residues and are identical in sequence except for about nine single amino acid differences.

The *cis–trans* test leads to ambiguous results in the remaining two types of trans-heterozygotes. At one extreme, in the case of complementing alleles (CA, case 2 of Fig. 2), the cistron seems to be a smaller unit than the structural gene. The basis of this once-puzzling phenomenon of intraallelic complementation (Fincham, and Pateman, 1957; Giles et al., 1957; review by Catcheside and Overton, 1958) has now been clarified. The structurally abnormal polypeptides produced by each allele are known

to aggregate even *in vitro* and to produce a polymeric product with some wild-type activity (Woodward, 1959). For example, in *Neurospora* a series of amination–deficient (*am*) mutants are known which have structural defects in the enzyme glutamate dehydrogenase (GDH). Fincham and Coddington (1963) have shown that the proteins determined by heteroallelic mutant genes, such as am^1 and am^3, react *in vitro* under suitable conditions to form active GDH. The enzyme is known in this case to be a polymer composed of possibly eight identical subunits (Barratt, 1961). Evidently the active enzyme formed in the complementation reaction results from mixed aggregations of the protein subunits produced by the two complementing mutants. The CA case is exceptional in that only a protein with polymeric subunits, and not every such protein, shows the phenomenon of intraallelic complementation.

At the other extreme, in the case of NCP (case 3 of Fig. 2), the cistron seems to be larger than the unit coding for a single polypeptide. This phenomenon of "position pseudoallelism" (Lewis, 1955) may have more than one biochemical basis, but the one now favored for examples in phage and bacteria is based on the finding that in some gene complexes a mutant gene at one locus in the complex is able to reduce the rate of synthesis of proteins which would normally be produced by wild-type alleles of the genes lying beyond it in the same complex. Such "polarity" mutants (Jacob and Monod, 1961) are believed to be of at least two kinds: (1) reading-frame shift mutants (Crick et al., 1961), in which the insertion or deletion of a base pair disturbs the reading of the code-message from that point on; or (2) mutants with a base-pair substitution which results in a different or "modulating" codon that is then read with reduced efficiency (Itano, cited by Ames and Hartman, 1963). In either case the polarity mutant is assumed to exert its effect during translation of the RNA message into protein.

An example of the NCP case is taken from the work of Hartman et al. (1960). Mutant *hisC-202* is a point mutant of the *C* gene of the histidine operon of *Salmonella*. This polarity mutant only poorly complements mutants at the adjoining *B* locus, symbolized in Fig. 2 as *hisB-k*. In fact, *hisC-202* only poorly complements mutants at any of the six loci (*E*, *I*, *F*, *A*, *H*, and *B*) of the *his* operon that lie to the left of *C*. Enzymatic assay of the strain with mutant *hisC-202* by Martin, et al., 1965 (personal communication) shows that it has only about 15 % of the normal amounts of the enzymes made by the *B* gene and the other genes to the left, while it has normal amounts of the enzymes made by the two genes (*D* and *G*) to the right. Failure of complementation between certain mutants at two different loci of a gene complex may therefore have its biochemical basis in the coordinate and polarized synthesis of polypeptides which possibly takes place on a single polycistronic messenger RNA.

Recombinational Tests

A genetic criterion of first importance in arriving at a definition for a hereditary unit is the extent of divisibility of that unit by recombination. Mendel, of course, discovered one form of genetic recombination—independent assortment. If two

factors assort independently, there is little doubt that they belong in separate units; in most such instances, they probably will even be in different chromosomes. The discovery of linkage and crossing over led to a working definition of the gene as a unit within which crossing over does not take place. The discovery of an intragenic recombinational process (Mitchell, 1955), or "gene conversion," has tended to confuse the picture. As already noted, the occurrence of gene conversion has permitted resolution of the gene presumably into its ultimate subunits, the DNA base pairs. Even though intragenic recombination and crossing over may be different aspects of the same phenomenon of hybrid DNA formation followed by enzymatic excision and repair of the hybrid region, along the lines visualized by Whitehouse (1963), Holliday (1964), and Whitehouse and Hastings (1965), nevertheless these two types of recombination are operationally distinguishable to some extent.

Crossing over is characterized by reciprocality (i.e., production of wild-type and double mutant recombinants simultaneously in the same tetrad from a *trans*-heterozygote) and by positive interference. On the other hand, intragenic recombination is characterized by nonreciprocality (recovery of either wild-type or double mutant recombinants but usually not both from the same tetrad) and by negative interference. In view of these circumstances, it seems best to proceed on the basis that there may be two types of units of recombination, recon_{I}, the smallest unit recognizable by the test of crossing over, and recon_{II}, the smallest unit recognized by the test of intragenic recombination. The former unit may (in higher organisms) correspond to the structural gene; the latter unit, as has already been noted, seems to correspond to the muton.

Operationally, interference may turn out to be the more useful property for distinguishing between recon_{I} and recon_{II}. A critical test would involve studying the pattern of recombinational events in a heterozygote that has, ideally, several mutant alleles at each of two (or more) adjacent loci—e.g., a heterozygote of the type, $a + b + / + a^2 + b^2$, where a and a^2 are mutant alleles of one gene and b and b^2 are mutant alleles of an adjacent gene. (Of course, such a heterozygote must also carry closely linked, outside-marker mutants.) Stadler et al., (1965) have analyzed this type of heterozygote in *Neurospora* except that they have used only one mutant allele at each of the two loci. That is, they have studied recombination in a trans-heterozygote for *cys-1* and *cys-2*, which are mutants of two different but closely linked cistrons concerned with cysteine synthesis. In this case, the observed pattern of recombination events supports the hypothesis that intragenic recombination involving one of the genes strongly interferes with intragenic recombination at the other. It may therefore turn out that interference between units of the recon_{II} type will be strongly negative, whereas interference between units of the recon_{I} type will be strongly positive.

The remarkable discovery of polarization of intragenic recombination events in *Ascobolus* has led Rizet et al. (1960) to postulate the existence of the "polaron," a unit within which there is polarized nonreciprocal recombination. Reciprocal recombination, by this concept, is believed to occur at the linkage structure which unites two polarons. Polarized nonreciprocal recombination has been reported in

other fungi (Murray, 1963; Stadler and Towe, 1963). It is possible that the polaron will turn out to be equivalent to one of the larger units of the genetic code, perhaps to the gene or the gene complex.

Mutation Tests

It was implicit in the preceding discussion that the mutants which are employed in *cis–trans* or recombinational tests represent point mutations, in the sense of their having arisen from wild type by single base-pair substitutions. Operational means of recognizing such point mutations therefore become an essential part of the analysis of the fine structure of genes.

Yanofsky and coworkers (review by Yanofsky, 1963) have been remarkably successful in showing that in *Escherichia coli* certain mutant alleles of the structural gene for tryptophane synthetase cause single amino acid replacements to appear in the protein of the mutant strain. From knowledge of the genetic code (e.g., Nirenberg et al., 1965) it has been possible in many instances to infer that the mutant alleles responsible for such replacements probably differ from wild type by single base-pair substitutions.

In principle, certain analogs of the DNA base pairs and certain other chemical mutagens, such as nitrous acid, may be used to induce mutants which have specific kinds of single base-pair substitutions. "Forward" mutants induced in this way can be further characterized by retreatment with chemical mutagens to induce "reverse" mutations. From the pattern of response to such mutagens, the nature of the base-pair substitution is then inferred. Although this approach has had some success with certain phages and other viruses (review by Freese, 1963), much remains to be done before gene mutations in higher organisms can be induced at will and in a specified manner.

A surprising kind of operational test for characterizing at least certain kinds of base-pair substitutions has recently become available in microorganisms and may have general applicability to higher forms as well. This is the test of susceptibility of specific classes of mutant alleles of a gene to phenotypic reversal by certain suppressor genes. Such suppressor genes, which are allele-specific but not locus-specific, seem to act at the level of translation of the RNA message into protein. The best example is a suppressor strain of *E. coli* which can suppress, at least partially, the phenotype of "nonsense" mutants (Benzer and Champe, 1962). Nonsense mutants are believed to result from base-pair substitution in a "sense" codon (one coding for a particular amino acid) to form a nonsense codon (one which fails to code for any amino acid). In an especially favorable case involving nonsense or "amber" mutants for the head protein of phage T4, Stretton and Brenner (1965) showed that, in strains of *E. coli* without the suppressor, the amber mutant causes synthesis of the polypeptide chains of head protein to be terminated prematurely at the point of the nonsense codon. In a strain of *E. coli* with a specific suppressor gene (*Su-1*), the amber mutant is able to make functional head protein. However, at the point where the nonsense codon of the amber mutant would normally have occurred and where the wild-type head protein carries the amino acid glutamine, the head protein of the suppressed mutant strain

now carries the amino acid serine. Other examples of correction of the nonsense triplet in a suppressor strain of *E. coli* to yield serine, or other amino acids depending upon the nature of the original nonsense codon, have been reported independently by Notani et al. (1965) and by Weigert and Garen (1965) for mutants of bacterial enzymes. Brody and Yanofsky (1963) have described a suppressor which appears to correct the reading of a missense codon (a codon for the wrong amino acid).

It may be envisaged that the above types of suppressor mutants will be increasingly used to characterize mutant alleles of structural genes in terms of the probable kind of altered codon involved. Examples of such suppressors are known in yeast (Hawthorne and Mortimer, 1960) as well as in bacteria and phage. A possible example in Drosophila is discussed below.

Rearrangement Tests

A powerful and elegant method of resolving the genetic material involves the manipulation of chromosomal rearrangements to synthesize deficiencies and duplications for minute regions of the chromosome. This method, which is due to Muller (1935), may not permit so high a degree of resolution as do the recombinational methods already discussed; nevertheless, rearrangements can help to specify the limits of a gene or especially of a gene complex. (Indeed, Raffel and Muller (1940) employed the method to try to resolve the scute gene in Drosophila; whether they were successful or not is still unclear since the rearrangements they used are now known to have variegated-type position effects extending over the achaete-scute gene complex.)

In spite of the position effects which often accompany chromosomal rearrangements, there are many instances in which chromosomal breakage and reunion do not lead to detectable changes in gene action. Evidently there is some kind of punctuation between the code-message units which allows the reading of the message to be interrupted and then restarted without difficulty. That is, in addition to the main portion of the code-message unit which must be concerned with specifying an RNA molecule (be it messenger, transfer, or ribosomal RNA), and in addition to prefixes and suffixes which presumably are needed for starting and ending the reading of the message (discussed by Stretton and Brenner, 1965), the chromosomes may, as many have suggested, contain "linkers" (composed perhaps of protein) which separate individual DNA molecules. Such linkers might undergo breakage and new unions without causing a disruption in the reading and translation of the essential part of the code-message unit.

In a prophetic paper, Brink (1932) proposed that the position effects which are known to accompany certain rearrangements might be explained as breakages within gene complexes:

> It might be assumed that the chromosome in its essential make-up consists not of genes which are entirely distinct from each other in function but of aggregates of groups of genes which are physiologically interdependent. On this hypothesis it is supposed that propinquity of the genes within a group is essential to normal gene action. On this view translocations involving breaks

between groups of genes would not alter the genotype . . . . If, however, the chromosome is broken in such a way as to separate the members of a gene group more or less profound changes in the physiological properties of the complex would follow.

There is an increasing amount of evidence that rearrangement processes go on within the gene and produce alterations comparable in type to some of the familiar kinds of gross rearrangements which have been detected cytologically. Evidence for intragenic rearrangements comes from a number of sources: for example, the deletion (and addition) mutants of phage genes (Benzer, 1955; Crick et al., 1961); and the presumed translocation of a portion of one of the histidine genes of *Salmonella* to another part of the genome, accompanied by a breakdown of the histidine operon (Ames et al., 1963).

Tandem duplications either of the direct or reverse type form a class of rearrangements that have special relevance to the present discussion. Such duplications may involve groups of genes, single genes, or portions of a gene, and may arise by rare, nonhomologous exchange between sister or nonsister chromatids. The Bar duplication in *Drosophila* (Sturtevant, 1925; Bridges, 1936; Muller et al., 1936) is a typical example of the tandem direct type. Such a duplication is known to be unstable in the sense that unequal crossing over (the occurrence of homologous crossing over within unequally paired segments of the duplication) generates the original unduplicated state or the complementary triplicated state.

Peterson and Laughnan (1963) have shown that certain rare exceptional types which arise from the Bar duplication and which have lost or gained segments of that duplication are most readily interpretable as arising from intrachromosomal (sister strand) unequal crossing over. Such exceptional types are nonrecombinant with respect to end markers, and hence they mimic the multiple exchange events associated with intragenic recombination.

It seems likely that gene complexes may often represent instances in which tandem gene duplications or higher repetitions have become established in a species and then have become differentiated by mutation into clusters of functionally similar but no longer identical genes. Examples of gene complexes that have been interpreted in this way will be discussed below.

EXAMPLES AND PROPERTIES OF GENE COMPLEXES

The remainder of this paper will be devoted to the properties of gene complexes as revealed by specific examples. The cases chosen by no means exhaust the list of possible examples (for additional ones see reviews by Stephens, 1951; Lewis, 1951; Carlson, 1959b; and Green, 1963, 1965).

Bacteria

In bacteria the genes which control sequential reactions in a particular biosynthetic pathway seem usually to be organized into a single operon. The most thoroughly

studied case is the operon for the histidine pathway in *Salmonella,* already cited above. The genes controlling the arginine pathway in *E. coli* constitute an exception; although not organized as an operon, these genes may nevertheless have once been part of an operon (discussion and references in Horowitz and Metzenberg, 1965).

Ames and Martin (1964) have reviewed the numerous examples of operons in bacteria and have further elaborated the theory of the operon as first put forward by Jacob and Monod (1961). This theory or model states that the functioning of the genes of an operon is regulated by means of repressor substances which act on the operator region and which are the product of regulatory genes. However, the manner in which the regulation is carried out at the biochemical level has not yet been elucidated. In a few cases, there is evidence that the operon is transcribed into a single polycistronic messenger RNA which is apparently then translated as a unit by the ribosomes. Nevertheless, different enzymes of the same operon are known in some cases to be synthesized in respectively different amounts. In order to account for the latter finding, as well as for the behavior of polarity mutants, Ames and Martin (1964) have proposed a "modulation" model for the reading of the operonic messenger RNA. Their model states that each of the possible codons for a given amino acid determines a different rate of enzyme synthesis through assumed differences in the relative amounts of the corresponding species of transfer RNA's. For example, when a gene contains a "modulating" codon corresponding to one of the less abundant species of transfer RNA, the reading of the messenger RNA slows down, presumably because the ribosomes tend to fall off the messenger at that point. The model can thus explain the differential rate of synthesis of different enzymes of the same operon. As expressed by Ames and Martin (1964), "the relative molar quantities of each enzyme made would be determined by the spacing of modulating triplets. The sequence of genes in the operon (which need not correspond to the sequence of enzymes in the pathway, as appeared at first) would be related to the number of molecules (in terms of the fundamental subunits) of each enzyme made. Thus, there would have been an evolutionary selection so that the order of genes is from the least efficient enzymes (of which more molecules are needed) to the most efficient."

Polarity mutants, by this model, would represent changes of the original codon to nonsense, missense, or modulating codons. Such changes would either interrupt or slow down the reading of the messenger—hence accounting for the observed coordinate repression of enzymes controlled by the gene containing the polarity mutant and by all genes distal to it in the operon.

Not all of the regulatory phenomena associated with gene complexes in bacteria have been satisfactorily explained by the operon theory (review by Horowitz and Metzenberg, 1965). However, the utility of this theory as a working hypothesis is unquestioned. The way in which operons may have originated is discussed below in the section on fungi.

Bacteriophage

Whereas a bacterium is estimated to have in the neighborhood of 2,000 different gene functions, a bacteriophage such as phage T4D may have only on the order of 100 (Edgar and Epstein, 1965). With the aid of conditional lethal mutants, Edgar and Epstein and their collaborators have mapped perhaps one-half of the genome of this phage. When gene order is correlated with gene function, a striking result emerges. That part of the genome which has been mapped appears to be made up to a large extent of clusters of genes with similar phenotype effects (Epstein et al., 1963). Although these clusters bear a resemblance to the operons of bacteria, it is not known whether a true parallel exists since neither polarity mutants nor mutants of regulatory genes or operator regions have been detected.

Fungi

In spite of the large number of biosynthetic pathways that have been genetically analyzed in *Neurospora* and yeast, only a few cases have been found in which genes with related functions are clustered. Although most of the genes corresponding to those in the histidine operon of *Salmonella* have been identified in *Neurospora,* only the genes controlling steps 2, 3, and 6 of the histidine pathway form an operon in *Neurospora* (Ahmed et al., 1964). Giles et al. (1965) have recently found in *Neurospora* a group of polyaromatic auxotrophic mutants which form a "supragenic functional unit" having many of the characteristics of a bacterial operon. The genes of this "aromatic" (*arom*) operon control the activities of five enzymes in a pathway composed of at least seven steps. Four of the genes of the *arom* operon have been identified by a combination of complementation, recombinational, and enzymatic studies.

Operons may also be rare in yeast. For example, although most of the genes corresponding to the galactose operon of *E. coli* (review by Ames and Martin, 1964) have been identified in yeast, only three of these are closely linked in yeast (Douglas and Hawthorne, 1964): furthermore it is not clear whether these three form a typical operon.

The possible basis for the scarcity of operons in fungi relative to the number in bacteria has been discussed by Horowitz (1965). He postulates that operons have often arisen by a process of repeated tandem gene duplications accompanied by gradual functional differentiation of the daughter genes. He further postulates that operons which are concerned with biochemical functions of great antiquity have tended to remain intact in bacteria but have become fragmented in higher forms such as *Neurospora*. He suggests, as one possibility, that such fragmentation may have occurred because of the demands imposed by the greatly increased gene number in *Neurospora* and the associated development of a complement of seven pairs of chromosomes compared to the single bacterial "chromosome." It remains possible that operons associated with pathways which have evolved after the separation of the fungi from the bacteria will still tend to remain intact in *Neurospora*. Few such pathways

have been studied thus far in *Neurospora,* since the analysis has been restricted largely to those pathways which must have antedated the evolution of fungi from bacteria.

Higher Plants

In maize a number of mutant series have long been known to exhibit considerable functional complexity. Examples which seem likely to represent gene complexes are (1) mutants of the "*R*" series, which determine absence of anthocyanin pigmentation in the aleurone or the plant or both (Stadler and Nuffer, 1953; Stadler, 1954; Stadler, and Emmerling, 1956), and (2) mutants of the "*A*" series, which affect the distribution of anthocyanin pigmentation in the plant and pericarp. In recent years, Laughnan has found genetic evidence that the *A* series is associated with a tandem gene duplication which has become established in at least some strains of maize and which undergoes unequal crossing over (review by Laughnan, 1961).

Stephens (1951) has reviewed other possible examples of gene complexes in higher plants.

That intragenic recombination occurs in higher plants is suggested by Nelson's (1962) discovery that the waxy gene in maize can be mapped as a linear array of sites on the basis of the frequency with which wild-type recombinants arise from heterozygotes for different mutant alleles of this gene. Presumably, in this case, only a single enzymatic function is involved (Nelson and Tsai, 1964); however, it is not clear whether waxy is the structural gene or a regulatory gene for this function.

Mammals

The most thoroughly understood example of a gene complex, from the standpoint of biochemical characterization of the associated polypeptides, is the cluster of two closely linked genes, already referred to above, which determines the beta and delta polypeptide chains of normal adult human hemoglobins A_1 and A_2, respectively. This gene complex may also include the gene which determines the gamma chain of fetal or F hemoglobin. The sequence of amino acids in the gamma chain has been determined by Schroeder et al. (1963). The close similarity among the amino acid sequences exhibited by the beta, delta, and gamma chains suggests that the three genes may have arisen from a single ancestral gene by a process of repeated gene duplication (Ingram, 1961). However, proof that the gene for the gamma chain is linked to the other two genes is lacking. Evidence that the genes for the beta and delta chains are adjacent and to some extent homologous comes from the discovery of an abnormal hemoglobin, Hb-Lepore. This hemoglobin variant has arisen independently on at least two occasions and is remarkable in that it contains a new type of polypeptide chain in which the left (C-terminal) portion seems to correspond to the left portion of a beta chain while the right (N-terminal) portion seems to correspond to the right end of a delta chain. Since the amino acid sequences in the central regions of the beta and delta chains are virtually identical with one another over long stretches, Baglioni (1962) has postulated that the requisite unequal crossover event probably took place

at a time when the central region of a beta-chain gene was unequally paired with the central region of a delta-chain gene. Smithies (1964) has discussed in detail the probable regions within which a crossover must have occurred to produce the two known kinds of Hb-Lepore.

Smithies et al. (1962) have found that one of the human haptoglobin variant genes (Hp^2) produces an alpha chain that is almost twice as large as the alpha chains resulting from the two common haptoglobin genes, Hp^{1F} and Hp^{1S}. On the basis of preliminary peptide studies, these investigators speculate that Hp^2 arose by nonhomologous crossing over in a heterozygote for Hp^{1F} and Hp^{1S}. Hp^2 might therefore be an incipient gene complex that has not yet become established in the species.

According to Herzenberg (1964), the heavy chains of mouse gamma globulins may be under the control of two closely linked genes in a manner analogous to that described for the beta and delta chains of human hemoglobin; however, sequence data are lacking.

The extensive series of tailless mutants found in mice was one of the first known examples of clustering of genes with similar effects (Dunn and Caspari, 1945). When studied in paired combinations, the three dominant mutants of this series, Fused, Kinky, and Tailless, were found to be separated by several map units. This relatively high frequency of recombination suggests not only that three separate loci are involved (rather than three sites within a single gene) but also that the loci may not be contiguous. The dominant mutants are in turn closely linked to a series of recessive tailless mutants which Dunn and his collaborators have isolated from wild populations (review by Dunn, 1956). But the precise nature of the linkage is obscure, since many of the recessive mutants are apparently associated with chromosomal rearrangements which suppress crossing over throughout the cluster.

Drosophila

Only a few of the numerous examples of gene complexes in *Drosophila* will be reviewed in this chapter. Mention should first be made of two examples of allelic diversity in *Drosophila* that seem most simply interpretable on the basis of a single gene rather than a gene complex. These are the rosy and garnet series of eye-color mutants.

Biochemical genetic analyses of the rosy series suggest that rosy is the structural gene for the enzyme xanthine dehydrogenase or for a polypeptide component of that enzyme (Forrest et al. 1956). By the *cis–trans* test, all of the available rosy mutants fall into a single cistron. Recombinational analysis of the rosy mutants by ingenious selective techniques (Chovnick et al., 1964) indicates that many recombinationally separable sites may exist within the rosy cistron. These sites, moreover, can be linearly ordered with reference to the behavior of outside marker genes. Although the analysis of this series has not progressed to the point of determining whether recombination is of the crossing-over or the intragenic type, it seems likely that Chovnick and

his collaborators have succeeded in producing a linear map of a single gene quite comparable to the gene maps of phages, bacteria, and fungi.

The mutants of the garnet series also fall into a single cistron by the *cis-trans* test. In this case, half-tetrad analyses by Hexter (1958) and Chovnick (1961) suggest that recombination within this series of mutants may be of the intragenic type since both nonreciprocal recombination and high negative interference are found. Although biochemical data are lacking and very few sites have been identified, the garnet mutants, like the rosy mutants, seem to form a single allelic series rather than a pseudoallelic one.

In a number of other mutant series in *Drosophila*, there is morphological evidence for functional diversity which would suggest that gene complexes are involved, but a firm conclusion cannot yet be drawn in most instances. A brief account will be given of only a few extensively studied examples—namely, the white, lozenge, Notch, and dumpy series.

The white series of mutants, located at 1.3 map units in the X chromosome of *Drosophila*, form a single cistron by the *cis–trans* test. There is, however, evidence for functional diversity within the series. One group of mutants is characterized by producing a generally lighter color in the male than in the female (i.e., males fail to show the normal dosage compensation of sex-linked mutants of this organism) and by acting as suppressors of the zeste (*z*) mutant (Gans, 1953; Green, 1959); this group, to which the original white mutant belongs, is called the "white" group. To the left of this group, there is another group of mutants which is characterized by usually causing a darker color in the male than in the female (i.e., the gene dosage is overcompensated) and by failing to act as suppressors of zeste (Green, 1959). This is the "apricot" group. A third group of mutants is characterized by a variegated eye-color phenotype. This is the "spotted white" group of mutants (Lewis, 1956; Green, 1959). Mutants in this group partially complement all mutants of the apricot or white groups (i.e., the *trans*-heterozygote has a nearly normal eye color). This is not a case of additive dominant effects since the *cis*-heterozygote exhibits a wild-type eye color. Half-tetrad analysis of *trans*-heterozygotes for the white and apricot mutants indicates that the mutants are separable by recombinational events of the classical crossing-over type, and hence at least two loci, white and apricot, can be identified by this test (Lewis, 1952). Many of the mutants of the white series were first ordered into two groups corresponding to the apricot and white loci by MacKendrick and Pontecorvo (1952). The spotted white group seems to occupy a separate locus very near and to the right of the white locus (Lewis, 1956; Green, 1963).

Judd (1959) and Green (1959) have found evidence for further subdivision of the apricot group into either two sites or two loci. The occurrence of unequal intragenic recombination, involving certain mutants of the apricot group, has tended, however, to obscure the picture (Green, 1965; Judd, 1964). At the present time the most likely interpretation of the entire series of mutants is that it represents a gene complex composed of three or four pseudoallelic genes. Moreover, there is cytological evidence in this case that the series is associated with at least two or three bands, two of which

(3C1-2) form a "doublet"—a structure that has been interpreted as a tandem single-band duplication probably of the reverse type (*ABBA*) (Bridges, 1935).

The lozenge series of roughened eye mutants has been studied extensively (Oliver, 1940; Green and Green, 1949, 1956). The vast majority of mutants in this series fail to complement one another. Although half-tetrad analyses have not been made, recombination seems to be of the classical crossing-over type (i.e., interference seems to be strongly positive within this series). Although many mutants are known, only four loci or sites have thus far been demonstrated. In contrast with the white series, the loci of the lozenge series are not obviously functionally differentiated from one another. This does not preclude, however, the existence of separate pseudoallelic genes. For example, the known lozenge mutants may be mostly of the polarity type; if so, the diversity of morphological effects (on the eye, leg, antenna, and female reproductive tract) may be pleiotropic effects exerted by the most distal gene of the series.

The Notch series of sex-linked wing mutants form a functionally diverse series, which has been examined in detail by Welshons (1958) and Welshons and von Halle (1962). Mutants of this series exhibit more or less independently varying morphological effects on eyes, wings, and bristles. Recombinational analysis provides some evidence that exchange between mutant genes of this series is not always accompanied by end-marker recombination—i.e., interference is sometimes negative—suggesting that intragenic recombination occurs. However, half-tetrad analysis has not been undertaken. Eleven sites have already been identified, and these are linearly ordered. Scattered throughout the locus or region are extreme, dominant, Notch mutants which act as recessive lethals. These fail to complement either each other or the recessive viable mutants of the series. Cytological studies have suggested that perhaps only one band is associated with the Notch region. This does not, however, preclude the existence of many cistrons (Rudkin, 1965). The Notch series, in spite of its functional complexity at the morphological level, may well represent either a single allelic series or a gene complex involving only a few pseudoallelic genes.

Mutants of the dumpy series in the second chromosome of *Drosophila* produce more or less independently varying alterations in wing shape, thoracic structure, and viability. Historically, this was the first example found in *Drosophila* of an apparently allelic series in which some members of the series complement one another (Muller, 1922).

In recent years Carlson and his collaborators have studied the dumpy series extensively. The series can be divided into two groups of mutants: a "vortex" group, in which the mutants produce whorls of hairs or vortices on the thorax, and an "oblique" group, in which the mutants produce truncated wing effects which may or may not be combined with vortex effects and with recessive lethal effects. Carlson (1959a) finds that members of the vortex group lie consistently to the right of members of the oblique group, the recombination distance being about 0.04 map unit. Southin and Carlson (1962) find that the oblique group can be further subdivided by recombination into eight more sites or loci, as the case may be. When recombination was measured in heterozygotes that had two morphologically distinct types of dumpy

mutants, interference was generally strongly positive and there was no difficulty in ordering the mutants. In heterozygotes that had morphologically similar types of mutants of independent origin, either no recombination between the mutants was observed or, in one case, a possible gene conversion or revertant was found. The dumpy series is associated with section *25A* of the salivary gland chromosomes (Bridges and Brehme, 1944), a region which contains a four-banded repeat structure (Bridges, 1935).

Although the dumpy series may involve only a single locus, it seems more likely that the series is a gene complex composed of at least two closely linked and functionally somewhat distinct genes. The complex may owe its origin to repeated gene duplication in tandem but further cytogenetic studies are needed to clarify this point.

The bithorax (*bx*) pseudoallelic series is a possible example of an operonic type of gene complex (Lewis, 1951, 1955, 1963, 1964) and appears especially favorable for the study of genetic control of developmental pathways. It is made up of five groups of mutants, each group having characteristic phenotypic effects that are readily distinguishable from those of the other groups. Recombination studies have identified five loci which have a one-to-one correspondence with the five phenotypic classes. The five loci will be symbolized here simply by the letters *a*, *B*, *C*, *d*, and *e*. Mutants at the *a* and *e* loci cause the anterior and posterior portions, respectively, of the metathorax segment of the fly to change developmental courses such that the metathorax becomes a wing-bearing instead of a halter-bearing segment. Mutants at the *d* locus cause a transformation of the first abdominal segment toward a thoracic state and at the same time have effects characteristic of the *e* mutant. Mutants at the *C* locus are dominant and, except for one case, are lethal when homozygous; they seem from mosaic studies (Lewis, 1963) to combine the properties of the *a*, *d*, and *e* loci. A single mutant is known at the *B* locus. It transforms the wing-bearing segment in the direction of the halter-bearing segment, the inverse of the transformation controlled by the *a* and *e* genes. In some respects the *B* mutant formally resembles the operator-constitutive, or o^c, type of mutant of the bacterial operons. *Cis–trans* tests suggest that the action of the genes of the bithorax complex are polarized in the manner of the genes of bacterial operons (Lewis, 1963).

Cytological evidence suggests that the series is associated with a complex of two doublet structures of the salivary gland chromosomes. Several instances have been found in which chromosomal rearrangements appear to have separated these two doublets; such cases were detected because of an associated inactivation of the fourth and fifth genes. In such rearrangements the functioning of the first three genes of the series was found to be unimpaired, even when, in one of the rearrangements, the trio had been shifted by transposition to the left arm of the third chromosome. These polarity effects associated with rearrangements are consistent with the polarity effects shown by the mutants in *cis–trans* comparisons.

Mutants at the different loci of the bithorax complex recombine with low frequencies in the range of 0.005–0.01%. It has been relatively easy to identify all

possible double mutant combinations. In addition it has been possible to synthesize not only certain triplet mutant combinations but also a quintuple mutant combination. In females heterozygous for the quintuple mutant combination and a normal third chromosome, there has been no evidence of negative interference.

Until recently recombinational analysis of the bithorax complex has been handicapped because, in the autosomes of *Drosophila*, it has not been practical to recover more than one product of the meiotic tetrad. This difficulty has now been circumvented by the use of pseudoiso-chromosomes for the right arm of chromosome 3. Such "attached 3R" chromosomes are comparable to the well-known attached-X chromosomes of this organism and were synthesized some years ago in our laboratory by I. E. Rasmussen and E. Orias. These chromosomes permit a half-tetrad analysis of meiotic events; i.e., two of the four products of a tetrad can be recovered by virtue of their sharing a common centromere.

It has been possible to construct, and to measure recombination in, females with an attached-3R chromosome in which one arm carries the quintuple bithorax mutant type (*a B C d e*) and the other arm carries the corresponding set of five wild-type alleles. Females carrying attached left arms (3L's) homozygous for a marker mutant, *radius incompletus* (*ri*), and attached-3R's of the genotype shown in Figure 3 were mated individually to males with attached-3L's marked with *ri* and attached-3R's marked with stripe (*sr*). (In such matings the attached-3L's usually segregate from the attached-3R's in much the same way that the Y chromosome of attached-X females segregates from the attached-X chromosome; the only viable progeny are those which receive one attached-3L chromosome and one attached-3R chromosome.)

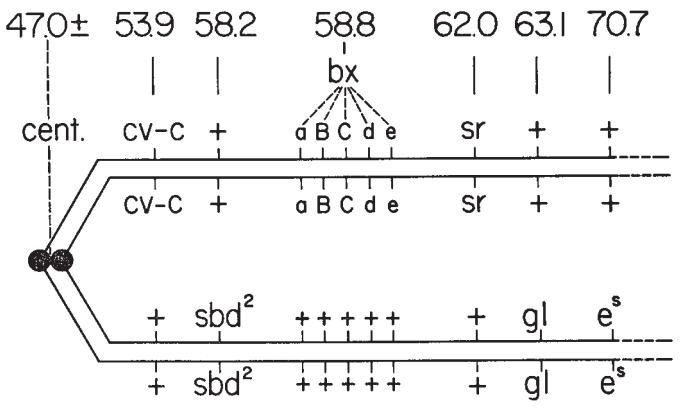

Figure 3. Diagram of the genetic constitution of attached-3R chromosomes heterozygous for a quintuple bithorax mutant combination and for closely linked marker genes. (Symbols: +, wild-type allele; *cent.*, centromere; *cv-c*, crossveinless-c; *sbd*^{2}, stubbloid-2; *sr*, stripe; *gl*, glass; e^S, ebony-sooty; *bx*, bithorax; *a*, bithorax-3; *B*, Contrabithorax; *C*, Ultrabithorax; *d*, bithoraxoid; and *e*, postbithorax.) The standard map locations are shown above the mutant symbols and are derived from Bridges and Brehme (1944), except that the location of *cv-c* is based on unpublished data kindly provided by Dr. Claude Hinton.

Owing to the existence of marked *cis–trans* effects in the bithorax series, it is possible to recognize the simultaneous recovery of reciprocal recombinants derived from any one of the four recombination regions of the bithorax complex. Thus, in region I, the recombinant *a/B C d e* (i.e., *a + + + +/+ B C d e*) has an extreme *a* phenotype; in region II, *a B/C d e* has a combination of *a* and *B* effects; in region III, *a B C/d e* is known to combine extreme *d* and *e* effects; and in region IV, *a B C d/e* has an extreme *e* phenotype. By contrast the parental, or *cis*, arrangement (*a B C d e/+*) is virtually wild type except for slightly enlarged halteres. Several other recombinant and nonrecombinant combinations can also be phenotypically identified, such as *a B C d e /e*, which has an extreme *e* phenotype, and *a B/+*, which has a moderate *B* phenotype. Moreover, certain types of events that would be indicative of gene conversion would be readily identifiable if they were to occur; for example, any of the above types of recombinants within the bithorax region which were not accompanied by recombination for the nearest outside-marker genes would represent possible gene conversions. Finally, certain types of double recombinants within the bithorax complex which would extract one of the single mutants, either *B* or *d*, would be readily detectable in certain combinations; for example, *B/+* or *B/a B C d e* is known to be viable and phenotypically easily identified, as is also *d/a B C d e*.

The results of the attached-3R experiment are shown in Table 1. In all, 16 recombinant strands have been verified by progeny testing. Such testing involved introducing the attached-3R's bearing the suspected recombinant types into triploids and isolating among the progeny of such triploids each strand of the attached-3R chromosome. In each case the occurrence of a recombinant strand within one of the bithorax regions was accompanied by a recombination of the outside-marker mutants. The marker-mutant recombination event was always consistent with the map order that had previously been deduced for the bithorax mutants from studies with nonattached-3R's. In this limited number of cases of recombination within the bithorax complex of attached-3R heterozygotes, interference was positive and complete. That is, there was no evidence of gene conversion. In this experiment, which involved approximately 221,000 progeny, the calculated frequency of recombination for regions I, II, and IV combined was roughly 1.5×10^{-4}. For technical reasons the frequency of recombination in region III could not be directly measured.

Observed recombination frequencies between different genes of the bithorax complex are so low that the detection of intragenic recombination might require examining many more progeny than has been done thus far. It is also possible that one or more of the mutants are associated with minute rearrangements which have precluded the occurrence of intragenic recombination. Although all of the mutants used in the half-tetrad analysis appear to be normal in the salivary gland chromosomes, more sensitive criteria for rearrangement than this cytological test are obviously needed. One such criterion, discussed above under mutational tests, is suppressibility of the mutants by allele-specific (but not gene-specific) suppressor mutants. A test of this kind has been carried out for the bithorax series with a recessive, allele-specific

Table 1 Recombinant progeny for the bithorax regions and verified recombinant strands, derived from a mating of attached-3R females of genotype *a B C d e*/ + + + + + with attached-3R males homozygous for *sr* (see Fig. 3 for the full composition of the parental female and for a description of symbols). The number of recombinant progeny for the bithorax region that were successfully tested is shown in parentheses. These latter progeny were the source of the verified recombinant strands.

	Recombination regions within the bithorax complex							
	I	II		III		IV		
Composition of attached 3R	*a* + + + + / + *B C d e*	*a B* + + + / + + *C d e*	*a B* + + + / + + + + +	*a B C* + + / + + + *d e*	*a B C* + + / + + + + +	*a B C d* + / + + + + *e*	*a B C d e* / + + + + *e*	Totals
Observed number of flies	8 (5)	2 (0)	4 (2)	0	1[a] (1)	2 (1)	2 (2)	19 (11)
Number of verified recombinant strands	10		2		1[a]	2	2	16

[a] Phenotype overlaps wild type; hence some specimens may have been overlooked. Since the observed specimen was selected because of an associated recombination of outside markers, this case is omitted from the total number of verified recombinant strands

suppressor—namely, the suppressor of Hairy-wing, located at 54.8 map units in the right arm of the third chromosome. The first known mutant allele, *su-Hw,* at this locus was found by Bridges, who showed that it had the ability when homozygous to suppress scute, Hairy-wing, cut, and, to some extent, forked and bar (Bridges and Brehme, 1944). Although this mutant was lost before tests of allele specificity were carried out, a recurrence, designated su^2-*Hw,* turned up during study of the bithorax complex.

The su^2-*Hw* mutant has been found to suppress only certain alleles of a wide variety of mutant genes. The loci at which almost complete suppression has been detected are as follows: yellow, Hairy-wing, scute, cut, lozenge, forked, bithorax, bithoraxoid (loci *a* and *d* in the *bx* complex), *cubitus interruptus,* and, as recently found in our laboratory by G. Del Campo, diminutive and Beadex. Usually only one of a number of different alleles that have been tested is suppressed. This is especially striking for the yellow, scute, and lozenge loci. Among a large number of alleles tested at each of these loci, only one allele apiece was suppressed by su^2-*Hw*—namely, y^2, sc^1, and lz^1, respectively.

Among the five mutants of the bithorax complex which were used in the attached-3R experiment described above, two (bx^3 at the *a* locus and bxd^1 at the *d* locus) are suppressed by su^2-*Hw* while the remaining three are not. It is of interest that bx^3 and *bxd* in homozygous lines have each on one occasion undergone reversion to wild type, apparently by back mutation. The suppressor and reversion results only suggest that bx^3 and *bxd* may be true point mutations.

A unique property associated with the bithorax complex is the occurrence of a "transvection effect" (Lewis, 1954). This effect was discovered when *trans*-heterozygotes for certain mutants of this complex were made structurally heterozygous for chromosomal rearrangements involving the right arm of the third chromosome. Under these circumstances, the mutant phenotype of a given *trans*-heterozygote (for example, *a* +/+ *C*) is frequently made more extreme than that of the corresponding heterozygote with structurally homozygous third chromosomes. Such phenotypes have been described elsewhere (Lewis, 1963). Only those rearrangements which cause a cytologically observable disruption in pairing of the bithorax regions (*89E* of the salivary gland chromosomes) give the transvection effect. The existence of this phenomenon implies that the wild-type alleles of the *trans*-heterozygote are able to cooperate with one another even though they are in opposite chromosomes; moreover, they cooperate more efficiently when the chromosomes are paired (i.e., in the absence of an associated rearrangement) than when the chromosomes are unpaired. It is not known whether this cooperation occurs at the level of transcription or translation of the DNA message or whether it occurs at some level that involves diffusion of products of chromosomally localized enzymatic reactions.

The bithorax complex promises to be a suitable system for studying the genetic control of developmental pathways. A model has been presented elsewhere (Lewis, 1963, 1964) that formally accounts for the manner in which the individual genes of the bithorax complex control the level of development achieved by certain body

segments of the fly. This model is based on an analogy with the behavior of inducible operons in bacteria (Jacob and Monod, 1961). The bithorax genes appear to control developmental pathways which recapitulate some of the phylogenetic steps that must have occurred during the evolution of the Diptera from primitive arthropod ancestors.

SUMMARY

At first glance, the genes of higher organisms appear to be distributed throughout the chromosomes in a more or less random fashion with respect to their functional role in development. An increasing number of exceptions to this rule have been uncovered as the gene concept has come to be scrutinized more and more closely. "Gene" is here used in the sense of the code-message unit that codes for the production of a specific polypeptide. Whenever an apparently allelic series of mutants exhibits functional diversity, it is often difficult to distinguish whether such diversity resides within different sites of a single gene or within different pseudoallelic loci of a gene complex. Attention is drawn to the various genetic methods that can be used to distinguish operationally between these two possibilities. The emphasis in this paper has been placed upon those examples of functionally complex mutant series which turn out to be of the gene-complex type. In bacteria, an entire sequence of reactions in a biochemical pathway is frequently found to be controlled by an operon—a gene complex in which the functioning of individual genes of the complex is regulated in a coordinate manner. Operons for the primitive biosynthetic pathways of living organisms seem to have become fragmented in higher organisms, as judged by the scarcity of operons of this type in *Neurospora* and yeast. Nevertheless genes involved in biosynthetic or developmental pathways of relatively recent origin may well turn out to be linked together as gene complexes in higher organisms. This will be especially likely if the genetic control of such pathways has evolved by a process of gradual functional differentiation of a set of tandemly duplicated genes. It seems likely that many, but not all, gene complexes have originated by such a process; however, supporting evidence is lacking, except in one case—namely, the two-gene complex which codes for the production of the nearly identical beta and delta polypeptide chains of human hemoglobin. The study of gene complexes that affect morphological or other kinds of developmental processes promises to advance our knowledge of the genetic control of developmental pathways in much the same way that the study of bacterial operons has advanced our knowledge of the genetic control of biosynthetic pathways.

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SECTION II: GENES AND DEVELOPMENT

LEWIS AND THE GENETIC CONTROL OF DEVELOPMENT

DEVELOPMENTAL GENETICS

In 1923, T. H. Morgan expressed the hope that "the combined attack on this problem of development both by genetics and by experimental embryology and especially by chemistry may lead to the discovery of the physiological action of the genes" (Morgan, 1923). He went on to argue in favor of the fundamental role that genes must play in controlling development: "The embryologist observes the developmental changes taking place in the cytoplasm in the presence of the entire chromosomal complex. He is therefore strongly impressed with the view that the cytoplasm of the cell is the active agent in development. In this he is, of course, entirely right, because his evidence is only concerned with what he can see and not with what takes place behind the scenes. The genetic evidence is the only means we have at present to show that the chromosomes influence what takes place in the cytoplasm. The physiologist is concerned with processes going on in the cell, but has no means of finding out at present how far the chromosomes may or may not have affected the process. He is, however, as a rule sufficiently broad-minded to concede at least that any change in the complex of factors present in a cell may affect the end result. Genetics supplies this evidence; it is not concerned with the processes that take place either before or after the postulated change—at least only secondarily interested. Whether or not genetics may in time develop new methods that make possible an approach to some

of the problems that at present belong to the field of experimental embryology and physiology remains to be seen . . . ".

In his presidential address to the Sixth International Congress of Genetics at Cornell University in 1932, he criticized experimental embryology ("Entwicklungsmechanik") for having failed to "bridge the gap between gene and character". Rather, it had produced "nothing that was really quantitative or mechanistic . . . Instead, philosophical platitudes were invoked rather than experimentally determined factors. Then too, experimental embryology ran for a while after false gods that landed it in a maze of metaphysical subtleties" (Morgan, 1932). He went on to emphasize again the role of genes in controlling development: "That the form of cleavage of the egg is determined by the kind of chromosomes it contained before the egg reached maturity has been sufficiently proven; and since the foundations of all later differentiation are laid down at this time, the demonstration is of first-rate importance for genetics, because it shows that we are not obliged to suppose the genes or chromosomes are functioning at the moment of the visible appearance of characters".

Donald F. Poulson, one of Sturtevant's graduate students at Caltech, was among the first to address the role of specific genes in regulating embryogenesis (Poulson, 1937; Poulson, 1940). He did so by analyzing the effects of X-chromosomal deficiencies on embryogenesis in Drosophila, in the process identifying a *Notch* deficiency that causes nervous system hypertrophy. He thus was in a position to begin his 1940 paper: "The problem of how genes act in development may be approached in several ways, more or less indirect. The effects of the dosage of genes on the end character, or upon the developmental morphology, as well as the differences in the effects of allelomorphs may be of the nature of cell size, shape, number or pattern, or rate of developmental reactions. In other cases they may be of the nature of chemical end- or intermediary-products. These are susceptible of qualitative and even quantitative treatment. The results of such studies . . . form the basis of our present knowledge of the genetical control of development . . . *Another, and more direct, approach is through the study of the effects of the absence of certain genes* [editor's italics]."

During the following decades there was, however, little systematic use of genetics to dissect developmental phenomena. One of the reasons for this was the increasing focus, starting in the 1940s, on fungi and prokaryotes to study fundamental genetic phenomena. Another was the growth of molecular genetics during the 1950s and early 1960s, when molecular analyses that were focused on defining the nature of the gene reached their peak. Following Watson and Crick's discovery of the double-helical nature of DNA, came the discovery of messenger RNA, transfer RNA and the triplet genetic code. Studies of the rII locus in phage indicated that the gene is in fact divisible by recombination, likely between any two base pairs along the double helix (Benzer, 1956). Analyses in *Escherichia coli* showed that enzyme induction results from an increase in the rate of the *de novo* synthesis of an enzyme from its constituent amino acids (Hogness et al., 1955). The combination of such biochemical analyses with genetic identification of different classes of mutants—notably in the lactose (lac)

utilizing genes—led to the operon model for regulation of gene expression (Jacob and Monod, 1961). Most significant for the present discussion was the discovery of "operator constitutive" mutants in which expression of the structural genes located in *cis* on the bacterial chromosome is no longer dependent upon an inducer.

During this period, genetic studies of development languished. The only model available was Drosophila—the use of *C. elegans*, mice and zebrafish lay well in the future. Many of the developmental mutants that existed in Drosophila were hard to study in depth because they affected the internal tissues (e.g., *Notch*) and most of the alleles were lethal early in development. It was only much later, following Lewis' pioneering analyses in the bithorax complex, that systematic isolation and categorization of embryonic lethal mutants were initiated (Nüsslein-Volhard and Wieschaus, 1980).

Lewis was one of the diehards who continued with his Drosophila studies during the 1950s and 1960s. Gradually his focus shifted from genes and their evolution to the genetic control of development. This shift was brought about largely through his ongoing analyses of the bithorax series of mutants. While his papers on the bithorax mutants in the 1950s had clearly spelled out that these genes are controlling the "level" of development of the different body segments of the fly, his rationale for studying them had remained "understanding . . . the gene—how it functions, how it mutates, how it evolves" (Lewis, 1955).

In a 1957 article written for Caltech's quarterly magazine *Engineering and Science*, and titled "Two Wings or Four?", Lewis wrote:

> What, if anything, can we learn from a four-winged fly? One thing we hope to learn is how genes affect the development of an organism. We know that the genes . . . not only account for the transmission of characteristics from the parents to the offspring, but they are also thought to control the whole course of development of the organism from fertilized egg to adult . . . We postulate that the development of a living organism is an orderly unfolding in time of many different sequences of biochemical reactions – each ultimately gene-controlled . . . If development is to be explained in terms of the action of genes, it becomes necessary to picture it as a gradual and orderly "turning on", so to speak, of systems of genes. The bithorax and postbithorax mutants probably represent part of one such system . . . We postulate that, during the course of development of a normal fly, this system of genes is present but effectively inoperative in the wing-bearing section. In the haltere-bearing section, on the other hand, these genes elaborate a series of substances which direct the pathway of that section from wing-formation toward haltere fomation . . . What determines the essential difference between the wing and haltere-bearing section? We postulate that there is a gradient in the concentration of some chemical substance during the development of the embryo, such that the concentration is relatively much greater in the haltere-forming region that it is in the wing-forming region. It would then be the function of the normal genes of the bithorax cluster to exploit this gradient– to amplify it into an "all-or-none" response.

Thus, Lewis' focus had shifted away from gene mutation and evolution to how genes control development. This change is highlighted by the fact that he begins both his 1963 and 1964 papers by announcing what is essentially the genetic approach to

development or what came to be known as the field of "developmental genetics" (only the 1963 paper is reprinted here): "The way in which genes control the growth and development of an organism is a central problem in biology, and one which is currently under study in diverse forms from phage to man. I would like to discuss an approach to this problem which makes use of a series of pseudoallelic genes in Drosophila. Such series of closely linked genes with related effects have . . . been profitably exploited in the bacteria to learn more about how genes control biochemical pathways . . . It may be anticipated that pseudoallelic series affecting morphological traits . . . can also be profitably exploited to learn more about how genes control developmental pathways" (Lewis, 1963a). "There are many experimental approaches to the problem of growth and development. We would like to discuss what might be called the genetic approach. The underlying concept is that the genetic mechanisms which are believed to control and regulate biosynthetic pathways may be applicable with relatively little modification to the control and regulation of developmental pathways" (Lewis, 1964).

Lewis was correct in understanding the validity of the genetic strategy but could not anticipate that new concepts would be required: more than "little modification" would be needed to proceed from biosynthetic pathways to developmental ones. Indeed, the bithorax complex genes and the genetic rules that Lewis inferred from their study were to provide the first insights into how genes control morphological traits. As is illustrated by the papers discussed in this and the following section, the genetic control of multicellular development cannot readily be extrapolated from studies of biosynthetic pathways in simple organisms.

GAIN-OF-FUNCTION AND LOSS-OF-FUNCTION BITHORAX ALLELES, AND THE OPERON MODEL

Along with Lewis' shift in focus from genes to developmental genetics, several major conceptual and technical achievements are evident in his 1963 and 1964 papers. He is still working with the same five mutants that he reported in 1955: *bx*, *Cbx*, *Ubx*, *bxd* and *pbx*. He has now, however, made the quintuple mutant (*bx Cbx Ubx bxd pbx*)—no mean feat!—and has used this to produce a linkage map where they are between 0.006 and 0.01 cM apart (0.006 cM = 6 recombinants among 100,000 progeny). He has also shown cytogenetically that *bx*, *Cbx* and *Ubx* map to 89E1,2 while *bxd* and *pbx* map more distally on the chromosome, possibly to 89E3,4. This was accomplished using X-ray induced rearrangements that break in the 89E region and asking which mutant phenotypes are associated with such breaks: breaks to the left of 89E1,2 inactivate all five genes while breaks between bands E1,2 and E3,4 inactivate only the *bxd* and *pbx* genes. This mapping satisfied one key incentive for studying the bithorax mutants, which was to investigate Bridges' hypothesis that doublets such as those in 89E represent tandem gene duplications and incipient evolution of new genes. Lewis had now shown that some of the genes mapped to one doublet while some mapped to the other.

Over 30 years earlier, Muller had defined a set of formal operational rules, based on gene dosage studies, that allowed functional categorization of mutants (Muller, 1932). If a dominant mutant phenotype worsens upon addition of copies of the wild-type gene, but becomes less severe when the wild-type copy number is reduced, the mutation is a hypermorph, behaving as if it results in excessive gene activity. If a dominant mutant phenotype is unaltered upon addition of copies of the wild-type gene, the mutation is a neomorph, behaving as if it results in a novel gene activity. If addition of wild-type copies of a gene return the phenotype of a mutant towards wild type, then the mutant must reduce the normal function of the gene (e.g., antimorph, hypomorph, amorph). The last three can be distinguished further by whether the mutant phenotype they produce becomes more severe upon removal of the wild-type gene copy (implying that some gene function remains; i.e., a hypomorph), is unaltered (implying that gene function is eliminated; i.e., amorph), or depends upon a wild-type gene copy (implying the mutant competes with the wild type in exerting its effect; i.e., antimorph).

In his 1963 paper, Lewis reports gene dosage analyses that allow him to define the nature of the bithorax series of mutants (Lewis, 1963a). He shows, using deficiencies and duplications, that *bx*, *Ubx*, *bxd* and *pbx* all behave as if they reduce gene function (he calls these "in the direction of loss of function"). In contrast he shows that the *Cbx* mutant phenotype is just as severe in the presence of two doses of the wild-type gene (*Cbx*/+/+) as one (*Cbx*/+). He calls *Cbx* a gain-of-function mutation: "it is as if the [*Cbx*] mutant causes the wild-type alleles of certain other genes in the series to begin acting in the MS [mesothorax], instead of acting only in MT [metathorax] or AB_I [abdominal segment I]." Lewis was exactly right, but this was not proved until molecular analyses were conducted two decades later.

Lewis invented the terms "loss-of-function" and "gain-of-function" as simplifications of Muller's classes. Perhaps because they are also more intuitively obvious Lewis' terminology remains in use today, although not all of those who use the terms have carried out the gene dosage analyses that underlie them. (Fortunately, the terms loss-of-function and gain-of-function are, in addition, easier to distinguish in an oral presentation than are hypomorph and hypermorph!)

The fact that *Cbx* behaves as a gain-of-function mutation, together with its dependence in *cis* on *Ubx* function (i.e., *Cbx* +/+ *Ubx* has the MS → MT transformation but *Cbx Ubx*/+ + does not) and the polarity of the effect of *Cbx* on *Ubx*, led Lewis to emphasize the close similarity to the operon model (Jacob and Monod, 1961). Thus, *Cbx* formally behaved as an "operator constitutive" mutation. Lewis does not fully explain the spatial effects of the mutant: i.e., why the *Cbx* phenotype should manifest itself only in the mesothoracic segment when all of the evidence is that the *bx*, *Ubx*, *bxd* and *pbx* genes function in the metathoracic and first abdominal segments. However, he for the first time presents an alternative to the sequential gene action model that he had proposed in the 1950's: an "operon model." In this model, the *bx* and *Cbx* pseudoalleles are presented as possible "operator" regions (although he is ambiguous regarding *bx*) that regulate the production of

mRNAs from the adjacent *Ubx*, *bxd* and *pbx* genes. These mRNAs would, in turn encode enzymes that lead to the production of "bithorax substances" S1, S2 and S3. Interestingly, this model is closer to the final molecular reality than Lewis' 1978 model, in that it has both *bx* and *Cbx* as regulatory regions, whereas in 1978 he has *bx* encoding one of the postulated bithorax complex substances.

By the time that Lewis wrote his 1963 paper it was known that mRNA is synthesized in the nucleus while protein synthesis occurs in the cytoplasm of eukaryotes (e.g., Palade, 1955). Thus, he points out that the operon model for bithorax gene function is consistent with this fact, while the "sequential reaction" model would "assume that enzyme synthesis occurs in the vicinity of the chromosome", a snag that has been alluded to in the "Genes" section of this book.

With the operon model in mind, Lewis begins, for the first time, to consider that gradients either of substrate (S) or of repressors/inducers might explain the spatial regulation of the bithorax genes along the antero-posterior body axis. Thus, "the presence of a repressor substance that combines with the operator would keep the genes turned off in such regions as MS . . . A posterior-anterior gradient in the concentration of the inducer is thus what is required on the operon model to interpret the normal pathway of development of MS, MT and AB_I." Fifteen years later he elaborated the gradient idea, postulating an anterior-to-posterior gradient of the *Polycomb* repressor substance (Lewis, 1978).

How might the bithorax "substances" function? "In the metathoracic region . . . it is necessary to suppose that the resultant concentrations of S1 and S2 [the *Ubx* and *pbx* substances] are sufficient to suppress the potential mesothoracic-like modification of the metathoracic region but that the concentration of S3 [the *bxd* substance] is still insufficient to bring about an abdominal type of development in the metathoracic region . . . Finally, in the cells of the first-abdominal region . . . the cellular concentration of S3 [would] build up to a point where it would suppress the potential thoracic-like development of this segment . . . The bithorax genes evidently . . . [produce] a whole set of new substances that repress certain systems of cellular differentiation and thereby allow other systems to come into play" (Lewis, 1964). Thus, in modern parlance, Lewis was in essence postulating that the bithorax genes function as regulators of target genes whose role is to elaborate cell fates. His focus on negative rather than positive regulation reflected the importance of repressors in regulation of the *lac* operon in *E. coli*, the only example at that time of a gene regulatory system (Jacob and Monod, 1961).

GENETIC MOSAICS: SPATIAL AND TEMPORAL ASPECTS OF BITHORAX FUNCTION

An additional conceptual and technical advance in the 1963 paper is Lewis' use of genetic mosaics to analyze the "autonomy" of bithorax gene function. He utilizes an unstable ring-X chromosome into which he had introduced a wild-type copy of the bithorax series of genes. The bx^+ ring-X chromosome also carried a copy of the

yellow^{+} (y^{+}) body color gene that produces a normal, brownish-gray body color. In contrast, in females, the other, rod-X chromosome carried the recessive, *y*, mutant allele that results in yellowish body color. Both copies of the third chromosome carried one or more bithorax series mutant alleles. Thus, when the ring-X is lost, a genetically mosaic gynandromorph fly results, in which the female (XX) tissue is brownish-gray and wild type for bithorax while the male (XO) tissue is yellowish and mutant for bithorax.

Lewis analyzed mosaics for *bx*, *pbx*, *bxd*, *Ubx* or the double mutant, *bx pbx*. In all cases he found that yellow tissue was transformed in segmental identity in the direction predicted from his previous studies in nonmosaic flies. Such transformations even occurred when there was only a single yellow hair or bristle, indicating that the role of the bithorax gene is autonomous at the level of a single cell (each hair is made by a single cell). What this meant was that the bithorax genes regulate the identity of each cell from within that cell, rather than by producing a diffusible substance that tells distant cells what identity to adopt. Over 20 years later it was shown that the bithorax complex encodes transcription factors that regulate other genes (Laughon and Scott, 1984; Shepherd et al., 1984), consistent with the autonomy of function that Lewis had defined in 1963.

In 1964, Lewis took the genetic mosaic analyses one step further, by using X-ray induced somatic crossing over at mitosis to analyze bithorax gene function. Here, he introduced a duplication carrying the wild-type bithorax genes into the X chromosome. In females, the other X chromosome, lacking the duplication, carried the *yellow* body color mutation (*y*) and a bristle marker mutation (*singed*, sn^{3}), while the third chromosome was again, homozygous for the relevant bithorax mutant(s). Thus, when the X-rays induced crossing over between the X chromosome homologs in a dividing cell, it was possible to produce a patch of tissue from which the wild-type bithorax alleles were missing. Such a patch could be recognized because it was yellow and had deformed bristles. As predicted from Lewis' gynandromorph mosaics, these data confirmed the autonomy of function of the bithorax genes. However, the fact that Lewis could control the developmental stage at which he produced the cell that gave rise to a mutant patch (by irradiating larvae at different developmental stages), enabled him to ask when during development bithorax gene function is required. He found that, when the mosaic was induced late in the larval period, just before cell divisions in the imaginal discs ceased, it was possible to obtain single bristles and hairs that were transformed. From this Lewis was able to conclude that, at least for *bx*, the wild-type alleles are still functioning late in larval development "preventing, so to speak, a mesothoracic-like transformation of even a few cells of the dorsal metathoracic imaginal disc."

Twelve years later, Antonio Garcia-Bellido was to collaborate with Lewis, and to culture mixtures of imaginal disc cells from wild type and bithorax complex mutants (Garcia-Bellido and Lewis, 1976). These studies confirmed the cell autonomous function of the bithorax series genes as well as implicating them in controlling "cellular affinities" that determine whether cells can mix within a developing tissue.

This last issue was of particular interest in light of the discovery of developmental "compartments" (Garcia-Bellido et al., 1973), whose boundaries correlate with the boundaries of the segmental transformations observed by Lewis for the bithorax series of mutants.

In summary, Lewis had found that the bithorax genes regulate cell fate on a cell-by-cell basis. Furthermore, the bithorax genes do not function as switches that, once flipped early in development, seal a cell's fate. Rather, bithorax gene function is needed—likely continuously—throughout a cell's development and differentiation, to tell it what fate to adopt.

In the final part of the 1963 paper, Lewis presents an explicit suggestion for the role of the bithorax pseudoallelic series in the evolution of Dipteran insects from primitive, multilegged and four-winged ancestors. Thus, he views the *bxd* gene as having evolved to suppress leg development on the abdominal segments, producing insects from multilegged ancestors. Similarly, the *bx*, *Ubx* and *pbx* genes would have evolved "since the insects appeared" to produce Diptera from four-winged insects. Lewis was to introduce his 1978 paper from this perspective.

THE 1978 PAPER: A PARADIGM FOR THE GENETIC CONTROL OF DEVELOPMENT

Thirty years' worth of analyses and concepts were brought together in Lewis' 1978 paper in *Nature* (Lewis, 1978). This is the paper that revolutionized our view of the way genes control development. Of all Lewis' papers it exemplifies an enormous compaction of decades' worth of research conveyed in a series of formal rules and an abstract model—a presentation that is quite alien to those expecting to see raw data first, model second.

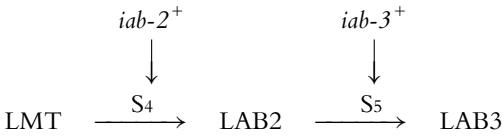
Lewis begins where he left off in 1963, by considering two major groups of genes: "leg-suppressing genes" that removed legs from the abdominal segments of millipede-like ancestors and "haltere-promoting genes" that suppressed the second pair of wings of four-winged ancestors. He now explicitly calls the cluster of position pseudoalleles the "bithorax complex" or "BX-C," which controls much of the diversification of the organism's thoracic and abdominal segments. He defines the extended BX-C gene series to include genes that promote the development of all segments of the fly posterior to the "primitive" mesothoracic segment: the metathorax (MT) and the eight abdominal segments. The genetic functions proceed from the proximal to distal on the chromosome: from the bx^+ gene, which promotes LAMS → LAMT, through *infra-abdominal-8* (*iab-*8^+), which converts LAB7 → LAB8. He postulates that each of these genes codes for "a BX-C substance (S) . . . which is presumed to act indirectly by repressing or activating other sets of genes which then directly determine the specific structures and functions that characterize a given segment."

Thus, Lewis has formally abandoned the operon-type model that he postulated in 1963 and returned to the simplest model available: each gene encodes a distinct substance, which regulates the development of characteristic segmental structures,

now through activation as well as repression of target genes. He has accumulated a collection of gain-of-function mutants throughout the complex—*Cbx*, *Hab*, *Uab*^{4}, *Uab*^{5}, *Mcp*—that behave as regulatory mutants of the *cis*-dominant type. He uses these to infer the presence of recessive functions in these regions, and postulates that each of these recessive functions (e.g., *iab-2*$^+$, *iab-3*$^+$, . . .) encodes a distinct substance, S_0, $S_1, \ldots S_x$.

In *Hyperabdominal* (*Hab*) mutants, the transformation is of MT → AB2 and of AB1 → AB2. Lewis reports *cis–trans* tests on all double mutant combinations of *Hab* with *bx*, *Ubx*, *bxd* and *pbx*. In no case does he see any effect (e.g., *Ubx Hab*/+ + has the same phenotype as + *Hab*/*Ubx* +). This differs, for example, from the situation with *Cbx*, discussed in the commentary on the section on Genes, where there is a strong *cis*-position effect of *Cbx* on the wild-type *Ubx* function (Lewis, 1955). He therefore infers that *Hab* does not derepress the wild-type alleles of *bx*, *Ubx*, *bxd* or *pbx*. Thus, he concludes that *Hab* must derepress a gene that produces substance S_4, which directs LMS → LAB2. He names this hypothetical gene, *infra-abdominal-2* (*iab-2*).

From the *Ultra-abdominal-5* (*Uab*^{5}) *cis*-dominant gain-of-function mutant, which causes the transformation of AB1 → AB3 and AB2 → AB3, Lewis infers the presence of a substance S_5, which directs LAB2 → LAB3. This substance is encoded by a hypothetical gene, which he calls *iab-3*. In the *Hab* +/+ *Uab*^{5} combination, he also sees MT → AB3. Thus the transformation is sequential:



Further evidence in support of the existence of *iab-3* comes from another dominant mutant, *Uab*^{4} (see below), which, when heterozygous with a translocation, *T(2;3)P10*, exhibits a new loss-of-function phenotype: AB3, AB4, AB5, AB6 → AB2. Such a transformation is consistent with the *Uab*^{4} mutation and the breakpoint of the translocation inactivating the postulated *iab-3* gene and thus eliminating S_5.

The final dominant gain-of-function mutation that Lewis reports is *Miscadestral pigmentation* (*Mcp*). Lynn Crosby had called the mutant *Male chauvinist pigmentation* when she identified it in Lewis' laboratory in 1977. It was so-named because it extends the male pigmentation from the fifth and sixth abdominal segments as seen in wild type, more anteriorly, onto the fourth segment! Its name, but not its abbreviation, was changed at Lewis' insistence prior to publication. The *Mcp* mutant causes the transformation: LAB4 → LAB5; thus Lewis infers the presence of a gene, *iab-5*$^+$, encoding substance S_7, which effects LAB4 → LAB5 (or, possibly, LMS → LAB5).

Thus far in the paper, Lewis' analyses proceed by inference from the nature of the mutations (loss-of-function or gain-of-function), their map positions within the

BX-C, their segmental phenotypes, and their phenotypes in *cis–trans* tests, to postulate the existence of particular genetic functions. Later it was to be found that almost all of these mutations affect *cis*-regulatory regions within the BX-C rather than genes that encode proteins (Bender et al., 1983a; Beachy et al., 1985; Hogness et al., 1985; Karch et al., 1985; O'Connor et al., 1988; Kornfeld et al., 1989; Irvine et al., 1991), disproving a model that hypothesizes substances $S_1 \ldots S_X$. In almost all instances, by substituting "*cis*-regulatory region" for "substance," the developmental functions exposed by Lewis' genetic inferences stand.

For the rest of the paper, Lewis bases much of his analysis on larval and embryonic phenotypes. These were crucial studies to affirm that his principles applied, as they should, to earlier developmental stages. They also set the foundation for rapid acceptance of the work of Christiane Nüsslein-Volhard and Eric Wieschaus in systematic identification of embryonic-lethal mutants that affect the cuticle pattern of the embryo (Nüsslein-Volhard and Wieschaus, 1980).

Lewis scores phenotypes according to certain embryonic structures: the Keilin's organs (hereafter abbreviated KO) and the ventral pits (VP), which are normally found only on the thoracic segments; the dorsal longitudinal tracheal trunk (DLT), which is normally continuous along the length of the embryo or larva; and the anterior and posterior spiracular openings (ASP and PSP), which are normally present only on the mesothoracic segment and the eighth abdominal segment, respectively.

Most importantly, Lewis introduces a novel method of analysis that turns standard genetic analysis on its head. Standard genetics proceeds from analysis of mutant phenotypes to infer the functions of the normal, unmutated genes as Lewis had done so successfully. Now he does the opposite, inventing a method that will be referred to here as "add-back genetics." He begins with a mutant situation in which the entire complex has been removed; then he adds back wild-type portions of the BX-C. To do so he capitalizes on the remarkable collection of deficiencies, translocations, inversions and transpositions that break in different parts of the BX-C, and which he had collected during the previous decades. Thus he is able to define where these wild-type functions lie within the BX-C as well as what these wild-type functions are.

To set the stage for his add-back genetic analysis, Lewis defines the phenotype of a homozygous deficiency that removes the entire BX-C, *Df P9*. These mutants survive to the late embryonic stage and so Lewis is able to show that the third thoracic segment, MT, and all eight abdominal segments are transformed to the second thoracic level of development, LMS. Thus, VPs, KOs, and incipient ASPs now appear, not only on the MS, but also on all nine more posterior segments. (The eighth abdominal segment is a little unusual in that certain head-like structures also appear.) Thus, all segments posterior to the MS are transformed to the "primitive," MS-like state.

When he adds back just the bx^+ Ubx^+ region of the complex using *Dp 100*, the third thoracic and eight abdominal segments become MT-like. Thus, the bx^+ Ubx^+ region does indeed promote LMS → LMT. When he adds back bx^+ Ubx^+ bxd^+ pbx^+ using *Dp P10*, all eight abdominal segments become AB1-like. Thus, the further

addition of *bxd*$^+$ *pbx*$^+$ region promotes LMT → LAB1. Together, then, *bx*$^+$ *Ubx*$^+$ and *bxd*$^+$ *pbx*$^+$ promote LMS → LAB1. These analyses are presented in terms of the ability of the regions to promote or repress development of segment specific structures. Thus, Lewis is able to infer that *bx*$^+$ *Ubx*$^+$ can produce a continuous DLT, while *bx*$^+$ *Ubx*$^+$ *bxd*$^+$ *pbx*$^+$ can suppress VPs and, partially, KOs. There is some redundancy in function, since for example, other BX-C genes share with *bx*$^+$ *Ubx*$^+$ the ability to produce a continuous DLT. However, this is accomplished in a spatially restricted fashion: for example, *bx*$^+$ *Ubx*$^+$ can produce a continuous DLT in all nine segments, while *iab-8*$^+$ can do so only in the posterior-most abdominal segments, AB7 and AB8.

The add-back genetic method also allowed Lewis to infer the existence of genetic functions in regions of the BX-C where he had yet to identify either gain- or loss-of-function mutations. For example, in the region of the BX-C represented by *Df Ubx*^{109}, an *iab-8*$^+$ function was inferred because that region can shift the identity of the eighth abdominal segment towards LAB8. Similarly, this same part of the BX-C is able to restore DLT continuity from abdominal segments 6–8. Thus, this region must also contain genetic functions to the right of *iab-3*$^+$ and to the left of the inferred *iab-8*$^+$ function (e.g., *iab-7*$^+$).

By adding back either *iab-8*$^+$ alone (*Df Ubx*^{109}); *iab-2*$^{+/-}$ (denoting partial *iab-2* function) with normal *iab-3*$^+$ to *iab-8*$^+$ function (*Df P10* in combination with *Df P9*); or *iab-2*$^+$ to *iab-8*$^+$ with full *iab-2* function (*Df 100*), Lewis shows that the *iab-2* function is to produce a continuous DLT as well as to suppress KOs in abdominal segment 2 and more posteriorly. A key observation was that *bxd*$^+$ suppresses VPs on all nine segments, and—of all the genes in the BX-C—only *bxd*$^+$ is able to do so. Thus, Lewis infers that the *bxd*$^+$ function operates, not only in AB1, but also in all segments posterior to AB1.

Lewis also presents the first evidence that the BX-C controls the segmental development, not just of the external structures, but also of certain internal tissues. He notes that *Uab*^{4} hemizygous mutants lack gonads, which reside in AB5. Since the gonads are mesodermal, not ectodermal in origin like the external cuticle of the fly, he infers that the BX-C genes control mesodermal as well as ectodermal tissues. The correctness of Lewis' conclusion was borne out later by molecular analyses (Akam, 1983; Akam and Martinez-Arias, 1985).

The final major advance that Lewis presents is the discovery of a *trans*-acting regulatory gene for the BX-C: *Polycomb* (*Pc*). *Pc* had been identified by Pamela Lewis (his wife) while working in his laboratory, in 1947; Ed Lewis subsequently identified a more extreme allele, *Pc*^{3}. Remarkably, hemizygotes or homozygotes for *Pc* alleles result in the thoracic and abdominal segments transforming towards LAB8, the opposite transformation to loss of the BX-C, which results in these segments transforming towards LMS. Lewis presents evidence in this paper, some of which has been summarized above, that the BX-C genes are first activated in a particular segment and then also in all segments posterior to it. Thus, the phenotype of *Pc* mutants indicates that the normal function of this gene is to repress BX-C genes

(a similar conclusion had been reached independently by Puro and Nygren [1975]). The mutant phenotype represents the derepression of all the BX-C genes and thus the posterior-most nine segments have all BX-C genes active, resulting in LAB8 transformation.

Lewis obtained additional proof that the *Pc* mutant phenotype is dependent on the presence of the BX-C by altering the number of doses of the complex from two to four in *Pc* mutants. As he did so, the LAB8 transformation became more severe. This is consistent with *Pc* normally repressing the BX-C; consequently additional doses of the BX-C worsen the *Pc* phenotype. Furthermore, the *Pc* phenotype is absolutely dependent upon the presence of at least one dose of the BX-C. Thus, in double homozygotes for Pc^3 and *Df P9*, the LMS transformation occurs rather than the LAB8 transformation (in modern parlance, removal of the BX-C suppresses the Pc phenotype). Lewis presents some additional supportive data and then reaches the conclusion that Pc^+ is "in all likelihood coding for a repressor of the BX-C".

Lewis ends by presenting several "rules" that emerge from the body of analysis:

- First, the expression state of any given BX-C gene is controlled by a *cis*-regulatory element. This is an important inference and remains true despite the fact that molecular analyses have reduced the number of protein-coding BX-C genes from 12 to 3.
- The genes tend to be individually rather than coordinately derepressed. This rule too, remains true, although molecular analyses indicate that it is *cis*-regulatory functions that are derepressed one at a time in a spatially regulated fashion. Exactly how this happens is still incompletely understood thirty years after Lewis' paper was published.
- The BX-C is negatively regulated in *trans* by *Pc* and, perhaps, other such genes. Molecular analyses have now refined this conclusion by showing that, while other *trans*-acting factors establish the expression state of the BX-C early in development, *Pc* and other genes are required to maintain the expression state of the BX-C throughout development.
- A gene derepressed in one segment is derepressed in all segments posterior thereto. With the caveat that "gene" should now largely be read "*cis*-regulatory region," this rule is an important one whose molecular basis remains only partially defined.
- The more posterior the segment the greater the number of BX-C genes that are in the derepressed state. Again this is true with "*cis*-regulatory region" substituted for "gene." The molecular mechanisms remain to be defined.
- Relative to their positions on the chromosome, the more proximal a gene is within the BX-C, the more likely it is to be in the derepressed state. This is the famous "colinearity" rule: that the order of the genes on the chromosome is the same as the order along the antero-posterior axis of the fly of the body segments whose

identity they control. It has proven true for all animals in which HOX complexes have been found, having been maintained for over 500 million years of evolution.

What made this paper so influential? It represents an inspired example of the remarkable power of genetic analysis to dissect biological phenomena. There had been nothing quite like it before. Here, for the first time, a framework for thinking about how genes control development is presented. No one could question that Lewis had shown the importance of the BX-C genes in regulating cell fates, the development of segmental morphology as well as of individual structures. The analyses in adults as well as earlier stages, together with Lewis' prior mosaic analyses, had proven that the BX-C functions throughout development to regulate cell fates and that these functions are carried out autonomously, cell by cell. Thus, the mutant phenotypes could not be dismissed as an indirect by-product of some more general physiological abnormality. Lewis had unquestionably defined a key set of developmental regulatory genes; in fact he had defined the first set of developmental regulatory genes outside of *E. coli*. And he had gone on to define a regulator of the regulators (*Pc*). This laid the groundwork for making a reality of the fantasy of a molecular explanation for development, proposed half a century earlier (Morgan, 1926). With the advent of recombinant DNA, the BX-C became the obvious place to launch these analyses.

TRANS-ACTING REGULATORS: POSITIVE AND NEGATIVE CONTROL OF THE HOMEOTIC COMPLEXES

With his postdoctoral fellow, Ian Duncan, Lewis followed up on the *trans*-acting genes that regulate the BX-C (Duncan and Lewis, 1982). They report in detail on the negative regulator, *Pc*. In addition, they present a positive regulator, *Regulator of bithorax* (*Rg-bx*), better known now as *trithorax*.

They use gene dosage analyses to show that Pc^3 behaves as an antimorphic allele ("dominant negative" in modern terminology) and use it to examine effects in adults that are genotypically $Pc^3/Pc^3/+$. In several instances they see transformations of segmental identity that mimic those produced by *cis*-dominant mutants within the BX-C (e.g., *Cbx*, *Uab*, and *Mcp*). Although these transformations are weak, they provide strong evidence that "several (perhaps all) of the BX-C genes are under negative control of Pc^+".

Between Lewis' 1978 paper and this one, a second complex of homeotic genes—the Antennapedia complex (ANT-C)—had been reported by Thom Kaufman and colleagues (R. A. Lewis et al., 1980). The ANT-C was shown to regulate the development of the more anterior segments of the embryo and adult, and thus was complementary in spatial control to the BX-C, which regulates the identity of more posterior segments. Duncan and Lewis present several lines of evidence that Pc regulates both the BX-C and the ANT-C. For example, the ventral setal belt of the eighth abdominal segment, which is somewhat prothoracic in appearance in

Df P9 homozygotes, becomes strictly mesothoracic-like in Pc^3 *Df P9* homozygotes. Transformation from prothoracic to mesothoracic identity had been reported for certain ANT-C mutants (Wakimoto and Kaufman, 1981), leading Duncan and Lewis to argue that *Pc* regulates one or more ANT-C genes as well as the BX-C.

Furthermore, Pc mutant adults show segmental transformations similar to those produced by dominant mutants in the ANT-C such as *Scx*, *Msc*, *Ctx* and *Antp* itself. Because the ANT-C mutants had not been studied in as much detail as the BX-C mutants, Duncan and Lewis were unable to make the generalization that *Pc* is acting as a negative regulator of all ANT-C genes. However, in the case of the *Antennapedia* gene itself, evidence had been presented that the antenna-to-leg transformation results from improper expression in the antenna (Struhl, 1981b). Thus the fact that a similar transformation is seen with *Pc* mutants, suggested to Duncan and Lewis that Pc^+ is likely to act as a negative regulator of ANT-C genes in the antenna.

The weak segmental transformations in $Pc^3/+$ mutant adults positioned Duncan and Lewis to carry out a screen for dosage-sensitive enhancers of this phenotype. In principle such genes might encode additional negative regulators of the BX-C and ANT-C. From this screen they identified the *Polycomb-like* (*Pcl*) gene that Duncan went on to describe (Duncan, 1982). Additional negative regulators included *extra sex combs* (*esc*) (Struhl, 1981a) and a collection of enhancers of *Pc* (Jürgens, 1985).

Of particular importance, Duncan and Lewis address the time of action of *Pc*. They show that *Pc* acts in the embryo (because mutant embryos exhibit segmental transformations). But genetic mosaic analyses of the type Lewis had used two decades earlier for BX-C mutants (Lewis, 1963a, 1964), also showed that *Pc* is still active late in development. Thus *Pc* could be inferred to be a negative regulator of the BX-C and ANT-C throughout development.

The *Rg-bx* mutant provided proof that the BX-C is not only negatively regulated, but is also under positive control. In a 1968 abstract for the Twelfth International Congress of Genetics in Tokyo (Lewis, 1968), Lewis had reported a positive regulator of the BX-C, *Rg-pbx* (now known to be an allele of *hunchback*, see Casanova et al., 1985; Bender et al., 1987). There he had mentioned the existence of "other, weakly dominant, homozygous lethal, regulator-like mutants which appear to be localized in the 88B region." These defined the *Rg-bx* locus which was subsequently identified independently and called *trithorax* (*trx*, see Ingham and Whittle, 1980). Duncan and Lewis showed that, unlike *Pc*, *Rg-bx* mutations cause anterior transformations that are similar to those produced by loss-of-function BX-C mutants. For example, embryos from which *Rg-bx* function is removed show anterior-directed transformations throughout the abdomen, consistent with a positive regulatory role on the BX-C. The effects of altering the dose of the BX-C were consistent with such a role.

Pc, *esc* and other *Polycomb* family genes, together with *Rg-bx* (*trx*) have now been shown to encode components of large protein complexes that are essential to maintain the correct state of BX-C and ANT-C expression, which is set in the early embryo,

through ensuing multiple cell divisions during development (reviewed in Ringrose and Paro, 2001). Interestingly, in humans, chromosomal translocations that break in trithorax homologs, cause acute leukemia (Gu et al., 1992).

CIS-REGULATION WITHIN THE BX-C: THE RULES OF ENGAGEMENT

Lewis' research in the years following the publication of his 1978 paper focused on two major areas. The first was identification of loss-of-function mutations affecting the postulated additional genetic functions in the BX-C (*iab-5*, *iab-6*, *iab-7*, etc.). Most of these were reported in the papers on the molecular cloning of the BX-C (Bender et al., 1983a; Karch et al., 1985) and are therefore part of the next section of this book. Lewis' second focus was on *cis*-regulation of the BX-C, which forms the major topic of the final two papers included here (Lewis, 1982, 1985).

By 1982, Lewis had identified loss-of-function mutations in the *anterobithorax* (*abx*) genetic function (Lewis, 1982). He reports that *abx* maps just to the left of *bx* (about 0.01 cM), and affects the presutural region of the third thoracic segment. The triple mutant, *abx bx pbx* produced a nearly complete transformation of the MT into the MS. This four-winged fly posed for the famous color picture, which appeared on the cover of *Science* on July 1, 1983 to accompany the positional cloning of the BX-C (Bender et al., 1983a), and has since appeared in most introductory biology textbooks. Interestingly, Lewis reports that he finds patches of muscles inside the transformed third thoracic segment that are never found in a normal MT. This observation was fully consistent with Lewis' report in 1978 that the BX-C controls mesodermal fates in addition to ectodermal fates (that conclusion had been based on loss of gonads from the fifth abdominal segment of Uab^4 homozygotes). The development of rudiments of what may be longitudinal flight muscles in the transformed third thoracic segment were later to be studied in detail by VijayRaghavan in collaboration with Lewis (Fernandes et al., 1994). Subsequently, additional internal tissues were shown to be affected by BX-C mutants, notably the central nervous system, which derives from the so-called neurectoderm (e.g. Ghysen and Lewis, 1986).

Lewis' 1982 and 1985 papers are focused upon identification of new *cis*-dominant regulatory mutants in the BX-C as well as the *cis*-regulatory rules that he was able to derive from analyses of both the loss- and gain-of-function mutants. He identifies three new *Cbx*-like dominant mutants, each of which has a somewhat different effect (see Table 1, Lewis, 1982). These mutants exert spatially restricted effects: *Hm* transforms dorsal regions of T2 only; *Cbx-2* transforms anterior and posterior parts of the mesothorax; in the T2 leg, *Cbx-1* operates in the anterior but not posterior region while *Cbx-2* operates in the posterior but not anterior portion. All of these new mutants are associated with visible chromosomal rearrangements that, in retrospect, almost certainly bring the *Ubx* gene under control of an ectopic *cis*-regulatory region causing expression in part of the MS. Lewis notes that the different *Cbx*-class mutants *cis*-dominantly activate different BX-C genetic functions: *Cbx* activates *Ubx* and *pbx* while *Cbx-3* activates *abx*, *bx* and *Ubx*. He tests which BX-C genetic functions might

be activated *cis*-dominantly by these mutants by carrying out *cis–trans*, gene dosage and transvection tests.

In his 1982 paper, Lewis reports a new *pbx* allele, pbx^2. He carries out transvection tests and finds, to his surprise, that *Ubx* +/+ pbx^2 and *bxd* +/+ pbx^2, exhibit transvection. (*Ubx* +/+ pbx^1 and *bxd* +/+ pbx^1 had, previously, not shown any such effect.) Thus, *bxd* +/+ pbx^2 is almost wild type while *bxd* +/R(+ pbx^2) or R(*bxd* +)/+ pbx^2 shows a moderate PMT → PMS transformation (he now calls this T3p → T2p). He is led to speculate that "the occurrence of transvection between a pair of mutants may be indicative of their representing separate functional units." Mathog was later to classify Lewis' mutants into "transvection groups" and to use these groups to assess the conditions necessary for transvection (Mathog, 1990).

The possibility that transvection represents an interaction between mutants representing separate functional units was strengthened in light of Lewis' more extensive tests in his 1985 paper. By then he had obtained one or more loss-of-function mutations in each of the predicted genetic functions within the BX-C: *abx*, *bx*, *bxd*, *pbx*, *iab-2*, *iab-3*, *iab-4*, *iab-5*, *iab-6*, *iab-7* and *iab-8* (Bender et al., 1983a; Karch et al., 1985). Thus, he was in a position to conduct cisvection and transvection tests among all of these. He finds that cisvection effects are invariably from proximal to distal in direction (relative to the centromere). For example, *abx cis*-inactivates pbx^+ (*abx* maps proximal, or to the left of, *pbx*) while *pbx* does not *cis*-inactivate abx^+. With respect to transvection, the same proximal-to-distal polarity applies throughout the BX-C. Thus *abx* and pbx^2 transvect (i.e., partially complement when homologous chromosomes are paired), but the effect applies to the more distal, *pbx* phenotype and not to the *abx* phenotype.

Work by others, to be discussed in the commentary on the section entitled Molecules and Development, had by 1985 defined three molecular and functional domains within the BX-C: *Ubx* (including *abx*, *bx*, *Ubx*, *bxd* and *pbx*), *abd-A* (including *iab-2*, *iab-3* and *iab-4*) and *Abd-B* (including *iab-5* to *iab-8*) (Regulski et al., 1985; Sánchez-Herrero et al., 1985; Tiong et al., 1985). Lewis' analyses had been conducted before the definition of these three domains of molecular and genetic function, but the fit is remarkable: In general, both the cisvection and the transvection effects of any particular mutation are restricted to the domain within which that mutation maps. For example, for mutations within the *Ubx* domain, there is no *cis*-inactivation or transvection with *iab-2*, *iab-3* . . . *iab-7*. The only apparent exception derived from the analyses of *iab-4* and *5*. However, this is the exception that proves the rule since the *iab-4* mutation ($iab\text{-}4^{DB}$) that exhibits cisvection and transvection with *Abd-B* domain mutations such as *iab-7*, is in fact a small deletion that also removes most of the *iab-5* region (Karch et al., 1985). Similarly, the transvection effect of *iab-2* on *iab-5* occurs with the $iab\text{-}4,5^{DB}$ double mutant.

In addition to the cisvection and transvection effects described above, Lewis describes the "*cis*-overexpression" effect (COE), in which mutation of a more distal genetic function causes an apparent overexpression of the adjacent, more proximal

function. He shows COE to occur between *abx* and *bx*, *iab-2* and *iab-3*, *iab-3* and *iab-4*, as well as *iab-4* and *iab-5*. In retrospect, as for the *cis*-inactivation and transvection effects, such *cis* overexpression tends to occur within each of the three BX-C domains rather than between them. The exception is for *iab-4* and *iab-5*, since *iab-4* lies in the *abd-A* domain while *iab-5* lies in *Abd-B.* This cross-interaction between *iab-4* and *iab-5* was later hypothesized to be caused by sharing of *cis*-regulatory regions by the *abd-A* and *Abd-B* genes (Duncan, 1987). Lewis and his postdoctoral fellow, Susan Celniker, in subsequent molecular analyses, were to refine this suggestion in terms of "enhancer sharing" (Celniker et al., 1990).

Lewis derives three rules for *cis*-regulation within the BX-C: the colinearity rule (COL), the *cis*-inactivation rule (CIN), and the *cis*-overexpression rule (COE).

- The COL rule had been presented in his 1978 paper and was now generalized to the entire BX-C: with the exception of *pbx* the proximal-to-distal order of the genetic functions along the chromosome are identical to the order along the antero-posterior body axis in which they control segmental identities.
- The CIN rule states that a mutant lesion in a given gene tends to inactivate in *cis* one or more neighboring genes that lie distal to that function. In light of molecular data this rule can now be restated by substituting "*cis*-regulatory function" for "gene." As described above, the CIN rule also can now be seen to apply largely within rather than between the functional domains of the BX-C.
- The COE rule states that certain lesions within a given gene lead to over expression of the gene lying immediately proximal. Again, as for the CIN rule, in general "gene" can be replaced with "*cis*-regulatory function"; and the effect is now seen to be largely within rather than between the three domains of the BX-C.

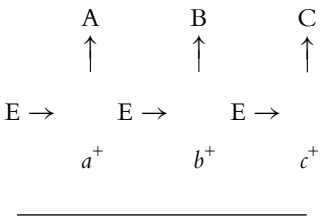
These rules remain as the final test for any comprehensive mechanistic explanation. To date, no molecular basis for the fundamental mechanisms by which the BX-C genes are regulated has passed this test. The challenge continues to motivate new experiments and insights.

Following his tradition of attempting to provide a formal structure within which to explain his genetic observations, Lewis ends his 1985 paper with an abstract model. The model attempts to encompass the recent molecular results showing that only three of Lewis' "genes" actually encode proteins, whereas the remainder are *cis*-regulatory regions. Thus he invokes a "cis-regulatory entity (E) . . . which could be pre-transcriptional or co-transcriptional." Most obviously, he states, E could track the other strand of the DNA from that which encodes the BX-C mRNAs, and so would affect genetic functions in the opposite direction. Here he is attempting to explain why the CIN rule acts in the opposite direction to the then known direction of transcription of mRNAs within the BX-C. For example, transcription within the Ubx domain proceeds in the direction from *pbx* → *bxd* → *Ubx* → *bx* → *abx* while

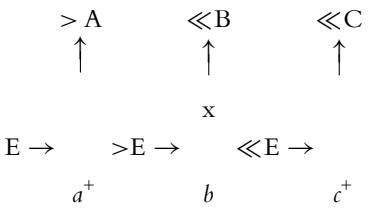
the direction of CIN is *abx* → *bx* → *Ubx* → *bxd* → *pbx*. Thus E would need to exert an effect in the latter direction rather than the former.

Lewis presents several possibilities as to the nature of "E", including a primasome-like molecule (Arai and Kornberg, 1981) or an antisense RNA, which "may be a plausible basis at the molecular level for the CIN effect." He goes on to suggest: "it is more profitable to pursue a model in which E is assumed to be a substance, as opposed to some type of construct that would involve, for example, structural deformation of the chromosome. To account for cisvection and transvection, it is necessary to assume that E has a nonrandom distribution in the nucleus and that its effective radius of action is very limited, suggesting that E is unstable or is produced in extremely minute amounts, or both. If cotranscriptionally produced, then E, or the effective part of E, is likely to be a RNA rather than protein, since the latter should act in trans, not in cis." At the time that he wrote this, Lewis was aware of the noncoding transcripts derived from the *pbx* and *bxd cis*-regulatory regions of BX-C (Hogness et al., 1985; Lipshitz et al., 1987). The direction of transcription of these transcripts was, however, the same as that of the *Ubx* mRNA, not the opposite as proposed for "E."

In wild type, Lewis pictures the situation as follows:



In the case of a mutation in gene *b*, two things happen: (1) less product B is produced (≪B) and (2) E cannot be transported and so less E reaches the more distal gene *c*. Since E is required for expression of gene *c*, expression of gene *c* is reduced (i.e., a mutation in gene *b* results indirectly in *cis*-inactivation of more distal gene *c*). Excess E accumulating near gene *b*, on the other hand, leads to hyperactivation of the more proximal gene, *a* (i.e., a mutation in gene *b* results indirectly in *cis*-overexpression of more proximal gene *a*). This Lewis figures as follows:



Lewis extends the basic ideas in the first two diagrams to show how the CIN effect would spread distally but become weaker with distance as well as to explain transvection.

Lewis' diagrams are an attempt to provide an abstract model for all of the phenomena that he had discovered: COL, CIN, COE, and transvection. At the time, they seemed very complicated compared with the idea that genetic functions such as *bx* and *bxd* simply represent *cis*-regulatory elements in the DNA that control expression of the adjacent protein coding transcription unit, *Ubx* (Beachy et al., 1985; Bender et al., 1985; Hogness et al., 1985). The simple molecular model did partially explain the results of Lewis' *cis–trans* tests. For example, *Ubx* +/+ *bxd* is phenotypically similar to + *bxd*/+ *bxd*. With the simple molecular model, this is because in the *Ubx* +/+ *bxd* case, the *Ubx* + chromosome will not encode functional Ubx protein (it is mutant for *Ubx*), and the + *bxd* chromosome will not express Ubx anywhere the *bxd cis*-regulatory region normally directs Ubx expression. Thus a *bxd* phenotype results. In the + *bxd*/+ *bxd* case, neither chromosome produces Ubx in the cells in which the *bxd cis*-regulatory region normally directs expression, so a *bxd* phenotype results.

What the molecular data did not—and still do not—explain is the *directionality* of the effects that Lewis summarized in the COL, CIN, COE, and transvection rules. It was speculated that the noncoding transcripts from the *cis*-regulatory domains might function in *cis* (Hogness et al., 1985; Lipshitz et al., 1987). However, the molecular data argued against those transcripts implementing the *cis*-regulatory genetic functions that Lewis had defined for those regions (Hogness et al., 1985). A model involving tethering of the 5′-end of those transcripts to the chromosome, proposed several years later, was able to explain the directionality of CIN and transvection (Mathog, 1990). Today, noncoding transcripts have been found to implement an increasing number of biological roles. It remains conceivable that an RNA-based explanation for the directionality of some, or all, of the COL, CIN, COE, and transvection rules that Lewis defined ought to be resurrected. Lewis' 1985 model, strict formalism that it was, may yet turn out to have been closer to the truth than any competing explanation. For more discussion of this topic, see the Molecules and Development commentary.

SUMMARY

The papers in this section of the book represent the transition for Lewis—and therefore the larger developmental biology community—from a focus on genes as entities worth studying in their own right, to genes as the regulators of animal development. The quarter century from the mid-1950s to early 1980s showed gradual progress in this regard. Lewis' famous 1978 paper appears to be saltational only because there was a gap of almost 15 years since his previous paper on the genetic control of development. There had, however, been a major leap in his understanding of the BX-C, and this was picked up by the broader community. The circle would be completed by molecular

analysis of the BX-C and of other developmental regulatory genes, yielding the deeper insights required for an understanding of how genes control development. As will be seen in the next section of the book, Lewis' contributions to molecular analyses were not seminal in the way that his genetic contributions had been. However, his genetic analyses and his collections of mutants made possible the first positional cloning of a gene—*Ubx*—and provided the raw material on which the field now known as functional genomics was founded.

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GENES AND DEVELOPMENTAL PATHWAYS[1]

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INTRODUCTION

The way in which genes control the growth and development of an organism is a central problem in biology, and one which is currently under study in diverse forms from phage to man. I would like to discuss an approach to this problem which makes use of a series of pseudoallelic genes in *Drosophila*. Such series of closely linked genes with related effects have, in several instances, been profitably exploited in the bacteria to learn more about how genes control biochemical pathways. Striking examples are the linked genes controlling histidine biosynthesis in *Salmonella* (Demerec and Hartman, 1959; Ames and Hartman, 1962), or lactose utilization in *Escherichia coli* (Jacob and Monod, 1961a,b). It may be anticipated that pseudoallelic series affecting morphological traits, such as the case to be described below, can also be profitably exploited to learn more about how genes control developmental pathways.

A number of levels of functional integration of the genetic material can now be recognized, with pseudoallelism being but one example (see reviews by Pontecorvo, 1958; Demerec and Hartman, 1959; Carlson, 1959). Moreover, the "genome" of phage and bacteria may be structurally organized in a manner different from the chromosomes of higher forms. In these circumstances it is not surprising that

[1]This work was supported in part by a grant from the US Atomic Energy Commission (AT-(04-3)-41).

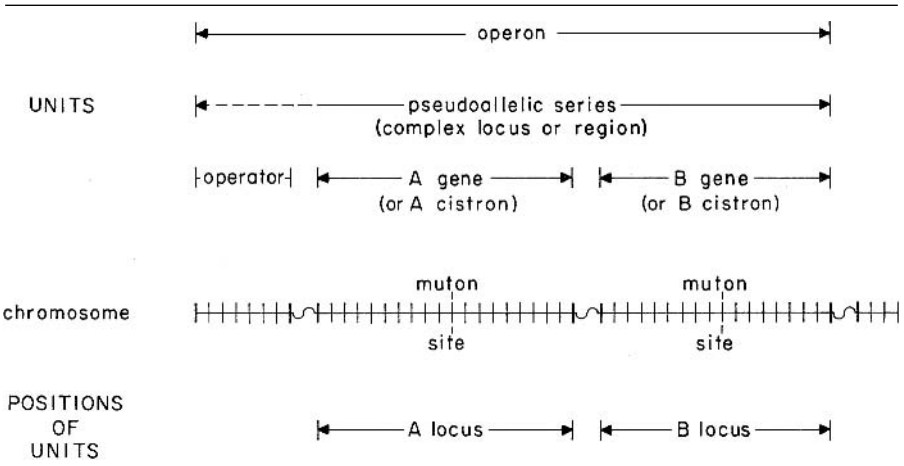

Figure 1. Diagram of the relationships between some of the terms in use for the elementary genetic units and clusters of functionally related units. The chromosome is represented, purely arbitrarily, as composed of a linear array of segments with linkers between them.

conflicting definitions and interpretations have sometimes arisen. At the outset it therefore seems desirable to review some of the basic methods used to resolve the genetic material and to show the relationship between some of the terms now in use for the elementary genetic units (Fig. 1).

A gene may be thought of as a portion of the chromosome or genome which contains the information needed to specify the sequence of amino acids in a single protein or polypeptide. It is useful to define the gene in this way since it allows one to retain the widely applicable principle of "one-gene one-enzyme" or "one-gene one-polypeptide." However, it is important to keep in mind that this does not provide an operational definition of the gene.

The problem of the nature of gene structure and function was considerably clarified by Benzer's (1957) introduction of the terms muton, recon, and cistron, to indicate the units which can be operationally defined by certain mutational, recombinational, and functional tests, respectively. The smallest element which, when altered, is able to produce a mutant effect Benzer called the muton. It presumably corresponds to an individual base pair of DNA. The position of the muton in the chromosome or genome is called a "site."

From an operational standpoint, the recon, or unit of recombination, is of first importance. One of the greatest advances in genetics in recent years has been the discovery by a number of investigators that genes in microorganisms can be resolved by a process of intragenic recombination into a linear array of mutons (e.g., in phage by Benzer, 1955; in bacteria by Demerec and Hartman, 1959; and in fungi by Pritchard, 1955, and by Case and Giles, 1958). (Intragenic recombination may be equivalent to, or associated with, a phenomenon discovered by Mitchell (1955), and now often

called gene conversion.) Some recent studies with *Drosophila* (see especially Welshons and von Halle, 1962; Chovnick et al., 1962) and with corn (Nelson, 1962) indicate that the genes of higher organisms can be similarly resolved.

Intragenic recombination usually exhibits a high degree of negative interference (i.e., more double recombinants than would be expected by chance); whereas, crossing over, the classical process of recombination shows a high degree of positive interference over short regions of the chromosome. In practice, this means that the ordering of the elements within the gene cannot be inferred directly from the behavior of outside marker genes, as in conventional mapping of chromosomes, but ordering in a statistical sense is still possible and a consistent linear ordering of intragenic elements has been achieved in a number of the studies cited above.

Pritchard (1955, 1960) has developed an ingenious hypothesis which accounts for intragenic recombination and crossing over on the basis of a single mechanism of exchange involving homologous chromosomes. On this hypothesis, certain types of multiple exchanges occurring within a very short segment of the chromosome, called the "effective pairing segment," give rise to intragenic recombination between elements which lie within the segment and to crossing over between say two elements which lie outside, and on either side, of the segment.

The other possibility is that intragenic recombination and crossing over are intrinsically different processes. For example, the occurrence of crossing over may be restricted to intergenic regions. Regardless of the model one has for these processes, crossing over and intragenic recombination are operationally distinguishable by a property such as interference. Hence, it may be possible to recognize two types of recons. One type would represent the smallest unit resolvable by intragenic recombination. That this recon may correspond to the muton is now made especially likely by Henning and Yanofsky's (1962) analysis of mutants affecting the tryptophane synthetase gene in *E. coli*. The other type of recon would be the smallest unit that can be resolved by the crossover test; it might turn out to be a much larger unit, possibly corresponding to the unit determining a single polypeptide or protein.

Thus, the gene may be pictured as a linear array of mutons (Fig. 1), but the intergenic regions. if they exist at all, remain undefined. It seems desirable to retain "locus" in its original sense to indicate the position that the gene occupies in the chromosome, and the term "allele" to designate one of the array of possible mutational states of the gene. It should be noted that the different alleles of a gene may represent mutational substitutions at different sites (heteroalleles), or different mutational substitutions at the same site (homoalleles)—to follow the terminology of Roman (1956).

Of primary concern in this paper are the tests which recognize different levels of functional integration in the chromosome, especially levels determined by clusters of two or more linked genes. One of the most important functional tests available is the *cis–trans* test, based on the cisvection effect (Lewis, 1955) or *cis–trans* position

effect as it has come to be called. Benzer (1957) used this test to reach a purely genetically defined unit of function, the cistron. Thus, two recessive mutants, *a* and *b*, are said to belong to the same cistron if the *trans* form of the heterozygote, or *a* +/+ *b*, has a mutant phenotype. To complete the test it must of course be shown that the *cis* form of the heterozygote, or *a b*/++, has a wild-type phenotype. Since, in practice, the double mutant *a b* must be derived by recombination, the *cis–trans* test is intimately tied to a recombination test. When one mutant is dominant, or both are dominants, they can be said to belong to the same cistron if the *cis* and *trans* types differ phenotypically. Conversely, if *cis* and *trans* types are identical phenotypically, there is a strong presumption that the mutants belong to different cistrons. If not applied with caution, the *cis–trans* test can be misleading. Thus, owing to the phenomenon of intraallelic complementation (review by Catcheside and Overton, 1958), the *trans* type between mutants affecting the same protein or polypeptide may approach wild type. However, in such cases the *cis* arrangement approaches wild type more closely than does the *trans* one. Since *cis* and *trans* are not equivalent in these cases it remains proper to speak of only one cistron.

The *cis–trans* test may tend to identify a cluster of different genes as a single cistron. This possibility sometimes arises in the case of a cluster of two or more closely linked and functionally related genes. Such a cluster has been called a pseudoallelic series, or a "complex locus" (Dunn, 1954), or simply a "region" (Benzer, 1957). Often there is independent evidence for a considerable degree of functional independence of the elements of the cluster, so that it seems best in such circumstances to consider a pseudoallelic series as a set of cistrons rather than one cistron.

With the discovery of intragenic recombination, confusion in usage arose between the terms allele and pseudoallele. Insofar as possible, it seems best to refer to the different alleles occupying different sites within the gene as "heteroalleles," as already noted above, and to refer to the different genes of a pseudoallelic series as "pseudoalleles."

Recently, Jacob et al. (1960) have used the term "operon" to designate a cluster of linked genes whose functioning is coordinated by an "operator." The operator is pictured as a segment of the DNA which is concerned with initiating the "transcription" or synthesis of messenger RNA by the adjoining "structural" (as opposed to "regulatory") genes. The operon concept provides the basis for a valuable working hypothesis applicable to pseudoallelic systems in general, as will be discussed later.

It is probably too much to expect that a unit defined purely by genetic tests will turn out to be identical with a chemical unit. Nevertheless, the *cis–trans* test, when carefully employed, does seem to reveal the existence of a genetic unit that may be equivalent to the region of the chromosome that codes for a single polypeptide or protein molecule. In other words, the cistron may well correspond to the structural gene. The *cis–trans* test is also of value, somewhat paradoxically, in testing functional interrelationships among the different genes of a pseudoallelic series, as discussed below, or among the different elements of the operon, as Jacob and Monod (1961) have shown.

There are now a number of pseudoallelic series in higher organisms which are known to affect developmental processes (see a recent review by Carlson, 1959). The example which I wish to discuss is the bithorax pseudoallelic series in *Drosophila*, which has striking and clearly delineated effects on the development of certain body segments of this organism (Lewis, 1951, 1954a,b, 1955, 1957). A summary of the principal findings together with the addition of some new data will be presented first. Then, a model will be developed that attempts to explain the observed developmental effects in terms of a set of three substances elaborated by three of the bithorax genes.

THE BITHORAX PSEUDOALLELIC SERIES

Description of the Bithorax Mutants

The bithorax pseudoallelic series comprises five groups of phenotypically distinguishable mutants, each group occupying a separate locus in the chromosome. For simplicity, the five loci are designated here as *a*, *B*, *C*, *d*, and *e*, corresponding to their order in the chromosome. (Capital letters in the case of genes *B* and *C* signify that the known mutant alleles are dominant.)

The various bithorax mutant phenotypes can be specified in terms of transformations of anterior and posterior portions of certain body segments into structures resembling the corresponding portions of homologous segments. The affected segments are: the mesothorax (MS), metathorax (MT), and first-abdominal segment (AB_I). For reference purposes, the wild type body segmentation pattern is shown diagrammatically in Fig. 2. For simplicity, this diagram shows the affected segments and their appendages (the wings, halteres, and legs) as being divided into anterior

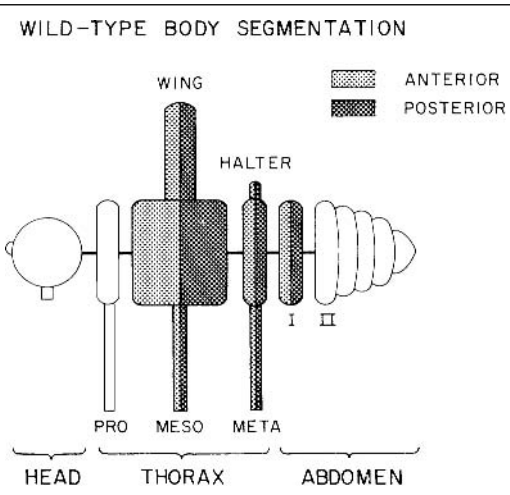

Figure 2. Highly schematic representation of the pattern of body segments in the wild type fly.

and posterior portions of equal size. Actually, the anterior portion is the larger. The line of separation between these portions is deduced from the mutant effects and is anatomically well defined in the case of the thorax and wing. It is less well defined in the case of the halter and leg, and is completely undefined in the case of the wild type first-abdominal segment. Diagrams of the body segmentation patterns of the principal mutant types, together with the names of the individual loci, are shown in Figs. 3 and 4. Photographs of mounted specimens of the wild type and of the principal mutant types are shown in Figs. 5–8.

The various levels or states of development of MS, MT, and AB_I that are found in wild type or in certain single or double mutant homozygotes are summarized in Table 1. Relationships, which will be discussed later, between such levels and the relative production in the developing body segments of hypothetical "bithorax" substances are also summarized in Table 1.

The *a* mutants cause the anterior portion of the metathorax (AMT) (including its appendages, the halteres and the third pair of legs) to be transformed into structures closely resembling the anterior portion of the mesothorax (AMS) (Figs. 4 and 5B). This change has been called the Type-I transformation (Lewis, 1955), and will be designated here simply as T1. The degree of this transformation depends upon the particular *a* allele present (four spontaneous mutants being known): an extreme form of this gene (bx^3), found by Stern, is able to effect an almost complete transformation of this kind, and is the one illustrated in Fig. 5B.

The *e* mutant, found by Bacon, is complementary to the *a* mutant in the sense that it causes the posterior portion of the metathorax (PMT) to resemble the corresponding portion of the mesothorax (PMS) (Figs. 4 and 5D). This is designated here as the T2 transformation.

The *d* mutant, found by Bridges, has two distinct kinds of effects. It has the T2 effect that is found in the *e* mutant and in addition it has a thoracic-like modification of AB_I or a change that will be designated T3. The latter effect is expressed in varying degrees, depending upon the mutant allele, there being three spontaneous alleles. The effect for a given allele is constant and symmetrically expressed. In compounds of the *d* mutant with certain X-ray induced changes, abdominal thoracic-like legs arise either singly or in pairs from AB_I (Fig. 6). These legs have the same symmetries as the other legs, and are clearly not reduplications of the thoracic legs. Rather rarely in such extreme types a small partially wing-like halter arises dorsally in the region of AB_I (Fig. 7). From an examination of such abdominal legs and halteres it can be inferred that in the *d* mutant the anterior portion of AB_I is metathoracic-like, while the posterior portion is partially mesothoracic-like.

The *B* mutant, found by Bacon, is dominant and viable when homozygous. It is of X-ray origin and arose simultaneously with the *e* mutant, from which it was later separated by crossing over (Lewis, 1954b). The phenotype of the heterozygote is virtually as extreme as that of the homozygote, and consists of a metathoracic-like modification of PMS (Fig. 8A). This remarkable transformation is designated as T4. There is some tendency, especially in the *B* homozygote, for the metathoracic

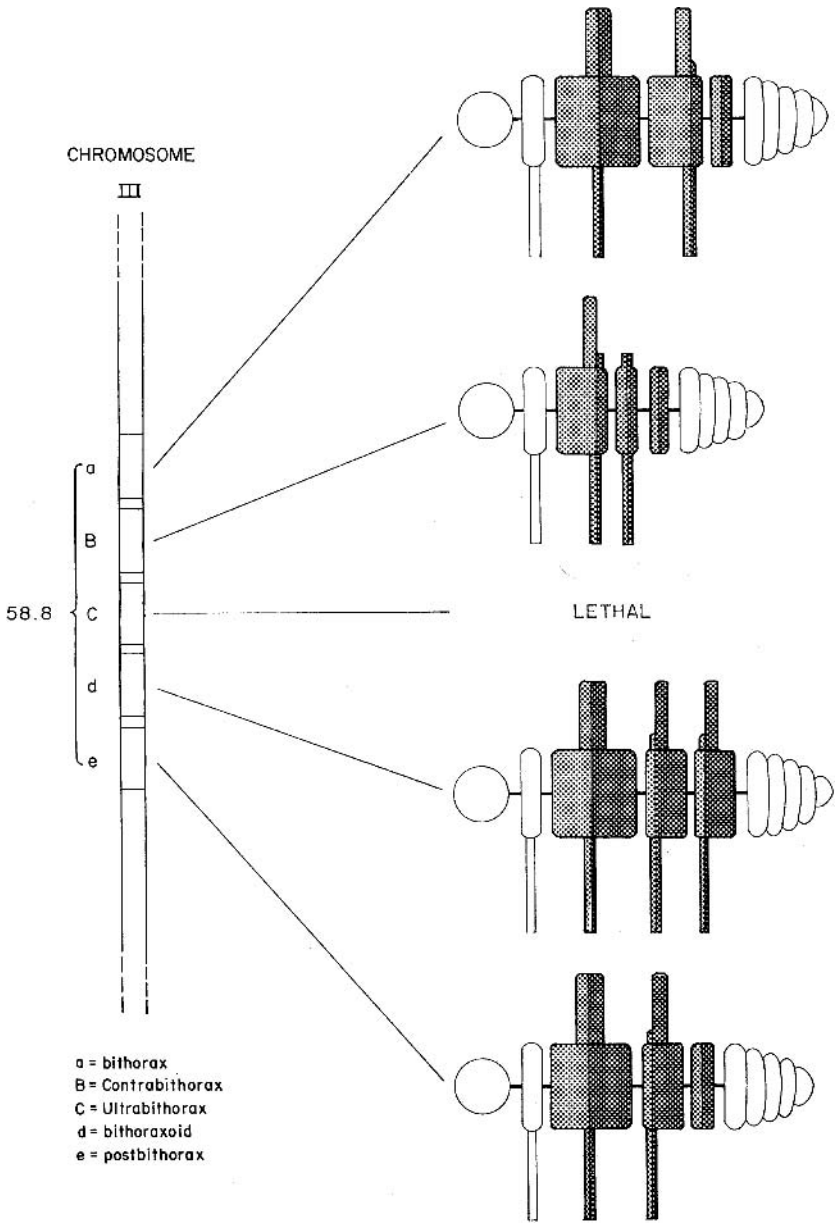

Figure 3. Chromosome map of the bithorax pseudoallelic series showing the location and names of five of the principal types of mutant genes and the body segmentation pattern associated with each. For reference to the wild-type pattern see Fig. 2.

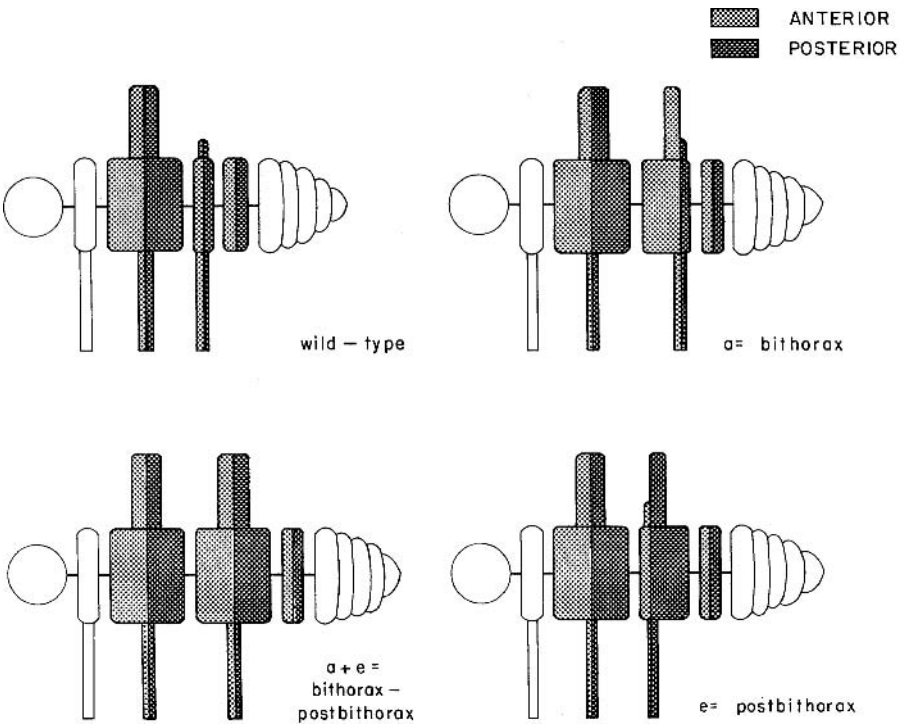

Figure 4. Diagram of the body segmentation pattern in the single mutants (*a* and *e*), compared with that in wild type and the *a e* double mutant combination.

transformation of MS to extend forward into the anterior portion so that in some flies the wing is converted almost completely into a halter and the dorsal MS is all but obliterated (usually asymmetrically), as shown in Fig. 8B.

Finally, the *C* mutant, found by Hollander, also has a dominant phenotype which consists largely of a slight enlargement of the distal segment of the halter (cf. Fig. 10C). Although the homozygote for the *C* mutant is lethal as far as the adult stage is concerned, it is viable in the larval stage and exhibits extra pairs of spiracles on MT and AB_I that are indicative of a mesothoracic-like modification of these segments (Lewis, 1951). Thus, *C* seems to combine T1, T2, and T3 effects. This is supported by preliminary studies of a probable new allele of *C* (kindly made available by Prof. Hans Gloor) which is homozygous viable and which expresses T1, T2, and T3 transformations, each to a mild extent. In its interactions with other pseudoallelic genes, the original *C* mutant acts as if it has rather extreme T1, T2, and T3 effects. Finally, studies described below of mosaics containing mixtures of wild type and homozygous *C* tissues also show that the *C* mutant has a combination of T1, T2, and T3 effects.

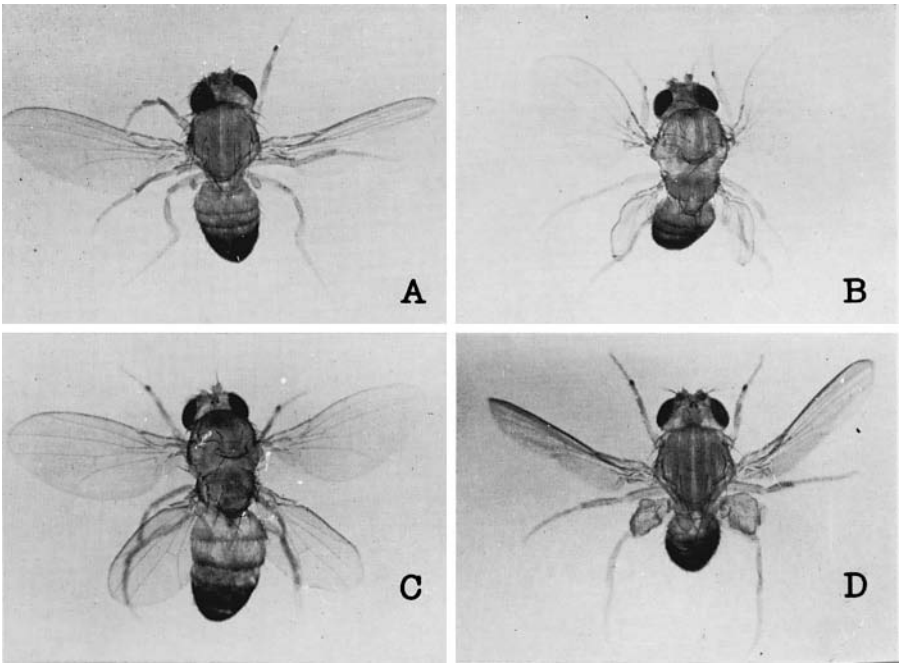

Figure 5. Photographs of male specimens of the following phenotypes: A, wild type; B, the *a* mutant (genotype: bx^3/Ubx^{105}); D, the *e* mutant (pbx/Ubx^{105}); and C, the *a e* double mutant ($bx^3\,pbx/Ubx^{105}$). The specimens are mounted in permount and the mesothoracic wings have been purposely outstretched to reveal the metathoracic and abdominal segments. Compare with Fig. 4.

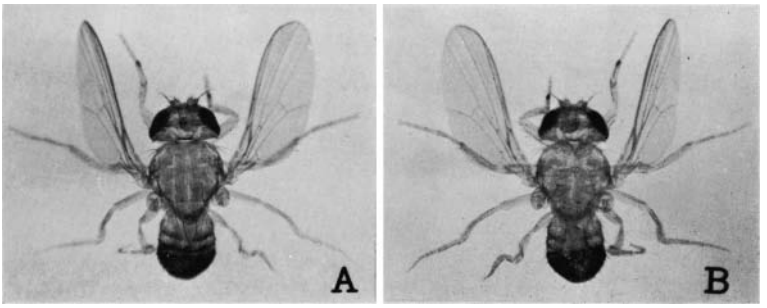

Figure 6. Photographs of an extreme example of the *d* phenotype (genotype: d/Ubx^{109}) with eight legs. A, dorsal view; B, ventral view of the same male. The first-abdominal legs although smaller than the thoracic pairs and somewhat crippled have the same symmetry and same number of segments as do the thoracic legs.

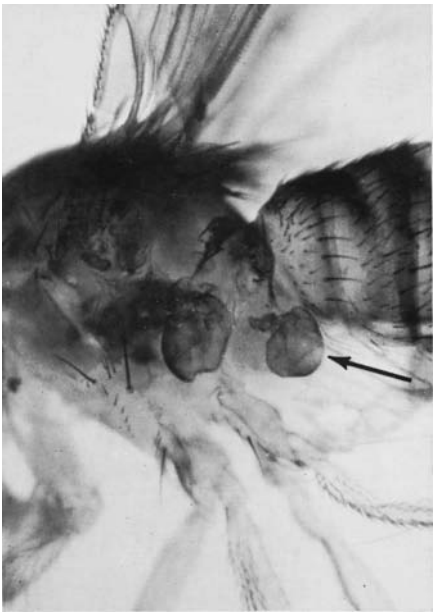

Figure 7. Photograph of a female (*lateral view*) having a first-abdominal partially wing-like appendage (see *arrow*) on the left side of the body (genotype: same as that in Fig. 6). This specimen has also one extra first-abdominal leg (not visible in the photograph) on the same side of the body. Just in front of the abdominal "wing-halter" is the partially wing-like metathoracic halter typical of the *d* mutant phenotype. In order to provide a "marker" for the dorsal metathorax, the female was made heterozygous for the dominant Tuft (*Tft*) mutant of Ritterhoff, which produces many extra bristles on the mesothorax and a streak of small bristles across the dorsal metathorax (normally devoid of bristles). The combination of *Tft* with the extreme *d* phenotype produces this streak not only in the metathoracic but also in the first-abdominal region indicating a thoracic-like modification of the latter segment.

Map of the Bithorax Region

Bithorax (*a*) is located at 58.8 in the right arm of the third chromosome (3R). A map of the bithorax region, based on the frequency of double mutant and wild type crossover products derived from pairwise crosses of *a*, *B*, *C*, *d*, and *e* mutants, has already been published (Lewis, 1954b). New data have been obtained making use of a quintuple mutant combination, *a B C d e*. Map distances calculated from recombination occurring in *a B C d e*/+++++ females are as follows: 0.008 between *a* and *B* (based on 6 *a* ++++ crossovers); 0.01 (based on 10 *a B* +++ and 5 ++ *C d e* crossovers); 0.006 between *C* and *d* (based on 1 *a B C* ++ and 5 +++ *d e* crossovers); and 0.006 between *d* and *e* (based on 5 ++++ *e* crossovers). These newer map distances are compatible statistically with the results from pairwise crosses. Pooling both sets of data gives the map shown in Fig. 9. It should be noted that in all of the recombination studies, parental females were heterozygous for closely linked marker genes on either side of the *bx* region, usually spineless (*ss*) at 58.5

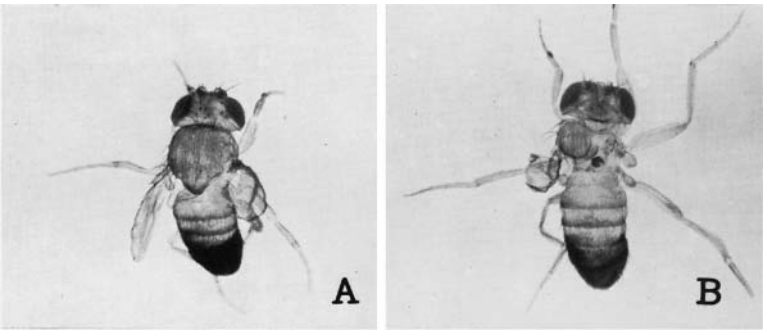

Figure 8. Photographs of male flies homozygous for the *B* mutant. In A, the posterior portion of the mesothorax, including the posterior half of the wing and the postnotum, or posterior portion of the dorsal mesothorax, is greatly reduced as in the metathorax. In B, anterior as well as posterior portions of the mesothorax on the right side have been reduced dorsally to a structure resembling the normal metathoracic, region. Most of the dorsal thoracic tissue which still remains in this individual on the right side is composed of the humerus or dorsal prothorax.

and Microcephalus (*Mc*) at 59.0. In addition, such females usually carried inversions in all of the chromosome arms other than 3R—this procedure resulting in about a threefold increase in crossing over in the *ss-Mc* region. The map distances shown in Fig. 9 were calculated on the assumption that the observed factor of increase in the *ss-Mc* region applied uniformly throughout the *bx* region; that is, observed frequencies

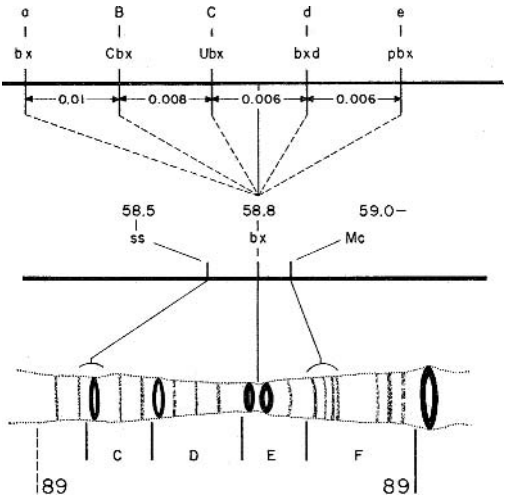

Figure 9. Correspondences between the linkage map and salivary gland chromosome map of the bithorax region of the third chromosome.

Table 1 Levels of development of body segments in wild type and certain bithorax single and double mutant homozygotes. Production in a body segment of postulated "bithorax" substances is indicated by presence of substance symbols, S1, S2, and S3, in parentheses; (—) indicates effectively no production of these substances. (ms = mesothoracic-like level; mt = metathoracic-like level; ab = first abdominal level of development; MS = mesothorax; MT = metathorax; AB_I = first abdominal segment; A = anterior; P = posterior.)

Genotype of homozygote a B C d e	AMS	PMS	AMT	PMT	AAB_I	PAB_I	Type of mutant transformation (T)
+ + + + +	ms (—)	ms (—)	mt (S1)	mt (S2)	ab (S1 + S3)	ab (S2 + S3)	None
a + + + +	ms (—)	ms (—)	ms (—)	mt (S2)	ab (S3)	ab (S2 + S3)	T1
+ B + + +	mt* (S1)	mt (S2)	mt (S1)	mt (S2)	ab (S1 + S3)	ab (S2 + S3)	T4
+ B + + e							
+ + C + +	ms (—)	ms (—)	ms (—)	ms (—)	ms (—)	ms (—)	T1, T2, T3
a + + d +							
+ + + d +	ms (—)	ms (—)	mt (S1)	ms (—)	mt (S1)	ms (—)	T2, T3
+ + + d e							
+ + + + e	ms (—)	ms (—)	mt (S1)	ms (—)	ab (S1 + S3)	ab (S3)	T2
a + + + e	ms (—)	ms (—)	ms (—)	ms (—)	ab (S3)	ab (S3)	T1, T2
+ B + d +	mt* (S1)	mt (S2)	mt (S1)	mt (S2)	mt (S1)	mt (S2)	T3, T4

*Variable and often asymmetrical expression

of recombination between the *bx* pseudoalleles were approximately three times the calculated map distances.

In some cases recombinational types were not directly recognizable phenotypically but had to be recovered by progeny tests of crossovers occurring between the outside marker genes. In other cases the crossover products could be directly detected phenotypically. In no case was there evidence for the occurrence of negative interference within the *bx* region. Moreover, the quintuple mutant cross provided the most powerful test, since in this case the occurrence of several kinds of multiple crossover types could have been detected but none was observed.

Functional Relationships

A variety of tests of functional relationships amongst the bithorax pseudoalleles have been carried out. The simplest involves making combinational studies of the various mutants. Some of the results are summarized in Table 1. In this way it has been possible to test the degree of independence of the various types of body segment transformations. Thus, it is possible to construct all possible combinations of weak, moderate, or strong T1 and T2 transformations. Of particular interest is the combination of a strong T1 with a strong T2 effect, since this results in a four-winged fly. The most complete transformation of this kind that has been achieved is shown in Fig. 5C

and involves the double mutant between an *a* mutant (bx^3) and the *e* mutant (see also Fig. 4). The expression of the T1 and T2 transformations in these flies is remarkably constant; the chief phenotypic variability consists in a failure in some individuals of the two halves of the MT to fuse properly down the midline; occasionally, in the struggle to emerge from the pupal stage, a wing, often of the MS, is torn off and remains behind in the pupal case. There is also a strong tendency for the second pair of wings to remain inflated with blood and the ability to fly is lost.

Independence of the T1 and T2 transformations is further shown by a study of the imaginal discs. Zalokar (1947) showed by means of experimental surgery that the pair of larval imaginal discs for the dorsal metathorax (DMT) gives rise to the halteres and other structures of the adult DMT: while the discs for the dorsal mesothorax (DMS) give rise to the wing and all of the other tissues of DMS. In wild-type larvae the DMT discs are appreciably smaller than DMS ones. In the *a* and *e* single mutant types the DMT discs are noticeably enlarged and begin to approach the DMS ones in size and structure: this is still more marked in the case of the *a e* double mutant type in which the DMT discs are virtually indistinguishable from the DMS ones. Thus, even though the anterior and posterior portions of the adult MT develop within single organs, these portions can be independently transformed towards the corresponding portions of the MS, depending upon the genotype with respect to the *a* and *e* mutants.

That the T3 transformation, or conversion of AB_I towards a thoracic-like state, is more or less independent of the T1 and T2 transformation is indicated from studies of certain double mutant combinations involving the *d* mutant. Thus, in the *a d* double mutant (which is viable as an adult when the weak and variable *a* allele, known as *bx*, is used) both anterior and posterior portions of AB_I are partially mesothoracic-like. On the other hand, in the *B d* double mutant, both portions are metathoracic-like.

The T4 transformation has been studied further by examining double mutants between *B* and mutants at the other pseudoallelic loci. The *a B* double mutant shows mutual partial suppression of each separate mutant phenotype. That is, the T1 and T4 effects are mutually weakened in this double mutant. The *B C* combination also shows such mutual partial suppression, except that the T4 effect is virtually eliminated while the T1 effect of *C* is only slightly weakened (in this case the effects of the *B C* mutant must be studied in combination with other mutants that permit a viable combination, since the *B C* homozygote is lethal). The *B d* double mutant type has the T2 effect of the *d* mutant completely suppressed, the T3 effect partially suppressed, and the T4 effect either unsuppressed or somewhat enhanced. Finally, the *B e* mutant has complete suppression of the T2 effect, even though it carries the extreme T2-producing *e* mutant, but little or no suppression of the T4 effect of the *B* mutant.

Gene Dosage Studies

All of the bithorax pseudoalleles, with the exception of the *B* mutant, act like hypomorphs. That is, they are more extreme in one dose than in two doses. This

can be shown by putting them opposite deficiencies. Although a joint deficiency for all the genes has not been obtained, separate deficiencies for the *a*, *B*, and *C* genes and for the *d* and *e* genes have been derived. Moreover, a common type of X-ray induced change occurs in which all of the genes of the series seem to be inactivated, although there is no visible deficiency. Extreme forms of the *a* and *e* phenotypes are most readily obtained by putting the *a* and *e* mutants opposite a deficiency for the *a*, *B*, and *C* genes, or opposite one of such X-ray-induced changes. In constructing the flies shown in Fig. 5 B–D, one of such X-ray-induced inactivations of the *bx* genes (Ubx^{105}) was used.

That the *a*, *C*, *d*, and *e* mutants are acting in the direction of producing less effect than the wild-type alleles is confirmed by the behavior of duplications containing the wild-type alleles of all of these genes (as well as of B^+). In the presence of two doses of any one of the recessive mutants, and one dose of the wild-type alleles, derived from such a duplication, the phenotype is wild type; this is true even for the triple mutant combination, *a d e* which, without the duplication, is lethal when homozygous.

In the case of the dominant *C* mutant, *C*/*C*/+ (see Fig. 13) is identical with *C*/+. Finally, *C*/+/+ is virtually wild type by contrast with *C*/+. That the *C* mutant acts in the direction of a loss of gene function is also shown by the behavior of the joint deficiency for the *a*, *B*, and *C* genes. This deficiency produces a heterozygous dominant effect exactly like that of the *C* mutant; namely, an enlarged posterior portion of the halter.

In the case of the dominant *B* mutant, on the other hand, two doses of the wild type alleles of all of the bithorax genes are unable to suppress its effect; that is the T4 transformation appears to be as marked in *B*/+/+ as it is in *B*/+. That is, *B* produces effects which are in the direction of a gain in, rather than a loss of, function. Indeed, it is as if the *B* mutant causes the wild type alleles of certain other genes in the series to begin acting in the MS, instead of acting only in MT or AB_1.

Rearrangement Effects at the Bithorax Region

When wild-type males are irradiated with X-rays, and new bithorax mutants are selected, the majority are associated with gross chromosomal rearrangements. Two categories in this rearrangement class can be readily recognized. The first consists of mutants which act as if all of the genes of the bithorax series are inactivated. In all of these cases, one of the breakage points appears to occur just to the left of two heavy doublet structures in section 89E of the salivary gland chromosomes (Fig. 9). The second category of X-ray-induced bithorax mutants associated with rearrangements acts as if only the wild type alleles of the *d* and *e* genes are inactivated. These have proved to be rearrangements in which one of the breakage points separates the 89E doublets. By the use of such rearrangements it has been possible to deduce that the *a, B*, and *C* loci lie in the first doublet structure of section 89E.

Table 2 Comparisons of *cis* and *trans* bithorax types with and without heterozygosity for certain chromosomal rearrangements (R) involving the right arm of the third chromosome. Legend: 0 = little or no; + = very slight; ++ = slight; +++ = moderate; ++++ = extreme transformations.

	Mutants in heterozygote	Types of heterozygotes	Types of body segment transformations			
			T1	T2	T3	T4
1	a–C	*cis* and R (*cis*)	+	0	0	0
		trans	+++	+	0	0
		R (*trans*)	++++	+	0	0
2	a–d	*cis* and R (*cis*)	0	0	0	0
		trans and R (*trans*)	0	0	0	0
3	a–e	*cis* and R (*cis*)	0	0	0	0
		trans	0	0	0	0
		R (*trans*)	0	+	0	0
4	C–d	*cis* and R (*cis*)	+	0	0	0
		trans and R (*trans*)	+	+++	+++	0
5	C–e	*cis* and R (*cis*)	+	0	0	0
		trans and R (*trans*)	+	++++	0	0
6	d–e	*cis* and R (*cis*)	0	0	0	0
		trans and R (*trans*)	0	++++	0	0
7	a–B	*cis* and R (*cis*)	0	0	0	++
		trans and R (*trans*)	0 to +	0	0	++++
8	B–C	*cis*	+	0	0	+
		R (*cis*)	+	0	0	0
		trans and R (*trans*)	++	+	0	+++
9	B–d	*cis* and R (*cis*)	0	0	0	++++
		trans and R (*trans*)	0	0	0	++++
10	B–e	*cis* and R (*cis*)	0	0	0	++++
		trans and R (*trans*)	0	0 to +	0	++++

Cis–Trans Tests

The powerful *cis–trans* test provides insight into the kinds of functional relationships which exist between the bithorax pseudoalleles. All possible pairs of double mutant heterozygotes, *cis* and *trans*, have been constructed and phenotypically compared (Lewis, 1955). Only a summary of the results is presented in Table 2.

An example of a *cis–trans* position effect involving a mild allele of *a* (bx^{34e} of Schultz) and the *C* mutant is shown in a comparison of the *cis* heterozygote, *a C*/+ + (Fig. 10C), with the *trans* one, *a* +/+ *C* (Fig. 10D). The *cis* type, which does not differ from the single *C* mutant heterozygote, *C*/+, uniformly had the distal segment of the halteres slightly enlarged compared to that of the wild-type fly (Fig. 10A), while the *trans* type invariably has small partially wing-like halteres. The *trans* type also usually has a few hairs in the region of the DMT, whereas in wild type this region is devoid of hairs and consists of little more than a line separating the MS from the first abdominal segments.

In some cases, such as comparisons involving *a* and *d*, or *a* and *e* mutants, no differences between *cis* and *trans* types are evident. This may be due to a lack of sensitivity of the phenotype to detection of slight differences—this will be especially true when the *trans* type is wild type—or to a real absence of a position effect. In the case of *a* and *e* mutants, if an extreme *a* allele (bx^3) is used and if the *cis* and *trans* types are reared at high temperature (28°C), the *trans* type shows a slight T2 transformation, consisting of a small patch of wing cells in the posterior region of the halter; while the *cis* type remains wild type. Another example in which the phenotype can be sensitized to the detection of *cis–trans* effects occurs in comparisons involving the *B* and *d* or *B* and *e* mutants. In these cases a recessive, sex-linked, partial suppressor of *B* can be used to bring out the effect. Thus, in the presence of this suppressor, the trans type between *B* and *d* shows slight T2 and T3 transformations while the *cis* type under the same conditions does not and is wild type in phenotype except for a slight T4 transformation. Similarly, in the presence of the suppressor, the *trans* type for *B* and *e* shows a slight T2 effect while the *cis* type does not. A third method of sensitizing the test is to use chromosomal rearrangements as described in the next section.

Transvection Effects

A unique type of position effect arises if certain *trans* (or, in one case, *cis*) heterozygotes for bithorax pseudoalleles are made heterozygous for any one of a class of chromosomal rearrangements involving the third chromosome (Lewis, 1954a). Cytological studies of the salivary gland chromosomes show that the rearrangements which evoke this position effect are those which tend to disrupt somatic pairing in the bithorax region of the third chromosome. The effect of such a rearrangement (R) is to intensify the mutant effect associated with the *trans* type. To illustrate, R(*a* +)/+ *C* or *a* +/*R*(+ *C*) individuals, which are designated "R(*trans*)" types, are identical in phenotype and have a more extreme T1 transformation than does the corresponding *trans* type lacking the rearrangement. This example of the position effect phenomenon has been designated the transvection effect (Lewis, 1954a). A specific example using one of the *a* mutants (bx^{34e}) and the *C* mutant is illustrated in Fig. 10. Thus, in the *trans* type (Fig. 10D) the DMT is represented by only a few hairs or bristles which arise near the outer edges of the "line" which dorsally separates MS from AB_I; in the R(*trans*) type (Fig. 10B) DMT is represented by a broad strip of MS-like tissue containing numerous hairs and bristles. The rearrangement in this case, *T*(2:3)*P*23, has one breakage point in the extreme proximal region of 3R some 500 "bands" to the left of the bithorax region, and the other breakage point near the tip of the right arm of the second chromosome. When heterozygous, this rearrangement shows failure of pairing (in some cells of the salivary glands) between the rearranged and nonrearranged right arms of the third chromosome.

A number of lines of evidence reviewed in detail elsewhere (Lewis, 1954a) show that the transvection effect is a position effect and not the consequence of a modifier

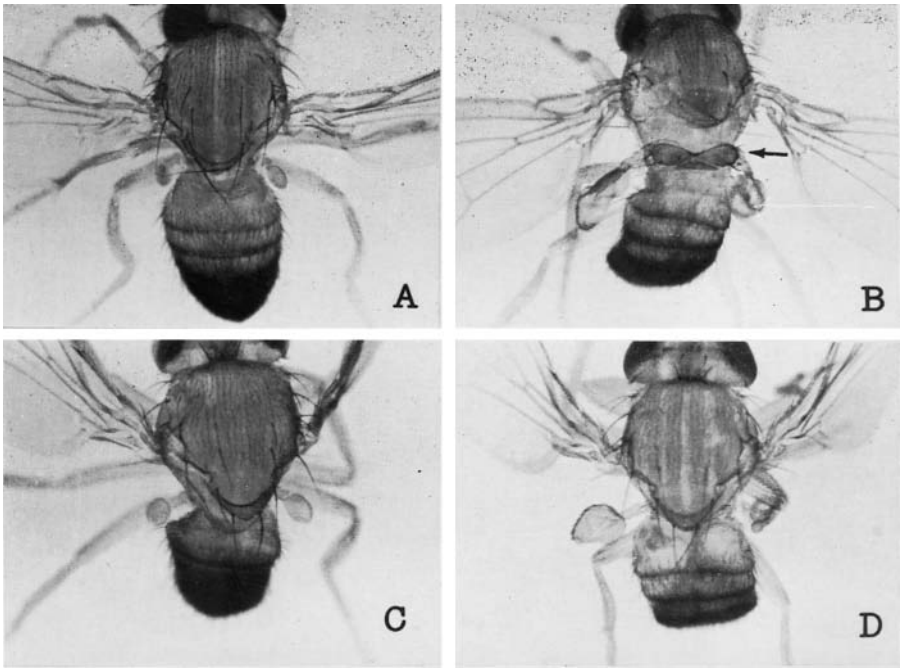

Figure 10. Photographs of male specimens illustrating A, wild type in relation to the following types of heterozygotes for an *a* (bx^{34e}) and the *C* (*Ubx*) mutant: B, R(*trans*); C, *cis*; and D, *trans*. Note in C the slightly enlarged halteres typical of single *C* mutant heterozygotes. By comparison, the still larger and partially wing-like halteres in D illustrates the *cis–trans* effect. The broad band of hairy tissue in the metathoracic region in B (see *arrow*) by comparison with its virtual absence in D illustrates the transvection effect.

gene associated with the rearrangement. Thus, the rearrangements which modify the phenotype of a given *trans* type do not modify the phenotype of the *cis* type nor that of either single mutant heterozygote or homozygote. The degree of intensification of the mutant effect is more or less in direct proportion to the degree of interference with somatic pairing. In cases in which rearrangements could be obtained in a homozygous viable state, or in which combinations of two rearrangements with different but nearly identical breakage points resulted in a return to nearly normal pairing relationships, the phenotype of the *trans* type is in general no longer modified. However, there are a few notable exceptions which show a slightly more extreme T1 effect than that in a *trans* type not carrying a rearrangement. Such exceptions have proved to be instances in which the bithorax-containing region of 3R is removed to remote distances from the centromere. The rearrangement described above, *T*(2:3)*P*23, is an example. Although in such cases there is structural homozygosity with respect to the rearrangement, somatic pairing is sometimes incomplete near the distal end of the long chromosome arm. It is presumed, therefore, that it is such occasional

failure of pairing that results in a slight intensification of the phenotype of the *trans* type.

In the case of the *a* and *e* mutants it is of interest that the *trans* type is wild type in the absence of a rearrangement, whereas the R(*trans*) type shows a slight T2 effect (Table 2). Since the *cis* and R(*cis*) types are found to remain wild type in this case, it is evident that heterozygosity for a rearrangement has served to reveal a *cis–trans* effect; i.e., R(*cis*) and R(*trans*) differ even though *cis* and *trans* do not. A similar degree of enhancement of expression of the *trans* type for the same *a* and *e* mutants has also been accomplished by high temperature as already noted.

A somewhat surprising transvection effect arises with the *B* and *C* mutants. In this case the *cis* and R(*cis*) differ slightly in regard to their effects on the T4 transformation, whereas the *trans* and R(*trans*) types are not obviously different in this or in other respects. Moreover, the T4 effect is less in the R(*cis*) than in the *cis* form. However, since inactivation of the bithorax region is not associated, as already noted, with a T4 effect, it is possible that the R(*cis*) type is producing a more extreme inactivation of the genes than occurs in the *cis* type.

In many cases no phenotypic difference between *trans* and R(*trans*) or *cis* and R(*cis*) types is evident. As already noted in the case of the *cis–trans* effect, this may be due to lack of sensitivity of the phenotype to the detection of slight differences; or it may be due to real differences between the genes in ability to have their action modified by structural heterozygosity.

Somatic Mosaics

In order to study more directly the nature of the bithorax mutant effects, mosaic individuals composed of mixtures of wild type and mutant tissue have been synthesized. The method adopted to produce such mosaics utilizes the important discovery of Brown and Hannah (1952) that a ring-X chromosome in *Drosophila* shows a relatively high rate of somatic elimination if introduced into aged cytoplasm. It was necessary, first of all, to construct a special ring-X chromosome into which the wild type alleles of the bithorax genes had been introduced. The technical details are as follows: an insertional translocation, *T(1;3)05*, of Oliver has a small segment of 3R containing the wild-type alleles of bithorax inserted into the X chromosome; by double crossing over this segment was transferred to a ring-X chromosome.

Matings were then carried out which resulted in the production of zygotes having the composition shown in Fig. 11. Such zygotes carry one free-X chromosome marked with the yellow body color mutant (*y*) and a ring-X chromosome bearing the wild-type alleles of all of the bithorax genes as well as of the yellow gene. The third chromosomes of such zygotes are constructed so as to have the particular bithorax mutant(s) desired (symbolized as bx^k in Fig. 11). Normally, such zygotes develop into females which have gray body color and a wild-type segmentation pattern. However, if the parental female has been aged, there is a tendency for the

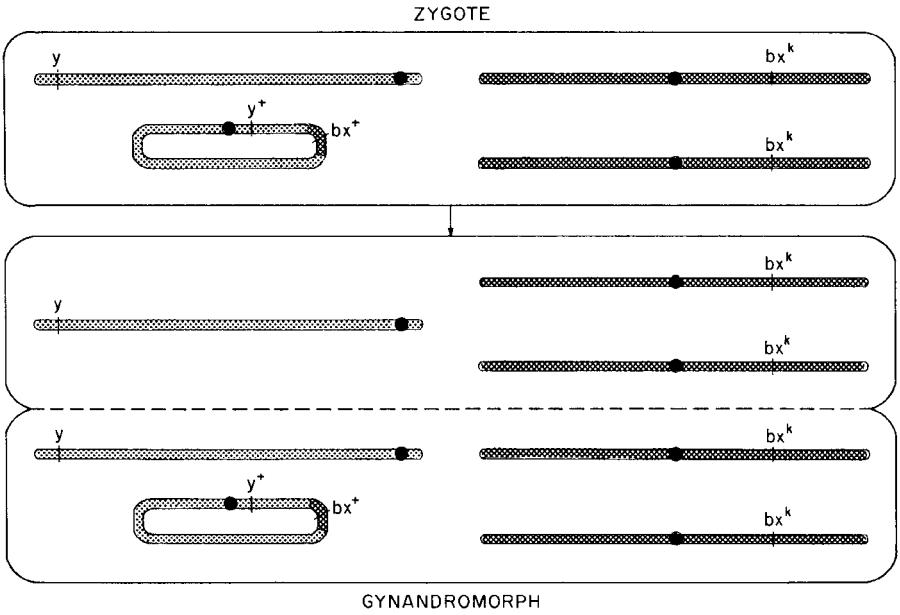

Figure 11. Diagram illustrating the origin, through loss of a special ring-X chromosome, of gynandromorphs mosaic for the yellow and bithorax mutants.

ring-X chromosome to be eliminated in early cleavage divisions and the zygote develops into a gynandromorph having the genetic constitutions shown in Fig. 11. Such gynandromorphs have been synthesized for a number of bithorax mutant genotypes.

Numerous gynandromorphs mosaic for the genotype which results in the extreme *a e* phenotype or four-winged condition (Fig. 5C) have been produced. Two examples of such mosaics showing large patches of *a e* tissue are illustrated in Fig. 12. In each case the entire DMT on one side is made MS-like and the extra mutant tissue that arises in this way is found to be yellow. In some specimens an occasional non-yellow bristle has been found. This may be due either to a slight induction of a bithorax effect in wild-type cells, or more likely, to a nonautonomous action of the yellow mutant such as Hannah (1953) has reported. The size of patches of bithorax tissues can vary from one hair or bristle to the large patches seen in Fig. 12.

Of particular interest are mosaics which contain homozygous *C* tissue, since the *C* mutant normally is lethal when homozygous. Such mosaics usually die before the adult stage is reached. However, several have proved viable and in this way it has been found that the *C* mutant expresses T1, T2, and T3 effects. Figure 13A shows such a mosaic in which the dorsal MT on one side is yellow and MS-like; also the dorsal part of AB_I on the same side is missing. This reduction in AB_I is typical of the T3

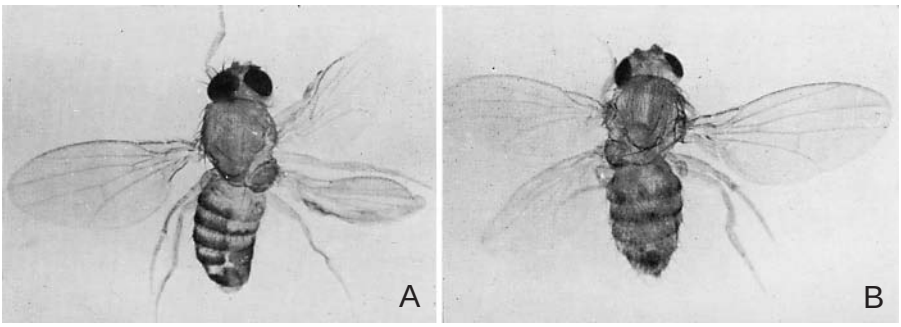

Figure 12. Photographs of "three-winged" gynandromorphs mosaic for the *a e* double mutant condition. In A, all of the dorsal right half of the body except the third abdominal segment is yellow and the dorsal metathorax on the same side can be seen to be mesothoracic-like. In B, only the left half of the thorax (and the entire head) are yellow; here again the metathorax on the yellow side can be seen to be mesothoracic-like.

transformation. Extra halteres or abdominal legs, however, have not been found in such mosaics. From these mosaic studies it has also been found that the *C* mutant when homozygous does not have effects on body segments other than MT and AB_I, with the following exception.

Of considerable interest is the finding in some gynandromorphs that homozygous mutant *C* tissue can result in a patch of bristles and hair in the region of the postnotum, a region of the posterior portion of MS immediately in front of MT. This patch of tissue can be seen in the specimen shown in Fig. 13B. It has not been seen in any combination of the *a*, *d*, or *e* mutants, and it thus may represent some special attribute of the *C* mutant. (One possibility that is being put to test is that it represents a development of an extra "humerus," i.e., prothoracic-like tissue.)

To summarize, the mosaic studies indicate a high degree of autonomy of the bithorax mutant effects, as far as the genes that have been studied are concerned, which include all the mutant types except for *B*. This independence of development of wild type and mutant tissue is consistent with the high degree of independence already described for the T1 and T2 transformation.

DISCUSSION

A model is required which will explain how the various bithorax mutant genes and combinations thereof are able to modify the pathway of development of certain body segments. Such a model must also explain how the corresponding wild-type alleles of these genes are able to determine the normal pathway of development of these segments. Since nothing is known about the biochemistry of gene action in the bithorax case, any model of the gene action can only be stated at this time in formal and abstract terms. Moreover, it is obvious that various models could be constructed.

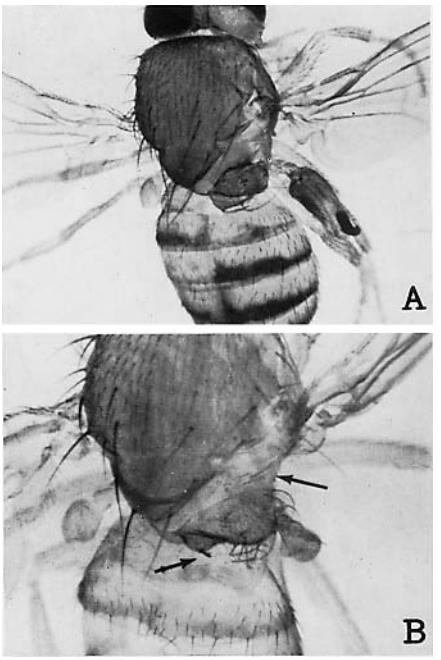

Figure 13. Photographs of gynandromorphs mosaic for homozygous mutant *C* tissue. In A, the anterior portion of the metathorax on the right side is yellow and mesothoracic-like. On the same side the first-abdominal segment is missing which is indicative of a partial thoracic-like transformation of that region. In B, the anterior portion of the metathorax proper is mesothoracic-like and yellow, while the halter remains largely metathoracic. The entire right half of the mesothorax in B is also yellow, except for a non-yellow scutellar bristle (*lower arrow*) and near its posterior margin on the area known as the postnotum there is a small patch of hairs and bristles (*upper arrow*), whose origin and homology is not known. The somewhat enlarged halter on the left (female) side of the specimens in A and B is typical of the duplication genotype, $+/C/C$, present in that side of the body.

What will be attempted here is to construct one along lines already presented in some detail before (Lewis, 1955), one of the main considerations being to eliminate insofar as possible assumptions of complex interactions of substances at a physiological level. First, certain assumptions are needed about the action of individual genes of the bithorax series. Then the problem of how such actions are regulated will be considered.

The Bithorax Substances

Five pseudoallelic genes have been recognized in the bithorax series by crossing over tests. However, only four distinct types of mutant-induced development effects have thus far been clearly identified, namely, the T1–T4 transformations. As already noted, the results of mutant interaction studies suggest that at least three of these, T1, T2, and T3, can vary more or less independently of one another. Moreover, the

results of gene dosage studies strongly suggest that each of these three mutant-induced transformations is the result of a loss of gene function.

From these considerations the simplest scheme for gene action is one that postulates that the wild-type alleles of three of the genes make three substances which in turn produce three developmental effects. Specifically, the wild-type alleles of the *C* (or *a*), *d* and *e* genes are assumed to control the production of "bithorax" substances which will be designated S1, S3, and S2, respectively. In turn, each of these three substances is assumed to determine certain levels of body segment development. Thus, S1 controls a change from an AMS-like level of development (L-AMS) to an AMT-like level (L-AMT). Similarly, S2 controls a change from L-PMS to L-PMT; and S3 a change from L-MS to L-AB_I (more exactly S3 controls L-AMS to L-AAB_I and L-PMS to L-PAB_I). These three transformations are conveniently symbolized IT1, IT2, and IT3, respectively, to indicate that they are the inverse of the T1, T2, and T3 transformations (except that T3 is defined as merely a thoracic-like modification of AB_I). These gene–substance–effect relationships are shown in Table 3 and Fig. 14. Conversely, absence or a reduction in amount of S1, S2, and S3 is assumed to lead to the T1, T2, and T3 transformations, respectively.

Given the above set of assumptions about the action of the bithorax genes, it is possible to interpret a given pattern of body segment development as the result of elaboration of different amounts of the three bithorax substances. This has been done for the patterns which arise in wild type and various mutant genotypes. The results are shown in Table 1. For simplicity of presentation, only presence or absence of a substance is indicated. Also the convention is followed in the case of substances S1 and S2 not to indicate their presence in regions where they are assumed to have no effect (i.e., in posterior and anterior portions, respectively, of a segment).

It should be noted that a completely consistent scheme of gene action involving the bithorax substances has not been devised. Some of the mutant effects that are not readily understandable without invoking the existence of additional substances have been discussed before (Lewis, 1955) and will not be considered here.

Table 3 Postulated substances and effects producd by wild-type bithorax pseudoalleles.

Bithorax pseudoallele	Corresponding substance	Effect of substance on levels of development (L)	Symbol of effect
C^+ (or a^+)	S1	L-AMS → L-AMT	IT1
d^+	S3	L-AMS → L-AAB_I L-PMS → L-PAB_I	IT3
c^+	S2	L-PMS → L-PMT	IT2

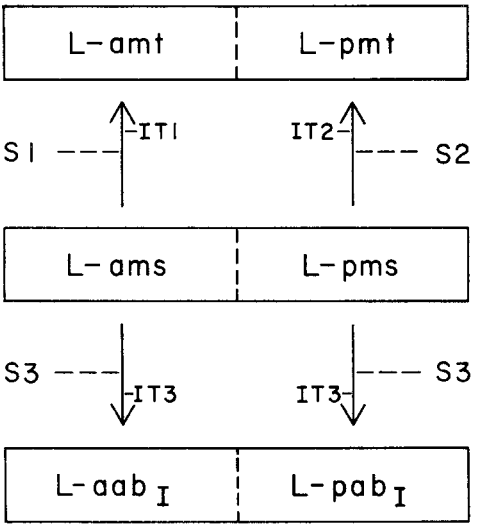

Figure 14. Scheme for the control of level of development of a body segment by the hypothetical *bx* substances (S). The segment, symbolized by a rectangle, is assumed to develop according to alternative pathways as follows: when the amount of S3 falls below a certain threshold, development of the segment proceeds toward the metathoracic level provided substances S1 and S2 are present; when the amount of S3 is above the threshold, development proceeds toward the abdominal level. If none of the substances is present, development remains at a mesothoracic level. (IT = inverse transformation; L = level of development: a = anterior, p = posterior; ms = mesothorax; mt = metathorax; ab_I = first abdominal segment.)

The Sequential Reaction Model

From the pattern of mutant effects (Table 1) and *cis–trans* effects (Table 2), it is evident that the *C* mutant acts as if it reduces the amount of S1, S2, and S3; while *d* reduces the amount of S2 and S3; and *e*, the amount of S2 only. (The *a* mutants act as if they reduce the amount of S1 and the most extreme allele acts as if it very slightly reduces the amount of S2, but not detectably the amount of S3.) To use the terminology of Jacob and Monod (1961) there is a "polarity" to the functioning of the bithorax genes. This polarity has been interpreted (Lewis, 1955) as evidence that the genes are controlling sequential enzymatic reactions according to the following scheme, which omits B^+ and arbitrarily puts a^+ before C^+:

$$X \xrightarrow{a^+} \xrightarrow{C^+} S1 \xrightarrow{d^+} S3 \xrightarrow{e^+} S2.$$

The *cis–trans* effects become readily understandable on such a model if it is assumed that the substances S1 and S3 do not diffuse readily, for one reason or another, from their site of production in one chromosome to the corresponding site in the homologous chromosome. That is, synthesis is assumed to proceed much more efficiently in the case of the *cis* arrangement of the wild-type alleles of these genes

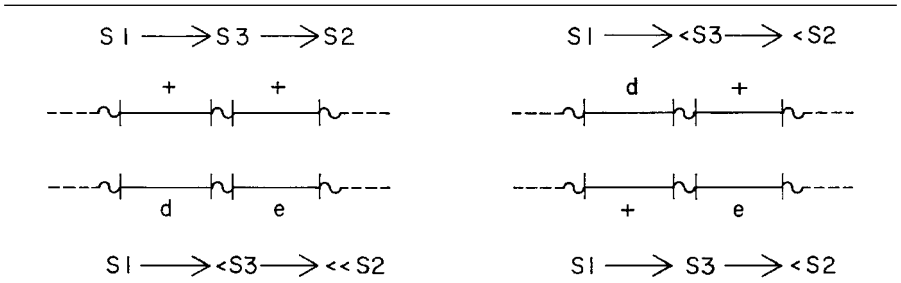

Figure 15. Sequential reaction model for interpreting the *cis–trans* effect in the case of the *d* and *e* mutants. The hypothetical bithorax substances (S) are assumed to be products of enzyme activity that are more efficiently transported along the chromosome than from one chromosome to its homolog. Greater production of substance S2 and therefore a more nearly wild-type phenotype is expected in the case on the left in which the wild-type alleles of the *d* and *e* genes are in the *cis* relationship.

than in the *trans* case (Fig. 15). Or, stated in another way, the reactions going on in one chromosome do not readily cross-feed those going on in the other.

The transvection effects are also readily understandable on the basis of a sequential reaction model. Thus, the degree of cross-feeding in the *trans* case would be expected to be still further reduced if somatic pairing of the homologous chromosomes is interfered with by the introduction of certain types of chromosomal rearrangements.

There is, however, at least one difficulty with the sequential reaction model. It assumes that protein synthesis and enzyme reactions go on close to the site of the genes in the chromosomes, whereas there is biochemical evidence that these processes go on in the cytoplasm. The resolution of this difficulty may be either that there are special cases, perhaps involving substances used only briefly in development, in which such synthesis does go on at the chromosomal level; or that the polarity is determined by a different mechanism entirely, such, for example, as that which Jacob and Monod (1961b) have proposed for the structural genes governing lactose utilization in *E. coli*.

The Operon Model

Formally, there is a close parallel between the polarized action of the *C*, *d*, and *e* genes of the bithorax system and that of the *z*, *y*, and hypothetical *x*, genes, respectively, in the lactose system. Thus, Jacob and Monod (1961b) find that certain mutants of the *z* structural gene not only reduce the level of activity of the enzyme controlled by that gene, but also reduce the levels of activity of the enzymes controlled by the *y* and *x* genes, these latter reductions occurring to a more or less parallel extent. In turn, certain mutants of the *y* gene behave similarly with respect to the *x* gene, but do not reduce the level of activity of the enzyme produced by the wild-type allele of the *z* gene.

Jacob and Monod (1961a,b) have proposed that this type of coordinated and parallel repression of gene function is the result of a mutation which alters the rate of transcription of messenger RNA by the structural gene, and a polarity in the

transcription process whereby the messenger RNA for each of the structural genes of an operon is "read" in only one direction.

Although the operon model readily accounts for the *cis–trans* effects, it requires modification if it is to explain the transvection effect. If this model is applicable to the bithorax case, then the transvection effect implies that homologous chromosomes cooperate with one another in the transcription process, perhaps through some type of copy-choice mechanism. However, further speculation along these lines does not seem profitable at the present time.

One test of the applicability of the operon model to the bithorax case would be the occurrence of a mutant of the "operator-constitutive" or o^c type. In the case of the lactose system such a mutant acts as a dominant when it adjoins the wild-type alleles of the structural genes, but as a recessive when next to mutant alleles of the structural genes. The o^c mutant is assumed to be one in which the operator region has lost its affinity for a repressor substance. As a result the operator region is no longer blocked by the repressor and the wild-type alleles of the adjoining structural genes function continuously or "constitutively." Such a mutant in the bithorax case would be expected to cause the production of the *bx* substances in the MS as well as in the MT and first abdominal segments and therefore to lead to a transformation of MS towards MT (or AB_I). To a considerable extent the *B* mutant fulfills all of these requirements for a mutation of the o^c type. It is dominant when next to the wild-type allele of the *C* gene and is virtually but not quite recessive when next to the *C* mutant. The dominance of *B* is also considerably weakened when it adjoins an extreme *a* mutant (but not when it adjoins a *d* or *e* mutant). Finally, the *B* phenotype is a transformation of PMS and occasionally AMS towards PMT and AMT, respectively.

Studies with the sex-linked partial suppressor of the *B* mutant suggest that *B* reduces the activity of the wild-type alleles of all of the other genes in the series. This would be compatible with the assumption that a mutation in the operator region has occurred, and that such a mutation reduces the rate of transcription of the structural genes of the operon. Unfortunately, it is not clear whether there is a parallel behavior of the o^c mutants in the case of the lactose system, since suppressors of such mutants have not been reported.

Although the operon model provides a rather attractive interpretation of the action of the *B* mutant, the sequential reaction model also provides a plausible interpretation as discussed elsewhere (Lewis, 1955); namely, the *B* mutant, by blocking a reaction near the beginning of the sequence, causes an accumulation of one of the precursors. In turn this accumulation is assumed to be responsible for the dominant effect of *B* in the mesothoracic region.

At the present time there is simply insufficient information on which to decide whether the sequential reaction or the operon model is a valid one for describing regulation of gene activity within the bithorax series. Indeed elements of both of these models may be involved, or it is possible that a different model entirely will be needed.

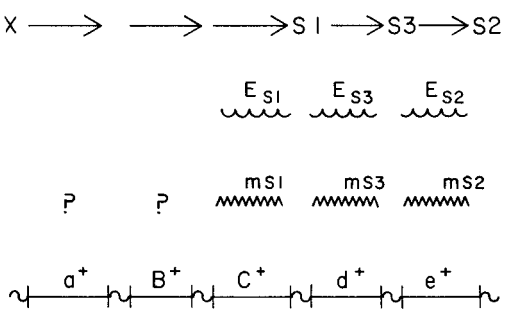

Figure 16. Diagram combining features of the sequential reaction and operon models as applied to the bithorax pseudoallelic series. The function of the wild-type alleles of the *a* and *B* genes is left undefined; as discussed in the text, B^+ may correspond to the operator region (see Fig. 1); or the a^+ and B^+ genes may be elaborating enzymes involved in steps which precede the production of the hypothetical bithorax substances (S1). It is assumed that the C^+, d^+, and e^+ genes, at least, produce specific messenger RNAs (mS1) which in turn specify the sequence of amino acids in enzymes (E_{S1}). On the sequential reaction (but not the operon) model it is also necessary to assume that enzyme synthesis occurs in the vicinity of the chromosome.

Regulation of Gene Action During Development

Another aspect of the problem of regulation of gene activity in the bithorax case is concerned with how, during development, the genes come to be turned on, so to speak, in certain segments of the body and turned off in other segments. Why, for example, in the wild-type individual do the bithorax genes appear to be inactive in MS but active in MT? Again an interpretation can be made using either the sequential reaction or operon models. On the former model a posterior–anterior gradient in the concentration of the initial substrate, X, (Fig. 16) can provide a basis for keeping the genes inactive in MS or active in MT and AB_I.

In the case of the operon model it is tempting to make an interpretation analogous to that offered by Jacob and Monod (1961b) for adaptive enzyme formation. Thus, as already noted above, the presence of a repressor substance that combines with the operator would keep the genes turned off in such regions as MS. This would correspond to the uninduced state of adaptive enzyme formation. In MT and AB_I, on the other hand, it would be assumed that the concentration of an inducer substance is sufficient to combine with and remove the repressor, thus allowing transcription to proceed. This would correspond to the induced state. A posterior–anterior gradient in the concentration of the inducer is thus what is required on the operon model to interpret the normal pathway of development of MS, MT, and AB_I.

To explain the difference between MT and AB_I in normal development, the assumption would be made that the gradient in concentration of X or of the inducer would be such that a sufficient quantity of substance S3 would not be made until the abdominal region is reached. Once having been made, it is necessary to suppose that

S3 takes precedence over S1 and S2 in causing development to proceed towards an abdominal rather than a metathoracic level (Fig. 14).

These interpretations of the way in which the bithorax genes control the normal pathway of development of certain body segments pose a fundamental question. What mechanism could determine the postulated gradient in concentration of substance X (or of the inducer)? Although a number of mechanisms are possible, the following one is believed to be more plausible than many.

Initially, the concentration of X or of the inducer is assumed to be uniform throughout the egg, and the substance itself is assumed not to diffuse effectively between cells. If now cell division occurs at a faster rate in the anterior region of the egg than in the posterior region, there would automatically be a relatively higher cellular concentration of the substance posteriorly than anteriorly. Moreover, the nondiffusible nature of the substance would make the concentration gradient relatively stable in time and space. In this way the remarkable stability of normal development under a variety of environmental conditions can be accounted for.

It is interesting in this connection that a rather drastic treatment (heavy etherization) of wild-type eggs produces phenotypic effects comparable to the *a* type of mutant effect, as shown by Gloor (1947).

Evolution of the Bithorax Pseudoallelic Series

The evolutionary significance of pseudoallelism has been discussed in some detail elsewhere (Lewis, 1951). It seems likely that pseudoalleles represent instances in which genes have become duplicated in tandem order in the chromosome and then diverged from one another in function. Recent evidence indicating close structural similarity between the different peptides in human hemoglobins has led Ingram (1961) to postulate that the various hemoglobin genes have evolved by repeated gene duplication. Moreover, it is possible that several of these genes may still lie in tandem repetition, thus forming a pseudoallelic series; while others have clearly become separated, presumably as the result of translocation or the occurrence of polysomy.

A rather unique feature of the bithorax pseudoallelic series is that it affects developmental processes whose evolutionary history is rather clearly defined. Since it is virtually certain that the flies evolved from four-winged ancestors, it is likely that the *a*, *C*, and *e* genes have evolved since the insects appeared. The *d* gene, on the other hand, would presumably have been the most ancient one of the series, since the insects certainly came from ancestors with more than six legs. That is, the original *d* gene was presumably concerned with suppressing abdominal leg development. It is of interest that a mutant comparable to the *d* mutant occurs also in Bombyx where it produces extra abdominal legs and small wing-like appendages in the adult moth (Itikawa, 1955); here also there is a complex pseudoallelic series, the mutants of which produce a variety of transformations of body segmentation patterns in the larval stage, as Tsujita (1955), Itikawa (1952), and others have shown. The above speculations about the evolution of the bithorax series of genes are incorporated in Fig. 17.

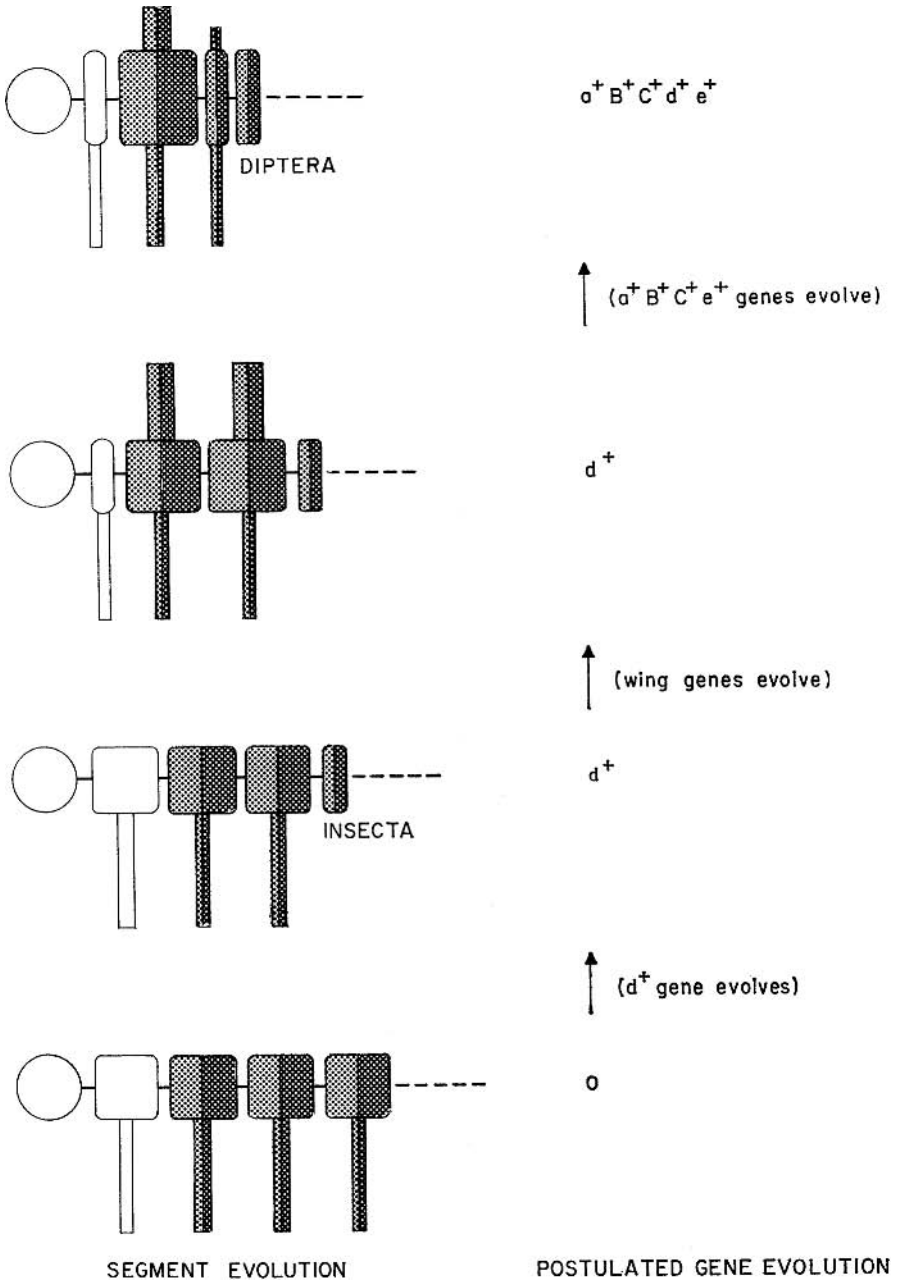

Figure 17. Scheme for the evolution of the bithorax pseudoallelic series. Initially, the d^+ gene (and possibly B^+, if it should represent the "operator") is presumed to have been involved in developing the segmentation pattern characteristic of the insects. After the insects developed wings, the remaining genes in the series are presumed to have arisen by repeated tandem duplication of the d^+ gene accompanied by divergence in function (through mutation) of the new genes thus formed.

SUMMARY AND CONCLUSIONS

A cytogenetic study of the bithorax mutants of *Drosophila melanogaster* suggests that the level of development attained by certain body segments in this organism is controlled by a cluster of five closely linked genes or pseudoalleles. At least three of the five genes appear to have specific functions that can be accounted for in terms of three hypothetical "bithorax" substances. At the same time the functioning of all of the genes of the cluster is closely coordinated, as evidenced by the occurrence of several types of position effects; namely, *cis–trans* effects, transvection effects, and changes resulting from physical separation of some of the genes of the series from others by chromosomal rearrangement. These position effects can be interpreted in a reasonably consistent way on the basis of either a sequential reaction model involving enzymatic reactions occurring in proximity to the chromosome, or a model which uses the "operon" concept of Jacob et al. (1960). It is possible that elements of both models may apply. The way in which the action of the bithorax genes is regulated during development can be formally understood if it is assumed that a posterior–anterior gradient exists in the concentration of a relatively nondiffusible substance which serves as the initial substrate (sequential reaction model) or as the inducer (operon model). Such a gradient would arise if there exists in turn an anterior–posterior gradient in mitotic division rates in the developing embryo.

The possible evolutionary significance of the bithorax pseudoallelic series is briefly discussed.

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A GENE COMPLEX CONTROLLING SEGMENTATION IN *DROSOPHILA*

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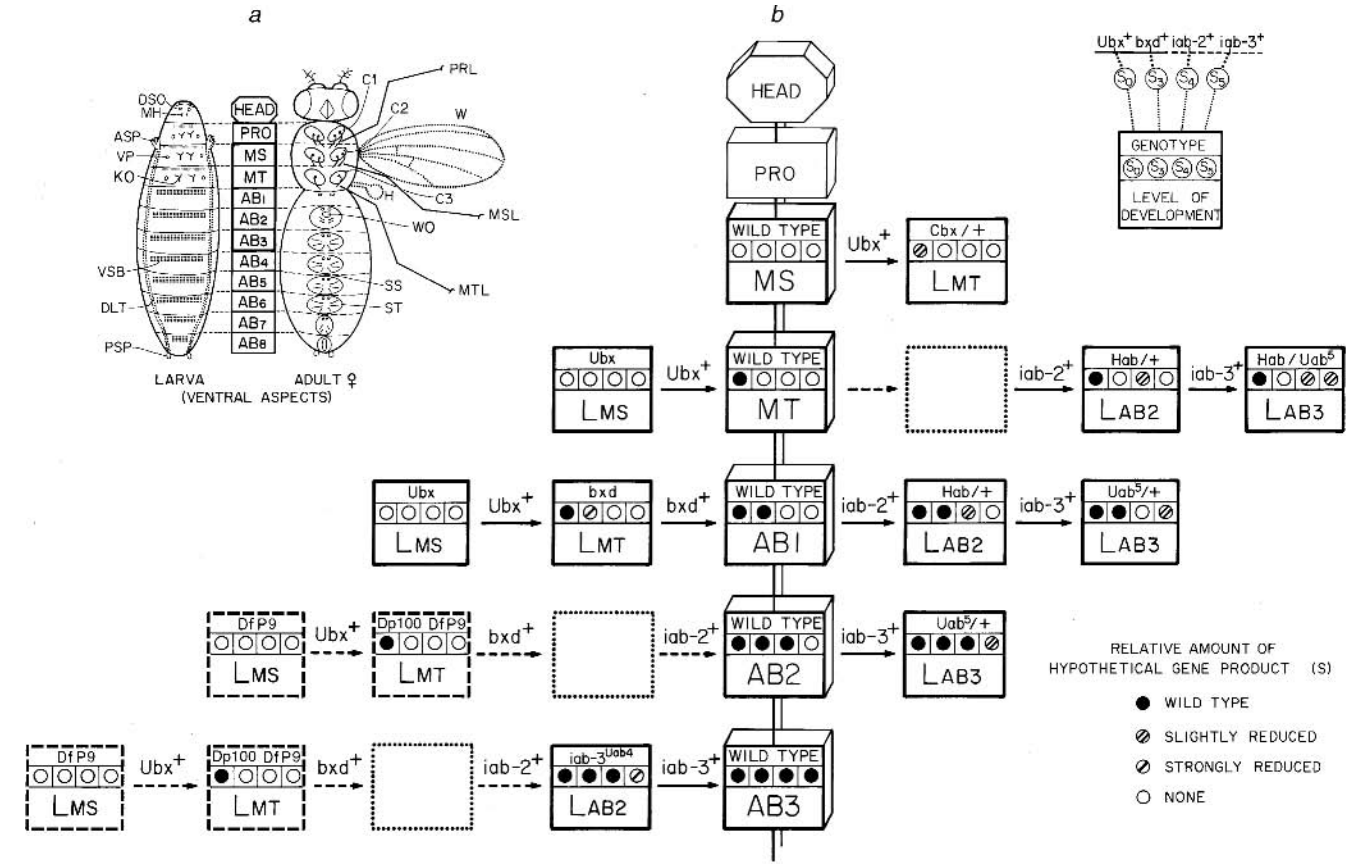
The bithorax gene complex in Drosophila *contains a minimum of eight genes that seem to code for substances controlling levels of thoracic and abdominal development. The state of repression of at least four of these genes is controlled by* cis*-regulatory elements and a separate locus (Polycomb) seems to code for a repressor of the complex. The wild-type and mutant segmentation patterns are consistent with an antero–posterior gradient in repressor concentration along the embryo and a proximo-distal gradient along the chromosome in the affinities for repressor of each gene's* cis*-regulatory element.*

The segmentation pattern of the fly provides a model system for studying how genes control development. The phylogeny, although not the ontogeny, of that pattern is reasonably well understood. Flies almost certainly evolved from insects with four wings instead of two and insects are believed to have come from arthropod forms with many legs instead of six. During the evolution of the fly, two major groups of genes must have evolved: "leg-suppressing" genes which removed legs from abdominal segments of millipede-like ancestors followed by "haltere-promoting" genes which suppressed the second pair of wings of four-winged ancestors. If evolution indeed proceeded in this way, then mutations in the latter group of genes should produce four-winged flies and mutations in the former group, flies with extra legs. In *Drosophila*, not only have both types of mutation been observed, they have been shown to involve a single cluster of pseudoallelic genes known as the bithorax complex (BX-C).[1–7] During evolution a tandem array of redundant genes presumably diversified by mutation to produce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GENOTYPE
LEVEL OF DEVELOPMENT
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LMS
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iab-2+
Hab/+
LAB2
iab-3+
Hab/Uab5
LAB3
Ubx
LMS
Ubx+
bxd
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WILD TYPE
AB1
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LAB2
iab-3+
Uab5/+
LAB3
DfP9
LMS
Ubx+
Dp100 DfP9
LMT
bxd+
iab-2+
WILD TYPE
AB2
iab-3+
Uab5/+
LAB3
DfP9
LMS
Ubx+
Dp100 DfP9
LMT
bxd+
iab-2+
iab-3Uab4
LAB2
iab-3+
WILD TYPE
AB3
RELATIVE AMOUNT OF
HYPOTHETICAL GENE PRODUCT (S)
WILD TYPE
SLIGHTLY REDUCED
STRONGLY REDUCED
NONE

this complex.[1,4] During development the BX-C genes seem, on the basis of evidence to be summarised in this article, to control much of the diversification of the organism's thoracic and abdominal segments. It is as if during ontogeny the BX-C genes recapitulate their own phylogeny.

Each of the wild-type thoracic and abdominal segments has a unique pattern of differentiated structures (Fig. 1a) which constitutes a morphologically defined state or "level of development" (L). Each BX-C mutant phenotype can be described in terms of the degree to which one or more segments, or portions thereof, are transformed from one such level to another. As shown in Fig. 1b a striking feature is that the metathoracic (MT) and first three abdominal segments (AB1, AB2, and AB3) can approach or achieve almost all segmental levels of development from a mesothoracic level (LMS), or "primitive" level,[1] to a third abdominal level (LAB3), depending upon the genotype. The wild-type allele of each BX-C gene (Fig. 2) will be assumed to code for a BX-C substance (S) which controls one or more components of an intersegmental transformation (Table 1). The various BX-C substances are presumed to act indirectly by repressing or activating other sets of genes which then directly determine the specific structures and functions that characterise a given segment. On such a model the level of development which a segment achieves depends upon the particular array of BX-C substances elaborated in that segment. When the wild-type and mutant phenotypes are interpreted in accordance with this model, it can be seen from Fig. 1b that the attainment of any level more advanced than LMS is a stepwise process in which each step requires the presence of a specific BX-C substance. In keeping with the model, all of the genotypes involving recessive loss of function lie to the left or LMS side of the wild-type segments while those involving dominant gain of function lie to the right or LAB3 side of those segments.

←

Figure 1. Genetic control of segmental levels of development in thoracic and abdominal segments. a, Anatomy of third instar larva. Head: dorsal sense organs (DSO) and protruding portion of mandibular hooks (MH). Thoracic segments: each bears a pair of Keilin's organs[13] (KO), three sensory hairs in a depression; ventral pits (VP) and ventral setal bands (VSB) with fine tooth-like setae. Abdominal segments: VSB with coarse tooth-like setae; KO and VP lacking. Each dorsal longitudinal trunk (DLT) terminates in an anterior (ASP) and posterior (PSP) spiracle. Anatomy of adult female. Thoracic segments: coxae (C1, C2 and C3) of prothoracic (PRL), mesothoracic (MSL) and metathoracic (MTL) legs; wing (W) and haltere (H), being dorsal, are shown in dotted outline. Abdominal segments: AB1–AB7 bear sternital sensillae (SS); AB2–AB7 bear bristled sternites (ST); AB2 has a raised lump, or Wheeler's organ[10] (WO). b. Levels of development (L), achieved in wild-type segments (vertical array of connected boxes) and approached in mutant segments, are depicted in panels containing genotype, phenotype and status with respect to relative amounts of hypothetical BX-C substances, S_0, S_3, S_4 and S_5, products of the wild-type alleles of Ultrabithorax (*Ubx*), bithoraxoid (*bxd*), infraabdominal-2 (*iab-2*) and infraabdominal-3 (*iab-3*). Panels to the left of wild type depict hemizygous genotypes for hypomorphic or amorphic mutants, the designated chromosome being opposite *Df-P9* (see Fig. 2) in all cases. For simplicity, a slight transformation of the posterior region of AB1 towards LMS in the *bxd* hemizygote is ignored. Panels to the right of wild type involve heterozygotes for hypermorphic (dominant-constitutive) mutants, Contrabithorax (*Cbx*), Hyperabdominal (*Hab*) and Ultraabdominal-5 (Uab^5). Panels in dotted outline represent theoretically possible LAB1 transformations for which definitive mutant genotypes are not available. Panels in dashed outline are genotypes dying in the first instar. All other genotypes shown survive in the adult stage except *Ubx* hemizygotes which die in either third instar or early pupal stages.

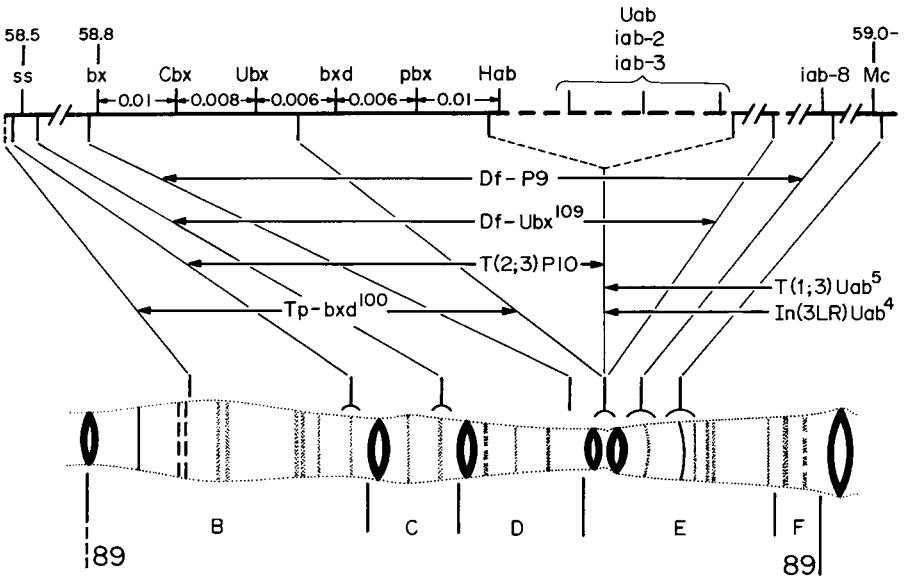


Figure 2. Correlation of the linkage and salivary gland chromosome maps of BX-C and immediately surrounding regions in the right arm of the third chromosome. The solid line portion of the linkage map is based on half-tetrad,[6] as well as on conventional, linkage analysis. The dashed line portion is based on unpublished cytogenetic studies of the designated rearrangements, the relative order of *Hab*, *Uab*, *iab-2* and *iab-3* with respect to each other being uncertain. The complex spans approximately 0.05–0.1 centimorgan and lies within the region of two heavy doublet structures and possibly an adjoining faint band of section 89E. Not shown is a spontaneous dominant mutant, Miscadestral pigmentation (*Mcp*), recently found by Crosby, which maps between *Hab* and Microcephalus (*Mc*).[15] *Df-P9* in its interactions with BX-C mutants and rearrangements acts as if it is deleted for all of the BX-C genes. *Df-P9*/+ has reduced male pigmentation in AB5 and AB6, is sterile, and has deformed genitalia in both sexes, which suggests insufficient gene dosage in the distalmost region of BX-C. Since *Df-Ubx*^{109}/+ lacks these effects and is fertile in both sexes, it is likely that such genes are not deleted in *Df-Ubx*^{109} which on the basis of other interactions and of larval studies (Fig. 6d) behaves as a deficiency extending from *bx* to *iab-3*, associated with a weakened dominance of genes to the right of *iab-3*. *Mc* is apparently a very small tandem duplication which partially restores fertility when *trans* to *Df-P9* but does not otherwise modify the *Df-P9*/+ phenotype. *T(2;3)P10* is an insertional translocation of the designated region of 89 to section 29C of chromosome 2; the breakage point in 89E is likely on genetic grounds to lie between *pbx* and *iab-2* and to be accompanied by polar position effects that weaken the dominance of *iab-2*$^+$ and *iab-3*$^+$. *Tp(3)bxd*^{100} is a transposition of the designated 89 region to 66C of chromosome 3L; the breakage point lies between *Ubx* and *bxd* and is accompanied by a weakened dominance of *bxd*$^+$ and *pbx*$^+$ (ref. 1) but not, as far as can be detected, of genes to the right of *pbx*. *T(1;3)Uab*^{5} is a reciprocal translocation with a breakage point in section 1E of the X chromosome; a synthetic deletion analysis indicates that the *Uab*^{5} phenotype is associated with the distal portion of the translocation; i.e., the breakage point in 89E lies to the left of *Uab*^{5} and falls either to the left or right of *iab-2*$^+$. *In(3LR)Uab*^{4} is a complex inversion with additional breaks in 80C and in or near 85A or 3R. Analysis of X-ray induced revertants of *Uab*^{4} indicates that the breakage point in 89E probably lies just to the left or right of *iab-2* and is associated with a weakened dominance of *iab-3*$^+$ (designated in Fig. 1 *iab-3*^{Uab4}) and possibly of *iab-2*$^+$, as well. *Hab* was originally designated as the F locus of the complex[7]; and has also appeared in the literature as Contrabithoraxoid (*Cbxd*). The first two *Uab* mutants found, *Uab* and *Uab*^{2}, although cytologically apparently normal, are associated with a reduction in crossing over within the complex and are accompanied by position effects or point mutations at other loci within BX-C. Their dominant phenotypes resemble that of *Uab*^{4} much more closely than that of *Uab*^{5}. Cellular determination of *Uab* has been extensively studied by Kiger and Davis.[20,21]

Table 1 Summary of the roles of BX-C substances (S) in controlling specific types of body segment transformations and specific structures in one or more segments of the larva.

Gene	Substance	Segmental transformation	Larval structures affected	Genotypic comparisons (from Fig. 6)
bx^+	S_1^a	LAMS → LAMT		
pbx^+	S_2	LPMS → LPMT		
Ubx^+	S_0^*	LMS → LMT	DLT	*a* vs *b*
				f vs *h*
bxd^+	S_3	LMS → LAB1	DLT[b], VP, KO, VSB	*g* vs *h*
$iab\text{-}2^+$	S_4	LMS → LAB2	DLT, KO, VSB	*b* vs *c*
				c vs *h*
$iab\text{-}3^+$	S_5	LAB2 or LMS → LAB3		*e* vs *f*
$iab\text{-}5^+$	S_7	LAB4 or LMS → LAB5		
$iab\text{-}8^+$	S_x	LAB7 or LMS → LAB8	DLT, PSP, CP, VP[c], KO[c]	*a* vs *d*

[a] Substance, S_1, was originally postulated to be coded for by either bx^+ or Ubx^+ (ref. 4); in this article S_1 is assigned to bx^+ and S_0 to Ubx^+

[b] Comparison of genotype e and *f* indicates that bxd^+ and/or $iab\text{-}2^+$ may be responsible for the continuity of dorsal tracheal trunk in AB1

[c] Suppression of VP and KO may result from the presence of $iab\text{-}8^+$ and/or one or more *iab* genes located between *iab-3* and *iab-8*

Segment MT transforms towards LMS even in larval stages of homozygotes[1] and hemizygotes for Ultrabithorax (*Ubx*). The most prominent feature is the occurrence of an extra set of spiracles on MT (and also AB1) (Fig. 3a) compared to the usual single set on MS. Although such animals die in late larval or early pupal stages, adult *Ubx/Ubx* cuticular tissue in MT has been shown by somatic mosaic analysis to transform autonomously towards LMS.[4,8] A substance, S_0, effecting LMS → LMT, will be assumed to be the Ubx^+ product. The inability of MT to achieve LMT in *Ubx* hemizygotes or homozygotes is then consistent with the expected reduction in amount of S_0 in that segment. The adult MT cuticle, but not the larval tracheal system, strongly transforms toward LMS in a double mutant combination involving bithorax-3 (bx^3) and postbithorax (*pbx*), notably producing a four-winged fly.[5,8] Substances S_1 and S_2 are assumed to be the products of bx^+ and pbx^+, respectively (Table 1), and to be involved in effecting LMS → LMT; however, for the sake of simplicity, they are omitted from Fig. 1b. The wild-type phenotype, on these assumptions, corresponds to that expected if bx^+, Ubx^+, and pbx^+ are repressed in MS and derepressed in MT.

Segment MS takes on MT characteristics in flies heterozygous or homozygous for Contrabithorax (*Cbx*), the most conspicuous effect being a partial conversion of wings into halteres.[5,9] *Cbx* is known to regulate Ubx^+ in a *cis*-dominant fashion; i.e., MS transforms towards LMT in *Cbx* +/+ *Ubx* but is virtually wild type in *Cbx Ubx*/ + +.[2,5] *Cbx* presumably damages a regulatory element adjacent to Ubx^+ causing S_0 to be made constitutively in MS but in a relatively reduced amount compared with that made in MT when Ubx^+ is in the wild-type configuration.

Segment AB1 transforms primarily towards LMT in hemizygotes for bithoraxoid (*bxd*), producing flies with one or two MT-like legs on AB1, and on rare occasions a tiny haltere-wing as well.[5] This phenotype has been accounted for by postulating that bxd^+ codes for a substance, S_3, effecting LMS → LAB1.[4] Evidence to be

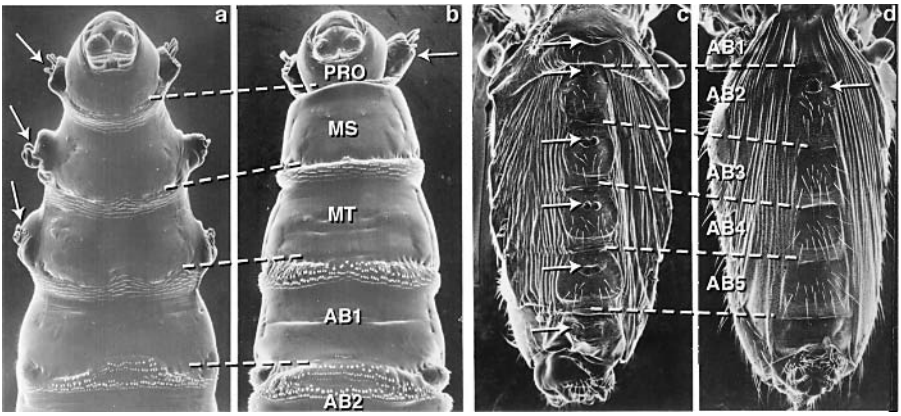


Figure 3. Mutant phenotypes resulting from recessive loss of function compared with wild type. See Fig. 1(a) for legend. a, Late third instar larva hemizygous for *Ubx* (*Ubx/Df-P9*). Note extra pairs of anterior spiracles (ASP) (arrows) on MT and AB1, protruding into MS and MT, and thoracic type of ventral setal bands (VSB) on AB1 compared with abdominal type on AB2. b, Corresponding stage of homozygous wild-type (Canton-S) larva. Note single pair of ASP (*arrow*) which arises in MS but protrudes into the region of the prothorax (PRO). Note setae of VSB are coarse in AB1 and AB2, fine in MS, and intermediate in MT. (Specimens a and b were fixed in hot water and photographed with SEM, X40.) c, Ventral aspect of male abdomen of genotype *In(3LR)Uab*^{4}*/T(2;3)P10* (see Fig. 2). Note presence of WO (*arrows*) and sternital bristle pattern of AB2 type on segments AB1 through AB5. Gonads are rudimentary in specimens of this genotype or lacking entirely in *Uab*^{4}*/Df-P9*. d, Corresponding region of adult wild-type (Canton-S) male. Note that AB1 lacks a sternite and that only AB2 (*arrow*) has WO. (Specimens c and d were unfixed and photographed with SEM, ×50.)

represented later suggests that *Ubx*$^+$ is derepressed not only in MT, but also in AB1 and segments beyond. Hence, in the *bxd* hemizygole, there is presumably enough S_0 in AB1 to transform that segment towards LMT. That AB1 remains at LMS in the *Ubx* hemizygote (Fig. 3a) is expected since *Ubx* is known to exert a strong polar effect on *bxd*$^{+1}$ (ref. 1); that is, neither S_0 nor S_3 is expected to be made in sufficient amounts to transform AB1 appreciably towards LMT or LAB1. In wild type, S_0 as well as S_3 is presumably being produced in AB1; although S_3 function may override that of S_0, a more attractive possibility is that LAB1 actually corresponds to a mosaic of structures and functions controlled jointly by S_0 and S_3.

Segments MT and AB1 approach LAB2 in heterozygotes for a second type of dominant regulatory mutant, Hyperabdominal (*Hab*), producing flies with missing halteres and/or MT legs and with an AB2-type of sternite pattern[10,11] on MT and AB1 (Fig. 4b). These effects are variably expressed within a fly even though the penetrance of *Hab*/+ exceeds 99% in certain backgrounds.

Cis–trans studies involving all possible double mutant combinations between *Hab* and *bx*^{3}, *Ubx, bxd*, and *pbx* indicate that *Hab* does not derepress the wild-type alleles of any of these genes. *Hab* will be assumed to cause partial derepression of a wild-type infraabdominal-2 (*iab-2*$^+$) gene, whose coding product, S_4, effects LMS → LAB2. On

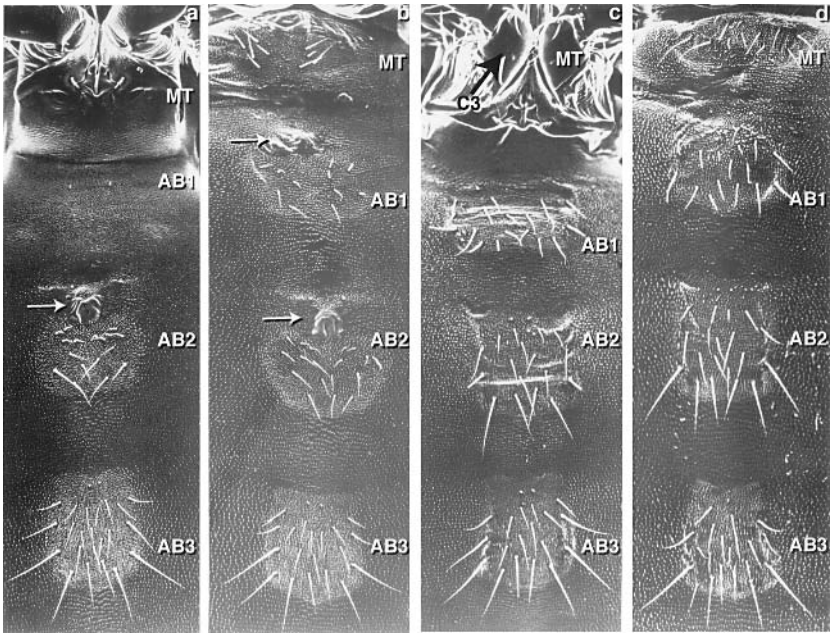


Figure 4. Mutant phenotypes resulting from dominant gain of function compared with wild type. Midventral cuticular regions of adults female with MT, AB1, AB2, and AB3 approximately parallel to one another. (All specimens unfixed and photographed with SEM.) a, Wild type. A portion of coxa-3 (C3) is visible on MT; AB1 lacks a sternite; AB2 has WO (arrow) and a reduced (compared to AB3) sternital bristle pattern, ×80. b, *Hab*/+. The specimen had four legs and no halteres. WO (*arrows*) and bristle pattern on MT and AB1 approach those of AB2, ×80. c, Uab^5/+. C3 resembles that of wild type. AB1, and AB2 sternites lack WO and approach in bristle pattern that of AB3. ×90. d, *Hab*/Uab^5. The specimen had four legs and no halteres. Sternites on MT, AB1, and AB2 have little or no trace of WO and approach in their bristle patterns that of AB3, ×80.

this assumption, the wild-type phenotype is that expected if *iab-2*$^+$ is repressed in MT and AB1 and derepressed in AB2. Similarly, the *Hab*/+ phenotype is that expected if *iab-2*$^+$ is partially derepressed in MT and AB1 and fully derepressed in AB2.

The failure of MS to be modified in *Hab*/+ requires explanation. The *Hab* mutation presumably damages a regulatory element adjacent to *iab-2*$^+$ in such a way as to reduce its affinity for a repressor. If there is an antero–posterior concentration gradient in that repressor, then conceivably the threshold concentration for keeping *iab-2*$^+$ repressed in the *Hab* chromosome is exceeded in MS but not in MT and segments beyond MT.

Segments AB1 and AB2 partially transform toward LAB3 in adults heterozygous for Ultraabdominal-5, Uab^5, a third type of dominant constitutive mutant, inseparable from an X-3 translocation (Fig. 2). The transformation is most easily seen in the ventral regions of AB1 and AB2 which develop sternite patterns approaching those

found in AB3 (Fig. 4c). The effect on AB2 can be accounted for if *Uab*^{5} derepresses a gene, *iab-3*$^+$, which codes for a substance, S_5, effecting LAB2 → LAB3. The wild-type pattern then corresponds to that expected if *iab-3*$^+$ is repressed in AB1 and AB2, but derepressed in AB3. The penetrance of *Uab*^{5}/+ is complete but the transformation of AB1 towards LAB3 is variable with that segment often progressing only towards LAB2. If, as certain larval findings discussed below suggest, *iab-2*$^+$ is normally weakly derepressed in AB1, then the presence of S_4 in AB1 even in subthreshold amounts may facilitate the transformation to LAB3 in *Uab*^{5}/+. Another possibility is that the *Uab*^{5} rearrangement in some way partially derepresses *iab-2*$^+$ as well as *iab-3*$^+$; however, although MT transforms to LAB2 in *Hab*/+, MT is not modified in *Uab*^{5}/+.

Segment MT is able to approach LAB3, along with AB1 and AB2, in *Hab/Uab*^{5} animals (Fig. 4d). Transformation to LAB3 in MT thus seems to require two sequential steps: LMS → LAB2 → LAB3, the former controlled by S_4 and the latter by S_5. The observed transformation of all three segments towards LAB3 is readily accounted for, since both S_4 and S_5 are expected to be produced in MT, AB1, and AB2 of *Hab/Uab*^{5}.

Additional evidence for an *iab-3*$^+$ gene comes from an analysis of another rearrangement-associated *Uab* mutant, *Uab*^{4}. When heterozygous, *Uab*^{4} shows a dominant gain of function in which AB1 partially transforms towards LAB2. When hemizygous, or opposite *T(2;3)P10* (Fig. 2), *Uab*^{4} exhibits a new type of recessive loss of function; namely, the sternites of not only AB1 but AB3 to AB6, inclusive, transform towards LAB2 (Fig. 3c). These latter effects are attributed to a reduction in the amount of S_5 which would be expected if the *Uab*^{4} and *T(2;3)P10* rearrangements are accompanied by position effects on *iab-3*$^+$.

Gonads, normally located in AB5, are absent in *Uab*^{4} hemizygotes, suggesting that BX-C genes control mesodermal as well as ectodermal tissues. Gonadal examination of *Uab*^{4} genotypes was prompted by the report of gonadal defects in homozygotes for certain *Uab*-like mutants of the homologous "E-locus" complex of Bombyx (see review by Tazima[12]).

Segment AB4 transforms strongly towards LAB5, both in pigmentation and bristle patterns in heterozygotes and homozygotes for Miscadestral pigmentation (*Mcp*), a dominant mutant recently discovered by M. Crosby (unpublished). Analysis of X-ray induced revertants indicates that *Mcp* probably derepresses a gene, *iab-5*$^+$, which codes for a substance, S_7, effecting either LAB4 → LAB5 or LMS → LAB5.

A new dimension has been added to the analysis of BX-C with the discovery that a homozygous deficiency for the entire complex, *Df-P9* (Fig. 2) survives to the late embryonic or early first instar stage and exhibits a transformation of MT and all eight AB segments towards LMS. Pairs of Keilin's organs[13] (KO), ventral pits (VP) and thoracic-type ventral setal bands (VSB) appear on those segments (Fig. 5a and b) and in place of a continuous dorsal longitudinal tracheal trunk (DLT) each segment bears a section of DLT terminating presumably in an incipient anterior spiracle (ASP)[14] (Fig. 5c and d); finally, the ventral nerve cord tends to retain its primitive embryonic pattern in that it extends to segment AB6 or AB7 instead of foreshortening to segment

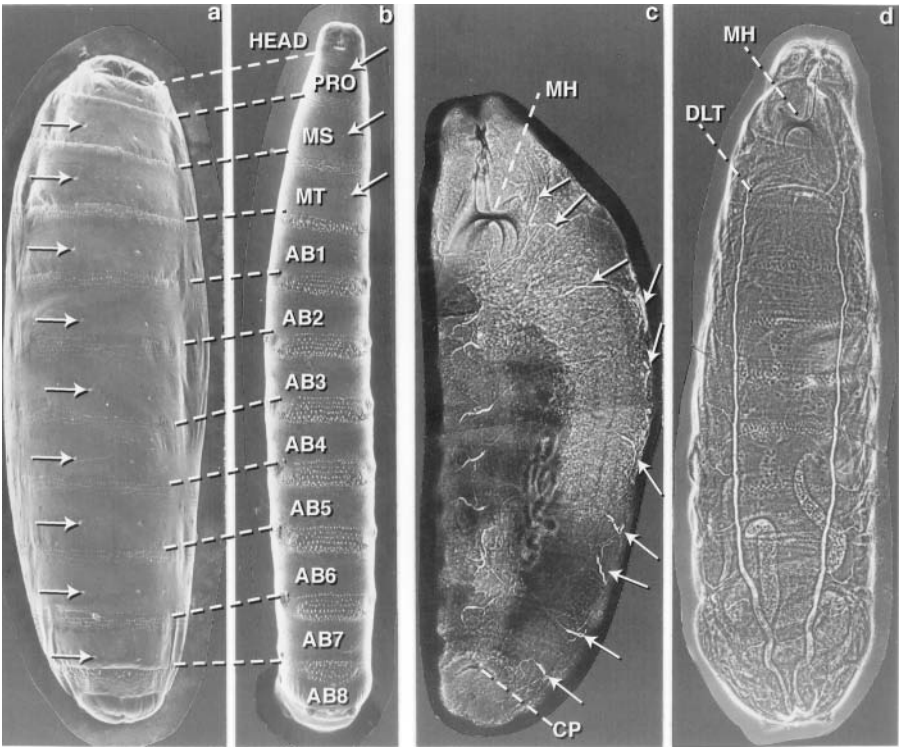


Figure 5. Comparison of cuticular and tracheal patterns in homozygotes for a deletion of BX-C (*a* and *c*) compared with those of wild type (b and d). See Fig. 1 for legend. *a, Df-P9* homozygote. Animals die within the egg or after partly emerging from egg membranes. Note thoracic-like VSB, KO (*arrows*) on segments AB1 through AB7, as well as on thoracic segments; AB8 has a reduced VSB. KO not visible in AB8 of specimen shown but are visible in whole mounts examined under phase contrast, ×160. b, Wild-type first instar larva. Note more prominent VSB on abdominal vs. thoracic segments, with VSB on MT somewhat intermediate between those on MS and AB1. KO (*arrow*) are restricted to thoracic segments as are also VPs, which are not resolved at the magnification shown, ×60. (Specimens a and b were fixed in hot water and photographed with SEM.) c, Whole mount of mature embryo of *Df-P9* homozygote. Note separate tracheal sections (arrows) of DLT occur in each segment from MS through AB8, and tiny chitinized plates (CP) in AB8. d, Whole mount of wild-type first instar larva. Note continuous DLT. (Specimens c and d mounted in Zeiss W15 medium and photographed under phase contrast. ×120.)

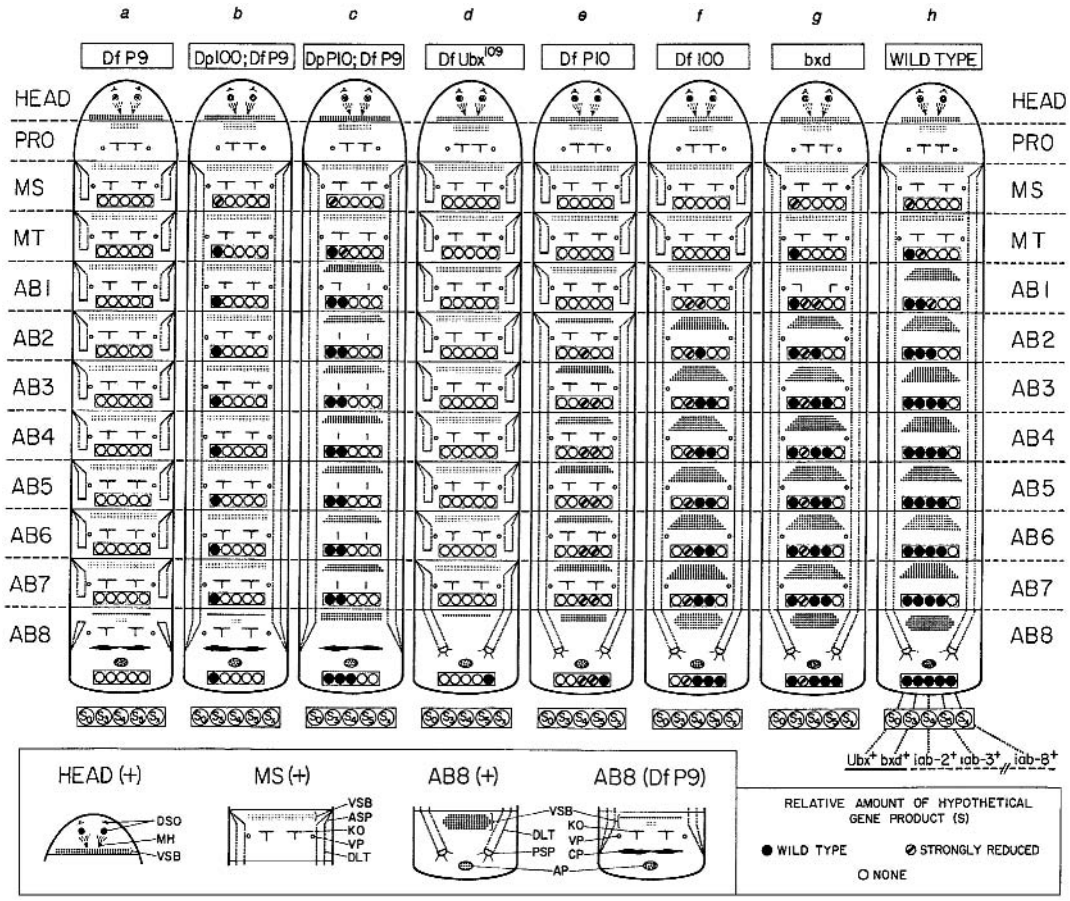
AB3 or AB4 as in wild type. Tiny chitinised plates (CP), resembling rudiments of mandibular hooks (MH), occur in AB8 and imply a head-like rather than LMS transformation of one or more of the embryonic segments that normally fuse to form AB8.

In order to delineate regional differences in morphological functions within BX-C, hemizygotes for deficiencies of portions of BX-C have been constructed. Figure 6 displays stylised phenotypes associated with genotypes hemizygous for five such deficiencies (*b–f*), for *Df-P9* (a), for *bxd* (*g*) and for wild type (*h*).

A direct demonstration that Ubx^+ effects LMS → LMT can be seen by comparing genotypes *a* and *b*, which lack all BX-C genes except that *b* retains one dose of Ubx^+ (and bx^+). All nine segments from MT to AB8 remain at LMS in *a* and attain LMT in *b*; i.e., in *b* they acquire a pattern of attributes unique to the larval MT (namely, a continuous DLT, VP, KO, and thoracic-type VSB). Furthermore Ubx^+ behaves as if derepressed not only in MT and AB1, but in the remaining abdominal segments as well. That many other BX-C genes share with Ubx^+ the ability to establish continuity of DLT is evidenced by the presence over several segments of continuous DLT in larval genotypes, *d*, *e*, and *f*, which lack Ubx^+ but have one or more other BX-C genes still present.

All abdominal segments approach, but do not quite achieve, LAB1 in animals of genotype *c*, which is a hemizygote for bxd^+ (and pbx^+) as well as Ubx^+. Such animals are seen to have most of the characteristics of wild-type AB1 (absence of ASP and VP and presence of DLT and VSB, but incomplete suppression of KO). Failure of bxd^+ to effect a perfect transformation to LAB1 is not understood. One possibility is that $iab\text{-}2^+$ and bxd^+ are both involved in the suppression of KO and that $iab\text{-}2^+$ is weakly derepressed in AB1 (depicted in Fig. 6h by indicating a strongly reduced amount of S_4 in that segment of wild type). Complete suppression of KO can occur in genotypes which lack full bxd^+ function; namely, *f* and *g*. In such cases, it seems

Figure 6. Stylized phenotypes detectable in first instar larvae or mature embryos. See Fig. 1(a) for legend. Fig. 2 for description of rearrangements and Table 1 for roles of postulated BX-C substances (S). All drawings based on whole mounts of animals examined with phase contrast microscopy. Mounting fluid: 9 parts lactic acid to 1 part 95% ethanol for revealing cuticular structures; and Zeiss W15 for tracheal structures. *a, Df-P9* homozygote (see also Fig. 5a and c). *b, Df-P9* homozygote containing. one dose of *Dp-100*, abbreviated symbol for the duplication derived from $Tp(3)bxd^{100}$ (Fig. 2). Note identity to a except for presence of DLT. c, *Df-P9* homozygote containing one dose of *Dp-P10*, abbreviated symbol for the duplication derived from *T(2;3)P10* (Fig. 2). Note suppression of VP and partial suppression of KO in segments AB1–AB7. Although VP and KO appear to be absent in AB8, they may be obscured by CP and other structures in that segment. *d, Df-Ubx*^{109}*/Df-P9*. Note DLT between segments AB7 and AB8, and presence of PSP in AB8; and absence of CP in AB8; occasionally DLT also forms between AB6 and AB7. *e, Df-P10/Df-P9. Df-P10* is an abbreviated symbol for the deficiency derived from *T(2;3)P10* (Fig. 2). DLT restored in AB2 through AB8. The pair of separate sections of that trunk in AB1 frequently lie in AB2, parallel to DLT in that segment. Note partial restoration of abdominal-type VSB, weakly developed in AB2 and moderately developed in AB3 through AB8. *f, Df-100/Df-P9. Df-100* is an abbreviated symbol for the deficiency derived from $Tp(3)bxd^{100}$ and has moderately weakened dominance of bxd^+ as a position effect of the 89E breakage point. Note retention of DLT and failure of suppression of VP in AB1 through AB7 (with VP status in AB8 uncertain) and full restoration of abdominal type of VSB in all segments from AB2 through AB8. *g, bxd/Df-P9*. Although not deleted for any known genes of BX-C this genotype is depicted since it has effects on several larval (as well as adult) cuticular structures. Note failure of suppression of VP on AB1 through AB7; thoracic-type VSB on AB1; and only partial suppression of KO on AB1. *h*, Wild-type homozygote or hemizygote (the latter having possibly somewhat narrower VSB on AB segments than the former) (see Fig. 5b and d). All individuals of genotype *a* through *f* die in the late embryonic or early first instar stage. ASP are lacking in first instar larvae according to Bodenstein.[14] Separate sections of DLT send out a branch to the cuticle but no anterior spiracles as such have been resolved. A number of larval phenotypic expressions are not readily explained unless it is assumed that there is weak derepression of Ubx^+ (to establish continuity of DLT between MS and MT), bxd^+ (to make very slightly larger VSB teeth in MT than MS) and $iab\text{-}2^+$ (to suppress KO in AB1) in the segment anterior to that in which each is fully derepressed. Whether $iab\text{-}3^+$ or $iab\text{-}8^+$ are also similarly weakly derepressed is uncertain.


a
b
c
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e
f
g
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Df P9
Dp100; Df P9
Dp P10; Df P9
Df Ubx109
Df P10
Df 100
bxd
WILD TYPE
HEAD
PRO
MS
MT
AB1
AB2
AB3
AB4
AB5
AB6
AB7
AB8
Ubx+ bxd+ iab-2+ iab-3+ iab-8+
HEAD (+)
DSO
MH
VSB
MS (+)
VSB
ASP
KO
VP
DLT
AB8 (+)
VSB
KO
DLT
VP
PSP
CP
AP
AB8 (Df P9)
RELATIVE AMOUNT OF HYPOTHETICAL GENE PRODUCT (S)
● WILD TYPE
⊘ STRONGLY REDUCED
○ NONE

likely that *iab-2*$^+$ and possibly *iab-3*$^+$ are able to bring about suppression of KO when fully derepressed.

Suppression of VP seems to be exclusively controlled by *bxd*$^+$, these organs being absent from abdominal segments only when *bxd*$^+$ is present, as in genotypes c and *h*. It is therefore directly evident from *h* that *bxd*$^+$ is normally derepressed not only in AB1, but in all segments posterior to AB1.

The larval effects of *iab-2*$^+$ can be indirectly inferred by comparing tracheal and cuticular patterns in AB2 of genotypes *d*, *e*, and *f* (Fig. 6), which involve loss, weakened dominance and dominance, respectively, of *iab-2*$^+$. Continuity of DLT is seen in both *e* and *f*, suppression of KO occurs only in *f*, and VSB become progressively more abdomen-like in proceeding from *d* to *e* to *f*. These effects are not necessarily solely due to the *iab-2*$^+$ gene since it is possible that *iab-3*$^+$ is weakly derepressed in AB2 of the larva. Unfortunately, definitive genotypes are not available that would resolve *iab-2*$^+$ and *iab-3*$^+$ larval functions.

A comparison of larvae of genotypes *a* and *d* (Fig. 6) shows that the latter retain the wild-type cuticular pattern in AB8 (except for thoracic-type VSB) and have DLT continuity between AB7 (or AB6) and AB8. These effects are most simply accounted for by assuming that in contrast to *Df-P9, Df-Ubx*^{109} still retains an *iab-8*$^+$ gene, whose product, S_x, effects LAB7 → LAB8 or LMS → LAB8. The ability of genotype *d* to restore a tracheal trunk in the region from AB6 to AB8 suggests that *Ubx*^{109} retains additional *iab* loci to the right of *iab-3*, besides *iab-8*.

The above findings point to an antero-posterior gradient within the organism in BX-C gene action. A regulatory gene(s) might therefore be expected to exist which, when inactivated, would derepress or activate BX-C genes in all segments of the body. The properties of Polycomb (*Pc*), a mutant found by P. H. Lewis,[15] and of a more extreme allele, *Pc*^{3}, suggest that *Pc*$^+$ (locus 3-47.1)[16] is such a gene. Thus, hemizygotes and homozygotes for *Pc* or *Pc*^{3} have recently been found to have their thoracic and first seven abdominal segments partially transformed towards LAB8. Such animals, which die as late embryos, also have reduced head structures, including poorly developed and incompletely sclerotised MH. As the doses of BX-C are increased from two to three to four, the transformation towards LAB8 becomes increasingly more extreme with many of the abdominal segments containing rudimentary posterior-type spiracles. The LAB8 transformation is not observed unless at least one dose of BX-C is present; i.e., *Pc*^{3} *Df-P9* homozygotes closely resemble *Df-P9* homozygotes (Fig. 5) except that the former have reduced head structures and incompletely sclerotised CP in AB8 (suggesting that the latter structures are indeed rudiments of MH). A dependence of BX-C phenotypic effects on BX-C dosage is seen also in *Pc*^{3} heterozygotes: e.g., *Cbx*-like effects occur in *Pc*^{3}/+ animals (with two doses of BX-C) and become increasingly more extreme with three and four doses of BX-C. Gene dosage studies of R. Denell (personal communication) indicate that *Pc* represents the inactivated state of the gene. Therefore, *Pc*$^+$ in all likelihood is coding for a repressor of BX-C.

REGULATORY RULES

The BX-C genes are assumed to control the organism's thoracic and abdominal segmentation pattern by producing substances which in turn regulate other genes that actually determine segmental structure and function. Regulation of the BX-C genes, themselves, seems to be governed by the following rules: (1) state of repression or derepression of a given gene is controlled (in at least four instances) by a *cis*-regulatory element; (2) the genes tend to be individually, rather than coordinately, derepressed; (3) BX-C is under negative control maintained by a major regulatory gene, Pc^{+} (along with perhaps other regulatory genes[7,17]); (4) a gene derepressed in one segment is derepressed in all segments posterior thereto; (5) the more posterior the segment (starting with MT) the greater the number of BX-C genes that are in the derepressed state; and (6) the more proximal the locus of a gene in the complex the more likely it is that that gene is in a derepressed state. Rules (5) and (6) suggest that two types of gradient are involved in BX-C regulation; an antero-posterior gradient within the organism in repressor concentration and a proximo-distal gradient along the chromosome in relative affinity for repressor of *cis*-regulatory elements. Presumably in MT repressor concentration is so high that the only genes derepressed are those whose *cis*-regulatory elements have relatively low repressor affinities, while in AB8 repressor concentration is so low that all of the genes of the complex escape repression.

During ontogeny the above rules presumably result in each segment having a specific array of BX-C substances, at the right time, at the right place. In AB8, for example, the rules predict a minimum of eight such substances, the number of BX-C genes being very likely to exceed the eight identified thus far. Whether such a multiplicity of substances act in a hierarchical or a compartmentalised manner[18,19] to establish the final pattern or level of development of a segment may well be accessible to analysis at the cellular level.

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GENETIC CONTROL OF BODY SEGMENT DIFFERENTIATION IN DROSOPHILA

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INTRODUCTION

Drosophila, like many other higher organisms, begins development as a tandem array of more or less identical body segments. During later development, these gradually diverge in morphology until the extensive differentiation of body segments seen in the adult is achieved. The genetic control of this divergence is, by now, moderately well understood. The differentiation of the third thoracic and of all abdominal segments appears to be under the control of a cluster of genes known as the bithorax complex (BX-C) (Lewis, 1978). As reviewed below, the genes of this complex appear to be differentially regulated along the body axis so that each body segment is characterized by a unique subset of active BX-C genes. The activities of these genes are thought to directly determine segmental identity. Recently, it has been proposed that more anteriorly located segments, including those in the head as well as the first and second thoracic segments, are under the control of another gene cluster that has been named the Antennapedia complex (ANT-C) by Kaufman et al. (1980). These authors suggest that the ANT-C genes may function in a manner analogous to that of the BX-C genes.

How the BX-C and ANT-C genes come to be controlled in a spatially patterned way is of major interest. This report begins with a brief review of the BX-C genes

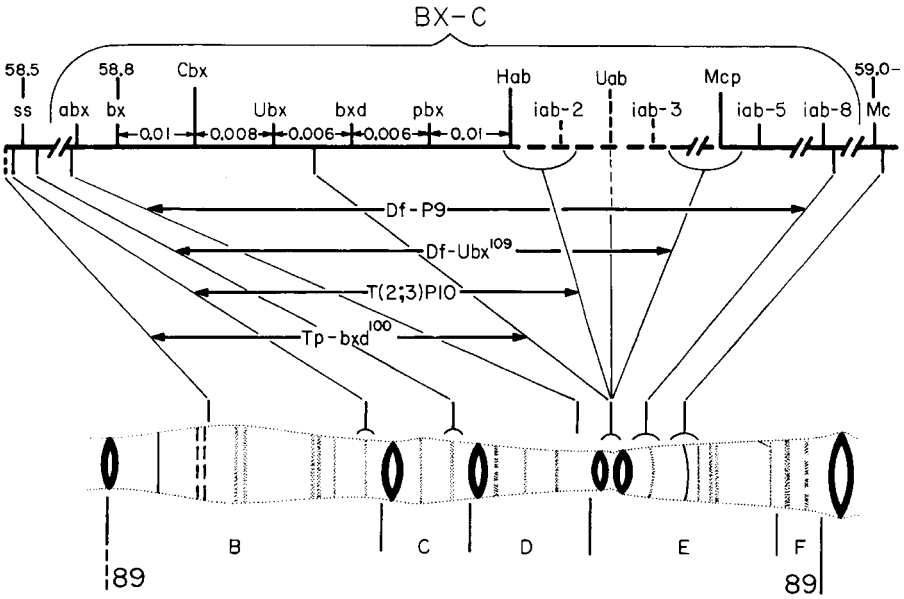

Figure 1. Correlation of the genetic and salivary gland chromosome maps of the BX-C. (Modified from Lewis, 1978). Loci within the dashed portion of the genetic map have not been ordered with respect to one another. *Mc* (Microcephalus) and *ss* (spineless) are flanking markers. Mutants within the complex include *abx* (anterobithorax), *bx* (bithorax), *Cbx* (Contrabithorax), *Ubx* (Ultrabithorax), *bxd* (bithoraxoid), *pbx* (postbithorax), *Hab* (Hyperabdominal), *iab-2* (infraabdominal-2), *Uab* (Ultraabdominal), *Mcp* (Miscadastral pigmentation), *iab-5* (infraabdominal-5) and *iab-8* (infraabdominal-8). For a description of the effects of these mutants on segmentation, see Lewis (1978, 1981). The mutants are shown on two levels, with gain-of-function mutants lying above loss-of-function mutants. Because existing *Uab* mutants are associated with reduced crossing over, the locus of *Uab* is uncertain. The *iab-3* gene is inferred to exist because of the transformations of first and second abdominal segments to third abdominal segments seen in $T(1;3)Uab^{5}$ heterozygotes (Lewis, 1978). Between the genetic and cytological maps are shown four chromosomal rearrangements that are discussed in the text. These include *Df(3R)P9*, which is deficient for the entire BX-C, and $Df(3R)Ubx^{109}$, whose probable extent is indicated. *T(2;3)P10* is an insertion of the indicated region into section 29C of chromosome 2; *Df(3R)P10* and *Dp (3;2)P10* are segregation derivatives of this translocation. $Tp(3)bxd^{100}$ is an insertion of the indicated region into section 66C. $Df(3R)bxd^{100}$ and $Dp(3;3)bxd^{100}$ are crossover derivatives of this transposition.

and proceeds to describe some of the properties of two genes, *Polycomb* and *Regulator of bithorax*, that appear to play important roles in this spatial control.

THE BITHORAX COMPLEX (BX-C)

Since the properties of the BX-C genes have recently been reviewed in detail (Lewis, 1978, 1981), only a summary is presented here. The genetic map of the BX-C is shown in Fig. 1.

Much of our knowledge of the BX-C has been derived from the examination of mutant embryos. These studies have shown that the BX-C genes are required

for the development of structures characteristic of segments posterior to the second thoracic segment. Thus, in zygotes completely deficient for the BX-C (*Df(3R)P9* homozygotes) the third thoracic through the seventh abdominal segments develop with the morphology of second thoracic segments. Since the Drosophila embryo contains a number of segment-specific structures (for a detailed description, see Lohs-Schardin et al. [1979]), these transformations can be easily recognized. Ventral pits and Keilin's organs, presumed sensory organs that normally occur only on the thoracic segments, are found on the posterior body segments of BX-C$^-$animals; the prominent ventral setal belts present on the abdominal segments of wild type are replaced by very narrow belts of fine denticles like those normally found only on the second and third thoracic segments. That the posterior body segments in BX-C$^-$animals are transformed to second thoracic segments and not to third thoracic segments is shown by examination of the embryonic longitudinal tracheal trunks. In wild type, these are continuous structures that terminate in spiracles located in the second thoracic and eighth abdominal segments. In *Df(3R)P9* homozygotes, each segment contains a separate piece of tracheal trunk, presumably because each section terminates in an incipient second thoracic spiracle.

Although not central to this discussion, the fate of the eighth, or most posterior, abdominal segment in BX-C$^-$embryos is very different from that of the other abdominal segments. In the posterior portion of this segment, sclerotized chitinous plates resembling portions of the normal cephalopharyngeal skeleton (a head structure) form both dorsally and ventrally. In the anterior portion, however, second thoracic-like setae form dorsally, whereas first thoracic-like setae develop ventrally. Recently, we have obtained evidence, which will be presented elsewhere, that this complicated behavior of the eighth abdominal segment results from the turning-off in this segment of one of the ANT-C genes.

Examination of embryos deficient for only portions of the BX-C has revealed three major properties of the genes in this complex. First, many (or all) of these genes appear to control the development of only particular structures within segments. Examples include the ventral pits, whose development in the abdominal segments is suppressed by the BX-C genes *bxd*$^+$and *iab-8*$^+$ only, and the ventral abdominal setal belts, whose formation appears to depend principally on the genes *bxd*$^+$ and *iab-2*$^+$. Because each BX-C gene's influence is restricted to a particular set of structures, BX-C mutant embryos frequently possess body segments containing sets of structures that are never seen together in a wild-type segment.

A second striking property of the BX-C is that the position of a gene within this complex appears to be correlated with the most anterior location in the animal of that gene's activity. Thus, genes that are located at the proximal (left) end of the complex have been shown to be active as far anteriorly as the third thoracic segment, while more distal BX-C genes appear to become sequentially activated in more posterior body segments. This pattern can be seen by comparing the effects on the embryonic tracheal system of three deficiencies that enter the BX-C from the proximal end and remove increasing numbers of BX-C genes. When homozygous, these deficiencies,

Df(3R)bxd^{100}, *Df(3R)P10*, and *Df(3R)Ubx*^{109}, cause separate pieces of tracheal trunk to form in segments anterior to the second, third, and seventh abdominal segments, respectively. Within the resolution of these experiments, then, it appears that the proximodistal order of genes within the BX-C is the same as the order on the body axis of the most anterior segments in which those genes become active. As discussed below, studies of adult animals have supported this generalization, although an exception (*pbx*) is known.

A third major property revealed by studies of embryos is that, once activated in a particular segment, each of the BX-C genes appears to remain active in all more posterior segments. Thus, the addition of *Dp(3;3)bxd*^{100} (which carries only the leftmost genes of the complex) to otherwise BX-C^{-} animals results in restoration of continuity to the tracheal trunks not only between the second and third thoracic segments, but down the entire length of the embryo as well. Another example is provided by a slightly larger duplication, *Dp(3;2)P10*, that carries the gene *bxd*$^{+}$. When added to otherwise BX-C^{-} animals, this duplication causes the suppression of ventral pits and the development of a first abdominal ventral setal belt on the first and all more posterior (except the eighth) abdominal segments.

The three properties of the BX-C described above have been summarized in a model (Lewis, 1978), according to which the genes of the BX-C are activated in sequence along the chromosome as one proceeds posteriorly in the early embryo. Once activated, each gene remains active in all more posterior segments. The resulting array of BX-C genes active within any particular body segment determines the set of structures that will develop in that segment.

Many loss-of-function mutants in the BX-C survive to adulthood when homozygous or hemizygous. All of these cause anteriorly directed segmental transformations, which is consistent with the idea that BX-C gene activity is required for the development of posterior characteristics. Several of these mutants have proven susceptible to analysis by crossing over and have been shown to define at least six loci that map, with one exception (*pbx*), in the same order as the body segments that they affect. Evidence from clonal analysis experiments with inactivation-type mutants at several of these loci (Morata and Garcia-Bellido, 1976) indicates that BX-C genes are expressed autonomously and are required throughout most, or all, of zygotic development to maintain particular segmental identities.

In addition to loss-of-function mutants, four dominant gain-of-function mutants (*Cbx, Hab, Uab*, and *Mcp*) are located within the BX-C. When heterozygous with wild type, each of these causes a particular posteriorly directed segmental transformation. Since a deficiency for the BX-C does not cause such transformations when heterozygous with wild type, these mutants are thought to cause BX-C genes to be active in some abnormal way and are postulated (Lewis, 1978) to be regulatory mutants that cause particular structural genes (defined by loss-of-function mutants) to become active in segments anterior to those in which such genes would normally become active. In two instances (*Cbx* and *Mcp*), these mutants have been shown to be *cis*-acting and to map adjacent to the genes they are thought to control (Lewis, 1981).

Finally, the genes of the BX-C show two peculiar genetic properties that may be related to their clustered organization. One such property is that rearrangement breakpoints within the complex have been found to partially inactivate neighboring genes that are distally located. This polarized impairment of function when genes are separated from their normal neighbors indicates that the clustering of genes within the BX-C is functionally important. Another unusual property, called transvection, is the apparent dependence of BX-C gene activity on the state of homologous chromosome pairing; several BX-C genotypes are known in which the severity of segmental transformations is increased or, in one case, decreased by the introduction of rearrangements that disrupt somatic pairing of the chromosomal region containing the BX-C (Lewis, 1954). The basis of this phenomenon (see also Ashburner, 1967; Gelbart, 1971; Jack and Judd, 1979) is unknown.

NEGATIVE CONTROL OF THE BX-C BY *POLYCOMB (PC)*

Evidence That Pc^+ Acts as a Repressor of BX-C Functions

Many, or all, of the genes within the BX-C appear to be under both positive and negative control by genes located elsewhere. Although several genes appear to be involved in negative regulation of the BX-C, the most important of these is probably *Polycomb* (*Pc*), a gene discovered by P. H. Lewis (1947). When heterozygous with wild type, all *Pc* mutants known cause partial transformations of the second and third legs into first legs. These transformations are easily seen in males, where they result in the development of sex comb teeth (which normally occur only on the first legs of males) on the posterior legs. Denell (1978) has reported that animals heterozygous for a deficiency that includes the *Pc* locus also show these leg transformations, indicating that the known *Pc* point mutants exert their effects by inactivating Pc^+. By inducing with X-rays a number of deletions that include the *Pc* locus, we have confirmed Denell's observations and localized *Pc* to 78E on the polytene chromosome map. For the work described below, we have chosen to study Pc^3, a strongly mutant and cytologically normal allele described by Lewis (1980).

The major lines of evidence that the wild-type allele of the *Polycomb* locus (Pc^+) acts as a negative regulator of the BX-C have already been presented (Lewis, 1978). The first indication of such a role for Pc^+ was the observation that embryos hemizygous or homozygous for *Pc* mutants (these survive until the late embryonic stage) show posteriorly directed transformations in the cuticle of segments in the head, thorax, and abdomen. Recently, we have found that embryos deficient for the *Pc* locus are similarly affected. The segmental transformations seen in *Pc* mutant embryos are illustrated in Fig. 2a and b, where anteroventral views of a wild type and of a Pc^3 homozygous embryo are shown. In extremely affected Pc^3 homozygotes, head involution appears to be blocked so that head segment derivatives develop externally. An abdominal-like ventral setal belt often forms on what may be the labial segment of such animals (see Turner and Mahowald [1979]) for the position of wild-type head segments prior to involution) and a prominent setal belt of unknown character

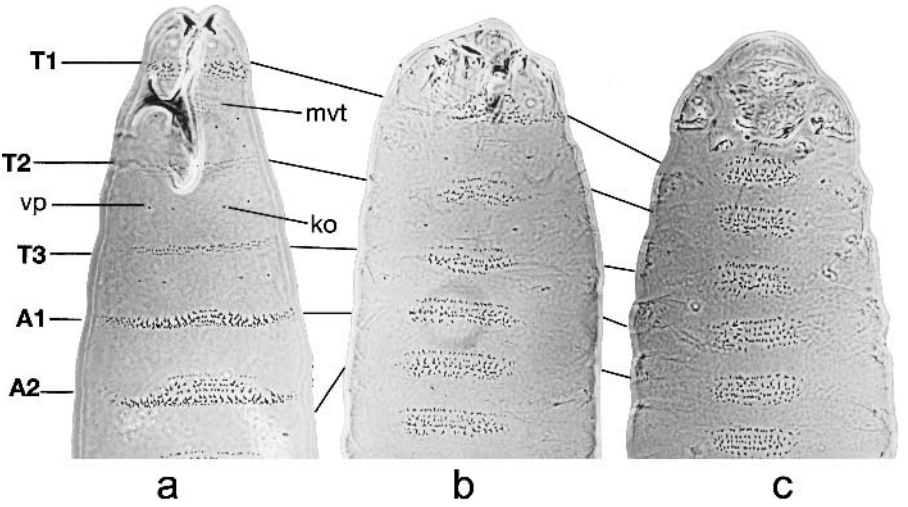

Figure 2. Segmental transformations in Pc^3 homozygotes. Embryos were fixed and cleared according to the procedure of Van der Meer (1977). (a) Anterior of a wild-type (Canton-S) embryo. The ventral setal belts of the thoracic (T1, T2, T3) and first two abdominal (A1, A2) segments are indicated as well as the midventral tuft (mvt), ventral pits (vp), and Keilin's organs (ko). (b) A typical Pc^3 homozygous embryo. All thoracic and abdominal segments are transformed towards the most posterior (eighth) abdominal segment. (See Fig. 4 for the posterior abdominal segments of wild type.) Note the incomplete suppression of midventral tuft in T1 and of Keilin's organs in T1 and T2. Head structures are partially involuted. (c) A Pc^3 homozygote which, owing to homozygosity for *Dp(3;3)P5*, carries four doses of the BX-C. All thoracic and abdominal segments show extreme transformations. A few setal belt teeth are present just anterior to T1, in what may be the labial segment. Head involution is completely blocked.

forms dorsally on the head. The components of the cephalopharyngeal skeleton are very reduced. The first thoracic segment is usually partially transformed towards the abdominal state, as indicated by suppression of ventral pits, Keilin's organs, and midventral tuft, and by the occurrence of rows of anteriorly directed denticles in the main setal belt of this segment. Ventral pits and Keilin's organs are also often absent from the second and third thoracic segments, which show prominent posterior abdominal-type ventral setal belts. Posterior transformation of the first abdominal segment is indicated by the development of rows of anteriorly directed ventral setal belt denticles. Transformations of the second through the seventh abdominal segments towards the eighth abdominal segment can be seen both by changes in the width (measured transversely on the animal) of the ventral setal belts of these segments (in wild type the eighth abdominal setal belt is narrower than those from the second through the seventh abdominal segments) and by changes in the intensity of the anterior denticle rows in these setal belts (in wild type this row is very fine in the second abdominal segment, grading to prominent in the eighth abdominal segment). Occasionally, Pc^3 homozygous embryos show a tiny extra ventral setal belt between the eighth abdominal segment and the anal pads. The development of this extra belt, which is never seen in wild-type animals, is strongly enhanced in Pc^3 homozygotes

that are also homozygous for *Df(3R)C4*, a deficiency that deletes, at minimum, all BX-C genes distal to *iab-5*$^+$. This extra setal belt may, therefore, represent a body segment whose development in wild type is suppressed, at least in part, by genes located at the right end of the BX-C.

In addition to ventral setal belt transformations, *Pc* homozygotes often show the development of posterior spiracles in the thoracic and abdominal segments. These extra posterior spiracles in *Pc* homozygotes are poorly developed and globular in shape. More nearly normal extra posterior spiracles of a tubular shape arise in *Pc* homozygotes that are also homozygous for partial BX-C deletions that extend into the complex from the left end (*Df(3R)bxd*^{100}, *Df(3R)P10*, *Df(3R)Ubx*^{109}). These tubular spiracles develop only in segments that would be second thoracic ones in *Pc*$^+$ animals. Hence, it appears that the activities of proximally located BX-C genes partially suppress the formation of posterior spiracles. In wild type, the failure of these genes to suppress the development of full-size posterior spiracles in the eighth abdominal segment suggests that at least some BX-C genes are inactive in the portion of this segment (the posterior) that gives rise to these structures. Further evidence that this is the case will be presented elsewhere.

It seems very likely that the posteriorly directed segmental transformations described above for *Pc* mutant homozygotes result from the activities in anterior segments of BX-C genes normally active only more posteriorly. That this apparent hyperactivity of BX-C genes is caused by *Pc* deletions strongly suggests that *Pc*$^+$ functions in normal development to repress BX-C genes in anterior body segments. Lewis (1978) has proposed a model in which the ordered expression of the BX-C genes comes about as a consequence of a body axis gradient in *Pc*$^+$ activity and of a proximodistal chromosomal gradient in the affinities of BX-C genes for *Pc*$^+$ repressor. In this scheme, as one proceeds posteriorly in the embryo, *Pc*$^+$ activity decreases in a graded fashion, allowing BX-C genes with increasing affinities for repressor to be sequentially activated. Thus, in the third thoracic segment *Pc*$^+$ activity is considered to be so high that only those BX-C genes postulated to have very low repressor affinities are active, whereas in the most posterior abdominal segment *Pc*$^+$ activity is effectively zero, with the result that all BX-C genes are expressed. While this model has proven to be a useful working hypothesis, the apparent inactivation of many of the BX-C genes in the posterior portion of the eighth abdominal segment suggests that the real situation is more complex.

An important piece of evidence supporting the above model is that the severity of the posteriorly directed transformations seen in *Pc* mutant embryos is dependent on the dosage of the BX-C. Thus, the addition of extra doses of the BX-C to *Pc*^{3} homozygotes strongly enhances these transformations (Fig. 2c), whereas homozygosity for a deficiency of the entire BX-C abolishes them. Lewis (1978) has described *Pc*^{3} *Df(3R)P9* homozygotes as closely resembling *Df(3R)P9* homozygotes, with the exception that the former show incomplete sclerotization of the cephalopharyngeal skeleton and of the eighth abdominal chitinous plates. More recently, a few denticles resembling those found in the midventral tuft, which is normally present

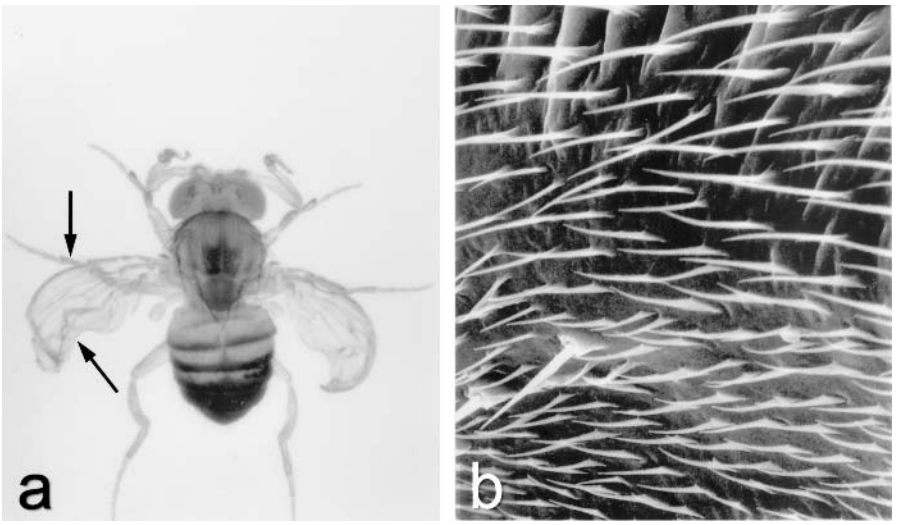

Figure 3. Segmental transformations in Pc^3 heterozygotes. (a) A $Pc^3/Pc^3/Dp(1;3;4)7$, Pc^+ male. Several transformations can be seen. In addition to the obvious conversion of antennae into legs, a few sex-comb teeth (normally found only on the first legs) are present on the second legs (*top arrow*), haltere tissue is present in the posterior of the wing (*bottom arrow*) and a patch of fifth abdominal segment cuticle (recognizable by pigmentation) is present in the fourth abdominal segment. (b) A scanning electron micrograph showing the transition zone between wing and haltere cuticle in an animal of the same genotype as (a). Wing hairs are normally very long, while haltere hairs are short. Although the transition between the two is often abrupt in Pc^3 heterozygotes, intermediate length hairs are also often seen. In the wing shown here, hairs grade from normal wing length at the top of the figure to normal haltere length at the bottom. Note sensilla trichodea (haltere structures) in the area with short hairs.

only in the first thoracic segment, have been observed in most of the body segments of such animals. Furthermore, the ventral setal belt of the eighth abdominal segment, which in *Df(3R)P9* homozygotes is first thoracic, appears to have a predominantly second thoracic character in Pc^3 *Df(3R)P9* homozygotes. Because similar conversions are caused by certain mutants in the ANT-C (Wakimoto and Kaufman, 1981), these transformations of first and second thoracic structures suggest that Pc^+ plays a role in regulating one or more genes in that complex.

Adult animals heterozygous for *Pc* mutants show a number of segmental transformations that have provided support for the idea that Pc^+ acts as a negative regulator of the BX-C. For close examination we have chosen to study adults of the genotype $Pc^3/Pc^3/Dp(1;3;4)7$, Pc^+. [*Dp(1;3;4)*7 is a fourth chromosome that has the material from 61A to 62E and 78D to 79F as well as $Dp(1;3)sc^{J4}$ appended to its distal tip.] A male of this genotype is shown in Fig. 3a. Such adults show much more extreme segmental transformations than do those of the genotype $Pc^3/+$, indicating that Pc^3 is not simply a null allele, but is able in some way to compete with and partially inactivate a Pc^+ allele. That is, in the terminology of Muller (1932), Pc^3

appears to be antimorphic. In a similar test, Puro and Nygren (1975) have shown that the Pc^2 allele is also antimorphic. Not all *Pc* mutants are of this type, however. We have induced a number of cytologically normal *Pc* mutants with X-rays and at least four of these appear to be null alleles (that is, $Pc^x/+$ is equivalent to $Pc^x/Pc^x/Dp(1;3;4)7$ in phenotype). Presumably, the Pc^2 and Pc^3 alleles were originally noticed because of their antimorphic nature; i.e., $Pc^2/+$ and $Pc^3/+$ animals show a more extreme extra-sex-comb phenotype than do animals heterozygous for a *Pc* deficiency.

Close examination of $Pc^3/Pc^3/Dp(1;3;4)7$ adults has revealed several posteriorly directed segmental transformations that mimic those caused by the known dominant gain-of-function mutants in the BX-C. Usually, patches of haltere cuticle are present in the wing, and the mesonotum and postnotum are reduced in size, indicating a partial transformation of the second thoracic segment to third, similar to that caused by the BX-C mutant *Cbx*. Sectors of second, third, or possibly fourth abdominal segment cuticle (i.e., patches with large bristles) occasionally develop in the first abdominal tergite, a transformation caused by BX-C mutants of the *Uab* type; patches of fifth abdominal tergite (recognizable in males by black pigmentation) often occur in the fourth abdominal segment, a transformation caused by the BX-C mutant *Mcp*. Partial transformations of the fifth abdominal segment to sixth (recognizable by the loss of hairs in the fifth tergite) and of the sixth abdominal segment to seventh (indicated by a reduction in size of the sixth segment) have also been seen in $Pc^3/Pc^3/Dp(1;3;4)7$ adults, although no BX-C mutants causing these transformations have yet been found. Although the weak posteriorly directed segmental transformations described above are consistent with the extreme transformations seen in Pc^3 homozygotes, their importance lies in indicating that several (perhaps all) of the BX-C genes are under the negative control of Pc^+.

Pc mutant adults also show segmental transformations that closely correspond to those caused by the known dominant mutants in the ANT-C. The transformations of second and third legs into first legs seen in *Pc* mutant heterozygotes are very similar to those caused by the ANT-C mutants *Extra sex comb* (*Scx*) and *Multiple sex comb* (*Msc*). The weak and variable leg transformations seen in *Scx*/+ or *Msc*/+ animals, like those seen in $Pc^3/+$ animals, occur almost entirely in the anterior compartments of the second and third legs (see Steiner [1976] for locations of the leg anterior–posterior compartment boundaries). In *Scx/Msc* animals, which show extreme leg transformations, the anterior compartment of the second leg is almost entirely transformed to first leg, whereas the posterior compartment is only weakly affected (Duncan, unpublished; see also Hannah-Alava, 1958). The third leg anterior compartment is strongly transformed distally, but is almost unaffected proximally. No effect has been seen on the third leg posterior compartment. Although weaker, the leg transformations seen in $Pc^3/Pc^3/Dp(1;3;4)7$ adults are similar to those seen in *Scx/Msc* animals.

Pc mutant adults frequently show a weak antenna-to-leg transformation that also corresponds well with transformations caused by dominant *Antp* mutants of the ANT-C. Such mutants cause the antenna, or parts of it, to transform to second

leg. $Pc^3/Pc^3/Dp(1;3;4)7$ adults frequently show an almost complete antenna-to-leg transformation and microscopic examination has shown that these antennal legs are mostly second leg in character, although a few sex-comb teeth (in males) and other first leg structures also occur.

Finally, $Pc^3/Pc^3/Dp(1;3;4)7$ adults occasionally show the development of what appears to be mesonotum on the posterior of the head. This transformation is also caused by a dominant mutant called *Cephalothorax* (*Ctx*) (Lewis, unpublished) that appears to be located within the ANT-C, based on its failure to complement the recessive lethality of *Scx* and $Antp^B$ and on cytological observations (Duncan, unpublished) showing that *Ctx* is a complex chromosome rearrangement with one breakpoint in or just distal to 84B1,2, which is known to be the locus of other ANT-C dominant mutants (Denell, 1973; Duncan and Kaufman, 1975).

$Pc^3/Pc^3/Dp(1;3;4)7$ adults, therefore, show segmental transformations that mimic those caused by certain dominant mutants in the ANT-C, suggesting that Pc^+ plays a role in regulating one or more genes in this complex. It is not clear, however, whether that role is positive, negative, or perhaps both. The results of Denell et al. (1981) and Struhl (1981a) indicate that the antenna-to-leg transformation observed in *Antp* mutants is caused by the improper activation of an ANT-C gene in the antenna, where it would normally be inactive. This implies that, at least for this gene, Pc^+ exerts negative control. For the transformation of second and third leg to first leg, arguments for either positive or negative control of the responsible ANT-C gene by Pc^+ can be made. An argument for negative control was presented by Puro and Nygren (1975), who suggested that Pc^+ acts as a repressor of "first leg genes."

As might be expected from the above discussion, when heterozygous with wild type, *Pc* mutants enhance the expression of certain dominant mutants in the BX-C and ANT-C (Lindsley and Grell, 1968). We have found that Pc^3 enhances the BX-C gain-of-function mutants *Cbx*, Uab^2, *Hab*, and *Mcp* and the ANT-C mutants *Scx*, *Msc*, $Antp^{Yu}$, $Antp^B$, $Antp^{73}$, and *Ctx*.

Effects of Extra Doses of Pc^+

Segmental transformations opposite in direction to those described above for Pc^3 heterozygotes might be expected to occur in animals carrying extra doses of Pc^+. Indeed, such an expectation is implicit in a model (Lewis, 1978), in which the level of Pc^+ expression is considered to be the primary determiner of segmental identity. In fact, however, it has been shown that animals carrying up to five doses of Pc^+ survive to adulthood and are morphologically wild type. Such animals have been constructed using *Dp(1;3;4)7*, which has already been described, and *Dp(3;3)C126*, a direct tandem duplication of the material from approximately 78D–79B.

The apparent lack of effect of increasing Pc^+ dosage can be explained in at least two ways. One possibility is that extra doses of Pc^+ are dosage compensated. Another possibility is that Pc^+ is required to cooperate with other genes to repress the BX-C. In this case, segmental transformations would not be expected in animals carrying extra doses of Pc^+, because the dosage of these other regulatory genes would limit the

extent of possible BX-C repression. Segmental transformations would be expected in animals carrying only one dose of Pc^+, however, because in this instance Pc^+ activity would itself be limiting. In fact, other genes apparently similar in function to Pc^+ have been identified. One of these is *1(4)29*, a gene discovered by Hochman (Lindsley and Grell, 1968). As described by Gehring (1970), the second and third legs of *l(4)29/l(4)29* adults are weakly transformed to first leg and the proximal antennal segments are replaced by leg tissue. In these animals, we have also observed posteriorly directed transformations of the abdominal segments, which indicates that $l(4)29^+$ is an ANT-C and BX-C regulator similar in function to Pc^+. Another such gene has been identified by selecting mutants that, when heterozygous with wild type, enhance the segmental transformations seen in Pc^3/+ adults. Mutants in this gene, which has been called *Polycomb-like* (*Pcl*) (2-84), cause almost all of the same segmental transformations that are seen in adults heterozygous for *Pc* mutants. Moreover, Pcl^+, like Pc^+, has been shown in clonal analysis experiments to be active until late in development. Finally, Struhl (1981b) has recently found that a gene called *extra sex combs* (*esc*) appears to be very similar to Pc^+ in function, at least early in development. This gene exerts a maternal effect so that *esc/esc* zygotes from *esc/esc* mothers show extreme transformations of most of their body segments towards the eighth abdominal segment. As reported by Struhl, such animals show the development of an extra posterior body segment similar to that occasionally seen in Pc^3 homozygotes. Moreover, he has found that *esc*; *Df(3R)P9* homozygotes show transformations similar to those that we have seen in Pc^3 *Df(3R)P9* homozygotes.

Time of Action of Pc^+

The segmental transformations seen in zygotes homozygous for *Pc* mutants show that Pc^+ is expressed in the embryonic stage. The results of clonal analysis experiments indicate that Pc^+ is still active very late in development. In these experiments, clones of cells homozygous for Pc^3 were induced by X-irradiation (total dose of 1,000 R delivered at a dose rate of 400 R/min; machine set at 220 kV, 14 mA) of larvae 24–48 h prior to pupation. Such clones have been examined in the tergites of the adult abdomen, where, because the histoblast cells that form these tergites do not divide during larval life (Garcia-Bellido and Merriam, 1971), they are not segregated until the early pupal stage. These Pc^3/Pc^3 clones—which were recognizable because they were marked by *y* (owing to loss of $Dp(1;3)sc^{J4}$) and, in some experiments, were in twin to clones homozygous for the cell marker *trc* (3-46; A. Ferrus, unpublished)—uniformly lack hairs and contain long, wavy bristles. It is likely that this effect is cell-autonomous, because *trc* Pc^3/*trc* Pc^3 clones never show any *trc*-affected hairs. Pc^3/Pc^3 clones have the same appearance in each tergite and in both sexes. It is difficult to be sure what these clones represent; if, however, we assume that all such clones are transformed to a single type of structure normally present at the posterior of the fly, then that structure is almost certainly anal plate. The latter is usually devoid of hairs and is covered with long wavy bristles. A transformation to genitalia is unlikely, because Pc^3/Pc^3 clones in females contain long bristles, but the

female genitalia contain only short bristles. Conversions to sixth or seventh tergites are unlikely, because clones occurring in the normally hairy regions of these tergites are bald. Transformation to other abdominal tergites can be ruled out because they are covered with hairs. Struhl (1981b) has recently reported that clones of cells homozygous for the Pc^2 allele show a similar transformation to anal plate when they occur in the eye-antennal and thoracic disks.

The transformation of *Pc/Pc* clones to anal plate is entirely consistent with the idea that Pc^+ acts as a negative regulator of the BX-C. Because anal plate is thought to be the most posteriorly derived structure in the adult fly (Schüpbach et al., 1978), it is the structure that one would predict tergite cells would transform to if all of their BX-C genes were to become active. The results of these clonal analysis experiments indicate, therefore, that Pc^+ exerts negative control over the BX-C until very late in development, at least until the early pupal stage. It should be kept in mind, however, that the above observations relate to regulation of the BX-C only. The nature and timing of ANT-C control by Pc^+ are not yet known.

The above results suggest that Pc^+ is active throughout zygotic development. Evidence of earlier, maternal activity of Pc^+ has come from an experiment in which Pc^3 hemizygous embryos from mothers carrying one dose of Pc^+ have been compared with similar embryos from mothers carrying two doses of Pc^+. The former show significantly more extreme segmental transformations than do the latter, indicating a maternal effect of Pc^+ (Lewis, in preparation).

An Anterior–Posterior Gradient in the Wing

Evidence suggesting a gradient in Pc^+ activity in the wing has been derived from an examination of the partial wing-to-haltere transformation seen in *Pc* mutant heterozygotes. Patches of haltere tissue can be easily recognized in the wings of such animals, because haltere cells produce much smaller hairs than do wing cells and because these patches frequently contain small bristles (sensilla trichodea) characteristic of the haltere. Haltere cuticle forms almost exclusively at the posterior wing margin, and the extent of the transformation varies from animal to animal. In weakly affected individuals, haltere tissue appears primarily at the margin of the wing and, in more extreme animals, extends for a variable distance anteriorly. In strongly affected wings, cells from both anterior and posterior compartments may transform to haltere. A poorly understood feature of this transformation is that partially transformed wings frequently contain more haltere tissue than is present in an entire normal haltere. Counts of sensilla trichodea show that normal halteres contain around 20 of these bristles, whereas the haltere territory in partially transformed wings often contains over 30.

An experiment has been done to determine if the patches of haltere cells occurring in the wings of $Pc^3/+$ animals have a clonal basis. Larvae of the genotype *Dp(3;Y;1)M2,mwh*$^+$ *y cv v f* ^{36a}/+; *Dp(3;3)P5,mwh* Pc^3/*mwh ve h* [where *Dp(3;Y;1)M2* carries the wild-type allele of the cell marker *mwh* at the distal tip of the

X chromosome (Garcia-Bellido and Ripoll, 1973) and *Dp(3;3)P5* (= Dp89E1-2;90A) is a direct tandem duplication for the BX-C and surrounding material] were irradiated with 1,000 R (220 kV, 14 mA, dose rate 400 R/min) 24–48 h prior to pupation and the wings of emerging adults were examined microscopically. *Dp(3;3)P5* was used in this experiment because its presence markedly enhances the wing-to-haltere transformation in *Pc* mutant heterozygotes. Of 34 *mwh* clones (resulting from mitotic recombination in the X chromosome) located in the posterior portions of these wings, 23 included both wing and haltere tissue and none filled all of the haltere cuticle present. These observations indicate that cells within the wings of Pc^3/+ animals do not transform to haltere because of their clonal ancestry. Rather, the distribution of haltere tissue in Pc^3/+ wings shows that the position of a cell within the developing wing determines whether that cell will transform to haltere or not. This nonclonal, position-dependent transformation of wing to haltere seen in Pc^3 heterozygotes is very similar to that described for *Cbx*/+ animals by Morata (1975).

When taken together, the nonclonality of the wing-to-haltere transformation in Pc^3/+ animals and the extension of this transformation for a variable distance forward from the posterior wing margin demonstrate the existence of some kind of anterior–posterior gradient across the wing blade. Although the basis of this gradient is not clear, it seems likely that it results from the graded activity of one or more genes involved in regulating the portion of the BX-C responsible for haltere development. A good candidate for such a regulatory gene is Pc^+. This locus is implicated not only because the wing gradient is revealed in animals heterozygous for *Pc* mutants, but also because Pc^+ has been shown to be active late in development, when this gradient is observed. The gradient described here for the wing is compatible with the proposed existence (Lewis, 1978) of a Pc^+ body axis gradient in Pc^+ activity. It is not clear, however, whether the wing gradient represents a small portion of an entire body axis gradient, or whether it represents a segmentally repeated gradient like the one discovered by Locke (1959). That the BX-C genes responsible for haltere development respond to the proposed regulatory gradient in a continuously variable fashion is suggested by the frequent occurrence in Pc^3/+ wings of a transition zone containing hairs intermediate in length between those of a normal haltere and those of a normal wing (see Fig. 3b). The possibility that these intermediate hairs result from short-range nonautonomy of BX-C gene activity has not been ruled out, however.

Possible Regulation of Certain Genes in the BX-C by a Gene in or Near the Antennapedia Complex

The partial transformation of fourth to fifth abdominal segment in Pc^3/+ animals has also been studied and appears to differ in two important ways from the wing-to-haltere transformation. In the first case, unlike patches of haltere tissue in the wing, patches of fifth abdominal cuticle in the fourth abdominal segment have been found to represent clones of cells. This was shown by examining fourth abdominal tergites from males of the genotype *pwn*/+; Pc^3/+ (*pwn* is a cell marker described by Garcia-Bellido and Dapena [1974]) that were irradiated with 1,000 R (220 kV, 14 mA, 400 R/min)

Table 1 Effects of varying BX-C and ANT-C dosage on selected segmental transformations in Pc^3 heterozygous males.

Male[a] genotype	# BX-C doses	# ANT-C doses	Transformation			
			Abdominal 4 →[b] abdominal 5	Wing →[c] haltere	Leg 2 →[d] leg 1	Leg 3 →[d] leg 1
Pc^3/+	2	2	0.40	0.65	2.83	1.17
Pc^3/*Dp(3;3)P5*	3	2	0.00	1.98	5.06	1.85
Pc^3/*Df(3R)P9*	1	2	1.10	0.00	1.10	0.54
Pc^3/*Dp(3;3)D1*	2	3	0.88	0.69	2.60	0.83
Pc^3/*Df(3R)Scr*	2	1	0.00	0.81	0.81	0.04

[a] These are progeny produced by crosses of Pc^3/*TM3, Sb Ser* sisters to +/+ (Canton-S),*Dp(3;3)P5/Df(3R)P9*, or *Dp(3;3)D1/Df(3R)Scr* males. A total of 52 males of each genotype were scored

[b] Values given are average number of hemitergites per fly that show transformation (maximum possible = 2)

[c] Values given are the average rank per fly where only the more extremely affected wing of each animal was scored. Ranking assignments were: 0 = wild type; 1 = posterior wing margin slightly serrate; 2 = small amount of haltere cuticle visible at posterior wing margin; 3 = large amount of haltere cuticle present

[d] Values are average number sex comb teeth present per pair of second or third legs

24–48 h prior to pupation. Of 42 *pwn* clones found in hemitergites showing transformed patches (recognizable by presence of black pigment), none contained both transformed and untransformed tissue and seven were included entirely within transformed patches. In such cases, the outline of the *pwn* clone closely followed that of the transformed patch, indicating that this transformation has a clonal basis. In three cases, the *pwn* clone occupied only a portion of the transformed patch, indicating that, at least in these instances, the commitment to become fifth abdominal segment was made by histoblast cells in the larval or embryonic stages.

A second major way in which the fourth abdominal and the wing transformations differ in Pc^3 heterozygotes is that variations in the dosage of the BX-C have opposite effects on the two transformations. The dependence on BX-C dosage of these and of other segmental transformations is shown in Table 1. It is unlikely that the differences seen between animals carrying one, and others carrying three, doses of the BX-C are due to differences in genetic background or in culture conditions, because these animals are siblings whose fathers are from a stock maintained for many generations as *Dp(3;3)P5/Df(3R)P9*. As shown in Table 1, the transformation of wing to haltere is almost undetectable when only one dose of the BX-C is present and it is markedly enhanced by the addition of an extra BX-C dose. This effect is consistent with the idea that the wing-to-haltere transformation in Pc^3/+ animals results from a reduced amount of BX-C repressor relative to wild type. In contrast, the frequency of transformation of the fourth abdominal segment to fifth is very high in Pc^3/+ animals with only one dose of the BX-C and is reduced to almost zero when three doses are present. (Pc^+/Pc^+ animals carrying one dose of the BX-C also often show fifth abdominal cuticle in the fourth abdominal segment.)

Why the fourth abdominal and the wing transformations differ in the two ways described above is not clear. A possible explanation is suggested by the results of

experiments in which the dosage of the ANT-C has been varied in Pc^3/+ animals (see Table 1). Rearrangements used in these experiments were *Df(3R)Scr*, a deficiency for the material from 84A1 to 84B1 (Kaufman, 1978), and *Dp(3;3)D1*, a direct tandem duplication for the material between 84A and 85A (Duncan, unpublished). The same reasons described above for the BX-C dosage tests make it unlikely that the differences observed here in one and three dose animals are due to differences in genetic background or in culture conditions. These experiments show that the frequency of the fourth to fifth abdominal segment transformation is very high in animals carrying three doses of the chromosomal region containing the ANT-C and is almost zero in animals carrying only one dose. That is, this transformation is enhanced by increasing ANT-C dosage, but is suppressed by increasing BX-C dosage. Variation in ANT-C dosage has little, if any, effect on the wing-to-haltere transformation.

These observations suggest the existence in or close to the ANT-C of a positive regulator of the BX-C gene required to differentiate the fifth from the fourth abdominal segments. That this positive regulator controls at least one other BX-C gene function is suggested by the finding that Pc^+/Pc^+ animals carrying three or four doses of the ANT-C frequently show a partial transformation of the first abdominal segment to second, third, or fourth abdominal segment. If this positive regulator is under the negative control of Pc^+, then the transformed patches in the fourth abdominal segment of Pc^3/+ animals may be an indirect result of the hyperactivity of this positive regulator. Such an indirect scheme would explain why increasing BX-C dosage suppresses this transformation, and if the positive regulator were to control heritable cellular commitments, it could also explain the clonal basis of the transformation.

One problem with the above experiments is that *Dp(3;3)D1* is a large duplication that includes many genes outside of the ANT-C. That the supposed positive regulator is in or very close to the ANT-C, however, is argued by the observation that, while *Dp(3;3)D3*,Pc^3/+ [*Dp(3;3)D3* is a direct tandem duplication almost identical in extent to *Dp(3;3)D1*] animals show very frequent posteriorly directed transformations of the first abdominal segment (30/115 animals affected), $Dp(3;3)D^3$,Pc^3/*Df(3R)Scr* animals do not (3/118 affected). It appears, therefore, that the gene responsible for promoting these transformations is located within *Df(3R)Scr*, a small deficiency which removes most or all of the ANT-C as well as probably a few surrounding genes (Lewis et al., 1980a).

POSITIVE CONTROL OF THE BX-C BY *REGULATOR OF BITHORAX*

Many, perhaps all, of the genes that are under the negative control of Pc^+ appear to be positively controlled by the wild-type allele of a gene called *Regulator of bithorax* (*Rg-bx*) (Lewis, 1968), originally identified by means of two deficiencies ($Df(3R)red^{P52}$ and $Df(3R)red^{P93}$) and an apparently amorphic point mutant, *Rg-bx*, for the locus (88B). When heterozygous with wild type these mutants cause patchy, anteriorly directed transformations that correspond to loss-of-function mutants in the BX-C.

Patches of wing or of mesonotum occasionally form in the third thoracic segment, and the fifth, sixth, and seventh abdominal segments frequently show patches of cuticle transformed to the next most anterior segment. These transformations, which are opposite in direction to those caused by *Pc* mutants, suggest that *Rg-bx*$^+$ is a positive regulator of many or all of the BX-C genes. Ingham and Whittle (1980) have reported that, in addition to severe BX-C-related transformations, adults homozygous for a hypomorphic allele of *Rg-bx*, which they have named *trithorax* (*trx*), often show the development of wing or of mesonotum near the humerus and a partial transformation of first leg to second leg. Since this leg transformation is caused by certain loss-of-function mutants in the ANT-C (Lewis et al. 1980b), it seems likely that at least one gene in this complex is also under the positive control of *Rg-bx*$^+$.

Zygotes that are deficient for *Rg-bx*$^+$ (i.e., *Df(3R)red*^{P52}/*Df(3R)red*^{P93} zygotes) die primarily as late embryos that often show anteriorly directed transformations of their posterior abdominal segments. The major indicator of such transformations is the size of the denticles in the anterior rows of the ventral setal belts of these segments. In wild-type embryos, these denticles are very small on the second abdominal segment and become progressively larger as one proceeds posteriorly. In strongly affected *Rg-bx*$^-$ embryos, these denticles are fine even on the eighth abdominal segment. *Rg-bx*$^-$ embryos also frequently show, in the posterior of the eighth abdominal segment, the development of chitinous plates, which are very similar to those found in embryos lacking genes in the right-hand portion of the BX-C. Both of these transformations are considerably enhanced in *Rg-bx*$^-$ embryos that carry only one dose of the BX-C (see Fig. 4a and b), which supports the idea that they result from reduced BX-C activity. *Rg-bx*$^-$ embryos that are entirely deficient for the BX-C *(Df(3R)red*^{P52}*Df(3R)P9/Df(3R)red*^{P93}*Df(3R)P9* embryos) frequently show weak transformations of the second thoracic through the seventh abdominal segments towards the first thoracic segment, as shown by the development of midventral tuft denticles. Occasionally, a few large denticles like those that form in the main setal belt of the first thoracic segment also form in these segments. These transformations, which are similar to those caused by certain ANT-C mutants (Wakimoto and Kaufman, 1981), support the idea that *Rg-bx*$^+$ is involved in the positive regulation of the ANT-C.

CONCLUSIONS

Our working hypothesis is that the differentiation of most or all body segments is under the direct control of genes located in the BX-C and ANT-C. These genes appear to be controlled in a way such that each of them is activated only in a particular portion of the body. As a result of this spatial regulation, each body segment comes to be characterized by a unique subset of active BX-C and ANT-C genes. The activity of each of these genes causes the development of specific segmental attributes, and the final state of differentiation of any particular segment is determined by the number and kind of BX-C and ANT-C genes that are active in it. How the genes of these complexes are regulated is, therefore, a specific example of one of

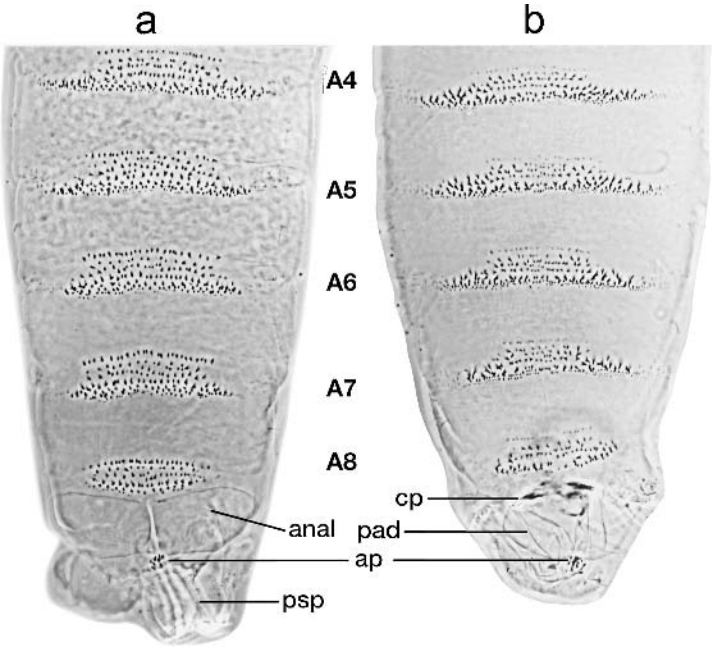

Figure 4. Segmental transformations in animals deficient for *Rg-bx*. Embryos prepared by the method of Van der Meer (1977). (a) Ventral view of the posterior abdominal segments of a wild-type (Canton-S) embryo. Shown are ventral setal belts, anal pads, anal papilla (ap) and posterior spiracles (psp). An embryo carrying only one dose of the BX-C is not figured but would be very similar, except that the anterior row of denticles on the ventral setal belts would be slightly less well developed than the corresponding row shown in (a). (b) Ventral view of the posterior abdominal segments of an embryo deficient for *Rg-bx* and carrying only one dose of the BX-C [genotype: *Df(3R)red* ^{P52}/*Df(3R)red* ^{P93} *Df(3R)P9*]. Several anteriorly directed transformations can be seen. The intensity of the anterior row of denticles in each setal belt as well as the overall shape of these belts indicates that the posterior abdominal segments are transformed towards second abdominal segments (see Fig. 2 for the morphology of the second abdominal segment of wild type). Animals of this genotype also show the development of chitinous plates (cp) in the posterior of A8, which are similar to those seen in animals deficient for the right-hand portion of the BX-C. Anal pads, anal papilla and posterior spiracles (out of the plane of focus) are present as in wild type.

the major problems in developmental biology: how genes are turned on in specific body parts and not in others. The study of Drosophila body segmentation has yielded considerable information as to how such spatial patterning of gene activity may be controlled. A number of loci that appear to regulate the positioning on the body axis of BX-C and ANT-C gene activity have been identified. One of the major loci with such a regulatory role is *Polycomb*, which, along with other similar genes, appears to be required for the normal repression of BX-C functions in anterior segments. A positive regulator, called *Regulator of bithorax*, is also known and appears to be required for the normal activation of the BX-C genes. Our current model is that the spatial pattern of BX-C and ANT-C gene expression comes about as a consequence of a gradient in

activity of one or more of these regulatory genes along the body axis. Experimental evidence supporting the existence of such a gradient is presented in this report.

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CONTROL OF BODY SEGMENT DIFFERENTIATION IN DROSOPHILA BY THE BITHORAX GENE COMPLEX

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INTRODUCTION

The bithorax complex (BX-C) in Drosophila is a giant cluster of closely linked genes that plays a major role in determining the segmentation pattern of the organism. From developmental and cytogenetic studies of this complex it now appears that orderly derepression of BX-C genes controls the orderly differentiation of body segments commencing with the thorax and proceeding posteriorly. The available evidence also suggests that the level of development which a given one of these segments achieves is a function of the particular subset of BX-C genes that is active in that segment. This paper summarizes the current status of our knowledge of what the BX-C genes do during development and how they are regulated.

GENE FUNCTION IN RELATION TO MAP LOCATION

Only a brief survey of cytogenetic correlations and mutant phenotypes will be presented since they have been described in more detail elsewhere (Lewis, 1978, 1981). The complex is restricted to two heavy doublet structures located in section 89E of the salivary gland chromosomes (Fig. 1) and includes nine genes which behave as if they code for substances involved in controlling the level of development which a segment achieves. In general, mutations of these genes act as partial or complete losses

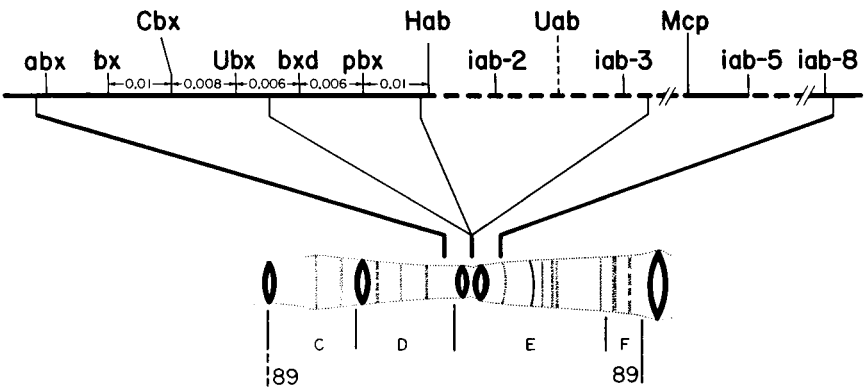

Figure 1. Correlation of genetic and cytological maps of the bithorax complex. *abx* = anterobithorax; *bx* = bithorax; *Cbx* = Contrabithorax; *Ubx* = Ultrabithorax; *bxd* = bithoraxoid; *pbx* = postbithorax; *Hab* = Hyperabdominal; *iab* = infraabdominal; *Uab* = Ultraabdominal; *Mcp* = Miscadastral pigmentation. Gene order within dashed portions of the genetic map has not been determined except that *iab-2* lies to the left of *Mcp* and to the right of *bxd*. The locus of *Uab* is based on an analysis of a translocation having a breakage point which appears to separate *iab-2* from *iab-3*; namely, T(1;3)Ultraabdominal-5 (Lewis, 1978). The *iab-3* gene is inferred to exist on the basis of the behavior of overlapping deficiencies, dominant effects of Ultraabdominal-5, and recessive effects of Ultraabdominal-4; however, no mutations of *iab-3* unassociated wtih chromosomal rearrangement are known. See Lewis (1978, 1981) for a more detailed account of cytogenetic correlations. Map units are in centimorgans. See Kuhn et al. (1981) for a description of the *iab-2* mutant.

of function and are recessive. Although the ultrabithorax (*Ubx*) mutant can be scored as a dominant mutation it is essentially a loss-of-function mutant, acts as a recessive lethal, and is recessive in the presence of two doses of the wild-type complex. In addition, at least four *cis*-regulatory regions can be recognized by means of certain dominant gain-of-function mutants. In Fig. 1 the symbols for the loci of these latter mutants are raised above those of the nine recessive loss-of-function loci.

The exoskeleton of the late embryo, larva, and adult Drosophila shows a wealth of cuticular detail that enables each of the thoracic and abdominal segments to be characterized in a unique manner (Fig. 2). As a result the degree to which one segment can be transformed towards another can be analyzed in terms of sets of morphologically defined limits or endpoints. In this way it has been found that BX-C mutants of the recessive loss-of-function type transform structures within a given segment towards homologous structures in a more anterior segment. On the other hand, mutants of the dominant gain-of-function type transform structures of one segment towards homologous ones in a more posterior segment. For example, wing and haltere are homologous, yet widely divergent, organs that normally arise as dorsal appendages of the second thoracic (T2) and third thoracic (T3) segments, respectively (Fig. 2). The recessive bithorax mutants transform halteres in the direction of wings, while the dominant Contrabithorax mutant transforms wings towards halteres. When mutants of BX-C are described in this way, a considerable degree of genetic

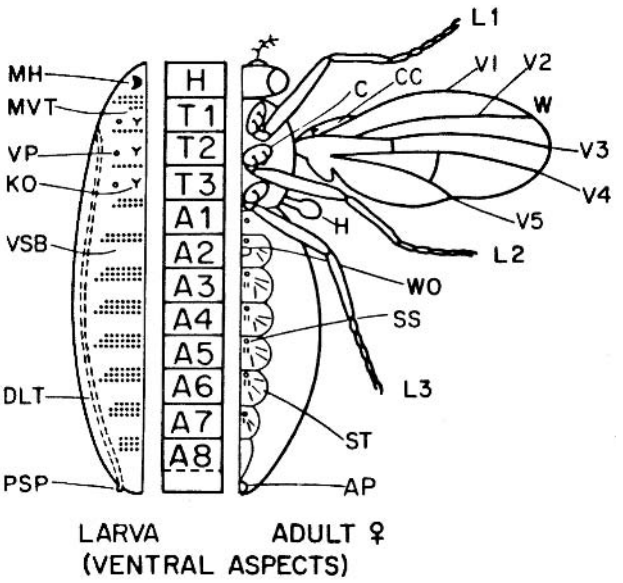

Figure 2. Comparison of the ventral cuticular pattern of the first-instar larval (or late embryonic) stage with that of the adult stage. MH = mandibular hooks; MVT = midventral tuft; VP = ventral pits; KO = Keilin's organ; VSB = ventral setal belts; DLT = dorsal longitudinal (tracheal) trunk; PSP = posterior spiracle; H = head; T = thoracic; A = abdominal; L = leg; W = wing; H = haltere; C = coxa; CC = costal cell (of wing); V = vein; WO = Wheeler's organ; SS = sensillum (on segments A1–A7, inclusive); ST = sternite; AP = anal plate. In the late embryo or first-instar larva, the dorsal tracheal trunk terminates in an incipient anterior spiracle near the boundary of the first and second thoracic segments. In later instars, a visible anterior spiracle would be present at this point. Special sense organs on the three thoracic segments of the larval stage and on the first seven abdominal segments of the adult are depicted grossly enlarged. Additional abdominal segments beyond the eighth abdominal are greatly reduced and not well delineated but are believed to include such structures as the anal plates (Schupbach et al., 1978). See Lohs-Schardin et al. (1979) for a more detailed account of larval cuticular patterns.

complexity has been found to underlie even adjacent intersegmental transformations. The first indication that more than one gene of the complex is needed to bring about such a transformation was the recognition (Lewis, 1951) that anterior and posterior portions of segments T2 and T3 can develop virtually independent of one another with respect to whether they will become wing-like or haltere-like. Thus, bithorax mutants, especially the extreme allele of Stern, bithorax-3, transform anterior T3 and postbithorax (*pbx*) transforms posterior T3 towards the corresponding regions of T2. With respect to transformation of haltere into wing, the boundary between anterior and posterior portions was found to lie approximately along the fourth vein (V4 in Fig. 2). The double mutant, bithorax-3 postbithorax, was then constructed by recombination and was found to give a four-winged fly with not quite complete transformation of T3 towards that of T2 (figured in Lewis, 1963, 1964).

Further genetic complexity underlying the transformation of T3 into T2 was suggested by the failure of the bithorax-3 homozygote to have the extreme anterior portion of T3 fully transformed to the corresponding, or presutural, region of T2. Paradoxically, the bithorax-3 mutant also causes the presutural region of T2 to be slightly underdeveloped as well; however, this effect of bithorax-3 was found to act as a dominant gain of function. In other words, bithorax-3 acts as if it inactivates the wild-type allele of bithorax but causes an over-activity of the wild-type allele of another gene whose function would be to prevent a presutural region of the T2-type arising in T3. A possible candidate for a mutant of the latter gene was an X-ray induced mutant, anterobithorax (*abx*) (formerly bithorax-7; Lewis, 1978).

Three features of anterobithorax suggested that a hitherto unrecognized genetic locus might be present in the complex. First, although the anterobithorax homozygote has a highly variable and often asymmetrically expressed transformation of anterior T3 towards anterior T2, a well-developed presutural area of the T2 type frequently arises in T3 even when the latter segment is otherwise not well transformed; by contrast, bithorax-3 homozygotes show a uniformly extreme, symmetrically expressed, transformation of anterior T3 towards anterior T2 except, as already noted, for a somewhat underdeveloped presutural region. Secondly, anterobithorax and bithorax-3 partially complement each other. Finally, abx/bx^3 heterozygotes have a more extreme bithorax phenotype in the presence of structural heterozygosity for chromosomal rearrangements that presumably disrupt somatic pairing proximal to BX-C. This phenomenon of transvection was first found in the case of *trans* heterozygotes involving bithorax mutants and Ultrabithorax (Lewis, 1954). A recombination analysis has shown that *abx* maps extremely close to the left of the bithorax-3 locus (approximately 0.01 centimorgan on the basis of one wild-type and one reciprocal double-mutant crossover). The triple mutant, anterobithorax bithorax-3 postbithorax, when homozygous or hemizygous, produces a four-winged fly in which externally the third thoracic segment is now virtually identical in both size as well as structure with the second segment (Fig. 3). In particular, triple mutant homozygotes, and especially hemizygotes, have a much more nearly complete transformation of T3 into T2 in the costal cell (Fig. 2) and presutural regions than do the corresponding bithorax-3 postbithorax genotypes. The transformation is so extreme that many but not all of the animals fail to emerge from the puparium. Internally, patches of muscles are found that are not normally found in T3 of wild type or of bithorax-3 postbithorax homozygotes.

Many of the mutants of the BX-C are remarkable and possibly unique amongst known eukaryotic loci in exerting strongly polarized *cis–trans* position effects that may spread over several genes. Thus anterobithorax, like bithorax-3, shows a weak effect of this kind that extends to the postbithorax locus in that *abx/pbx* animals have a tiny flap of wing-like tissue in the posterior compartment of the haltere that is lacking in *abx pbx*/+ + animals. This *cis–trans* effect will be referred to here as a *cis*-polar effect of anterobithorax on the wild-type postbithorax gene located *cis* to anterobithorax. The effect is so weak, however, that in anterobithorax or bithorax-3 homozygotes the

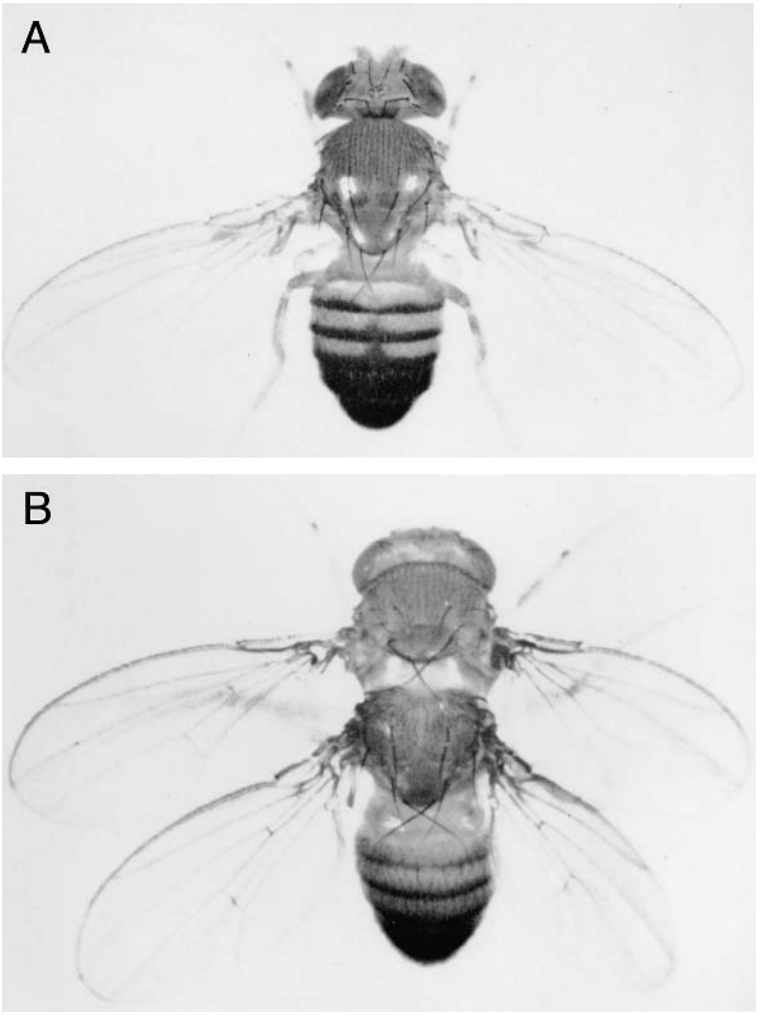

Figure 3. Two wings vs. four. The male shown in A is wild type except that it is homozygous for a recessive mutant, heldout, which spreads the wings and allows the halteres and abdominal segments to be readily observed. B is a hemizygote for the triple mutant, anterobithorax bithorax-3 and postbithorax.

mutant transformation is confined to anterior T3. Similar *cis*-polar effects are exerted in all combinations involving anterobithorax, bithorax and Ultrabithorax mutants. As a result it is not possible in these cases to dissect the individual contributions of the mutants to the transformation of T3 to T2.

Studies of homozygotes for the Ultrabithorax mutant suggest that the Ultrabithorax gene may act earlier in development than do the anterobithorax, bithorax and

postbithorax genes. Thus, the Ultrabithorax homozygote has the tracheal system of the late embryo and larva profoundly modified (Lewis, 1951), in contrast to a virtual lack of tracheal system effects in the case of homozygotes for anterobithorax, bithorax or postbithorax mutants or combinations thereof. Externally, an extra pair of spiracles of the type normally found in wild type only on T2 (Fig. 2) occur on T3 (and A1) (figured in Lewis, 1978). One caveat, however, is that Ultrabithorax, in contrast to anterobithorax, bithorax and postbithorax, has strong *cis*-polar effects on the adjoining wild-type bithoraxoid (*bxd*) gene which evidently is responsible for the thoracic-like transformation of a spiracle in A1. Double mutant homozygotes involving bithorax-3 and bithoraxoid have much less well-developed spiracles in T3 (and A1) than do Ultrabithorax homozygotes. Therefore, whether the spiracle transformations seen in the latter homozygotes is an effect of the Ultrabithorax mutant gene *per se*, or whether it arises from joint *cis*-polar effects of Ultrabithorax on the adjoining wild-type alleles of anterobithorax, bithorax, bithoraxoid, and postbithorax, cannot be determined. In any case, at least four genes of the complex are involved in the mutant transformation of T3 towards T2.

Although the Ultrabithorax homozygote is lethal in the late larval or pupal stage, homozygous Ultrabithorax adult cuticular tissue in T3 has been studied by the gynandromorph method and found to exhibit an extreme T2-like transformation whether it arises in the anterior or posterior portions of T3. However, in the case of certain weaker alleles of Ultrabithorax, such as Ultrabithorax-61d of Gloor (Lewis, 1981), there is an indication that the transformation from T3 towards T2 of the appendage portion of the segment is partially independent of the transformation of the thoracic tissue itself. Thus, the Ultrabithorax-61d homozygote, which survives to the adult stage, has small wing-like halteres but completely lacks notal tissue. By contrast, the homozygote for bithorax-34e has a conspicuous band of notal tissue but lacks wing-like transformation of the halteres, except for a swollen bristled appearance of those organs. (Ultrabithorax-61d when homozygous also has weak transformation of posterior T3 towards posterior T2 as well as a weak thoracic modification of A1.) Similar evidence for semi-independent control of appendage and thorax proper is provided by certain *cis*-regulatory mutants, as will be discussed below.

Mention should be made at this point of an important new finding by Morata and Kerridge (1981) suggesting further complexity at a morphological level exerted by the Ultrabithorax region. By somatic mosaic analysis, they demonstrate that homozygous Ultrabithorax tissue, when generated early in embryonic development (but not later on), produces in the posterior portion of the second pair of legs a transformation towards the corresponding portion of the first pair of legs. Whether this transformation is the result of a *cis*-polar effect of Ultrabithorax on an adjoining locus, which they tentatively suggest be called postprothorax (*ppx*), or whether it is an attribute of the Ultrabithorax mutant *per se* must await further dissection of this region of the complex at a molecular and genetic level.

Another possible morphological component of the transformation of T3 to T2 is the development of a humerus-like patch of tissue which arises ectopically on the

Figure 4. A recessive loss-of-function phentoype. Lateral view of the thorax, first abdominal segment and part of the second abdominal segments of an anterobithorax fly of genotype: *Df*(3*R*)*red-P*52, *abx*/*In*(3*R*)*Hu, Hu Ubx*. The deficiency-bearing chromosome, which is of maternal origin is deleted for the Regulator-of-bithorax gene. Note that an ectopic humerus (*arrow*) appears on the postnotum (PN) of the second thoracic segment and carries extra bristles. Such bristles appear only in the presence of the dominant humeral mutant (*Hu*) and are specific to, and visible on, the humerus (HU) in its normal location at the anterior lateral edge of the thorax. The normal wing (W) has been cut off near the base. The third thoracic segment has the notum (N_3) partially transformed towards that of T2 (N_2), and the anterior portion of the haltere (H) is transformed towards the wing.

posterior portion of T2 in the postnotal area near the boundary with T3 (Fig. 4). Such patches were first found on rare occasions as patches of homozygous Ultrabithorax tissue in gynandromorphs (figured in Lewis, 1963, 1964). Such patches were later found in an *abx*/*Ubx* heterozygote in which the anterobithorax chromosome was maternally derived and also carried a Deficiency (3R) red-P52. The latter rearrangement is a deficiency for approximately two bands in 88B and includes the locus of a positive regulator of bithorax, *Rg-bx* (Lewis, 1968). That dominant enhancement of BX-C mutants is a maternal effect of *Rg-bx* was later shown by Garcia-Bellido and Capdevilla (1978), so that it was a fortunate accident that the deficiency had been introduced maternally. To test the possibility that the ectopic patch was of humeral origin, the dominant Humeral (*Hu*) mutant (see Lindsley and Grell,

1968) was introduced into an Ultrabithorax chromosome. This mutant produces a reduplication of macrochaetes on the humeral regions (epaulet-like regions in the extreme anterior dorso-lateral regions of the Dipteran thorax) but not on any other portions of the body. A genotype was therefore constructed consisting of a maternally derived chromosome, containing the *red-P52* deficiency and anterobithorax, and a paternally derived *Hu Ubx* chromosome. Several animals of this genotype produced extra macrochaetes on the ectopic humeral-like tissue. An example is shown in Fig. 4.

The presence of an ectopic humerus in these special *abx/Ubx* genotypes would not be totally unexpected. It has long been known that the extra spiracles which arise in homozygous Ultrabithorax larvae have imaginal discs organized around them, as do the normal anterior spiracles in T2, whose imaginal discs are known to give rise to the normal adult humeral regions, as first demonstrated microsurgically by Zalokar (1945). Whether the ectopic humeral-like regions of the *abx/Ubx* and *Ubx/Ubx* genotypes represent functions specific to Ultrabithorax, or to anterobithorax, or to perhaps other genes of the complex, cannot be determined at this stage of the analysis.

CIS-REGULATION OF THE BITHORAX COMPLEX

The action of *cis*-regulatory mutants of BX-C is best illustrated by the Contrabithorax mutant. It has been shown to produce a strong T3-like transformation of T2 only when *cis* to the wild-type alleles of bithorax and Ultrabithorax (Lewis, 1955). Additional mutants somewhat resembling contrabithorax were subsequently found: Contrabithorax-2 of Kreber; Contrabithorax-3 of Akam; and Haltere-mimic (*Hm*) of Slatis (the latter being described in Lindsley and Grell, 1968). All but Contrabithorax are associated with visible chromosomal rearrangements. Haltere-mimic is a complex translocation between the second and third chromosomes (the probable new order being 2L tip to 29/88F to 3L tip; 3R tip to 89E3-4/32 to 29/89E1-2 to 88F/32 to 2R tip); Contrabithorax-2 is an inversion (89E/91C-E). Each of these two rearrangements is accompanied by recessive bithoraxoid and postbithorax position effects that typify rearrangements which have the Ultrabithorax and bithoraxoid loci separated from one another (see review of such cases in Lewis, 1981). Contrabithorax-3 is also associated with an inversion (89A/89E) in which the break in 89E occurs either just proximal to, or within, the first 89E doublet; this mutant is not associated with detectable position effects on bithoraxoid or postbithorax. The phenotypic effects of these four mutants on specified structures in anterior and posterior compartments of the second thoracic segment are summarized in Table 1 using a rough grading system.

Although the original Contrabithorax transforms both anterior and posterior regions of T2 towards the corresponding regions of T3, it is only the posterior portion that is strongly and uniformly transformed. The anterior portion is variably and rather weakly transformed in heterozygous Contrabithorax animals; however, it is more strongly but still variably transformed in homozygous Contrabithorax animals. In striking contrast, heterozygotes for Contrabithorax-3 have a variable and extreme transformation of the anterior portion of T2 towards T3, especially the presutural

Table 1

Mutant genotype	Thorax (notum) A	Thorax (postnotum) P	Wing A	Wing P	Leg (coxa) A	Leg (tarsus) P
$Cbx^3/+$	0 to ++++	0	0 to ++++	0	0 to ++	0
$Cbx/+$	0 to +	+ to ++	0 to +	+++	0 to +	0
$Cbx^2/+$	+	+ to ++	++	++	0	++
$Hm/+$	0	0	+++	++	0	0

Grades: 0 = no transformation, hence, wild type. + = slight, + + = moderate, +++ = strong, and ++++ = virtually complete transformations from T2 towards corresponding T3 structures.

area which is often the first region to be transformed; while the posterior portion remains untransformed or is T2-like. It is as if Contrabithorax activates in *cis* the wild-type alleles of postbithorax, and/or Ultrabithorax, and possibly those of bithorax and anterobithorax, as well; while Contrabithorax-3 acts as if it activates in *cis* the corresponding wild-type allele of anterobithorax, and possibly those of bithorax and Ultrabithorax, but not the wild-type allele of postbithorax.

The Haltere-mimic mutant is particularly striking and unique in that its Contrabithorax mutant effects are exclusively confined to the wing. Thus, in *Hm*/+ animals the wings resemble halteres, except that the extreme proximal portion of the organ remains partially wing-like in both anterior and posterior portions. Although lethal when homozygous, two doses of Haltere-mimic in the presence of one dose of the wild-type allele, result in flies which have virtually perfect halteres on T2 except for a few bristles on the stalk of the haltere (Fig. 5). It is as if there is a gene in BX-C which when activated in T2 can transform the dorsal appendage of the thorax and not other adult cuticular structures.

The Contrabithorax-2 mutant is unique in that it moderately and uniformly transforms anterior and posterior portions of the thorax proper as well as the haltere. Particularly interesting is the presence in Contrabithorax-2 heterozygotes of a "brush" on the proximal tarsal segment of the second (T2) pair of legs. Since such a structure is normally only found on the third (T3) pair of legs, this transformation indicates that the posterior portion of T2 is transformed towards T3. Such a transformation fails to occur, however, not only in Contrabithorax-1 heterozygotes, but also in Contrabithorax-1 homozygotes even though the latter show a much stronger transformation of the posterior portion of T2 dorsally in the wing than does the Contrabithorax-2 heterozygote. Thus, in bringing about a transformation of T2 towards T3, Contrabithorax-1 operates in the anterior, but not the posterior, portion of the second leg, while Contrabithorax-2 operates in the posterior, but not the anterior, portion of that leg. Apparently these two dominant gain-of-function mutants differ qualitatively either because each operates on a different functional unit of the complex, or because they operate differentially on the same functional unit.

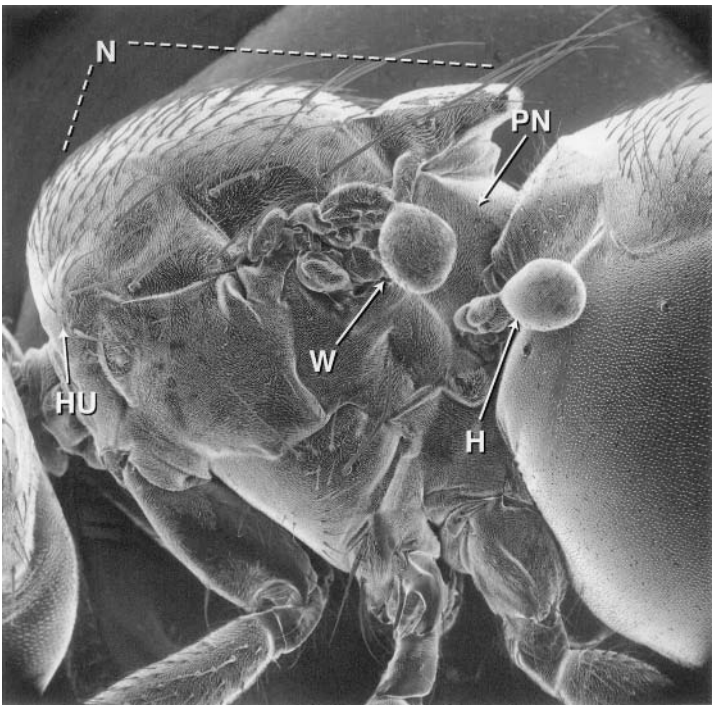

Figure 5. An extreme dominant gain-of-function phenotype. Lateral view of the thorax and first and second abdominal segments of a fly having two doses of the Haltere-mimic mutant and one dose of wild type. Genotype: *Dp(Y;2;3)Hm/T(2;3)Hm/+*. Note that the wing (W) is transformed into a structure which is similar to, but slightly larger than that of, the haltere (H). Some wing bristles remain on the stalk of the haltere-wing. The haltere itself is somewhat smaller than that of wild type and the distal segment, or capitellum, is often greatly reduced. The notum (N) and postnotum (PN) are also derived from the dorsal second thoracic segment but appear wild type; that is, are not affected by Hm. The humerus (HU), of uncertain segmental origin, is also unaffected by Hm.

The possibility that in the *Hm/+* genotype Haltere-mimic causes a dominant gain of function of the wild-type postbithorax gene in *trans* is ruled out by studies of *Hm/pbx* and *Dp,Hm/pbx/pbx* animals. The latter two genotypes fail to have the extreme wing-like transformation of the posterior haltere that characterizes postbithorax homozygotes or heterozygotes involving postbithorax and a rearrangement with a position effect on postbithorax. Instead *Hm/pbx* and *Dp,Hm/pbx/pbx* show only a residue of wing-like tissue in the posterior portion of the haltere in T3; in the posterior portion of T2 the appendage remains strongly haltere-like and comparable to that seen in *Hm/+*. (Not unexpectedly, *Hm/pbx* and *Dp,Hm/pbx/pbx* animals have a strong postnotal development of the posterior portion of T3 towards T2 in the thorax proper, as opposed to the haltere; thus, such heterozygotes have a wide band of postnotal tissue similar to that seen in postbithorax homozygotes

or heterozygotes of postbithorax and a rearrangement with a position effect on posibithorax.)

Finally, the Haltere-mimic hemizygotes (*Hm/Df-P9*, where *Df-P9* is a deficiency for the entire complex) have a strong transformation of the anterior and posterior portions of the wing blade towards haltere tissue, very similar to the dominant gain-of-function transformation effected in *Hm/+* and *Hm/pbx* wings. (In addition, such hemizygotes, which tend to die as pharate adults, show recessive loss-of-function transformations of two types expected from the postbithorax and bithoraxoid position effects accompanying Haltere-mimic; namely, a posterior transformation of the thorax of T3 towards that of T2, as already described for *Hm/pbx*, and a thoracic modification of the first-abdominal segment.) Since the hemizygote has by definition no BX-C genes in *trans* to Haltere-mimic, little if any of the dominant phenotype of *Hm/+* can be attributed to a *trans*-dominant effect of Haltere-mimic on BX-C genes. Similar kinds of analyses indicate that the Contrabithorax-2 mutant also fails to exert a *trans*-dominant effect.

It remains, then, to consider what gene or genes within BX-C undergo *cis*-dominant regulation when next to Haltere-mimic, or to Contrabithorax-2. As already noted, because of chromosomal rearrangements associated with these mutants, neither appears to have a functioning wild-type postbithorax gene distal to (namely, in Fig. 1, to the right of) the *bxd* locus. Yet, these mutants clearly transform specific portions of the posterior portion of the wing towards haltere, and in the heterozygote with postbithorax each exerts suppression in the haltere of much of the postbithorax type of transformation of that organ. Hence, Haltere-mimic and Contrabithorax-2 each behaves as if it exerts a *cis*-dominant effect on one or more genes proximal to the *bxd* locus. One possibility is that there is a gene (or genes) in the latter region of the complex which can act under certain conditions as if it has a similar function to that of the wild-type postbithorax gene; it might even be an existing gene, such as bithorax or Ultrabithorax. Another possibility is that Haltere-mimic and Contrabithorax-2 each arose with an invisible transposition of the wild-type allele of postbithorax to the proximal region where it now is abnormally regulated. This latter possibility would be identical to that invoked (Lewis, 1968) as one possible explanation for the dominant gain of function of the Contrabithorax-1 mutant.

TRANSVECTION WITHIN THE BITHORAX COMPLEX

Early in the analysis of the BX-C certain pairs of partially complementing mutants were found to exhibit a unique type of cooperative interaction across homologous chromosomes. This phenomenon of transvection (Lewis, 1954) has also been detected at the cytological level in certain cases of puffing of salivary gland chromosome bands (Ashburner, 1967); it has also been detected between the zeste and white loci in the X chromosome (Gelbart, 1971; Jack and Judd, 1979). An example of transvection is illustrated in Fig. 6. A *trans*-heterozygote between Ultrabithorax and postbithorax-2 shows a weak postbithorax phenotype (Fig. 6A) when the two homologous third

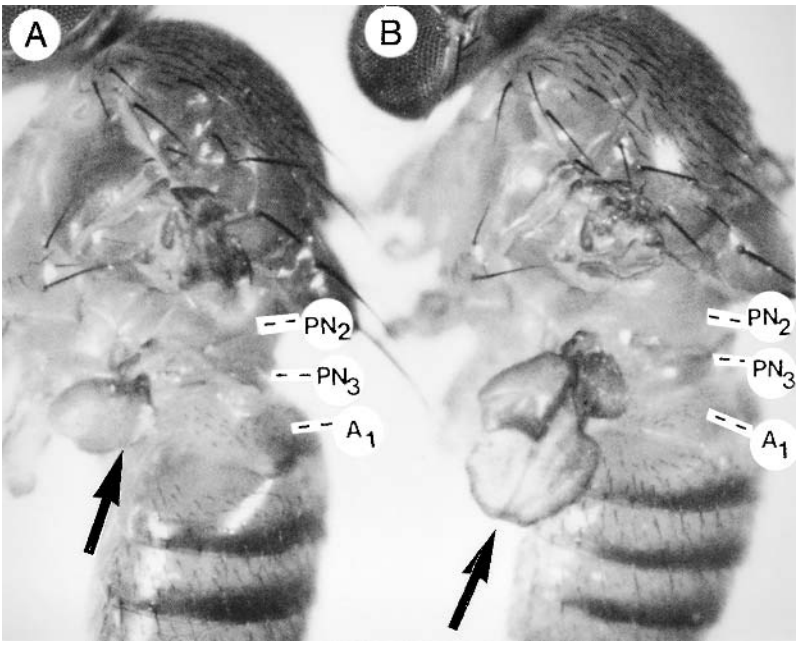

Figure 6. Transvection of the postbithorax phenotype. Lateral views of the third thoracic and surrounding segments of female *trans* heterozygotes for postbithorax-2 and Ultrabithorax. A. A genotype having structurally normal third chromosomes shows only a relatively weak postbithorax phenotype. The arrow points to a small patch of wing-like tissue in the posterior haltere. Only a rudimentary third thoracic postnotum (PN_3) is present between the second thoracic postnotum (PN_2) and the first abdominal tergite (A_1). B. The same genotype as that shown in A except that it is heterozygous for a translocation, P36 (see text for description), which results in a greatly enhanced postbithorax phenotype. Note the wing-like transformation of the posterior portion of the haltere (*arrow*) and the large postnotum on the third thoracic segment. The two specimens shown were reared under similar cultures conditions at 25°C. Legs and wings were removed under etherization and the specimens photographed together.

chromosomes are free of chromosomal rearrangements. In the presence of certain rearrangements that are expected to disrupt pairing proximal to the complex, the postbithorax phenotype becomes greatly intensified (Fig. 6B).

Although the underlying basis of transvection is unknown, it may be useful at this time to examine the degree to which the phenomenon shows locus or allele specificities. Curiously, the *trans* heterozygote involving Ultrabithorax and postbithorax-1 does not show a detectable transvection effect; in this case, both structurally homozygous and structurally heterozygous genotypes have a very extreme postbithorax phenotype. Nor do the *trans*-heterozygotes, *Ubx/bxd* and pbx^1/bxd exhibit the phenomenon. It came, therefore, as a surprise that the postbithorax-2 mutant (Lewis, 1981) showed transvection when tested against bithoraxoid as well as against Ultrabithorax. Thus, when structurally homozygous, bxd/pbx^2 is virtually wild-type except for a weak and variable postbithorax transformation of the posterior

haltere; while, when structurally heterozygous (for the *P36* translocation, or for two other tested rearrangements of the *P36* type), it has a moderate, only slightly variable, postbithorax transformation of T3, resembling but somewhat less extreme than that shown in Fig. 6B.

In the early analyses (Lewis, 1954, 1955) most of the mutants which showed transvection were of spontaneous origin. However, both postbithorax and postbithorax-2 are of X-ray origin. Moreover, the latter mutants each exhibit transvection when in *trans* with the spontaneous bithorax-3 or the X-ray induced anterobithorax; while only postbithorax-2 exhibits transvection, as already noted, with the spontaneous Ultrabithorax-1 and bithoraxoid mutants. Numerous chemically induced (ethyl methane sulfonate) mutants which closely resemble Ultrabithorax-1 in their phenotypic interactions with other mutants of the complex behave like that mutant in transvection tests.

The occurrence of transvection between a pair of mutants may be indicative of their representing separate functional units. Thus, mutants which act like alleles of the same functional unit (namely, give a phenotype in *trans* intermediate between that of the two homozygotes, as opposed to giving a partially (or fully) complementing phenotype) have failed to give evidence of a transvection effect. For example, the *trans* heterozygote involving bithorax-34e and bithorax-3 has an intermediate phenotype that is not further intensified by structural heterozygosity for rearrangements (such as the *P36* translocation). Yet, such rearrangements exert strong transvection effects on *trans* heterozygotes involving either bithorax-34e, or bithorax-3, with Ultrabithorax (Lewis, 1954). It was, as already noted, the occurrence of transvection, as well as partial complementation, in the case of anterobithorax and bithorax-3 that led to the recognition that these mutants occupy separate loci.

RULES GOVERNING THE REGULATION OF BX-C GENES

A new dimension was added to the analysis of BX-C when it was recognized that genotypes lacking portions or all of the complex survive until the end of embryonic life, at which point in development patterns of cuticular and tracheal structures (Fig. 2) can be readily scored in whole mounts. When such genotypes are examined in this way a number of rules governing the behavior of BX-C genes are apparent: (1) a gene active in a given segment is active in all segments posterior thereto; (2) the more posterior is the segment in the animal, the greater the number of genes that become active in that segment; and (3) the more proximal is a gene in the complex, the more likely it is to be active. Rules (1) and (2) apply to all segments commencing with the third thoracic and extending posteriorly to the seventh abdominal. (The eighth abdominal segment on the basis of recent unpublished evidence, cited in Duncan and Lewis (1982), appears anomalous in that genes in the proximal portion of the complex may be inactive at least in the posterior portion of the segment). Rule (3) applies to all genes which can be studied in late embryonic stages; to the limited extent that it can be examined in adult stages using recessive mutant genes, the rule remains applicable

except that the postbithorax locus would have been expected to be proximal, not distal, to that of bithoraxoid.

Two types of gradients have been invoked (Lewis, 1978) to explain the pattern of segmental differentiation which arises in the wild-type organism. The first is an antero-posterior gradient along the organism in concentration of one or more repressor-like molecules elaborated by *trans*-regulatory genes. Two major genes of the latter type have been characterized thus far for their effects on the BX-C genes; namely, Polycomb (Lewis, 1947, 1978; Duncan and Lewis, 1982) in the left arm of the third chromosome, and extra sex combs (*esc*) (Struhl, 1981) in the left arm of the second chromosome.

A second type of gradient has been invoked to explain why it is that within a given segment some genes appear to be repressed and others derepressed in a definite pattern. Thus, Rule (3) can be understood if there exists a proximodistal gradient along the chromosome in the affinity of each gene's *cis*-regulatory region for repressor substance(s), such that the lowest affinity occurs in the most proximal region, and the highest in the most distal region. On this concept, the behavior of a given dominant gain-of-function mutant can be interpreted as an alteration in a *cis*-regulatory region that lowers the affinity of that region for repressor. The role of another *trans*-regulatory gene, Regulator of bithorax, already cited above, may be to produce either a positive regulator or an inducer-like substance. Recently, Ingham and Whittle (1981) have shown that the "trithorax" mutant exerts profound maternal effects on BX-C gene functions and it is presumably a recessive allele of the Regulator of bithorax.

THE ROLE OF THE BX-C GENES IN DEVELOPMENT

It would be premature to speculate on the specific mode of action of the BX-C genes during development. In a formal sense, each gene (in the case of the bithoraxoid and infraabdominal genes) presumably produces a specific substance that tends to "abdominalize" structures that would otherwise remain at a thoracic level of development. In this view, the greater the number of BX-C genes that are derepressed in a given segment the more posterior is the type of abdominal segment that arises and the more structures within a segment that become abdominalized. The actual state of differentiation of a segment would then depend upon the particular subset of BX-C genes that are derepressed in that segment.

SUMMARY

The BX-C complex is a gene cluster whose function is to regulate still other genes in such a way that each segment of the body commencing with the second thoracic segment and proceeding posteriorly, develops a unique pattern of structures. Numerous *cis*-regulatory regions have been identified within the complex and are believed to bind to repressor-like substances elaborated by the wild-type alleles of at least two major *trans*-regulatory genes, Polycomb and extra sex combs. The more

proximal is a gene in the complex (with the exception of postbithorax) the more likely it is to be derepressed; the more posterior is a segment in the organism, the greater is the number of BX-C genes that become derepressed in that segment. A gradient in repressor substance(s) and a gradient in affinity of the *cis*-regulatory regions for repressor can account in a formal sense for the regulation of the BX-C genes themselves.

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REGULATION OF THE GENES OF THE BITHORAX COMPLEX IN *DROSOPHILA*

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The bithorax complex (BX-C) is a set of master control genes that regulates other genes outside the complex to program much of the development of the thorax and abdomen of the fly. The emphasis in this paper will be on how the genes of the complex are themselves regulated, rather than on how they regulate other genes.

The BX-C is regulated in *cis* and in *trans*. Only a brief review of its regulation by *trans*-acting loci will be given, since our primary concern in this paper is to identify the rules that govern regulation in *cis* and to outline a model for *cis*-regulation based on these rules.

Regulation of the BX-C in *trans* appears to be chiefly negative in the sense that loss-of-function alleles at several loci outside the complex are known to result in derepression of BX-C genes in regions of the body where the latter are normally repressed. Examples are Polycomb, *Pc* (Lewis, 1978; Duncan and Lewis, 1981; Denell and Frederick, 1983); Polycomb-like, *Pcl* (Duncan, 1982); extra sex combs, *esc* (Struhl, 1981); super sex combs, *sxc* (Ingham, 1984); and polyhomeotic, *ph* (Dura et al., 1985).

Only one gene outside the BX-C has been found to act as a positive regulator of the complex in the sense that loss-of-function alleles fail to activate the wild-type functions of BX-C. For example, animals heterozygous for a dominant Regulator-of-bithorax (*Rg-bx*), have slightly reduced pigmentation of the tergite of the fifth abdominal segment (A5); a similar phenotype is seen in deficiencies, which include *Rg-bx* and

are deleted for several bands in 88B, namely, *Df(3)red^{P52}*/+ and *Df(3)red^{P93}*/+ animals (see Lewis, 1968, 1982). Garcia-Bellido and Capdevila (1978) and Capdevila and Garcia-Bellido (1981) have shown that *Rg-bx* acts maternally, as well as zygotically, in repressing BX-C genes. A recessive mutant, trithorax (*trx*), has been shown by Ingham and Whittle (1980) and Ingham (1981) to have strong maternal and zygotic repression of many gene functions of the BX-C and to fail to complement with *Rg-bx* or with the two small red deficiencies in 88B cited above. Thus, *Rg-bx* and *trx* evidently occupy a single locus that codes for a regulatory product; *trx* might have inducer-like properties or it could operate as an activator of the BX-C genes. However, Ingham (1983) has shown that the BX-C genes are still differentially expressed in animals that are doubly mutant for *trx* and *esc*.

In the egg, a gradient in the concentration of an inducer (Lewis, 1963) or of a repressor (Lewis, 1978) has been invoked to account for sequential derepression of the BX-C genes. Specifically, in the latter case, an antero-posterior gradient in the concentration of repressor along the body axis is assumed to be coupled with a proximo-distal gradient along the chromosome in the affinities for repressor of *cis*-regulatory elements of the BX-C genes. The proximo-distal gradient has been proposed to account for the preliminary finding that the order of the genes in the chromosome is colinear with the order of their expression along the body axis of the organism. Evidence that this type of colinearity extends to the distal half of the complex is reviewed below.

The remainder of this paper deals with *cis*-regulation of the BX-C. Two highly unusual genetic phenomena have been shown to characterize such regulation in the proximal portion of the complex: (1) cisvection, or *cis*-inactivation of one gene function by a mutant allele of a neighboring gene; and (2) transvection, or synapsis-dependent complementation of neighboring gene functions (for review, see Lewis, 1955, 1982). In this paper evidence is summarized which indicates that cisvection and transvection also occur in the distal portion of the BX-C.

METHODS

Two genetic screens have been devised for the purpose of identifying as many as possible of the functional units within the BX-C. The first, or MCP screen, involves searching for revertants of the dominant gain-of-function mutant Miscadastral pigmentation (*Mcp*). The *Mcp* phenotype consists of a strong transformation of the fourth abdominal segment (A4) toward the fifth abdominal segment (A5) and is readily scored in males as they have solid black pigmentation on A4, A5, and A6, whereas wild-type males have such pigmentation only on A5 and A6. M. Crosby, who found and mapped *Mcp* (see Lewis, 1978), showed that several types of revertants can be induced, one of which results from a loss of function in an adjoining infraabdominal-5 (*iab-5*) gene. In this latter case, the revertant proved to be a second mutant, *iab-5^{C7}*, very close to the right of *Mcp* (M. Crosby, unpublished); this and several other revertants have been mapped by molecular methods (Karch et al., 1985). To

apply the MCP screen, one mutagenizes *Mcp* homozygotes (either males or females, as desired) and mates to wild type. The F_1 males are then scored for partial or complete loss of the male-type pigmentation on A4.

The second, or global rearrangement (GR) screen, is designed to detect virtually all gross chromosomal rearrangements that have one breakage point within the BX-C. This screen employs a *cis*-arrangement of two dominant mutants, Contrabithorax (*Cbx*), a dominant gain-of-function mutant that transforms the second thoracic segment (T2) toward the third thoracic segment (T3), and Ultrabithorax (*Ubx*), a dominant loss-of-function mutant that transforms T3 toward T2. The *cis*-heterozygote, *Cbx Ubx*/+ +, has nearly, but never quite entirely, complete suppression of the *Cbx* phenotype when the third chromosomes are structurally homozygous for the wild-type sequence, but it does have complete suppression of the *Cbx* phenotype when those chromosomes are structurally heterozygous for a transvection-suppressing rearrangement (TSR) of the type that suppresses transvection of the bx^{34e}/*Ubx* genotype (Lewis, 1954).

The GR screen involves mutagenizing the wild-type males and mating to females homozygous for *Cbx Ubx* and for a duplication, *Dp(3;1)68*, which is an insertion of region 89D–89E into the proximal portion of the X chromosome. Such females have wild-type wings and halteres. Thus, this duplication fully rescues the otherwise lethal *Cbx Ubx* homozygote. These tester-strain females are also homozygous for a recessive marker in X (yellow-2) and one in the third chromosome (glass-3). The F_1 flies that are *Dp(3:1)68; Cbx Ubx*/+ + have weak traces of the *Cbx* phenotype, consisting of spread wings and partially reduced alulae; however, when flies of this genotype are structurally heterozygous for a TSR, their wings are normal (Lewis, 1955). It should be noted that the extra copy of Ubx^+ is without influence on the wing phenotype for two reasons: (1) The slight *Cbx* effect is a dominant gain of function not suppressible by an extra dose of wild type; and (2) the Ubx^+ gene in the duplication is apparently too remotely located to be affected by the *Cbx* mutant. F_1 males with normal wings are therefore selected and backcrossed to the tester strain to check for transmission of suppression of transvection, or, since the vast majority of normal-winged flies in the F_1 of the screen do show such transmission, the F_1 males are often mated directly to a homozygous bithoraxoid (*bxd*) line. From this latter mating, male larvae that have eight rows of abdominal setal bands (see Fig. 1) are heterozygous for the mutagenized wild-type chromosome, whereas those with seven rows have a *bxd*/*Cbx Ubx* genotype; the latter are therefore discarded and the former are analyzed cytologically by examining their salivary gland chromosomes.

Rearrangements detected in the GR screen obey the same rules as those found in the original study of TSRs (Lewis, 1954). Although the breakage points of such rearrangements may occur anywhere in a region from the centromere to the BX-C and slightly beyond, a distance of over 500 bands in the salivary gland chromosomes, only those having breakages within the 89D–89F region are generally saved for further study; they, in turn, are tested in a variety of ways, including determining their phenotypes when opposite deletions for portions or all of the BX-C.

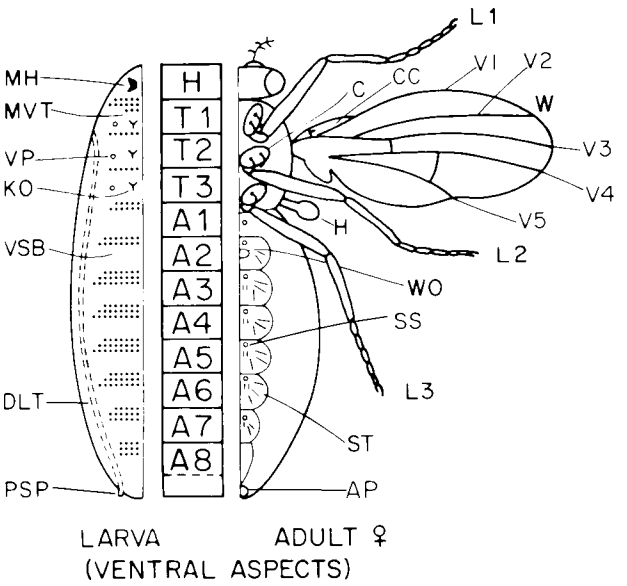


Figure 1. Comparison of ventral cuticular patterns in the larval first-instar and adult stages of *Drosophila*. (MH) Mandibular hooks; (MVT) midventral tuft; (VP) ventral pits; (KO) Keilin's organ; (VSB) ventral setal belts; (DLT) dorsal longitudinal (tracheal) trunk; (L) leg; (W) wing; (H) haltere; (C) coxa; (CC) costal cell (of wing); (V) vein; (WO) Wheeler's organ; (SS) sensillum (on segments A1–A7, inclusive); (ST) sternite; (AP) anal plate. For a detailed account of larval cuticular patterns, see Lohs-Schardin et al. (1979). (Modified from Lewis, 1982.)

The MCP screen detects in the F_1 both lethal and nonlethal mutants that weaken the dominance of *Mcp*. The GR method detects in the F_1 generation not only lethal and nonlethal mutants, but also dominant sterile mutants that are made fertile by covering with *Dp(3;1)68*; in addition, the GR method can detect breakages within the BX-C that have little or no detectable effect on any of the BX-C genes.

The GR screen detects the chromosomal rearrangements, almost exclusively. Point or pseudopoint mutations are not detected, except in the case of *Ubx* mutants. The latter are detected as would be expected on the basis that substitution of such a mutation for Ubx^+ in the *Dp-68; Cbx Ubx*/++ genotype would be expected to suppress the remaining slight *Cbx* phenotype. The GR method also detects tandem duplications of the BX-C, evidently because the pairing of the two BX-C regions within the duplication-bearing chromosome tends to exclude their interaction with the homologous *Cbx Ubx* chromosome.

RESULTS

Relevant cuticular patterns of the wild-type organism are shown in Fig. 1. The current status of the correlation of cytological, genetic, and molecular maps of the BX-C is

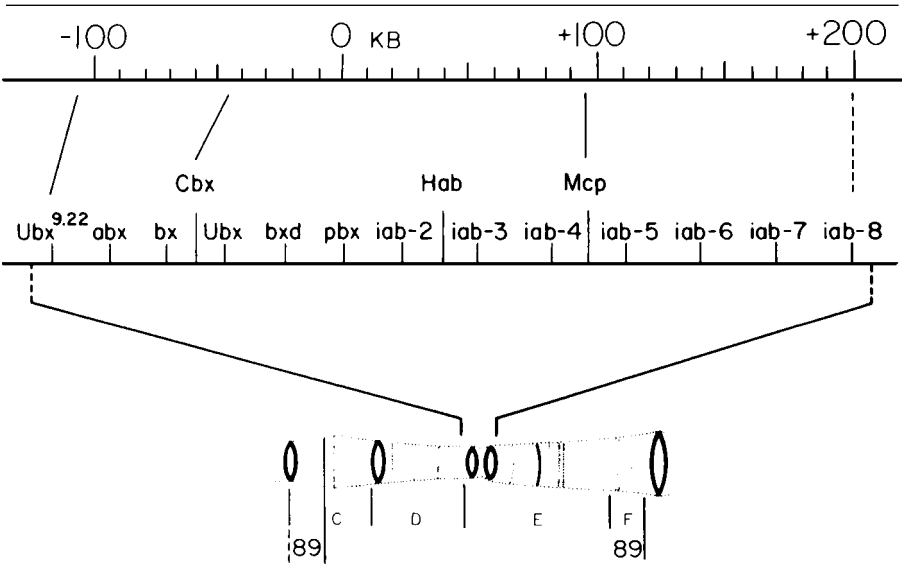


Figure 2. Correlation of the cytological, genetic, and molecular maps of the BX-C. On the DNA map, the location of *pbx* is 0.0, since it was the starting point of the walk (Bender et al., 1983); for reference points, only the approximate locations of the *9.22* allele of *Ubx, Cbx* insertion point, and a pseudopoint small deletion mutant *Mcp* are shown. For details of the genetic mapping of the region, see Lewis (1982) and for the molecular maps see Karch et al. (1985).

shown in Fig. 2. The entire complex appears to be contained within the first two doublets of section 89E.

Several revisions and additions are shown in the genetic map since the last map was published (Bender et al., 1983). The location of $Ubx^{9.22}$ (Kerridge and Morata, 1982) on the genetic and molecular maps is based on the work of Akam et al. (1984), who found it to be a pseudopoint mutation in the major leftmost, or 3′, exon of the *Ubx* transcripts. The location of Hyperabdominal (*Hab*) has been found to be to the right of an *iab-2* allele, $iab\text{-}2^{C53}$ (I. Duncan, personal, communication); previously, its position was only known to be at the right of *pbx*. Evidence for the existence of additional loci beyond *iab-2* is based on genetic and recombinant DNA studies summarized by Karch et al. (1985) and results reported in this paper.

From a screen for detecting lethals, Sanchez-Herrero et al. (1985) reported finding two lethal complementation groups in the distal half of the complex. They have suggested that this implies only two functional domains in that half. From the work of Karch et al. (1985), one of their lethal complementation groups is confined to the *iab-2* region, whereas the other includes one or more loci distal to *iab-5*. A lethal complementation screen fails to detect lesions in *iab-3*, *iab-4*, and *iab-5*, since inactivations of these genes, including deletions thereof, are viable as adults. However, each has a specific function, and $iab\text{-}4^{+}$ turns out to control whether gonads will

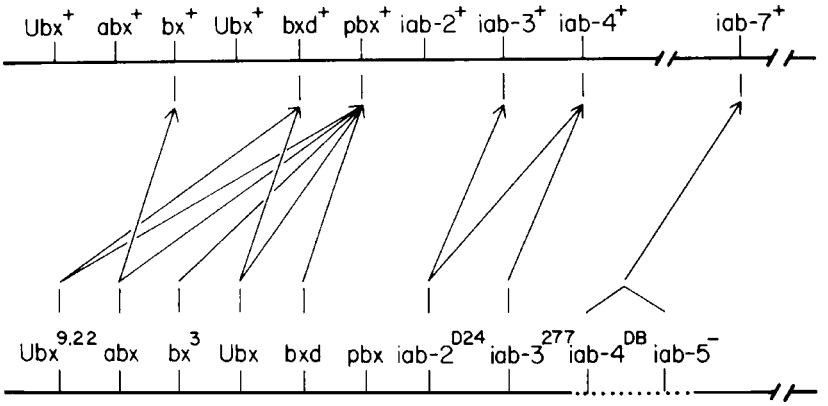


Figure 3. Direction of *cis*-inactivation of wild-type genes (*top line*) imposed by individual mutant genes whose locations are shown on the bottom line is indicated by means of sloping lines ending in an arrow and is from a proximal to a distal direction in every case. Only cases in which the direction of *cis*-inactivation has been established are indicated, as explained in the text. For descriptions of mutant symbols, see Fig. 2 and text.

form. The utility of using survival to adulthood as a criterion for defining higher functional units with the BX-C is open to question, since animals totally lacking the BX-C complete embryonic development and die as first-instar larvae (Lewis, 1978); however, Sanchez-Herrero et al. (1985) have done an important service in identifying what may turn out to be a significant correlation between adult lethality and the presence of homeo boxes within the complex (see below).

Cisvection in the Proximal Half of BX-C

Cisvection in the region from anterobithorax (*abx*) to postbithorax (*pbx*), inclusive, has been discussed elsewhere (Lewis, 1952, 1955, 1982). Examples shown in Fig. 3 are those cases of cisvection in which the direction of the effect can be deduced. Consider the two mutants *bxd* and *pbx*. The *cis*-heterozygote *bxd pbx*/+ + is wild type, whereas the *trans*-heterozygote *bxd* +/+ *pbx* has a *pbx* phenotype. Thus, in the latter case, the first abdominal segment (A1) fails to transform toward a thoracic one, as it does in homozygous *bxd* genotypes, whereas the posterior compartment of the third thoracic segment (T3) partially transforms toward that of the second (T2), but to a slightly lesser degree than it does in *bxd* or *pbx* homozgyotes. In other words, *bxd* partially *cis*-inactivates pbx^+, but *pbx* does not show detectable *cis*-inactivation of bxd^+.

Cisvection in the region from the position of the $Ubx^{9.22}$ mutation to that of Ubx^1 is of special interest, since these mutants occupy different exons in the same transcription unit, as described above. The two mutants are indistinguishable from one another phenotypically in a large number of combinations tested, including those shown in Fig. 3. Ubx^1 and $Ubx^{9.22}$ each shows partial complementation with either *abx* or *bx* mutants. Since these four types of mutants express the same type

of transformation (anterior T3 toward anterior T2), direction of *cis*-inactivation cannot be unambiguously determined; therefore; these cases of cisvection have been omitted from Fig. 3. Although the *abx* and *bx* mutants show a very similar type of transformation of the anterior compartment T3 toward that of T2, *abx* differs qualitatively in that it causes, with variable expressivity, a more complete transformation in the anteriormost region of that compartment than does bx^3. In *abx* +/+ bx^3 animals, there is no trace of these effects of *abx*; however, such animals show only very weak and variable transformations of T3 toward T2. The direction of *cis*-inactivation can nevertheless be deduced indirectly in the sense that the bx^3 homozygote acts as if it causes overactivity of the adjoining abx^+ gene, rather than inactivation of that gene, as described in more detail elsewhere (Lewis, 1981). Hence, in Fig. 3, *abx* is shown as *cis*-inactivating bx^+.

Cisvection in the Distal Half of BX-C

Analysis of cisvection in the right half of the complex has been complicated by the lack of dramatic differences in some of the segments, such as A3 and A4, and by the finding that hemizygotes for a wild-type third chromosome express slight dominant loss-of-function phenotypes for many of the loci, especially *iab-5* and *iab-6*, as judged from partial loss of male pigmentation on A5 (A5 transforming toward A4) and extra bristles on the male sternite of A6 (A6 transforming toward A5). The analysis has also been handicapped by a shortage of point or pseudopoint mutations that would permit *cis–trans* tests to be performed. Suggestive evidence for cisvection can nevertheless be inferred from analysis of chromosomal rearrangements having breakages in that portion of the complex. The first example involved the *iab-2* and *iab-3* loci.

Previously, only one example of a recessive loss of function corresponding to that predicted for an *iab-3* gene had been found, namely, Ultraabdominal-4 (Uab^4) (Lewis, 1978). Although this mutant was originally detected as a dominant transformation of A1 toward A2, it exhibits, when hemizygous, a very strong transformation of the predicted phenotype for an *iab-3* mutant, namely, transformation of A3 toward A2.

A second *iab-3* mutant, $iab\text{-}3^{277}$ (found by D. Baker, California Institute of Technology) is a revertant of *Mcp* and has not been separated from *Mcp*. It is associated with a transposition of a region from 94 to 96 of the right arm of the third chromosome into the BX-C approximately at +65 kb (Karch et al., 1985), presumably within or adjacent to the *iab-3* gene. This mutant is associated with a strong transformation of A3 toward A2, not only when hemizygous, but also when in *trans* with *iab-2* mutants, including $iab\text{-}2^{D24}$ that Karch et al. (1985) find may be a true point mutation. Thus, *iab-2* is inferred from these results to *cis*-inactivate $iab\text{-}3^+$.

The $iab\text{-}3^{277}$ mutant lacks the strong, dominant *Uab* effect associated with the Uab^4 mutant; however, when hemizygous, it expresses a very weak transformation of A1 toward A2, or, in other words, a very weak *Uab* effect. This transformation was first noticed by B. Weiffenbach and W. Bender (personal, communication).

Five *iab-4* mutants have been identified. Two, $iab\text{-}4^{45}$ and $iab\text{-}4^{302}$ (found by R. Baker, California Institute of Technology) are from the GR screen and three are from

the MCP screen. Two of the latter, *iab-4^{125}* (found by J. von der Ahe, California Institute of Technology) and *iab-4^{166}* (found by R. Baker), are partial revertants of *Mcp*, and the third (found by D. Baker) is a complete revertant of *Mcp* and has a deletion extending from +80 to +118 kb on the DNA map (Fig. 2); it is designated *iab-4,5^{DB}* to indicate its dual loss of function in the *iab-4* and *iab-5* regions.

All five *iab-4* mutants are viable when hemizygous. Externally, in males hemizygous for the *45* and *302* alleles, the black band bordering the rear margin of segments A3 and A4 fails to reach the edge in A4 and in A3, whereas in wild-type males, it reaches the margin in A4 but not in A3 (the latter subtle difference was noticed by I. Duncan (personal, communication); for further details and illustration of this effect, see Karch et al. [1985]). Since the *125* and *166* alleles still have residual *Mcp* effects, they are not useful for analyzing loss-of-function effects in the A4 tergite.

Internally, hemizygotes for four of the *iab-4* mutants show loss of gonads in both sexes, whereas the *iab-4^{45}* hemizygote shows only partial loss, or reduction in size, of the gonads. Since loss-of-function mutants of *iab-5*, such as *iab-5^{301}* (described below), do develop gonads, the absence of gonads in *iab-4* mutants is taken to mean that *iab-4^{+}* has the important function of controlling the initiation of gonadal development.

Partial to complete loss of gonads has been reported for heterozygotes involving the *P10* allele of *iab-2* and the *Uab^{4}* mutant (Lewis, 1978). Such loss has also been observed in the case of *iab-3^{277}* when opposite several *iab-2* mutants, including *iab-2^{D24}*. Finally, *iab-3* mutants in *trans* with several *iab-4* mutants tested also show partial to complete loss of gonads. Thus, *iab-2* and *iab-3* mutants *cis*-inactivate *iab-4^{+}*. The *iab-4^{302}* mutant is viable when homozygous, and in addition to showing strongly reduced gonad development, the ventral sternite of segment A2 is converted to one resembling that of A3. A weak transformation of this type in hemizygotes for the *iab-4^{302}* allele was first noted by F. Karch and W. Bender (personal, communication). The *iab-4^{45}* and *iab-4^{302}* rearrangement-associated mutants show only partial *cis*-inactivation of *iab-5^{+}* in the sense that in the hemizygotes, A5 transforms much more strongly toward A4 than do wild-type hemizygotes (*Df-P9/+*), whereas in the homozygote, which is viable only in the case of *iab-4^{302}*, there is little or no effect on A5. (Since the *125* and *166* alleles of *iab-4* still carry the *Mcp* mutant, they are not useful for cisvection analysis.)

Cis-inactivation beyond *iab-5* is most clearly seen in the case of *iab-4,5^{DB}*. It is quite viable when homozygous and expresses a strong transformation of A4 toward A3 (already described above as an *iab-4* mutant effect) and of A5 toward A4 (an *iab-5* mutant effect expected because of the associated deletion of DNA in the *iab-5* region). In addition, the homozygote has partial transformation of A6 toward A5, principally dorsally in the middle of the tergite (a weak *iab-6* mutant effect). The hemizygote (*iab-4,5^{DB}/Df-P9*) is also quite viable and differs from the homozygote in having a much more extreme transformation of A6 toward A5 and in having a small tergite on A7 that is significantly larger than the tiny streak of A7 tergite tissue found in *Df-P9/+*. Thus, *iab-4,5^{DB}* shows partial *cis*-inactivation of *iab-6^{+}* and *iab-7^{+}* functions.

Two rearrangements from the GR screen, *iab-5*^{301} and *iab-5*^{843} (found by R. Baker), are viable as hemizygotes and express a strong transformation of A5 toward A4 but show only a weak transformation of A6 toward A5, detectable in the pattern of trichomes. The breakage points of these rearrangements within the BX-C occur in the region to the right of *iab-4* breakages and near the middle of the *iab-4,5*^{DB} deletion that extends from approximately 85 kb to 115 kb (F. Karch and W. Bender; cited in Karch et al., 1985).

In the case of the *iab-5*^{301} rearrangement, the homozygote and the hemizygote are viable. The former shows a transformation of the pigmentation and bristle pattern at the lateral edge of the tergite of A3 toward that found in A4. The direction of this transformation is that expected for a gain-of-function phenotype (Lewis, 1978). Again, as in the case of *iab-3* and certain *iab-4* mutants, there is gain of function for a gene just proximal to the gene exhibiting *cis*-inactivation of gene(s) immediately distal. Results indicating that *iab-5*^{DB} and another pseudopoint mutation, *iab-5*^{C7} (M. Crosby; cited in Lewis, 1981), show *cis*-inactivation extending to *iab-7*$^{+}$ are discussed below.

Transvection

The known instances of transvection are diagramed in Fig. 4. An example of transvection between *Ubx* and *bx*^{34e} has been described previously (Lewis, 1964), as well as one between *Ubx* and *pbx*^{2} (Lewis, 1982). Transvection between *iab-2* and *iab-4* has been assayed in *trans*-heterozygotes for the pseudopoint mutations, *iab-2*^{D24} and *iab-4,5*^{DB} (E. B. Lewis and R. Baker, in preparation). For example, among a total of 35 males of this genotype that were structurally homozygous for normal third chromosomes, only three had no trace of gonadal tissue, 16 had incompletely developed testes, and the remaining 16 each had one or both seminal vesicles pigmented, suggesting (see Stern and Hadorn, 1939) that traces of gonadal tissue may have been present. When the *iab-2*^{D24} +/+ *iab-4,5*^{DB} genotype carried a TSR, of the 65 males dissected, 49 had no trace of gonadal tissue, 1 had a testis that was one-half normal size, and the remaining 15 had one or both seminal vesicles pigmented. Hence, the TSR significantly reduced gonadal development.

Transvection involving the region from *iab-5* to *iab-7*, inclusive, has been detected by comparing *trans*-heterozygotes for *iab-5*^{C7} and *iab-7*^{D14} in the presence and absence of structural heterozygosity (E. B. Lewis and I. Duncan, in preparation). A significantly larger seventh tergite develops when a TSR is present than when the third chromosomes are structurally homozygous for the wild-type sequence. In this comparison, tergite size was measured by counting the number of bristles on that tergite on slides that had been coded and scored blindly. The results indicate that *iab-5*^{C7} *cis*-inactivates *iab-7*$^{+}$ and that the TSR when heterozygous enhances that effect. The results, however, are complicated by the unexpected finding that the *iab-5*^{C7} chromosome acts as if it carries a dominant and closely linked maternal modifier of the *Rg-bx* type. Thus, when the mother carries that chromosome and the father contributes the *iab-7*^{D14} chromosome, the *iab-5*^{C7} +/+ *iab-7*^{D14} male progeny have a

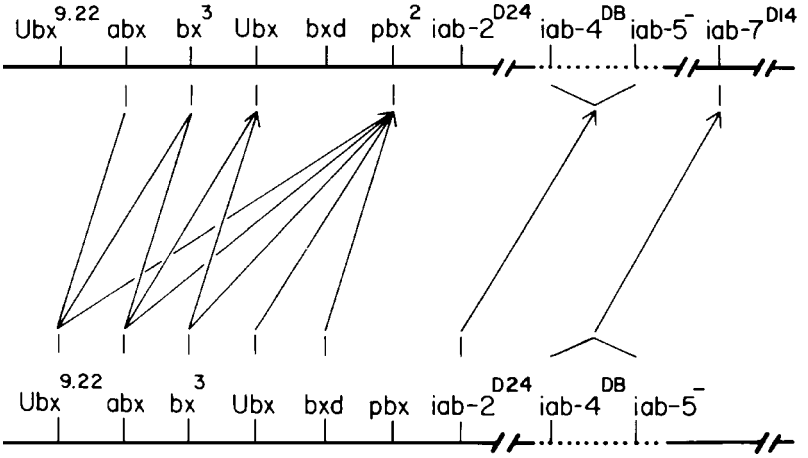


Figure 4. Established cases of transvection within the BX-C. When the direction of *cis*-inactivation accompanying the transvection phenomenon is known, it is indicated by an arrow and is always from a proximal to a distal direction. For a description of mutant symbols, see Fig. 2 and text.

significantly larger number of bristles on the seventh tergite than do males of the same genotype from the reciprocal mating. Nevertheless, transvection was demonstrable regardless of the maternal genotype. Care has therefore been taken to carry out transvection experiments with the mother of the same genotype; i.e., the TSR is always introduced through the male parent. Parallel analyses with *iab-5*^{DB} and *iab-7*^{D14} also indicate a significant effect of a TSR in enhancing tergite development in A7.

DISCUSSION

Rules for *Cis*-regulation

From the above results, three rules governing *cis*-regulation of the BX-C can be recognized: (1) The colinearity (COL) rule states that there is colinearity between the order of the genes in the chromosome and the order in which they show their effects along the body axis of the organism; (2) the *cis*-inactivation (CIN) rule states that a mutant lesion in a given gene tends to *cis*-inactivate one or more neighboring genes lying distal to that gene; and (3) the *cis*-overexpression (COE) rule states that certain lesions in a given gene tend to lead to an overexpression of the gene lying immediately proximal. Examples upon which these three rules are based are discussed below.

The COL rule continues to apply for much, and possibly all, of the distal half of the complex (Figs. 2 and 3). This conclusion is based on extensive molecular mapping studies of that region (Karch et al., 1985), as well as a limited number of genetic mapping and deletion experiments. The only known exception to this rule is the *pbx*

locus, which would have been expected to lie proximal to *bxd*, since *pbx* affects T3 while *bxd* affects A1 (as well as T3).

The first examples of the CIN rule involved the *bx, Ubx*, and *bxd* mutants (Lewis, 1951). Additional examples, reported in this paper, are summarized in Fig. 3. Exceptions to the CIN rule occur in several cases. The known *pbx* mutant alleles, *pbx*, and pbx^2, are pseudopoint mutations involving deletions of 17 and 15 kb, respectively, yet they fail to show detectable *cis*-inactivation of $iab\text{-}2^+$, the next most distally known gene in the complex (see Struhl, 1984). A critical test of whether the distal half of the complex can function independently of the proximal half requires analysis of rearrangements having breakages in the intervening region. The GR screen is capable of detecting such rearrangements even though they might lack any detectable phenotype, other than their ability to act as TSRs. None have yet been found in the GR screen; however, only nine TSRs that have breakages within the distal-half complex have been recovered. Whether it is chance that has kept the BX-C intact or whether the two halves are functionally interrelated cannot be determined from the existing data.

The first known example of the COE rule involves the *bxd* mutant. Thus, accumulation of the Ubx^+ product has been specifically invoked (Lewis, 1955) to explain the finding that bx^3 + bxd/bx^{34e} + + has a less extreme transformation of T3 toward T2 in the notal area than does the corresponding genotype in which *bxd* is replaced by bxd^+, namely, bx^3 + +/bx^{34e} + +. (To make explicit that Ubx^+ is homozygous, a plus sign is included as the middle symbol in these genotypes and in others that involve the *bxd* comparisons.)

That Ubx^+ overexpression is actually present both in T3 and in T2 is strongly suggested by two other comparisons in which the possibility for such an effect has been sensitized by the use of gain-of-function mutants (E. B. Lewis, unpublished). *Cbx* + *bxd*/+ + + has a more extreme transformation of T2 toward T3 than does *Cbx* + +/+ + +. That the effect involves *cis*-regulation is shown by the finding that the *trans*-heterozygote, *Cbx* + +/+ + *bxd*, fails to show such an enhancement of the *Cbx* phenotype. Second, a very weak *Cbx*-like wing effect that occurs in Pc^3/+ animals is intensified slightly but significantly when there is heterozygosity for a *bxd* mutant (whether in the *Pc* or + chromosome). Thus, in these two sets of comparisons involving, in the one case, *Cbx* as the sensitizer and, in the other, Pc^3, the *bxd* mutant effects a stronger transformation of T2 toward T3. Hence, *bxd* acts as if it causes overexpression of Ubx^+ in T2, which is one segment anterior to that in which the latter allele normally becomes fully expressed. Using indirect immunofluorescence, Beachy et al. (1985) have obtained direct evidence for this inferred effect of a *bxd* mutant on Ubx^+ expression by showing that in embryos homozygous for an extreme *bxd* mutant, bxd^{113}, specific staining of a part of the Ubx^+ protein could first be detected in nervous tissue of T2, compared with T3.

In the bx^3 case, weak transformation of extreme anterior T2, especially in the homozygous mutant, is consistent with there being overexpression of abx^+ in T2, one segment anterior to that (T3) in which abx^+ is normally assumed to be first expressed.

The utility of applying the CIN and COE rules to these mutants was demonstrated with the finding that the homozygous double mutant, *abx* bx^3, has the effect of such overexpression largely blocked in T2 and in T3, so that the triple mutant, *abx* bx^3 *pbx*, when homozygous, produces a much more nearly perfect four-winged fly (see Lewis, 1982) than does the bx^3 *pbx* homozygote (see Lewis, 1964).

In the case of *iab-3*^{277} (see above), there is evidence for weak transformation of the dominant gain-of-function type that implies overexpression of *iab-2*$^+$ in A1, one segment ahead of the segment in which that gene normally becomes fully expressed. Whether that overexpression occurs in A2, where that gene normally becomes fully expressed, has not been studied.

Strong overexpression of *iab-2*$^+$ has been invoked to account for the strong transformation of A1 toward A2 in the case of the Uab^4 mutant, which is associated with a recessive loss of function of the *iab-3* type, as already discussed above (Lewis, 1978); however, caution must be used in interpreting such examples because it was the dominant gain of function that led to the detection of that mutant, rather than the recessive loss of function in *iab-3* that was only shown later. Such strong dominant mutants of the gain-of-function type tend to occur extremely rarely compared with mutants of the recessive loss-of-function type, as if the former require special types of DNA association. For a discussion of the behavior of numerous *cis*-dominant mutants of the overexpression type involving genes in the BX-C, see Lewis (1978). Still other dominant gain-of-function mutants show gross misregulation of certain genes of the BX-C (see, e.g., Gausz et al., 1981; Celniker and Lewis, 1984; and other examples cited in Karch et al., 1985).

In the case of *iab-4*, the *iab-4*^{302}, *iab-4*^{45}, and *iab-4*^{125} mutants transform the sternite of A2 toward that of A3 and therefore are examples of mutants that obey the COE rule; i.e., they show an overexpression of the *iab-3*$^+$ gene. *iab-4*^{302} shows much greater overexpression in the homozygote than in the hemizygote. The remaining two *iab-4* alleles, *iab-4,5*^{DB} and *iab-4*^{166}, do not show this overexpression. In the case of *iab-5*, the *iab-5*^{301} allele obeys the COE rule since, as reported above, the homozygote survives and shows a transformation of A3 toward A4. This result is, of course, consistent with overexpression of *iab-4*$^+$ in A3.

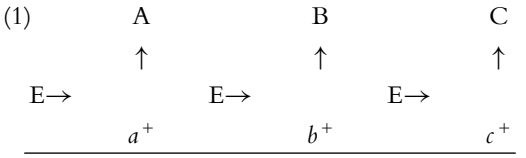
The CIN and COE rules have been found to apply not only to pseudopoint mutants, including deletions and gypsy insertion sequences, but also to gross chromosomal rearrangements, including wholly euchromatic and euchromatic-heterochromatic ones. Too few mutants, however, have been characterized at each locus within the complex to detect patterns related to specific types of DNA lesions.

A Model for *Cis*-regulation

The CIN and COE rules suggest a model for explaining cisvection and transvection in the case of the BX-C. It is sufficient to consider three closely linked genes, *a, b*, and *c*, to illustrate the main features of the model. (Gene will be used here in the broad sense of embracing an entire transcription unit, including all noncoding and coding regions.) These genes would make three specific *trans*-regulatory products (TRPs),

designated A, B, and C, respectively. Each gene would code for at least one TRP (or a group, if more than one spliced message were involved). TRPs in some cases would be translated into proteins. Thus, White and Wilcox (1984) and Beachy et al. (1985) have shown that *Ubx* codes for a protein, whereas the discovery of homeo boxes by McGinnis et al. (1984) and Scott and Weiner (1984) combined with the molecular mapping data of Karch et al. (1985) indirectly implicates the *iab-2* gene and either the *iab-6* or the *iab-7* gene as coding for proteins. However, for the TRP coming from a gene that is transcribed but appears not to be translated, as may be the case with bxd^+ (Peattie and Hogness, 1984), it is assumed that the RNA transcript still functions, albeit in an unknown way, as a *trans*-regulator of other genes.

The model invokes a *cis*-regulatory entity (E) that is assumed to be an essential ingredient in the machinery needed by a gene to produce its final transcript(s). The interrelationships of the three genes with E is depicted in a series of diagrams, starting with the wild-type haploid complement (the chromosome is represented by the horizontal line at the bottom of the diagram):

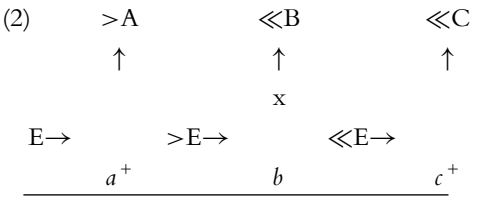


The source of E is not specified and could be pretranscriptional or cotranscriptional. In either case, it would be desirable to have a mechanism that would account for the finding that the direction of *cis*-inactivation appears to be opposite of that of transcription (based on analyses of *Ubx* transcripts [see Beachy et al., 1985] and of a cDNA from the *bxd* region [H. Lipshitz and D. Hogness, personal, communication], as well as indirect inferences based on molecular studies of *iab-2* and *iab-6* or *iab-7* regions that code for the two known homeo box sequences in the distal half of the complex [W. Bender, personal, communication]). The most obvious mechanism would be one that could track the other DNA strand from that used by DNA-dependent RNA polymerase. If E is made pretranscriptionally, then an analogy for such tracking would be the case of the hypothetical primosome of prokaryotes (Arai and Kornberg, 1981). If E is produced cotranscriptionally, then E might be (or contain) antisense RNA either from some portion of the coding or noncoding regions of the BX-C genes or from one or more *trans*-regulatory genes that would code for E. Such speculation is invoked here only to suggest that there may be a plausible basis at a molecular level for the direction of the CIN effect.

At this stage of the analysis, it seems more profitable to pursue a model in which E is assumed to be a substance, as opposed to some type of construct that would involve, for example, structural deformation of the chromosome. To account for cisvection and transvection, it is necessary to assume that E has a nonrandom distribution in the nucleus and that its effective radius of action is very limited, suggesting that E is

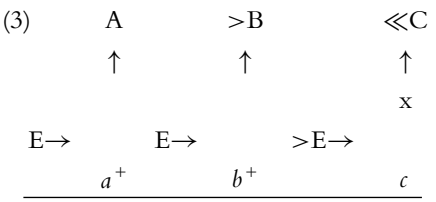
unstable or is produced in extremely minute amounts, or both. If cotranscriptionally produced, then E, or the effective part of E, is likely to be RNA rather than protein, since the latter should act in *trans*, not in *cis*.

In case a mutant lesion in one of the genes, such as *b*, obeys the CIN rule, the new pattern of relationships among the TRPs and E can be diagramed as follows:



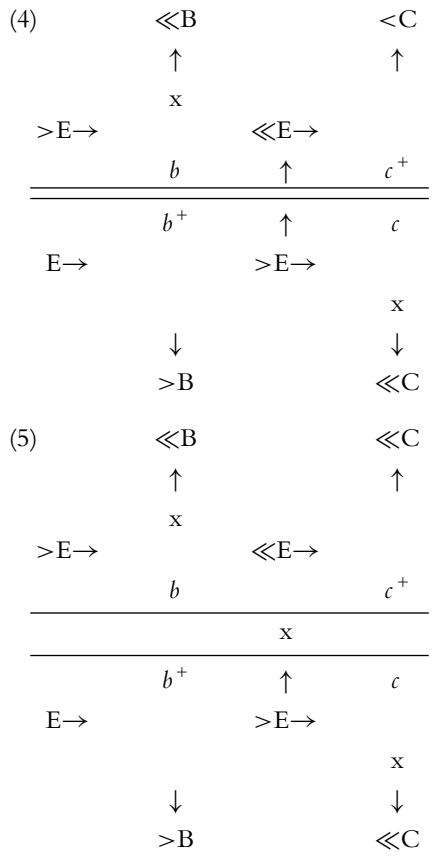
The DNA lesion in the *b* mutant is assumed to have two effects: (1) It leads to either a defective TRP or lowered quantity thereof (either possibility being symbolized by "≪B"; and (2) it tends to block the transport of E so that the effective concentration of E that reaches c^+ is greatly reduced ("≪E") relative to that found in the case (Diagram 1) of wild type. The postulated block in the transport of E to c^+ would be responsible for the *cis*-inactivation of c^+ leading to less C ("≪C"): the CIN rule. To account for the COE rule, it is further assumed that E accumulates ahead of *b* sufficiently in amount to cause overexpression of the a^+ gene and hence an increase in concentration of A (">A") relative to that in the wild type.

A corollary of the CIN rule is that the *cis*-inactivation tends to spread over several genes located distally to the mutant lesion; however, the farther the gene is from that lesion, the less is the CIN effect. Such a result implies that the concentration of E gradually builds up the more distal the gene from the lesion. Some type of restart mechanism for E production from either outside or within the complex is also needed in the case of *iab-2*$^+$, since neither *pbx* nor *bxd* mutant lesions have induced detectable *cis*-inactivation of that gene. Similarly, for the case of a *c* mutant:



Combining Diagrams 2 and 3 to give a *trans*-heterozygote, *b* +/+ *c*, with closely paired chromosomes, or with pairing reduced or eliminated by a TSR (Diagrams 4

and 5, respectively), yields the following two genotypes that are needed to examine the application of the model to cases of transvection:



In Diagrams 4 and 5, the concentration of C is expected to be lower than that in the *cis*-heterozygote, *b c*/++ (not diagramed), in agreement with the observed findings that the *trans*-heterozygotes in both cases express the "*c*" phenotype rather than the wild-type phenotype (or that of a slight dominant effect that arises whenever one of the mutants is incompletely dominant, as in the case of a mutant such as *Ubx*); i.e., *Ubx* mutants, deletions that include *Ubx* and such double mutants as *bx Ubx, Ubx bxd*, and *Ubx pbx*, all are virtually in identical in their causing a slightly enlarged haltere when tested opposite a wild-type chromosome.

To account for transvection, it is assumed that when the chromosomes are paired, as in Diagram 4, E diffuses from the lower "*c*"-bearing chromosome to a sufficient extent to permit the c^+ in the upper two chromosomes to function more efficiently, thereby producing more C, than when the chromosomes are unpaired, as in Diagram 5 in which E is assumed not to be able to so diffuse. The nature of the restriction placed

Table 1 *Cis*-overexpression or COE rule.

	Trans-regulatory products					
	genotype (1)			genotype (3)		
	$--a^+--$	$--b^+--$	$--c^+--$	$--a^+--$	$--b^+--$	$--c--$
Segment number	↓ A	↓ B	↓ C	↓ A	↓ >B	↓ «C
n-2	+++	++	+	+++	+++	±
n-1	+++	+++	++	+++	++++	±
n	+++	+++	+++	+++	++++	±

Levels of expression of TRPs coming from the chromosome genotypes (1) and (2) in progressively more anterior body segments; e.g., if *n*, *n-1*, and *n-2* correspond to abdominal body segments A4, A3, and A2, respectively, then a^+, b^+, and c^+ correspond to *iab-2*$^+$, *iab-3*$^+$, and *iab-4*$^+$, respectively, (±) Little or none; (+) very weak or incipient; (+ +) weak; (+ + +) wild type; (+ + ++) overexpression.

on the ability of E to diffuse readily over such short distances might reside in its being produced in very small amounts, in being unstable, or both. As a result, Diagram 4 is expected to have a less extreme "*c*" phenotype than that of Diagram 5: the observed result in the known cases of transvection involving such *trans*-heterozygotes.

To account for the COE rule, it is necessary to postulate that there is a gradient in the expression of many, if not all, of the BX-C genes. The application of this principle to the most relevant genotypes is shown in Table 1. To illustrate, let *n*, *n-1*, and *n-2* represent abdominal segments A4, A3, and A2, respectively, then the gene symbols *a*, *b*, and *c* correspond, respectively, to *iab-2*, *iab-3*, and *iab-4*. To explain why a lesion in *iab-4* can cause an overexpression of *iab-3*$^+$ in relevant cells of segment A2, it is necessary to assume that in wild type, *iab-4*$^+$ is very weakly expressed in cells of A2, or incipiently expressed in the sense that E would be assumed to have prepared it to be so expressed.

In a number of instances, it has been necessary to assume that a given gene is weakly expressed one segment ahead of the segment in which it is normally active in order to account for certain phenotypes (Lewis, 1978). Evidence that can be interpreted as pointing to an expression of this kind extending to two segments ahead has been found in one instance by Duncan (1982). Recently, evidence has been obtained (E. B. Lewis, unpublished) pointing to the conclusion that an *iab-3*$^+$ function in the embryo (namely, promotion of development of special denticles of a triangular shape in the dorsal posterior half of the abdominal segments [Lohs-Schardin et al., 1979]) appears to be weakly manifested in A1 of wild type and increases in strength posteriorly in proportion to the number of copies of the wild-type BX-C present in the embryo.

The present model, which is quite different from one recently proposed by Beachy et al. (1985), has a number of testable predictions. It would, for example, predict that each gene, including *iab-3*, *iab-4*, and *iab-5*, would make a transcript that would be expected to be appear in appropriate segments in accord with the COE rule. Specifically, a *bxd*$^+$ transcript would be expected to be primarily active in A1 and segments beyond, especially in genotypes such as *Dp-P10*; *Df-P9*, where other

transcripts from genes to the right of *bxd* would be absent and therefore not likely to interfere with the expression of the bxd^+ function (namely, suppression of sense organs in the abdominal segments and promotion of ventral setal bands). As pointed out above, molecular results obtained by Beachy et al. (1985) are consistent with the COE rule in showing that an extreme *bxd* mutant causes overexpression of Ubx^+ function in T2, one segment anterior to T3, i.e., the segment in which that function normally becomes fully expressed.

SUMMARY

The BX-C is a set of master control genes that *trans*-regulate other genes and thereby control much of the segmentation pattern of the fly. The BX-C genes are themselves regulated in *cis* and *trans*. Three rules governing *cis*-regulation of BX-C are applicable over a region extending from *Ubx* to at least *iab*-7, a distance of nearly 300 kb on the DNA map: (1) The colinearity (COL) rule: genes are colinear with respect to map location and order of expression along the body axis, the only exception thus far being pbx^+; (2) the CIN rule: a mutant lesion in one gene tends to *cis*-inactivate the wild-type gene(s) immediately distally; and (3) the COE rule: certain mutant lesions in a given gene cause the next most proximal gene to overexpress one segment more anterior to the one in which the latter gene normally expresses. A model is proposed that attempts to account for these rules by invoking a special *cis*-regulatory entity (E) that diffuses more efficiently along the chromosome than between chromosomes.

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SECTION III: MOLECULES AND DEVELOPMENT

LEWIS AND THE MOLECULAR CONTROL OF DEVELOPMENT

POSITIONAL CLONING OF THE BX-C

In the early 1970s methods were invented that revolutionized the study of individual eukaryotic genes by allowing molecular analysis of any gene of phenotypic interest, independent of the abundance of its products. This was particularly important in the case of developmental control genes, because such genes were expected to encode mRNAs and proteins of low abundance.

The foundations for the use of recombinant DNA methods to clone and analyze genes solely on the basis of their position on the chromosome were first proposed by David Hogness of Stanford University in a remarkable grant application in 1972. There Hogness presented revolutionary plans for what is now called "genomics"—plans that included production of recombinant DNA libraries representing entire chromosomes or genomes, ordering of overlapping genomic clones to produce physical maps of chromosomes, the use of these chromosomal walks together with chromosome rearrangements to clone genes identified solely on the basis of their mutant phenotype and genetic map position (what is now referred to as "positional cloning") and subsequent mapping of mutations and transcripts (now called "functional genomics"). The methods to allow the implementation of these plans were developed in Hogness' laboratory during the early to mid-1970s (the history of the development of these methods is given in greater detail in Rubin and Lewis, 2000; Burtis et al., 2003).

In 1968, when Hogness had changed the focus of his research from the genome of bacteriophage λ to that of *Drosophila melanogaster,* he had spent part of a sabbatical year with Lewis at Caltech. Thus he had become familiar with the fundamental importance of the BX-C in developmental control and the remarkable resource that Lewis' collection of BX-C mutants represented. Another key player in the positional cloning of the BX-C, Welcome Bender, was also familiar with the BX-C and Lewis' mutant collection. Bender had completed his graduate research at Caltech in the laboratory of Norman Davidson, before joining Hogness' laboratory as a postdoctoral fellow in 1977. There, he and Pierre Spierer, also a postdoctoral fellow, initiated the first chromosomal walk in the vicinity of the Ace locus (Bender et al., 1983b). In the process they cloned DNA that was likely to span the breakpoint in 87E1,2 of a mutant, Cbx^{+R1}. The other breakpoint of this inversion was in the BX-C at 89E1,2.

In 1978 Bender and Hogness proposed a close collaboration to Lewis: Lewis would provide his collection of genetic mutants. Bender, Spierer, and Hogness would use the inversion mutant to "jump" into the BX-C. They would then walk in both directions from this starting point, thus molecularly cloning the gene complex. As they did so they would map as many of Lewis' mutants on the genomic DNA as possible, correlating Lewis' genetic map with a physical map of the DNA. Subsequently, all transcripts produced from the BX-C would be mapped and cDNAs representing these transcripts sequenced. In this way it would be possible to determine the nature of the "substances" proposed by Lewis to regulate segmental identity. Lewis, with his characteristic generosity, made all of his mutants available and eagerly awaited molecular insights into the BX-C, which would serve as tests of the models that he had proposed on the basis of his genetic assays.

In an era where positional cloning is now routine, where the mapping methods developed in Hogness' laboratory have been applied to entire genomes, including the human genome, it is perhaps hard to imagine how exciting and risky the positional cloning of the BX-C was a quarter century ago. Bender and Spierer would face obstacles such as the presence of repetitive DNA that could lead them astray. Fortunately, it was known from previous work that these transposable elements are inserted at different sites in the DNA of different strains (Finnegan et al., 1978). Consequently, every step of the walk was taken in genomic DNA libraries from more than one strain. Each step was confirmed by analyses of the DNA on Southern blots as well as by *in situ* hybridization to polytene chromosomes. All along, they could have the confidence that Lewis' collection of mutants would provide landmarks that would confirm the success of their endeavor.

In their own words (Bender et al., 1983a): "As the overlapping DNA segments were collected in the walk, we began to look for the breakpoints of cytological rearrangements associated with various mutations. Such breakpoints can be located unambiguously on the DNA map by *in situ* hybridizations. Probes from along the walk were hybridized to a chromosome with an inversion, for example, to see where the probes switched from labeling one inversion end point to the other end point. Once the breakpoint was identified to within about 15 kb, Southern blots were done with

genomic DNA from the inversion strain to find the anomalous restriction fragments associated with the inversion breakpoint. We also began to examine, by Southern blot analysis, the DNA from cytologically normal, spontaneous, and X-ray-induced mutations. Most had anomalous restriction fragments indicative of DNA insertions or deletions. For many of these mutations we have constructed libraries of recombinant phage from the mutant DNA and isolated the region of interest. The mutant lesions were then characterized directly by restriction mapping and by electron microscopy of heteroduplex molecules." If Columbus had been as careful, he may have made it all the way to China!

The above section is quoted, not only to show how rigorous the analysis was, but also to illustrate the key role that Lewis' mutants played in the entire process. Without the mutants, there would have been no landmarks to tell them where they were or, indeed, whether they were making any progress at all.

The first walk in the BX-C (Bender et al., 1983a) netted 195,000 base pairs (195 kb) of DNA that extended from the proximal end of the complex (where the *Cbx-3* inversion breakpoint maps) through to roughly the middle of the complex. It therefore included all known *abx*, *bx*, *Ubx*, *bxd*, and *pbx* mutations, which comprise what is now called the "*Ubx* domain" of the complex. The cloning of the *Ubx* domain was accomplished by early 1979 and molecular mapping of many of the mutations in the left half of the BX-C was completed by 1980; however the data were not published until 1983. Subsequently, the remainder of the complex was cloned in Welcome Bender's own laboratory, spanning a total of more than 300 kb of DNA (Karch et al., 1985). At about the same time, it became clear that there are three functional genetic domains defined by lethal alleles in the complex, the *Ubx*, *abd-A*, and *Abd-B* domains (Sánchez-Herrero et al., 1985; Tiong et al., 1985). Furthermore, three homeobox-containing protein-coding genes map to these three regions, each encoding homeodomain-containing transcription factors (Regulski et al., 1985).

The molecular mapping of the BX-C mutations in the 1983 and 1985 papers was gratifying for both Lewis and Hogness. The physical map of the complex corresponds perfectly with the genetic map, validating over 35 years worth of Lewis' genetic results. Hogness had, two decades earlier, conducted experiments on bacteriophage lambda DNA that provided the first proof that genetic and physical maps are colinear (Kaiser and Hogness, 1960). Now, colinearity had been demonstrated for almost a third of a million base pairs in an eukaryote.

COMPLETING THE BITHORAX MUTANT SERIES

The papers reporting the positional cloning of the BX-C (Bender et al., 1983a; Karch et al., 1985) served as the forum for Lewis to report identification and phenotypic analysis of many of the genes that he had postulated in his 1978 paper (Lewis, 1978). Thus, alleles of *abx*, *iab-3*, *iab-4*, *iab-5*, *iab-6*, *iab-7*, and *iab-8* are reported and molecularly mapped together with several gain-of-function alleles. Additional BX-C alleles, called *abd-A* and *Abd-B* (Sánchez-Herrero et al.,

1985; Tiong et al., 1985), were also mapped and found to lie, respectively, in the vicinity of *iab-2* and *iab-6*/7. It was postulated that lethal *Abd-B* alleles, lesions for which had not yet been detected molecularly, would map to *iab*-7 on the basis of similar phenotypes. In essentially all cases, the position of the mutations respected Lewis colinearity (COL) rule—that the order of the genes on the chromosome parallels the order along the body axis in which they function. For example, *iab-6* mutations lie between *iab-5* and *iab*-7 mutations, and result in a transformation of AB6 → LAB5, and so on.

In addition, complementation tests for *cis*-inactivation (CIN) analysis were undertaken. Again, most of the results are consistent with Lewis' previous reports of a proximal-to-distal polarity in this effect. A question, raised in the commentary on the previous section of this book and in Lewis' 1985 paper, is whether CIN crosses the boundary between the *abd-A* and *Abd-B* domains (in the terminology of Karch et al., the *iab-2* and *iab*-7 regions). Three lines of evidence are presented that this boundary is not respected in terms of genetic effects:

- First, CIN crosses the boundary between *abd-A* and *Abd-B*, which is thought to lie between *iab-4* and *iab-5*. In *iab-2* +/+ *iab-5* adults there is a partial transformation of the fifth and sixth abdominal segments towards more anterior identity, consistent with *cis*-inactivation of *Abd-B* region functions by mutations in the *abd-A* region. Similarly, in the *iab-4,5* +/+ *iab*-7 mutant combination, there is a transformation of the fifth abdominal segment towards the fourth.
- Second, the gain-of-function *Mcp* allele (now shown to be a small deletion between *iab-4* and *iab-5*) causes AB4 → LAB5. Complete reversion of *Mcp* is always associated with lesions in the *iab-5* region. However, partial revertants included lesions mapping in the *abd-A* (*iab-2*^{C26}, *iab-3*^{277}, *iab-4*^{166}) or *Abd-B* (*iab*-7^{297}, *iab*-7^{380}) domains.
- Third, the combination *Df-bxd*^{121}/*Mcp Df-C4* results in loss of *iab-2* and *iab-3* on one chromosome and loss of *iab-6*, *iab*-7 and *iab-8* (along with the small *Mcp* deletion between *iab-4* and *iab-5*) on the other chromosome. If there were no interaction across the *abd-A* to *Abd-B* boundary, then the recessive functions in the abdominal region should show complementation in the *trans* combination. However, the flies showed a strong transformation of the fifth and sixth abdominal segments to LAB3 or LAB4, despite the presence of the *iab-5* region on both chromosomes.

THE MOLECULAR BASIS OF DEVELOPMENTAL CONTROL

The molecular analyses of Lewis' mutants reported in Bender et al. (1983a) and Karch et al. (1985) produced a series of remarkable discoveries (these are reviewed in more depth in Duncan, 1987):

- Many of the spontaneous mutations in the complex are not point mutations at all, but derive from insertions of mobile repetitive (transposable) elements. For

example, the first Drosophila homeotic mutation, *bx*^{1}, that Bridges had discovered in 1915 and from which the complex derived its name, is caused by insertion of a "412"-type transposable element. "Gypsy" transposable elements were found to have caused the *bx*^{34e}, *bx*^{3}, *bxd*^{1}, *bxd*^{55i}, *bxd*^{K}, *bxd*^{55i} mutations (Bender et al., 1983a) as well as *iab-2*^{K} (Karch et al., 1985). The "Doc" transposable element caused the *Ubx*^{1} mutation (Bender et al., 1983a), while the "Delta 88" transposable element had caused the *iab-8*$^{tuh-3}$ mutation (Karch et al., 1985). It was later shown that the *bx*^{8} allele, which Lewis had induced in 1965 using EMS, is caused by insertion of a "Harvey" transposable element (Peifer and Bender, 1986).

- Most of the remaining spontaneous, EMS and X-ray-induced mutations that are not caused by gross chromosomal rearrangements, involve small deletions or insertions of DNA. For example, *abx*^{1} and *abx*^{2} are small overlapping deletions, *pbx*^{1} is a 17 kb deletion that overlaps with the 15 kb *pbx*^{2} deletion, *Cbx*^{1} is an insertion of the 17 kb deleted in *pbx*^{1}, *Ubx*^{849} is a 110 bp deletion, *Ubx*$^{6.28}$ is a 50 bp deletion and *Ubx*$^{9.22}$—which had been discovered, mapped and characterized by Michael Akam (Akam et al., 1984)—is a 1.5 kb deletion, *iab-2*^{C26} is a 1.4 kb deletion, *abd-A*^{MX2} is a 3 kb deletion, *iab-4,5*^{DB} is a 28 kb deletion, *iab-5*^{C7} is an insertional translocation of over 10 kb of DNA derived from 90E, and *iab-7*^{D14} is a 0.4 kb deletion. Thus very few, if any, of the mutations in the BX-C are true point mutations, including those induced by the chemical mutagen, EMS.
- *Ubx* mutations that are cytologically normal cluster in two regions, separated by over 70 kb of DNA (*Ubx*^{1}, *Ubx*$^{6.28}$, and *Ubx*^{849} at position −32 kb on the walk; *Ubx*$^{9.22}$ 73 kb more proximal at position −105 kb). Subsequent analyses were to show that a giant, 75 kb "*Ubx*" primary transcript is produced in a distal to proximal direction from this region, and is spliced to give the homeodomain protein-encoding *Ubx* mRNAs, which are 3.2 and 4.3 kb in length (O'Connor et al., 1988; Kornfeld et al., 1989). Two other homeodomain protein encoding mRNAs are produced from the BX-C: *abd-A* and *Abd-B* (Regulski et al., 1985).
- Lewis had reported in the mid-1950s, the remarkable fact that the *pbx* and *Cbx* mutations had simultaneously been induced with X-rays in one fly, on the same chromosome, and that they produce reciprocal phenotypes (Lewis, 1954a, 1955): *pbx* causes transformation of T3p → T2p while *Cbx* causes transformation of T2p → T3p. Amazingly, the molecular analysis showed that the 17 kb segment of BX-C DNA that is deleted from −3 to +14 kb on the map in the *pbx*^{1} mutant, is actually inserted in inverted orientation at position −44 kb to give the *Cbx* mutation! It is now known that the *pbx* region DNA acts to *cis*-regulate the *Ubx* promoter (Beachy et al., 1985; Simon et al., 1990; Irvine et al., 1991). Somehow, moving it 40 kb proximally causes it to turn on *Ubx* expression abnormally in T2p, hence the *cis*-dominant effect on *Ubx* that Lewis had described (Lewis, 1955, 1963a, 1978). The exact mechanisms

remain to be determined although several models have been proposed (see, for example, Peifer et al., 1987).

- The *bxd* and *pbx* mutations map in an area of about 35 kb just distal to the *Ubx* cluster of mutations (between −23 and +14 kb on the map). The phenotypes of the *bxd* mutations correlate with their map positions in that those with breaks closest to the *Ubx* mutations (e.g., bxd^{55i}) have a strong mutant phenotype in the first abdominal segment (AB1 → T3) but little affect on the third thoracic segment; those that map further distally (e.g., bxd^{1}, bxd^{55j}) have a weaker effect on AB1 but a stronger effect on T3 (T3p → T2p); those that map even more distally (e.g., bxd^{K}) have an even weaker effect on AB1 and a stronger effect on T3p; and the most distal mutations, pbx^{1} and pbx^{2}, have almost no effect on AB1 but a very strong effect on T3p. It is now known that the 35 kb *bxd-pbx* region is a giant *cis*-regulatory region that controls the expression of the giant *Ubx* transcription unit and thus the Ubx protein (Beachy et al., 1985; Simon et al., 1990; Irvine et al., 1991). The gradient of phenotypic effects in fact was the first evidence for the presence of distinct *cis*-acting elements spread throughout the *bxd-pbx* region DNA. Without Lewis' large and carefully characterized collection of mutations such analyses would have been impossible.
- Lewis had reported that the *suppressor of Hairy-wing* [*su(Hw)*] mutation suppresses several BX-C mutants, notably certain *bx* and *bxd* alleles, as well as alleles at many other loci (Lewis, 1949, 1967). It now became clear that all such suppressible mutations are caused by gypsy retrovirus-like transposons. Furthermore, suppression did not involve excision of the transposable element, neither was the map of the gypsy insertions altered in any way. Thus, what Lewis had reported 15 years earlier turned out to be a remarkable genetic system that is now known to involve binding of the su(Hw) protein to the gypsy element, preventing it from exerting its mutagenic effects on the gene in which it is inserted (Parkhurst and Corces, 1985; Geyer et al., 1986; Spana et al., 1988).

The positional cloning and subsequent detailed molecular analyses of the BX-C—using methods that are now referred to as "functional genomics"—showed that most of the genetic functions that Lewis had identified reside within giant *cis*-regulatory regions that regulate where and when the three protein-coding mRNAs are expressed. While these regulatory regions produce transcripts, they do so only transiently in early embryos at the time that the BX-C genes are being activated for the first time (Akam and Martinez-Arias, 1985; Lipshitz et al., 1987; Sánchez-Herrero and Akam, 1989; Cumberledge et al., 1990). Surprisingly, these regulatory region transcripts were found to be nonprotein coding (Lipshitz et al., 1987; Cumberledge et al., 1990). Upon their discovery, it was argued that they are unlikely to mediate cis-regulatory activation of the *Ubx*, *abd-A* and *Abd-B* promoters. Consistent with this argument, recent studies have shown that the *bxd* noncoding RNAs actually act in *cis* to *downregulate* the adjacent, *Ubx*, transcription unit in cells that do not express *Ubx*, via a transcriptional interference-based mechanism (Petruk et al., 2006).

The molecular analyses were a mixed blessing from Lewis' perspective. His pseudoallelic series had been shown not to comprise the 12 tandemly duplicated protein-coding genes that he initially thought he had discovered. Instead the BX-C was shown to consist of three homeodomain-protein coding genes—fewer in number than Lewis had postulated, although clearly related by tandem duplication—along with nearly a dozen *cis*-regulatory regions, each represented by one of Lewis' genes. Lewis was, at first, disappointed that the molecular reality did not correlate more closely with his abstract model. However, as discussed above, it is not realistic to suppose that one can predict, solely on the basis of genetic analyses, the molecular reality.

Interestingly in retrospect, Lewis' graduate student, Gary Scheidt (1970), had begun genetic screens to identify lethal mutants that mapped in or near the BX-C two years after Lewis had devised efficient methods for chemical mutagenesis in Drosophila (Lewis and Bacher, 1968). As Lewis put it in his introduction to the summary of his laboratory's research in the 1970 Biology Division Annual Report: "One of the major lines of attack is to use chemical mutagens to identify as many as possible of the gene functions which can exist in this 'bithorax' region of the third chromosome." By 1970, Scheidt had identified some 20 lethal mutants. However he subsequently decided to leave graduate school, and Lewis never pursued the screens. Had he done so, perhaps he would have arrived at the idea that there are three functional genetic domains. Even then, there would have been no way to infer the molecular details. As it happened, the lethal screens that led to the definition of the *Ubx*, *abd-A*, and *Abd-B* functional domains (Sánchez-Herrero et al., 1985; Tiong et al., 1985) coincided with the molecular cloning and analysis of the BX-C (Bender et al., 1983a; Karch et al., 1985; Regulski et al., 1985), thus the two approaches synergized in ways that would have been impossible a decade earlier.

Almost 40 years after Lewis had initiated his studies of bithorax mutants because of his interest in tandem gene duplication and gene evolution, molecular analyses showed that tandem duplication did in fact underlie evolution of the BX-C (McGinnis et al., 1984b; Scott and Weiner, 1984). Furthermore, the homeodomains, encoded by the homeoboxes that are the hallmark of these tandemly duplicated genes, function directly in DNA-binding and gene regulation (Laughon and Scott, 1984; Shepherd et al., 1984) as Lewis had hypothesized in his earlier papers (Lewis, 1964; Lewis, 1978). Finally—and amazingly—it was discovered that the so-called homeobox (HOX) complexes date back to the beginning of metazoan evolution, as well as that the colinearity and other genetic rules Lewis had defined, may also have been conserved (see, for example, Duboule and Morata, 1994; Duboule, 1998; Kondo et al., 1998; Kmita et al., 2000)!

BITHORAX AND THE EVOLUTION OF SEXUALLY DIMORPHIC TRAITS

During the decade from 1985 to 1995 Lewis published a series of molecular biological analyses of the *Abd-B* region of the BX-C in collaboration with his

postdoctoral fellow and research associate, Susan Celniker (Celniker and Lewis, 1987, 1993; Celniker et al., 1989, 1990, 1993). As will be seen below, this close collaboration continued after Celniker left to join the Berkeley Drosophila Genome Project Sequencing Center. Several of the papers published during that decade reflect Celniker's interest in *Abd-B* molecular biology. However, two of the papers (Celniker and Lewis, 1987, 1993) addressed the issue of how BX-C genes might participate in the evolution of sexually dimorphic traits. They are therefore included here.

Sexual dimorphism presumably evolved to enhance the ability of animals to find mates. Many animals exhibit extreme sexual dimorphism while, in other cases, dimorphism is subtle or absent. *Drosophila melanogaster* derives its species name from the fact that male flies have dark pigment on abdominal segments 5 and 6. Female flies lack such pigment. The *Mcp* gain-of-function mutation that Lewis (1978) had reported in 1978, derives its name from the fact that, in males, it causes the fourth abdominal segment to become fifth segment-like in identity, thus gaining pigmentation.

In October 1982, Lewis discovered a curious gain-of-function mutant that he called *Transabdominal* (*Tab*), which causes two sets of pigmented longitudinal stripes to appear on the dorsal second thoracic segment (the notum). Most strikingly, in males the stripes are darkly pigmented while, in females, the inner pair is in fact lighter than the rest of the notum, and the outer pair has a dark medial boundary but is otherwise lighter than the surrounding wild-type tissue. The *Tab* phenotype is of interest for two major reasons: First, it is sexually dimorphic. Second, if its underlying cause is an alteration in *cis*-regulation of the abdominal gene(s) of the BX-C, then it is unusual in that it affects body segments far removed from the normal site of action of the gene (i.e., in T2 versus A5 and A6) and also very different in origin (derived from the T2 imaginal disc versus the abdominal histoblast clusters). This last point was of particular developmental interest since the growth dynamics of the imaginal discs and histoblast nests are very different: the discs divide, grow and differentiate largely during the larval and early pupal stages, while the histoblast nests are quiescent throughout the larval period and only begin to divide during the pupal period, reaching a fully patterned, differentiated state in the late pupa.

In this pair of papers, Celniker and Lewis carry out a molecular analysis of *Tab* and show that it is caused by misexpression of the *Abd-B* gene (referred to as *iab-7* in the 1987 paper and as *Abd-B* in the 1993 paper) (Celniker and Lewis, 1987, 1993). They show that *Tab* is caused by a chromosomal inversion (89E to 90E) and that DNA introduced from 90E causes ectopic expression in the wing disc of *Abd-B*. The expression of *Abd-B* in the disc correlates with the position of certain muscle-attachment sites, suggesting that the *cis*-regulatory elements from 90E are normally activated in or near these sites. Thus the pigmented regions of the notum are homeotic transformations of thoracic cuticle into abdominal cuticle, and the sexual dimorphism of the abdominal pigmentation has been transferred to the homeotically transformed stripes in the thorax.

A *Tab*/+ mutant fly therefore has misexpression of *Abd-B* in the presumptive notal region (because of the *Tab* chromosome) and normal expression of *Abd-B* in the

posterior abdomen (because of the presence of the + chromosome). In homozygotes, *Tab* is lethal because it disrupts expression of *Abd-B*. Thus, in the Discussion to the 1993 paper (Celniker and Lewis, 1993), an argument is presented reminiscent of Lewis' Introduction to his 1951 paper (Lewis, 1951), but with an emphasis now on dominant gain-of-function mutations and enhanced by the new molecular insights:

"*Tab*... results in an extension of sexually dimorphic traits into the dorsal thorax of the adult, where it could play a role in the sexual behavior of the organism and therefore might under some circumstances have a selective advantage...A macromutation will generally, as in the case of *Tab*, represent loss of an "old" gene function and gain of a "new" one. Since the old function will seldom be a dispensable one, such a mutant will tend to be lethal when homozygous and can be maintained in a population only over a chromosome containing the wild-type allele. If a duplication for the wild-type allele is already present in the species at the time the mutation to a new function arises, then obviously the resultant mutant can immediately become viable as a homozygote. Otherwise a duplication containing one copy of the mutant allele and one copy of the wild-type allele would need to arise later."

It was not lost on Celniker and Lewis that the change they had discovered is in the *cis*-regulation of a homeotic gene and that much of evolution might, as proposed many years earlier (King and Wilson, 1975), derive from alterations in *cis*-regulation rather than in the encoded protein. That over 98% of the DNA in the BX-C is *cis*-regulatory rather than protein coding, emphasizes the importance of *cis*-regulatory regions in gene evolution.

DNA SEQUENCE MOTIFS IN THE *CIS*-REGULATORY REGIONS OF THE BX-C

Lewis was an early and strong supporter of the human genome project, particularly the pilot projects that aimed to sequence invertebrate model organism genomes, such as that of *D. melanogaster*, prior to the larger, human, enterprise. While the *C. elegans* community readily embraced the idea, there was much resistance in the Drosophila community, in large part because of opposition to the coordinated, large-scale, machine-driven science that genome sequencing projects represent. Lewis is not one for making public pronouncements, thus it was surprising—and in many ways ironic—when he pointed out, to the 100 Drosophilists at the 1990 EMBO Workshop in Kolymbari, Crete on "Molecular and Developmental Biology of Drosophila," that failure to support a concerted effort to sequence the *D. melanogaster* genome might result in Drosophila ceasing to be a relevant experimental model. Whether or not Lewis' comments were decisive to the outcome of the debate, shortly thereafter Gerry Rubin agreed to head the Drosophila genome project and the rest—as they say—is history!

Because the BX-C remained the best-characterized gene complex in terms of genetic and molecular functions, particularly in the *cis*-regulatory regions, as well as because of its large size (over 300 kb) it was selected to test the methods then planned

for use in sequencing the entire Drosophila genome. By 1995, the complete BX-C sequence was obtained (Martin et al., 1995). Lewis' background in biostatistics had served him well 40 years earlier when he began his analyses of the effects of ionizing radiation in inducing human cancer (Lewis, 1957). It now provided the basis for his analysis of sequence motifs in the *cis*-regulatory 300 kb of the BX-C. An attraction for Lewis was the hope of finding sequence motifs in these regions, which might indicate evolution by tandem gene duplication beyond that already shown by the presence of three homeobox-containing genes in the complex (Lewis, 1951, 1978). With colleague, David Mathog—whose combination of talents in Drosophila genetics, mathematics and computer programming is unusual—Lewis searched for sequence motifs (Lewis et al., 1995). Using Markov chain theory it was possible to derive a table of hexanucleotides that are "overrepresented" and occur in clusters of two or more along with a map of where these occur within the BX-C (Lewis et al., 1995). Here "overrepresented" means relative to the expectation derived from pseudorandom control sequences, and the cumulative Poisson distribution was used to derive P values.

Lewis divides the results into two classes: overrepresented motifs of unknown function (of which there were six) and motifs that are known protein-binding consensus sites (four out of the ten that they analyzed were significantly overrepresented). Among the former, by far the most overrepresented sequence is AGATAC (and its complement GTATCT), which, particularly in clusters of two or more, is present 100 times more frequently than expected ($P < 10^{-15}$). This is the sequence that had been identified a decade earlier in a search for possible sequence complementarity between the noncoding *bxd* primary transcript and the primary transcript that is spliced to form the *Ubx* mRNA (Hogness et al., 1985; Lipshitz et al., 1987). In retrospect, given that the sequence had been discovered using reduced-stringency DNA blot hybridization, it is not surprising that this method found the most abundant repeated sequence in the complex! The function of the AGATAC repeat remains unknown, although, given its location in or near the *abx-bx* and *bxd-pbx* regions, it has been speculated that it might function together with appropriate binding-proteins, to bring distant *cis*-regulatory regions into proximity (Hogness et al., 1985).

Several proteins are known to regulate BX-C function either transiently in the early embryo (e.g., Hunchback, Tailless, Caudal, Fushi tarazu) or throughout development (e.g., Zeste). One problem faced in analyzing the BX-C sequence is that the consensus-binding sites for many of the transient early regulatory proteins are degenerate. Furthermore, the sites may function alone rather than as repeated motifs. Consequently, motifs for several of these candidates were not found to be overrepresented in the statistical analysis. In contrast, sites for Zeste were found to be overrepresented ($P < 10^{-3}$ or 10^{-4}) with almost 600 such sites throughout the complex. Zeste is known to be involved in pairing of homologous chromosomes and thus to participate in transvection within the BX-C and elsewhere (Benson and Pirrotta, 1987, 1988; Bickel and Pirrotta, 1990).

Thus Lewis had discovered the BX-C beginning in the mid-1940s, had used it to define the fundamental genetic mechanisms that control animal development, had

participated in a crucial way in the transition from genetic to molecular analysis of development, and now—50 years after commencing his analyses—had carried out the first search for DNA sequence motifs in part of the Drosophila genome, "his" part, the BX-C! As he points out in the Discussion, the analysis "should be viewed as an attempt to develop a 'signature' for each of the *cis*-regulatory regions of the complex . . . [A] powerful approach when coupled with biochemical and developmental approaches and ultimately with comparative sequence analysis of homologous genes in other organisms".

EVOLUTION OF THE HOX COMPLEXES

The final two papers in this section (Von Allmen et al., 1996; Lewis et al., 2003) partially fulfill Lewis' goal as set out in the 1995 paper to carry out comparative sequence analysis of HOX-C's from other organisms, also returning to an evolutionary question of major interest: How have the HOX complexes evolved? Of particular significance in light of the COL rule, is why—and where—the complexes have split in Drosophila while they appear to have remained contiguous in the vertebrate lineage for half a billion years.

Lewis had induced *Ubx* mutations in several species of Drosophila (*D. virilis*, *D. hydei*, *D. repleta*, *D. funebris*) in the late 1950s, using X-rays. His goal at the time was to obtain chromosomal rearrangement mutations that would enable him to define the polytene location of the bithorax gene cluster and thus whether it is located in a pair of doublet bands as in *D. melanogaster*. This was of interest because of his gene duplication and divergence hypothesis (Lewis, 1951), described in the commentary on the Genes section of this book. In *D. virilis*, he had recovered a rearranged chromosome with breakpoints in 24E, 25G, and 27F, which had an associated *Ubx*-type phenotype. With cloning of the *Antp*, *abd-A* and *Abd-B* genes of *D. virilis* in the laboratory of Francois Karch, it became possible to assess whether these genes all map to the same place (Von Allmen et al., 1996). Surprisingly, the *Ubx* and *Antp* genes map to chromosomal region 24E—the site of one of the breakpoints—while the *abd-A*, and *Abd-B* genes map to 26D. Thus, the *D. melanogaster* HOX-C is split between *Antp* (now located in 84B) and *Ubx* (now in 89E) while, in *D. virilis* these two genes are contiguous and a split has, instead, occurred between *Ubx* (now in 24E) and *abd-A* (now in 26D).

Subsequently, the almost complete sequencing of the genomes of *D. melanogaster* (Adams et al., 2000) and the mosquito, *Anopheles gambiae* (Devenport et al., 2000; Powers et al., 2000; Zdobnov et al., 2002), provided the first chance to compare the organization of the HOX-C's in a representative of a more recent (*D. melanogaster*) and a representative of a much older (*A. gambiae*) group of Diptera. In the latter, the HOX-C genes are all contiguous. Sequence analysis of the region extending from *Antp* to *Ubx* in *D. virilis* showed that, while *Ubx* and *Antp* are contiguous in *D. virilis*, a gene that is unlinked to the HOX-C in *A. gambiae* (CG31217) had transposed into the region between *Antp* and *Ubx* in the ancestor of *D. virilis* and *D. melanogaster*

(Lewis et al., 2003). This gene had remained linked to *Ubx* upon the splitting of the ancestral Drosophila HOX-C between *Ubx* and *Antp* in *D. melanogaster*. In *D. virilis*, a further transposition had occurred into the *Antp*-to-*Ubx* region, of a gene CG10013. Thus Lewis could propose a series of hypothetical ancestors and an evolutionary tree, explaining the splits in the Dipteran HOX-C's (Lewis et al., 2003).

It is ironic that the HOX-C's seem to be most fluid in the very lineage in which Lewis discovered them and derived the genetic rules describing why their component genes have remained contiguous in most animals for half a billion years!

SUMMARY

The papers in this section of the book brought Lewis full circle, back to his starting point in gene evolution, but with major new molecular and mechanistic insights. The molecular analyses of the HOX-C's in Drosophila had provided, for the first time, the ability to extend Lewis' paradigm for developmental control from an abstract genetic model to concrete reality. As described above, the molecular mechanisms did not always fit with Lewis' models, but this was in large part because the rules by which genes control the development of complex multicellular organisms could not be modeled using concepts of the day, which derived from studies of simpler organisms. Gratifyingly, Lewis' argument in favor of "genetic" rather than "functional" models had held up. These genetic models could now be reified in terms of master regulatory genes that encode transcription factors together with the giant *cis*-regulatory regions that control the spatial and temporal expression of these regulatory molecules. Furthermore, Lewis' ideas about gene evolution by tandem duplication had proved to be correct, albeit not in quite the way that he had originally proposed when he speculated about "position pseudoalleles" in the early 1950s. Experimental embryology could no longer be accused of invoking "philosophical platitudes... rather than experimentally determined factors", of being "a maze of metaphysical subtleties" (Morgan, 1932). Thanks to Lewis and the molecular pioneers who followed him, developmental biology had been placed on a firm mechanistic basis a century after the first experimental embryologists had begun their studies.

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MOLECULAR GENETICS OF THE BITHORAX COMPLEX IN *DROSOPHILA MELANOGASTER*

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The bithorax complex in Drosophila melanogaster *is a cluster of homeotic genes that specify developmental pathways for many of the body segments of the fly. The DNA of the bithorax complex has been isolated, and a region of 195,000 base pairs that covers the left half of the complex is described here. The lesions associated with many of the bithorax complex mutants have been identified, and most are due to DNA rearrangements. Most of the spontaneous mutants have insertions of a particular mobile element named "gypsy." This element affects the functions of sequences removed from the site of insertion. Mutant lesions for a given phenotypic class are distributed over large DNA distances of up to 73,000 base pairs.*

The bodies of insects are divided into a series of segments. The segments are formed very early in the development of the embryo, and cells from one segment do not, in general, mix with cells from other segments throughout the rest of development (1). In the fruit fly *Drosophila melanogaster,* there are mutations that transform parts of segments or entire segments into the form of other segments. These homeotic mutations define genes that direct cells into different developmental pathways in different segments. The bithorax complex in *Drosophila* is one of the best-studied clusters of such genes (2); these genes determine the developmental fate of many of the thoracic and abdominal segments of the animal. When the whole bithorax complex is deleted, the animal dies late in embryonic development and shows striking changes in the segmental pattern of the embryonic cuticle. The third

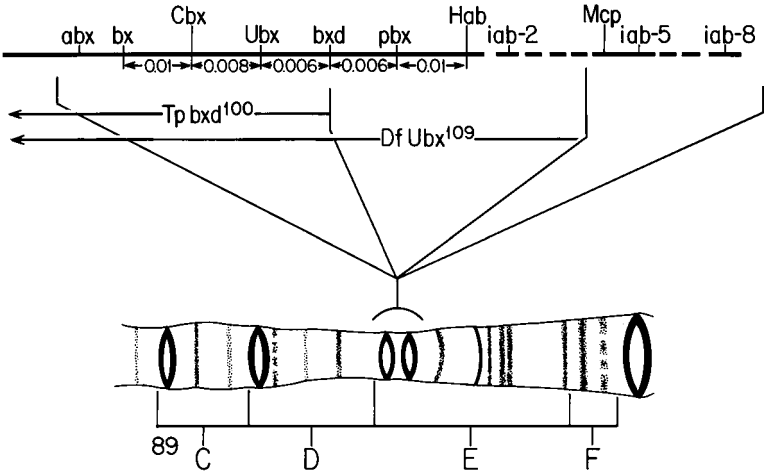


Figure 1. Genetic map of the bithorax complex. The alleles *abx (anterobithorax), bx (bithorax), Cbx (Contrabithorax), Ubx (Ultrabithorax), bxd (bithoraxoid)*, and *pbx* (*postbithorax*) are mutants in the left half of the complex covered by the DNA map. The mutants *Hab (Hyperabdominal), iab-2 (infraabdominal-2), Mcp (Miscadestral pigmentation), iab-5*, and *iab-8* define the abdominal half of the complex. *Mcp* and *iab-5* were isolated and mapped by Crosby (35). The dominant alleles are raised above the recessive ones. Recombination distances between some pairs of mutations are shown. The diagram also shows the approximate position of a *bxd* transposition breakpoint and the extent of a deficiency, Ubx^{109}, that removes the left half of the complex. The entire cluster maps on the third chromosome to the pair of doublet bands at the 89E constriction, as shown.

segment of the thorax and all eight abdominal segments resemble the normal second thoracic segment (2). Thus the second thoracic segment, which gives rise to the pair of wings and the second pair of legs in the adult fly, can be considered the developmental ground state, and the bithorax complex directs the more posterior segments to specialized developmental pathways. Individual recessive mutations within the complex give less extreme segmental transformations than those resulting from deletions of the whole complex. These mutations transform part of a segment or segments into tissue appropriate to a more anterior segment, toward the ground state. There are also dominant mutations, which transform a segment or part of a segment into more posterior structures, away from the ground state (3). These dominant mutations seem to upset the regulation of genes within the complex and turn on functions in an inappropriate segment.

A genetic map of the complex is shown in Fig. 1. Most of the recessive mutants and several dominant mutants show no cytologically visible rearrangements in the salivary gland polytene chromosomes, and they can be recombined with each other. The recombination distances between some pairs are shown. The recessive mutations *bx* and *pbx* affect development of the anterior and posterior halves, respectively, of the third thoracic segment. In the abdomen, *bxd*, *iab-2*, *iab-5*, and *iab-8* affect the first, second, fifth, and eighth abdominal segments, respectively. With the exception

of *pbx*, the alleles are arranged left to right in the complex in the order, anterior to posterior, of the segments which they affect. Some of the recessive alleles and most of the dominant ones are associated with cytologically visible rearrangements, which all have a breakpoint within the pair of doublet bands designated 89E1-4. These bands are in the middle of a prominent constriction, and so it is difficult to distinguish the cytological positions of various breaks within the complex. Some of the rearrangements, such as the bxd^{100} transposition, can be located on the genetic map by testing the resulting fragments of the complex for complementation with the various recessive mutants.

At present we know little of the molecular processes by which genes such as those of the bithorax complex control the developmental fates of cells. However, the large polytene chromosomes in the salivary glands of *Drosophila* have permitted the isolation of such genes when nothing is known of the gene products. We have used techniques called chromosome walking and jumping to isolate a small fragment of the bithorax complex in recombinant DNA molecules (4). That fragment was used to isolate adjacent DNA fragments in a chromosomal walk through the bithorax complex. The first objective of the molecular analysis of the complex has been to identify the DNA changes in the various bithorax mutants. For the mutations in the complex associated with cytologically visible rearrangements, the rearrangement breakpoints can easily be mapped on the DNA (5). However, many of the best studied mutations are cytologically normal. When such mutations are due to single base changes in the DNA sequence, they are very difficult to locate. As it happened, the majority of these cytologically normal mutations in the bithorax complex are associated with easily detectable insertions and deletions.

COLLECTING BITHORAX DNA

Our initial toehold in bithorax DNA was gained by "jumping" across an inversion. The inversion, called Cbx^{+R1}, breaks in the bands 87E1,2 and 89E1,2; the latter breakpoint is within the bithorax complex, as judged by its cytology and by its *Ubx* phenotype (see *Ubx* mutants below). One of the inversion fusion fragments was cloned and identified by homology to sequences in 87E, and the bithorax complex sequences from the fusion fragment were used as the starting point of a chromosomal walk (4). Figure 2 shows a composite restriction map covering 195 kb (1 kb is 1,000 base pairs), marked off in kilobases from the starting point of the walk. This DNA region covers the left half of the bithorax complex, from *abx* through *pbx*, plus sequences to the left of the complex. The walk has also been extended to the right through the region of abdominal mutations, but that half of the complex is less well characterized genetically and molecularly, and it will not be discussed here. The chromosomal orientation of the walk was initially determined by *in situ* hybridizations of bithorax complex probes to four rearrangements with *bxd* phenotypes (see *bxd* mutants below), and has been amply confirmed by the positions of the lesions associated with several genetically mapped mutants (6).

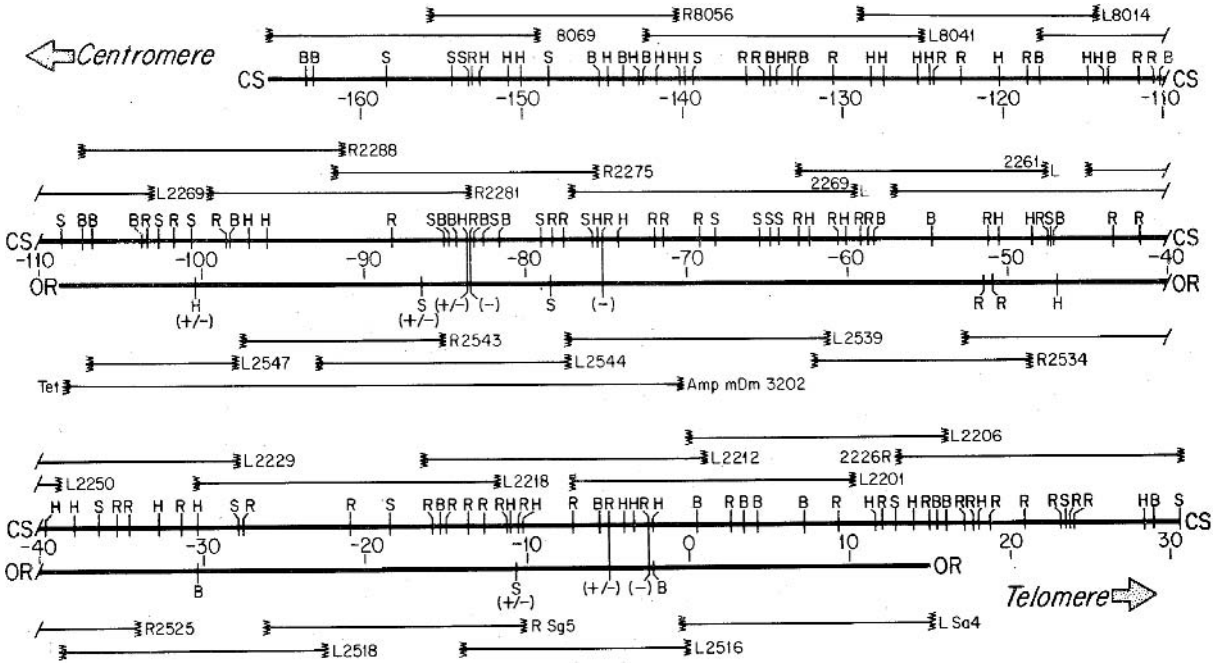


Figure 2. Composite restriction map of the DNA walk. The boldface lines marked *CS* show the map from the Canton S wild type for the four restriction enzymes Bam H1, Hind III, Eco R1, and Sal I (*B, H, R,* and *S*, respectively). The coordinates show the distance in kilobases from the starting point of the walk. The thinner lines above the *CS* map indicate the cloned DNA segments from the Canton S library of recombinant phage. The *L* and *R* designations indicate the orientation of the left and right arms of the lambda phage vector. The boldface lines marked *OR* show the map for the Oregon R wild type. The restriction sites are identical to *CS* except where indicated. The +/− symbol indicates that the site is present in some Oregon R chromosomes but not in others. Representative cloned segments from the Oregon library of recombinant phage are shown below the *OR* line, as well as one Oregon R segment cloned into a cosmid vector (mDm 3202).

The entire map of Fig. 2 consists of single-copy DNA, as can be judged by Southern blot analysis at standard criteria with representative recombinant phage from along the walk. (Repeated sequences that are poorly matched or shorter than a few hundred base pairs might be missed by this analysis.) Thus, there is as yet no evidence for large repeating DNA units corresponding to segments of the fly. DNA was collected from libraries representing two wild-type strains of *Drosophila*, Canton S and Oregon R, and restriction maps from the two strains are compared in Fig. 2. The number of restriction site differences between the two strains does not suggest tight evolutionary sequence conservation for the overall region (7).

As the overlapping DNA segments were collected in the walk, we began to look for the breakpoints of cytological rearrangements associated with various mutations. Such breakpoints can be located unambiguously on the DNA map by *in situ* hybridizations. Probes from along the walk were hybridized to a chromosome with an inversion, for example, to see where the probes switched from labeling one inversion end point to the other end point (8). Once the breakpoint site was identified to within about 15 kb, southern blots were done with genomic DNA from the inversion strain to find the anomalous restriction fragments associated with the inversion breakpoint. We also began to examine, by Southern blot analysis, the DNA from cytologically normal, spontaneous, and X-ray-induced mutations. Most had anomalous restriction fragments indicative of DNA insertions or deletions. For many of these mutations we have constructed libraries of recombinant phage from the mutant DNA and isolated the region of interest (9). The mutant lesions were then characterized directly by restriction mapping and by electron microscopy of heteroduplex molecules. The descriptions of the mutant lesions that follow are based on such clones from mutant libraries, unless otherwise noted.

BX AND *ABX* MUTATIONS

Calvin Bridges found the first spontaneous *bithorax* mutant (bx^1) for which the complex is named. Mutations of *bx* transform the anterior third thoracic segment into anterior second thoracic, so that anterior haltere becomes anterior wing tissue, anterior third leg resembles second leg, and anterior notal tissue appears on the dorsal surface of the third thoracic segment. Various alleles of *bx* differ markedly in the strength of the transformation, and also in which regions of the third thoracic segment are most strongly transformed. bx^1 is the weakest of the alleles considered here: its expression is variable and sometimes overlaps wild type (10). It is associated with an insertion of the mobile repetitive element named "412" (11) at the map position −60 kb (Fig. 3). Homology to 412 was suggested by the restriction map of the insert, and it was confirmed by comparison to a prototypic copy of 412 (12).

The mutation bx^{34e} is another spontaneous allele, intermediate in its phenotype; it usually produces a thin band of notal tissue in the third segment and halteres that are enlarged and bent downward (13). It is associated with the insertion of another mobile element at −63.5 kb (Fig. 3). This element is 7.3 kb in length and has

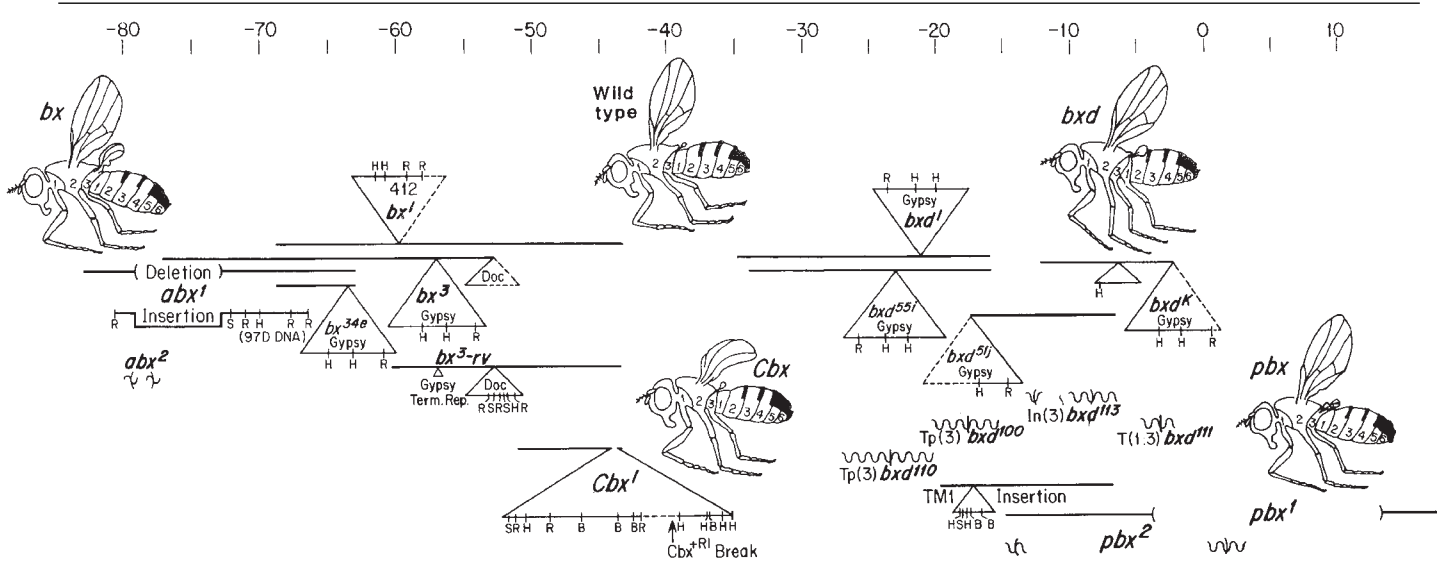


Figure 3. Lesions of the *abx*, *bx*, *Cbx*, *bxd*, and *pbx* mutations. The maps of recombinant clones derived from mutant libraries are shown on the coordinate scale of Fig. 2. The thin horizontal lines indicate DNA sequences cloned from the various mutants that are identical to the wild-type map. Triangles touching these lines show DNA insertions; parentheses show deletions. Dotted lines indicate sequences not recovered in recombinant clones but inferred from genomic Southern blots. Horizontal wavy lines indicate the limits of uncertainty in the positions of breakpoints identified by Southern blots. The drawings of male flies indicate the phenotypes of *bx*, *Cbx*, *bxd*, and *pbx* mutants. Three thoracic and six abdominal segments are numbered.

direct terminal-repeating sequences of about 0.5 kb; we have named the element "gypsy" (14). bx^{34e} reverted spontaneously to a less extreme phenotype. The partial revertant (15) was examined by Southern blot analysis and the restriction pattern of the gypsy insert had changed. The partial revertant has not yet been cloned and examined directly, but a change in the gypsy insertion in this revertant confirms our presumption that the gypsy element is responsible for the bx^{34e} mutation.

The mutation bx^3 is the strongest of the spontaneous *bx* alleles; homozygotes always show strong haltere to wing transformation and a wide band of notal tissue in the third thoracic segment (16). bx^3 also has a subtle dominant phenotype of slight shrinkage of the presutural region of the notum in the second thoracic segment (17). There are two mobile repetitive elements inserted in the bx^3 chromosome, a gypsy at −57 kb, identical in restriction map and orientation to the bx^{34e} gypsy, and a 4.3 kb element named "Doc" (4) at −53 kb (Fig. 3). We know the Doc element is irrelevant to the phenotype for two reasons. First, bx^3 was recombined onto a chromosome carrying the Cbx^1 mutant (see below); the Doc element was lost in the exchange. The bx^3 mutation was subsequently reisolated by recombination away from *Cbx*, and this laundered copy (18) of bx^3, with only the gypsy insertion, appears identical in phenotype to the original. Second, bx^3 reverted spontaneously with loss of the gypsy (19). DNA cloned from the revertant still contained the Doc element at −53 kb plus one 0.5 kb terminal repeat of the gypsy element at −57 kb (Fig. 3), which is consistent with excision of the gypsy by recombination between its terminal repeats. The reversion in this chromosome appears to be complete: no *bx* phenotype is present even when the revertant chromosome is heterozygous with a deficiency for the whole complex.

Other spontaneous *bx* alleles (bx^9, bx^{AV}, and bx^G) are associated with apparent insertions in the −75 to −55 kb region, but the mutant lesions have not yet been examined in cloned DNA.

The mutation abx^1 (*anterobithorax*) was initially designated bx^7, but was renamed when it could be distinguished from other *bx* alleles by a number of criteria (17, 20). abx^1 gives anterior leg and haltere transformations like bx^3, but it produces the more anterior presutural notal tissue in the third thoracic segment. abx^1 was X-ray–induced, and is associated with an insertional transposition of 6 kb of bithorax complex DNA (−79 to −73 kb) into the 97D region near the right end of the third chromosome, as determined by *in situ* hybridization (Fig. 3). The deletion in the bithorax complex at 89E and the insertion in 97D have been separated by recombination, and each has been made homozygous. The deletion alone gives a phenotype apparently identical to the initial abx^1 mutant; the insertion alone gives the wild type.

There is another X-ray–induced mutation (21), called bx^{SK}, with a phenotype like that of abx^1; we suggest that it be renamed abx^2. This mutation is associated with a 1.5 kb deletion (−79.5 to −78 kb) that overlaps the abx^1 deletion (Fig. 3). The abx^2 deletion was mapped by Southern blot analysis in which the mutant chromosome was compared with the marked chromosome on which it was induced.

PBX AND *CBX* MUTATIONS

The mutations *Cbx*^{1} (*Contrabithorax*) and *pbx*^{1} (*postbithorax*) arose together after X-ray exposure and were subsequently separated by recombination (22). The recessive *pbx* mutation transforms posterior haltere to wing, posterior third leg to second leg, and gives dorsal postnotal tissue in the third thoracic segment (22). Double mutant flies *bx pbx* have both anterior and posterior transformations of the third thoracic segment, and so they develop four wings (16). The dominant *Cbx*^{1} mutation transforms posterior wing into posterior haltere, and the posterior notal tissue is reduced. Thus the *pbx* and *Cbx* transformations are complementary, although not completely so (17). *pbx*^{1} is associated with a deletion of 17 kb from −3 kb to +14 kb (see Fig. 3). *Cbx*^{1} has an insertion of that same 17 kb segment, with its orientation inverted, into the map at position −44 kb (Fig. 3). The limits of the *Cbx* insertion are identical to the end points of the *pbx* deletion, to within about 0.5 kb, as judged by restriction mapping and heteroduplex analysis of the clones derived from the *Cbx* and *pbx* mutants.

The molecular events of the *Cbx* and *pbx* mutations suggest a simple model for the observed phenotypes. The −3 to +14 kb region encodes the information to specify posterior third thoracic segment. The loss of that information in *pbx* homozygotes causes the developmental path for the posterior third thoracic segment to mimic the second thoracic ground state (2). When this sequence is inserted at −44 kb, it is expressed in the second thoracic segment, so that third thoracic structures are produced. This interpretation is reinforced by the phenotype of the double mutant. *Cbx pbx/pbx* flies have normal halteres and lack any postnotal tissue in the third thoracic segment, as if the insertion rescues the deletion. The *pbx* transformation in the third leg is not altered by *Cbx*, however. An alternative model (2) postulates that the *Cbx* insertion causes inappropriate expression of the *bx*$^+$ or *Ubx*$^+$ products, which causes the dominant *Cbx* transformation, and substitutes for the loss of *pbx*$^+$ in the double-mutant animal.

The only other *pbx* allele, *pbx*^{2}, was also X-ray–induced and is associated with a large deletion from about −14 to +1 kb. The phenotype is similar to *pbx*^{1}, except in transvection (17). This deletion has not yet been cloned, but analysis of genomic DNA by the Southern technique is unambiguous, since restriction fragments from within the deletion show no homology to the DNA of *pbx*^{2} flies.

There are several other dominant mutations analogous to *Cbx*^{1}, including *Cbx*^{2}, *Haltere-mimic (Hm), Cbx*^{3}, and *Cbx*^{Tiwt}, although each of these is phenotypically quite distinct (17). *Cbx*^{3} and *Cbx*^{Tiwt} are discussed below.

BXD MUTATIONS

The *bxd* (*bithoraxoid*) mutations transform the first abdominal segment to third thoracic; the first abdominal tergite is reduced or absent, and strong alleles, when hemizygous, generate an extra leg or pair of legs from the first abdominal segment and, rarely, an extra haltere. In addition, the posterior third thoracic segment is partly transformed to posterior second thoracic, which has been described as *cis*-inactivation

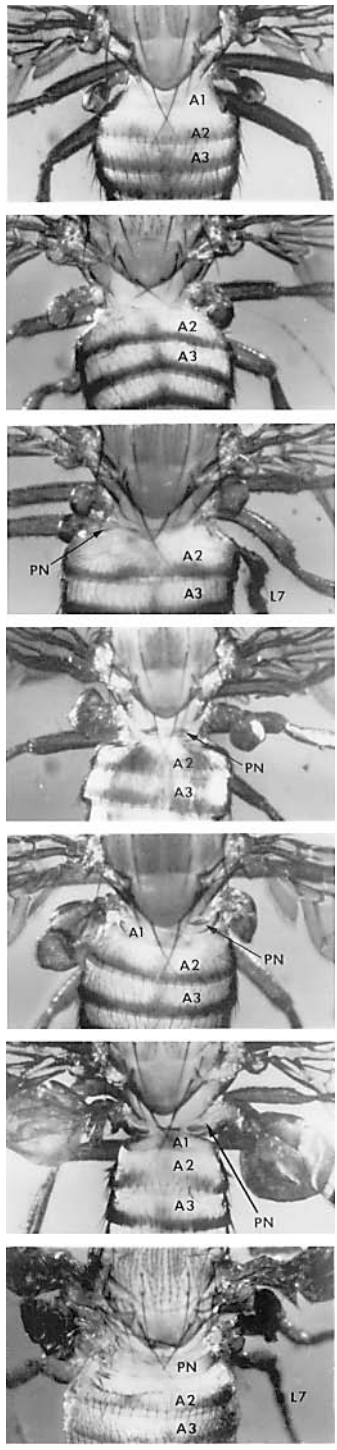
of the adjacent *pbx* region (16). Like *bx* alleles, *bxd* alleles vary in the severity of the segmental transformation and somewhat in the spectrum of transformed structures observed.

The mutation bxd^1 occurred spontaneously and is associated with an insertion of the gypsy mobile element at −21 kb (Fig. 3). The gypsy is identical in restriction map to the bx^3 and bx^{34e} gypsies but is in the opposite orientation. bxd^1 has spontaneously reverted to wild type twice. In both instances (23), Southern blots of the whole genome show that 0.5 kb of extra DNA remains at about −21 kb, which presumably represents a copy of the gypsy terminal repeat.

Another spontaneous mutation, bxd^{55i}, has an insertion of the gypsy mobile element at −23 kb, identical in restriction map and orientation to the bxd^1 gypsy (Fig. 3). The mutation bxd^{51j}, also spontaneous, is associated with an insertion of the gypsy element at −17.5 kb, opposite in orientation to that of bxd^1 (Fig. 3). bxd^K is also spontaneous and has two insertions, an unidentified 3.4 kb repetitive element at −6.5 kb and a gypsy element at −2.5 kb (Fig. 3). The gypsy is identical in its restriction map to that of bxd^1 but opposite in orientation. We presume that the element at −6.5 kb is silent because bxd^K is completely suppressible by *su(Hw)* (see below). Other spontaneous *bxd* alleles (bxd^9 and bxd^{SR}) are also associated with apparent insertions in the −20 to −5 kb region, but these mutant lesions have not yet been cloned. The balancer chromosome TM1 has an insertion of about 3 kb of unidentified repetitive DNA at about the same position as the bxd^{51j} insertion (Fig. 3), but the TM1 insertion has no detectable *bxd* phenotype.

Another large class of strong *bxd* alleles are X-ray–induced and are associated with cytological rearrangements that split the bithorax complex. Four such breakpoints have been mapped by *in situ* hybridizations and Southern blots of genomic DNA; their positions are shown in Fig. 3. bxd^{100} is a transposition of the left half of the complex into 66C, bxd^{110} is a transposition of the 91D-92A region into the bithorax complex, bxd^{111} is a translocation of the right half of the complex into 4D, and bxd^{113} is an inversion to 69 (apparently associated with a small deletion of bithorax material at the breakpoint). bxd^{111}, which maps farthest to the right, can be distinguished from the other rearrangement alleles by its larval phenotype (20).

The phenotypes of the different *bxd* alleles can be correlated somewhat with their position on the DNA map. Figure 4 shows the more extreme phenotypes of several *bxd* alleles, all heterozygous with a deficiency. Farthest to the left is bxd^{55i}, which causes complete loss of the first abdominal tergite (rarely with a seventh leg) but very little transformation of the posterior third thoracic segment. bxd^1 also always removes the first abdominal tergite, and there is slightly more enlargement of the haltere, with an occasional thin band of postnotal tissue in the dorsal third thoracic segment. About 20% of flies with this allele have seven or eight legs. bxd^{51j} again removes the first abdominal tergite, the posterior haltere is more swollen than in bxd^1, and there is consistently a band of dorsal postnotal tissue. About 40% of these flies have extra legs. bxd^K shows a variable reduction of the first abdominal tergite, rarely complete. Flies with this mutation never have extra legs, but the


A1
A2
A3
A2
A3
PN
A2
A3
L7
A2
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PN
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A1
A2
A3
PN
PN
A2
L7
A3

posterior haltere is variably enlarged, sometimes like a *pbx* haltere, with occasional patches of postnotum in the third thoracic segment. pbx^1 and pbx^2 give consistent transformation of posterior haltere to wing with extra dorsal postnotal tissue, and both give variable slight reduction of the first abdominal tergite. Thus, the insertions of the gypsy elements, going from left to right, show a graded effect on the first abdominal segment (strongest on the left) and on the posterior third thoracic segment (strongest on the right). A *bxd* breakpoint consistently gives strong expression of all of the above transformations (the flies usually die before eclosion), as if causing complete inactivation of the whole region.

SUPPRESSOR OF HAIRY-WING AND GYPSIES

Many of the spontaneous *bx* and *bxd* alleles are suppressed by the recessive second-site suppressor, *suppressor of Hairy-wing* [*su(Hw)*]. This suppressor affects particular spontaneous mutations at several other loci, such as *scute, cut, forked*, and *lozenge*. Of the bithorax complex mutations we have recloned, bx^3, bx^{34e}, bxd^1, bxd^{51j}, bxd^{55i}, and bxd^K are suppressible. These are all of the mutations that have insertions of the gypsy element, suggesting that a gene product of *su(Hw)* might interact specifically with this element. Thirteen other suppressible alleles at other loci have since been checked for gypsies, and all except two alleles of *rudimentary* have the gypsy element at the site of the mutation (14). Several other spontaneous mutations in the bithorax complex are also suppressed, including bx^9, bx^G, and bxd^9; we expect the cloning of their DNA will reveal gypsy insertions.

The gypsy element is apparently not excised in suppressed animals. DNA was extracted from suppressed adult flies homozygous for bxd^1 or hemizygous for bx^3, and was examined by Southern blot analysis. The band from the gypsy insertion remained unchanged in both cases. We have little other information on the mechanism of suppression since most of the suppressed alleles are in "complex loci" (24) for which the gene products have not been identified.

UBX MUTATIONS

Ubx (Ultrabithorax) mutations fail to complement with *bx*, *bxd*, and *pbx* alleles, and the *Ubx* recessive phenotype is equivalent to the sum of these three phenotypes (3). Animals homozygous for *Ubx* die as larvae or early pupae, but it is clear from

←

Figure 4. Photographs of *bxd* and *pbx* mutants. All pictures show the backs of female flies with the wings extended to reveal the dorsal abdomen. All flies have one chromosome with deficiency Ubx^{109} (Fig. 1); the second chromosome is (from top to bottom): Canton S wild type, bxd^{55i}, bxd^1, bxd^{51j}, bxd^K, pbx^1, and Tp bxd^{100}. Some parts of the cuticle are designated as follows: *A*1, *A*2, and *A*3, the first, second, and third abdominal tergites: *PN*, new postnotal tissue appearing in the third thoracic or first abdominal segments: *L*7, a seventh leg from the first abdominal segment. The bottom fly (Tp bxd^{100}) had to be dissected out of the pupal case; it shows a band of cuticle between *A2* and *PN* which lacks hairs and pigment. This cuticle is presumed to be scar tissue which fills the space normally taken by the first abdominal tergite.

larval cuticular structures that the third thoracic and first abdominal segments are both transformed to copies of the second thoracic segment (2).

Most of the available *Ubx* alleles are associated with cytological rearrangements with a break in the bithorax complex. These breakpoint alleles are nearly equivalent in phenotype to deletions for the left half of the complex; all give very strong transformations when heterozygous with *bx*, *bxd*, or *pbx*. The breakpoints of 12 such *Ubx* mutations are shown in Fig. 5: Ubx^{780} and Ubx^{882} were induced by ethyl methane sulfonate (EMS) and the rest by X-rays. All were induced on defined background chromosomes, and so most of these breakpoints were identified by comparing Southern blot patterns of their genomic DNA with those of the background chromosomes. The leftmost breakpoint (actually the end point of a cytological deficiency for 89D-E) is at about −105 kb; the rightmost breakpoint is at −32 kb. Thus the Ubx^{+} function apparently requires continuity of the chromosome for a region of at least 73 kb.

Ubx^{1}, a spontaneous mutant, is a medium-strong allele; it gives less extreme transformations when heterozygous with *bx*, *bxd*, or *pbx* than does a deficiency for the bithorax complex. It is associated with an insertion of the Doc mobile repetitive element at −32 kb (Fig. 5). This Doc element is identical in its map but opposite in orientation to the silent Doc insertion at −53 kb in the bx^{3} chromosome.

Ubx^{849} is also similar in phenotype to Ubx^{1}: it is associated with a deletion of about 110 base pairs at −32.4 kb, as judged by comparison of the mutant and background chromosomes by the Southern technique with four restriction enzymes. Ubx^{849} is one of a group of nine *Ubx* mutations induced by EMS (25); the Ubx^{780} translocation was also from this group, but no lesions were found for the others.

Among a set of X-ray–induced *Ubx* mutations, two had phenotypes very similar to that of Ubx^{1} (26). (The remainder were stronger alleles associated with the rearrangements mentioned above.) One of these, $Ubx^{6.28}$, is associated with a deletion of about 50 base pairs at −31.5 kb, very close to the site of insertion of the Doc element in Ubx^{1}. The other, $Ubx^{9.22}$, has not yet been cloned, but a comparison of the mutant and background chromosomes analyzed by the Southern technique with five restriction enzymes showed a 1.6 kb deletion at −105 kb and no other detectable change in the left half of the bithorax complex.

These four cytologically normal mutations (Ubx^{1}, Ubx^{849}, $Ubx^{6.28}$, and $Ubx^{9.22}$) therefore map at the ends of the 73 kb region defined by the *Ubx* rearrangement breakpoints—three at the right end and one at the left end. The location of $Ubx^{9.22}$ at the left end has been confirmed by genetic mapping that places it 0.02 unit to the left of the bx^{34e} mutation.

There are several *Ubx* alleles that are weaker than Ubx^{1}. Most of them were induced with EMS. One (Ubx^{51}) (27) has been mapped by recombination to be between *Cbx* and *bxd*, in the Ubx^{1} region. This allele shows no anomalous bands when the whole genome is analyzed by the Southern technique with probes covering this region. We presume that it is a true point mutant, and other EMS-induced alleles for which we found no lesions may also be point mutations.

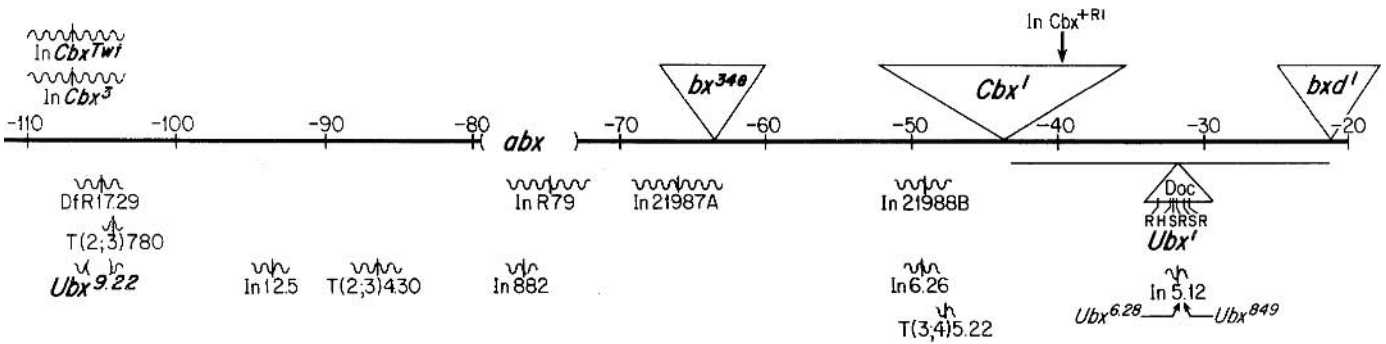

Figure 5. Lesions of *Ubx* mutations. The boldface line represents the DNA map of Fig. 2, with some of the mutant lesions of Fig. 3 included for orientation. Df R17.29, In R79, In 21987A, and In 21988B are a *Ubx* deficiency and three inversions induced on a Cbx^1 chromosome by E. B. Lewis and T. Ramey. The Cbx^{+R1} inversion was induced by T. Kaufman, also on a *Cbx* chromosome. The 780 translocation was induced by Lewis on a $su(Hw)^2\ sbd^2$ chromosome. $Ubx^{9.22}$, In 12.5, T 4.30, In 882, In 6.26, T 5.22, and In 5.12 are inversions, translocations, and a deficiency induced on a marked third chromosome by S. Kerridge and G. Morata (26).

THE LEFT END

The mutations *Cbx*^{3} (also called *Cbx-like*) (17) and *Cbx*^{Tiwt} (*Twin-thorax*) (28) are associated with lesions in the leftmost region of the complex. Both are X-ray-induced inversions to 89A and 87EF, respectively. Both have a dominant phenotype with a variable reduction of anterior wing and notum. Neither breakpoint has a *Ubx* phenotype and both are viable over deficiencies for the complex. Both breakpoints fall in the region of the leftmost *Ubx* lesions, between −110 and −103 kb (Fig. 5), as judged by hybridization *in situ* and Southern blot analysis. These breakpoints will have to be cloned and examined in detail, but the phenotypes suggest that the inversions remove from the left end of the complex a negative regulatory region which keeps the *abx*$^{+}$ or *Ubx*$^{+}$ functions repressed in the second thoracic segment.

We continued to walk farther to the left, looking for rearrangement breakpoints clearly outside the complex. One of the TE transpositions isolated by Ising (29), TE77, inserted the *white* to *roughest* region of the X chromosome into 89D. This translocation produces no segmental transformation and, since the insertion is so large, it is assumed to lie outside (proximal to) the bithorax complex. The site of insertion has recently been recloned by Paro et al. (30). The 35 kb region around the TE77 insertion site that they isolated is identical to that shown on our map (Fig. 2) between −154 and −119 kb. The TE77 insertion site is at −136 kb.

CONCLUDING REMARKS

We are confident that the DNA changes identified in this article are responsible for the mutant phenotypes for several reasons. The mutations *abx*^{1}, *bx*^{3}, *Cbx*^{1}, *Ubx*^{1}, *bxd*^{1}, and *pbx*^{1} have each been recombined with the adjacent mutations in the series, and the lesions we describe cosegregated with the mutant phenotypes. The changes in the gypsy elements in the revertants of *bx*^{3}, *bx*^{34e}, and *bxd*^{1} confirm that the gypsies are responsible for these mutations, and we have found a general correspondence between mutations suppressible by *su(Hw)* and insertions of the gypsy element (14). The DNA map locations of the other lesions correlate well with their phenotypes and their positions on the genetic map (31).

We were surprised that all of the cytologically normal spontaneous mutations (*bx*^{1}, *bx*^{3}, *bx*^{34e}, *Ubx*^{1}, *bxd*^{1}, *bxd*^{51j}, *bxd*^{55i}, and *bxd*^{K}) are associated with insertions of mobile elements. For comparison, only 3 out of 5 spontaneous alleles in the *rosy* locus (32) and 8 out of 13 spontaneous alleles in the *white* locus (33) are associated with mobile element insertions. Likewise, the cytologically normal X-ray–induced alleles have large DNA deletions (*Ubx*$^{9.22}$, *abx*^{1}, *abx*^{2}, *Ubx*$^{6.28}$, *pbx*^{1}, and *pbx*^{2}) or insertions (*Cbx*^{1}), whereas the large majority of X-ray–induced *rosy* mutations are apparent point mutations (32). It was also unexpected that these mutant lesions would be so spread out, the *bx* alleles over 7 kb and the *bxd* alleles over 20 kb. This spread of the mutant lesions could reflect mutations that inactivate coding regions distant from the site of the mutant lesion. This hypothesis is most obvious for the gypsy insertions because the gypsy elements remain in place in suppressed flies. Likewise, the revertants

of bx^3 and bxd^1 leave insertions in the DNA at the site of the original gypsy element; since the flies are completely reverted, it is unlikely that these insertions interrupt essential coding sequence. Insertions of most other mobile elements into the *bx* and *bxd* regions may also be silent, as in the TM1 insertion and the Doc element in bx^3; this might account for the predominance of gypsy insertions among the available mutants. The failure to find true point alleles of *bx*, *bxd*, and *pbx* suggests that single-base changes in these regions may be invisible. Perhaps these regions do not encode functions, or perhaps the functions are not inactivated by most single-base changes, as might be true if the gene products are folded RNA molecules. Alternatively, there may be many subtle functions encoded so that only lesions that inactivate many functions can give a noticeable phenotype. Unfortunately, this action at a distance makes it difficult to locate the regions responsible for normal bithorax functions, or to guess how many distinct functions there are.

The *Ubx* lesions have a peculiar arrangement. The $Ubx^{9.22}$ deletion lies 73 kb to the left of the other cytologically normal *Ubx* alleles (Ubx^1, Ubx^{849}, and $Ubx^{6.28}$), on the other side of the region of *abx* and *bx* lesions. The distribution of the *Ubx* rearrangement breakpoints indicates a requirement for chromosome continuity over the same 73 kb. For the *bxd* function, there is a similar requirement for continuity over some 20 kb. The need for such continuity may reflect RNA transcripts spanning these regions, or tissue-specific rearrangements of the DNA. Several complementary DNA clones generated from embryonic RNA have recently been isolated by homology to genomic bithorax clones (34). Preliminary mapping of these complementary DNA's indicates that exons are spread out over the 73 kb *Ubx* region in some clones and over the 20 kb *bxd* region in others. *Ubx* mutations, including the alleles with small deletions at −105 or −32 kb, inactivate in *cis* the function of *abx*, *bx*, *bxd*, and *pbx*. Long transcripts might be processed differently to give the RNA products for each of these functions, with sequences from the −105 and −32 kb regions included in all the different products.

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(6) The orientation dictated by the fusion fragment was initially misleading. The Cbx^{+R1} inversion was induced on a Cbx^1 chromosome, and the inversion breakpoint fell within the Cbx^1 insertion (see section on *pbx* and *Cbx*). Until the inverted orientation of the *Cbx* insertion was discovered, the implied orientation of the DNA map was reversed.
(7) In the DNA region recovered from both strains, 88 restriction sites have been identified in Canton S, of which five are missing in the Oregon R clones. Thus five out of 528 base pairs are changed, which implies about 1% sequence divergence. The reciprocal comparison is complicated by variation in the restriction maps among clones isolated from Oregon R. The same method of comparison of 167 restriction sites in the 87DE region showed about 0.4% divergence [see (4)].

(8) Such rearrangements were usually made cytologically homozygous so that the two breakpoints of the rearrangement were well separated in squashed preparations. When the rearrangement had a recessive lethal *Ubx* phenotype, the lethality was covered by a duplication for the bithorax complex in the X or second chromosome.
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THE ABDOMINAL REGION OF THE BITHORAX COMPLEX

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SUMMARY

The homeotic mutations in the right half of the bithorax complex of Drosophila cause segmental transformations in the second through the eighth segments of the fly. A chromosomal walk in the bithorax complex has now been extended 215 kb through the right half of the complex, and lesions for over 40 mutations have been located on the DNA map. The mutations can be grouped in a series of phenotypic classes, one for each abdominal segment, although each mutation typically affects more than one segment. The mutant lesions of each class are clustered, and they are aligned on the chromosome in the order of the body segments that they affect. Complementation tests suggest interactions between widely spaced DNA regions; indeed, the right half cannot be split anywhere without some loss of function.

INTRODUCTION

The bodies of many higher organisms are built in segments. In the fruit fly *Drosophila melanogaster* a tandem array of similar body segments forms early in embryonic development. Each segment then develops distinct tissues and structures in the larva and in the adult fly. This differentiation of the segments is under the genetic control of at least two gene clusters, the Antennapedia complex and the bithorax complex (for reviews see Kaufman and Abbot, 1984; and Duncan and Lewis, 1982). These genes

have been identified through homeotic mutations that transform one segment of the fly into another.

The bithorax complex primarily controls the differentiation of the third thoracic and the first through eighth abdominal segments. When the whole bithorax complex is deleted, the third thoracic and all eight abdominal segments look similar to the normal second thoracic segment. In our working model (Lewis, 1978), the different genes of the complex are expressed in different segments. They function to suppress the structures characteristic of more anterior segments and to promote the development of appropriate posterior structures. Thus, loss-of-function mutations in individual genes transform one or more segments to resemble a more anterior segment. Genetic analyses suggest that as one proceeds from anterior to posterior in the animal, bithorax complex genes become activated in sequence along the chromosome. Once activated, each gene appears to remain active in many or all of the more posterior segments.

The Left Half of the Bithorax Complex

The bithorax complex can be divided into two sections of nearly equal size. The mutations affecting differentiation of the third thoracic segment and the first abdominal segment (namely *abx, bx, Ubx, bxd*, and *pbx*; see Lewis, 1978) map in the left half of the bithorax complex. The chromosomal walk covering the left half of the complex and the molecular nature of the lesions there have been reported (Bender et al., 1983b). The DNA walk through the complex began in the *pbx* region; the starting point was chosen as the zero point on the DNA coordinate scale.

The left half of the complex is about 120 kb and can be divided into two DNA regions. The *Ubx* region is defined by rearrangement break points that split the region between −105 and −32 kb, and that all have a strong lethal *Ubx* phenotype. In *Ubx* homozygotes, the third thoracic and first abdominal segments are transformed towards the second thoracic segment (Lewis, 1978). (It has been suggested recently that bithorax mutations transform units composed of the posterior half of one segment and the anterior half of the next segment [Hayes et al., 1984; Struhl, 1984; Martinez-Arias and Lawrence, 1985]. We see some transformations that respect such parasegmental boundaries, and some that respect normal segment boundaries. We will discuss the transformations here as if all transform normal segments.) Within the *Ubx* DNA region, there are a number of viable recessive *abx* and *bx* mutations. These mutations fail to complement with *Ubx* mutations and show a subset of the recessive *Ubx* phenotype. They transform the anterior third thoracic into the anterior second thoracic segment. All of these alleles are cytologically normal, and most result from insertions of transposable elements.

The second DNA region is the *bxd* region, from −30 to +10 kb. Breaks in this region give very strong *pbx* and *bxd* recessive phenotypes, in which the posterior third thoracic segment is transformed into the posterior second thoracic segment, and part or all of the first abdominal segment becomes like the third thoracic segment. This is again a subset of the *Ubx* recessive phenotype. Unlike in the *Ubx* breaks, the

phenotypes of these *bxd* breaks are not all identical. Break points further to the right show successively less severe *bxd* transformations (Bender et al., 1985). Within the *bxd* DNA region, there are also a number of cytologically normal mutations designated as *bxd* or *pbx* alleles. Again, most of the *bxd* alleles are caused by insertions of transposable elements. The *bxd* and *pbx* mutations also fail to complement with *Ubx* mutations. This is because *Ubx* mutations *cis*-inactivate the *bxd* region on the same chromosome. In other words, the *Ubx* and *bxd* regions comprise a single DNA domain that cannot be split apart without losing some wild-type functions.

Molecular data suggest that there are two very large, nonoverlapping transcription units corresponding to the *Ubx* and *bxd* DNA regions. Each of these might produce multiple products, perhaps by alternate patterns of splicing (Rob Saint and Michel Goldschmidt-Clermont, personal communication; Beachy et al., 1985), but the *cis*-inactivation of the *bxd* region by *Ubx* alleles remains unexplained. There is also synapsis-dependent complementation, or transvection, between alleles of these two regions (Lewis, 1954; 1955; 1981a). When, for example, a cytologically normal *Ubx* allele like Ubx^1 is heterozygous with the pbx^2 mutation (also cytologically normal), we observe a weak *pbx* phenotype. When there is a chromosomal rearrangement proximal to the bithorax complex, which disrupts the pairing of chromosome homologs in the salivary glands, the *pbx* phenotype is greatly enhanced. This weak interaction between chromosomes could be a weak correlate of a strong intrachromosomal interaction in a wild-type chromosome between sequences or transcripts from the *Ubx* and *pbx* regions.

In addition to these loss-of-function alleles, there are several dominant gain-of-function mutations in the left half of the complex. These mutations, such as *Cbx* and *Hm*, appear to have abnormal expression of bithorax complex functions in the second thoracic segment.

The Right Half of the Bithorax Complex

The phenotype of embryos deficient for the whole bithorax complex indicates that there are also bithorax genes that control the fate of the abdominal segments. Indeed, recessive *infra abdominal* (*iab*) mutations have been described (Lewis, 1978, 1981b; Kuhn, 1981b; Sanchez-Herrero et al., 1985; Tiong et al., 1985) that transform particular abdominal segments to more anterior segments. Many more *iab* mutations have been recently found; their phenotypes and patterns of complementation are described here. The chromosomal walk has been extended through the abdominal half of the complex, and DNA lesions have been identified for most of the *iab* mutations. The mutant lesions are arranged on the chromosome in the order *iab*-2, 3, 4, 5, 6, 7, and 8, reflecting the order of the segments on the body of the fly. There are two large DNA regions with strong failures of complementation between widely spaced alleles. Weaker interactions are seen between the two regions. Thus, the abdominal half of the bithorax complex, like the left half, appears to require chromosome continuity for wild-type function.

RESULTS

Abdominal Segments

The abdominal segments are more similar to one another than are the thoracic segments, and thus the phenotypes of transformations caused by mutations in the *iab* genes are often subtle (for a description of the adult abdominal segments, see Bryant, 1978). The dorsal surface of each abdominal segment of the adult fly has a rectangular plate of hard cuticle called the tergite. Each tergite is connected to the next by a membrane of soft cuticle usually folded underneath the tergite. The ventral surface of the abdominal segments is composed of soft cuticle called the pleura. On the ventral midline of the second through sixth abdominal segments in the male (second through seventh in the female), there are small plates of harder cuticle called sternites. Tergites, membranes, and sternites are covered with trichomes, which are very small hairs.

The differences between abdominal segments will be described for the adult male, where they are most apparent. The first abdominal segment is easy to distinguish from the other segments because its tergite is small, unpigmented, and covered with only a few short bristles. On the ventral side, there is no sternite. The second through fifth abdominal segments have sternites with 10–16 bristles; the sixth sternite has no bristles. There is a raised patch of smooth cuticle without trichomes and bristles called the Wheeler's organ on the anterior second sternite (Lewis, 1978). The second through fourth tergites are larger than the first and are covered with bristles. The posterior edge of these tergites has a band of pigment and a row of longer bristles. This band of pigment does not quite reach the junction of the tergite with the pleura on the second and third tergites. However, this pigmented band covers the whole posterior edge of the fourth tergite and helps us to distinguish between the third and fourth abdominal segments. It should be noted that the width of this band varies among strains, and it is sometimes impossible to distinguish third and fourth tergites. The fifth and sixth tergites are pigmented black, but they can be distinguished because most of the sixth tergite is clear of trichomes. The sixth sternite in the male differs in shape from the fifth sternite, and it lacks bristles. The development of the seventh abdominal segment is suppressed in the male adult (but not in the female; Santamaria and Garcia-Bellido, 1972). More primitive insects have ten clear abdominal segments, and early Drosophila embryos also show ten segments (Turner and Mahowald, 1979). The eighth, ninth, and tenth segments give rise to the genitalia and analia in the adult. Much of the abdominal cavity is occupied by the gonads composed both of somatic tissue and of the germ cells. The rest of the internal reproductive system derives from the genital disc.

Sources of the *iab* Mutants

We have used several schemes to induce mutations that affect the abdominal segments. A large number of alleles has been isolated as revertants of the dominant gain-of-function mutants *Hab* (*Hyperabdominal*) and *Mcp* (*Miscadestral pigmentation*) (see Table 1). The *Hab* mutation transforms the third thoracic and the first abdominal segment toward the second abdominal segment (Lewis, 1978). It is postulated to

Table 1 Abdominal mutants.

Mutants	Cytology	Screen	Position	Discoverer
iab-2				
$abdA^{S3}$	In (89E/93F)	*γ*-ray mre^{c}	24–28[a]	S. Tiong (Tiong et al., 1985)
$iab\text{-}2^{Kuhn}$	Normal	Spontaneous	27.5[b]	D. Woods (Kuhn et al., 1981b)
T(2;3)P10	89C1-2 to 89E1-2 inserted into 29A-C	X-rayed C.S.	35–36[b]	N. Shaw (Lewis, 1978)
$iab\text{-}2^{C26}$	Normal	X-ray *Mcp*	34.5–36[b] deletes 1.5kb	M. Crosby
$iab\text{-}2^{C51}$	In (89E/80) to 3R heterochromatin	X-ray *Hab*	41.5–43[a]	M. Crosby
$abdA^{S2}$	Complex rearr. incl. 69D/89E/37C	*γ*-ray mm^{c}	50–55	S. Tiong (Tiong et al., 1985)
$abdA^{MX1}$	T(2;3) 89E/2L heterochromatin	X-ray *mm*	54–56	J. Casanova (Sanchez-Herrero, et al., 1985)
$abdA^{MX2}$	Normal	X-ray *mm*	55–58.5[b] deletes 3.5kb	J. Casanova (Sanchez-Herrero, et al., 1985)
iab-3				
Uab-4	Complex inversion with breaks in 80C, 85A, and 89E	EMS C.S.	58.5–61.5	E. B. Lewis (Lewis, 1978)
$iab\text{-}3^{277}$	94A to 96F inserted into 89E	X-rayed *Mcp*	63–64.5[a]	D. Baker
iab-4				
$iab\text{-}4^{166}$	In (79DE/89E)	X-rayed *Mcp*	76–83	J. Von Der Ahe
$iab\text{-}4^{45}$	Complex with breaks in 89E, 41 and 32	X-rayed C.S.	78.5–82	R. Baker
$iab\text{-}4^{125}$	T(3;4) 89E to 4 heterochromatin	X-rayed *Mcp*	81–83	R. Baker
$iab\text{-}4^{302}$	Complex with breaks in 60D, 81, 89E, 100F, and 101	X-rayed C.S.	83–86.5	R. Baker
$iab\text{-}4,5^{DB}$	Normal	X-rayed *Mcp*	85–113[b] deletes 28 kb	D. Baker
iab-5				
$iab\text{-}5^{843}$	81E to 89E inserted into 23	X-rayed C.S.	93–99.5	R. Baker
$iab\text{-}5^{301}$	Complex w/breaks in 86E, 89E, and 40	X-rayed C.S. female	95–104 deletes 9kb	R. Baker
Mcp^{1}	Normal	Spontaneous	94–97.6[b] deletes 3.6 kb	M. Crosby (Lewis, 1978)
$iab\text{-}5^{75}$	T(2;3) Complex	X-rayed *Mcp*	103–108	R. Baker
$iab\text{-}5^{Vno}$	94A to 96F inserted into 89E	X-ray	108–11[a]	E. H. Grell (Lindsley and Grell, 1972)
$iab\text{-}5^{C1}$	T(2;3) included 60/89E-81/89E-100	X-rayed *Mcp*	108–111[a]	M. Crosby

(*Continued*)

Table 1 (Continued)

Mutants	Cytology	Screen	Position	Discoverer
iab-5^{105}	T(2;3) incl. 89E/60B	X-rayed *Mcp*	108–111	E. B. Lewis
Camel	In (89B-89E3,4)	X-ray	113–121	J. Gausz
iab-5^{C7}	Normal	EMS *Mcp*	DNA from 90E inserted at position 124	M. Crosby
iab-6				
Abdb^{MX1}	T(Y;2;3)	X-ray *mm*	126–129	J. Casanova (Sanchez-Herrero, et al., 1985
SGA62	In (88C-89E)	X-ray *bw*	133–139.5[a]	H. Gyurkovics (Awad et al., 1981)
iab-6^{Spth}	In (89A-89E)	X-ray	133–139.5[a]	K. Kemphues
Abdb^{MX2}	In (3LR) incl. breaks in 64A, 89A, and 89E	X-ray *mm*	139.5–142	J. Casanova (Sanchez-Herrero, et al., 1985)
iab-7				
D14	Normal	EMS C.S.	157–157.4[b] deletes 0.4 kb	I. Duncan
iab-7^{65}	T(2;3) 89E/41	X-rayed C.S.	163–166.5[a]	R. Baker
iab-8				
Uab^{1}	Normal	EMS *su(Hw) sbd*^{2}	In from -14 +185 kb[b]	E. B. Lewis
Tab	In (89E-90D)	X-ray *Mcp Sab*	187–188[b]	E. B. Lewis
tuh-3	Normal	Spontaneous	200[b]	C. M. Woolf (Gardner and Woolf, 1949)
Deficiencies				
Df SX1		X-ray *mre*	27.5–35	S. Tiong (Tiong et al., 1985)
DfP10	see T(2;3)P10	X-rayed C. S.	35–36[b]	N. Shaw
Df bxd^{121}	Normal	X-rayed *bx*^{34e}	deletes from −15 to +60-65	E. B. Lewis
DfP13	Df(89CD-89E)	X-rayed *Cbx*	78–81	T. Ramey
DfP2	Df(89E1-32)	X-rayed C.S.	75–81	E. B. Lewis
DfUbx^{109}	Df(89D1-89E1-2)	X-rayed *gl e*	86–93	E. B. Lewis (Lewis, 1978)
DfC4	Df(89E-90A)	X-rayed *Mcp*	133.5–137	M. Crosby
DfP9	Df(89E1-4)	X-rayed *p*^{p}	225–230	E. B. Lewis (Lewis, 1978)
Apparent Points				
Hab	Normal	EMS O.R.		(Lewis, 1978)
iab-2^{D24}	Normal	EMS C.S.		I. Duncan
iab-7^{D6}	Normal	EMS C.S.		I. Duncan
iab-7^{D16}	Normal	EMS C.S.		I. Duncan
iab-7^{D3}	Normal	EMS C.S.		I. Duncan
iab-7^{297}	Normal	ENU on *Mcp*		E. B. Lewis
iab-7^{380}	Normal	ENU on *Mcp*		E. B. Lewis
Other				
S485	89F to 96A into 21C	X-ray C.S.	205–216	N. Shaw

[a] indicates that *in situ* hybridizations were done to confirm the position of the break point
[b] indicates that the mutation was cloned
[c] *mre* indicates the *mwh red e* chromosome
mm indicates the *mwh jv st red sbd e*^{11} *ro ca* chromosome

be a regulatory mutation that turns on the *iab*-2^+ function in the two transformed segments. In *Mcp* flies, the fourth abdominal segment is transformed into the fifth, so *Mcp* is presumed to turn on the *iab*-5^+ function in the fourth abdominal segment. Recessive *iab* mutations have been isolated by inducing mutations that revert the dominant *Hab* or *Mcp* effects, presumably by inactivating *iab*-2^+ or *iab*-5^+. Several partial revertants of *Mcp* have also been found; these are spread over a large region and cause different *iab* transformations (see below). A screen employing the transvection effect (Lewis, 1954) was used to isolate chromosomal rearrangements of the right arm of the third chromosome. These breaks were examined cytologically, and rearrangements affecting the 89E region were further examined for recessive phenotypes (R. Baker and E. B. Lewis, personal communication). Other *iab* mutations have been isolated in a screen for failure to complement *iab*-5^{C7}.

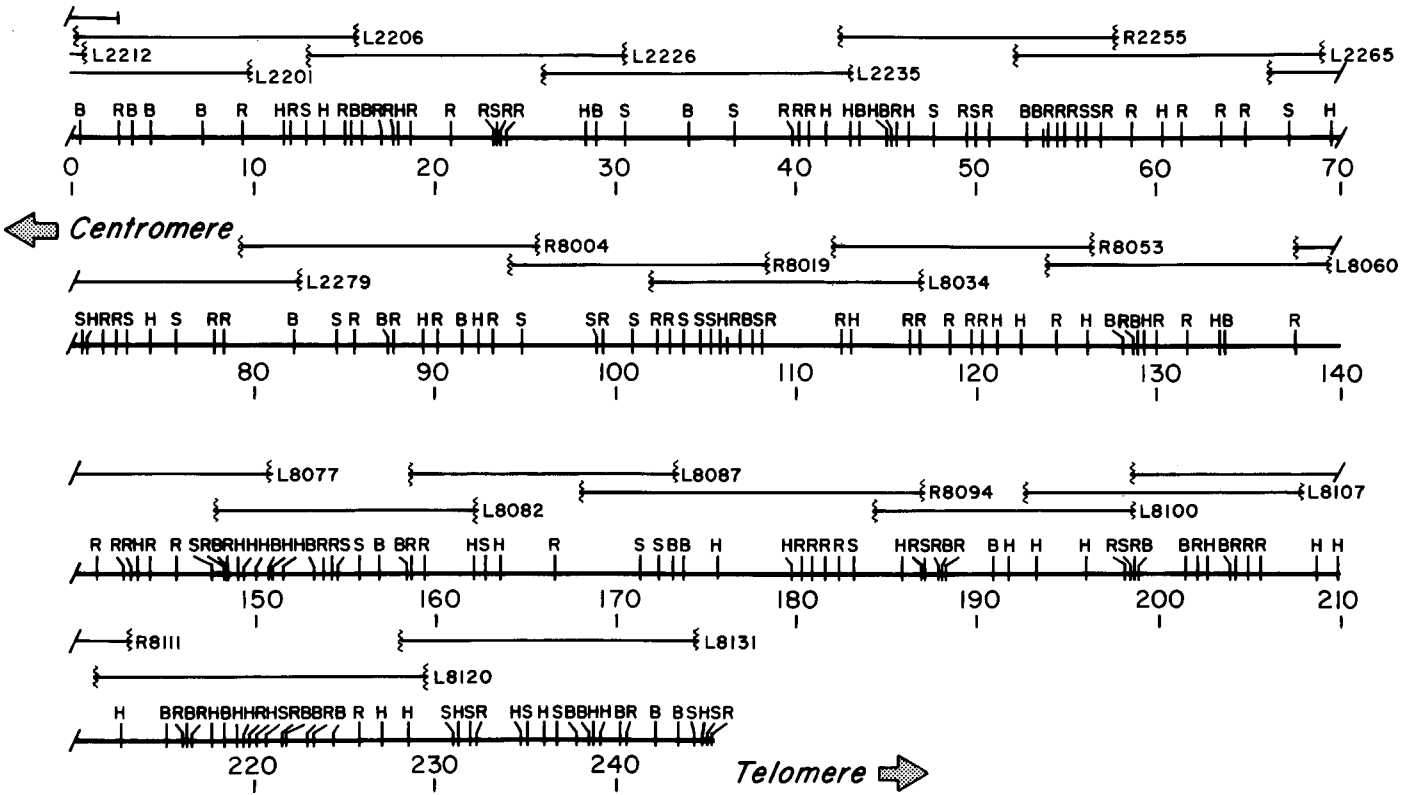
Some of the *iab* mutations we have studied were isolated by Sanchez-Herrero et al. (1985) and Tiong et al. (1985). These were selected by their lethality over a deficiency of the whole complex (*DfP115*) or by their dominant, haplo-insufficient phenotypes. Finally, several mutations that were discovered because they are associated with a dominant phenotype (*Camel*, *SGA62*, *iab-6*^{Spth}, *Uab*^{1}, *Uab*^{4}, *Vno*, and *tuh-3*) also have recessive abdominal phenotypes; their origins are listed in Table 1.

The loss-of-function mutations have been assigned *iab* designations on the basis of which segment is most clearly transformed. This is usually the anterior-most of the segments affected, although this is not true for *iab*-7 alleles (see below). Mutations in any one group, such as *iab-2*, can differ markedly in their severity or in their polarity on neighboring DNA regions. The phenotype of one group (such as *iab-5*) can grade continuously towards the phenotype of the adjacent group (like *iab-6*). This convention for nomenclature seems appropriate, in retrospect, since lesions for each mutant class are found to be clustered and nonoverlapping, and since the mutant classes lie on the chromosome in the order of the segments.

DNA Map

We have extended the chromosomal walk from the left half of the bithorax complex (see Bender et al., 1983b) into the abdominal region. The whole complex is now cloned, and the complete walk covers 410 kb. The overlapping phage shown in Fig. 1 were isolated from the Canton S library (Maniatis et al., 1978) and were mapped by restriction digestion and heteroduplex analysis. A restriction map shown in Figs. 1 and 2 covers 240 kb and includes the whole abdominal region of the complex. The DNA maps are marked off in kilobases (kb) from the starting point of the walk

Figure 1. Composite restriction map of the right half of the complex.
The boldface line shows the map from Canton S wild type for the four restriction enzymes Bam HI (B), Hind III (H), Eco RI (R), and Sal I (S). The coordinates show the distance in kilobases from the starting point of the walk in the left half (see Bender et al., 1983b; Figs. 2, 3, and 5). The thinner lines above the map represent overlapping cloned DNA segments from the Canton S library of recombinant phage. The L and R designations indicate the position of the left and right arms of the lambda phage vector.


L2212
L2206
L2201
L2226
L2235
R2255
L2265
0
10
20
30
40
50
60
70
Centromere
R8004
R8019
L8034
R8053
L8060
L2279
80
90
100
110
120
130
140
L8077
L8087
L8082
R8094
L8107
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R8111
L8131
L8120
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230
240
Telomere

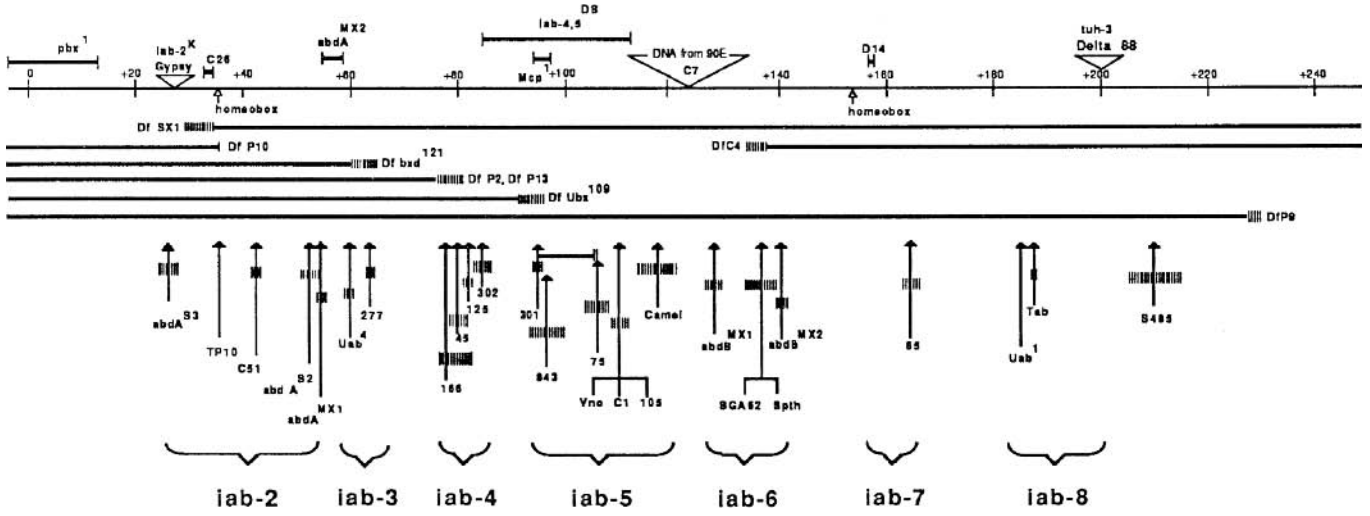


Figure 2. Molecular map of *iab* lesions.
The thin line represents the DNA walk in kilobases, as in Fig. 1. The thick lines represent the sequences deleted in various deficiencies. The vertical arrows indicate the location of mapped chromosomal rearrangements. Hatched horizontal lines indicate the limits of uncertainty in the positions of break points identified by Southern blots. *iab-5*^{301} has a deletion associated with the cytological break point, as indicated. Triangles indicate DNA insertions. The various classes of *iab* transformations are indicated under the lesions.

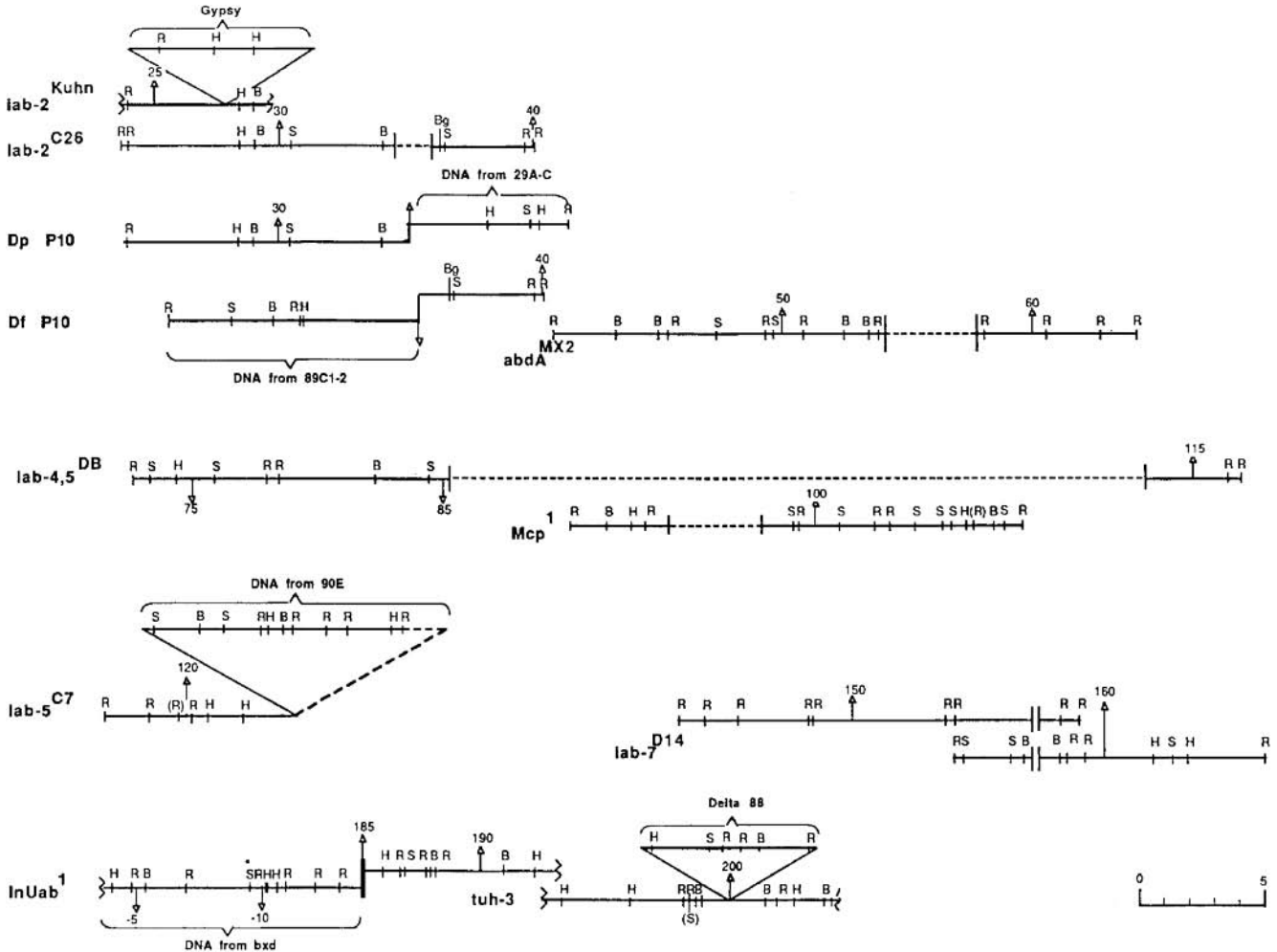
(Bender et al., 1983b). Two short regions were found to contain short middle repetitive DNA when used as probes against whole genome Southern blots. They correspond to M repeats (or opa repeats) near positions +35 and +157 kb, respectively (McGinnis et al., 1984; Regulski et al., 1985; Wharton et al., 1985). Regulski et al. (1985) have also found two sequences in the abdominal half of the complex with homology to *Ubx* and *Antp* homeo box probes. These map near positions +35 and +153 kb in the *iab-2* and *iab-7* regions respectively (Figs. 1 and 2). We have mapped these two homeo boxes in heteroduplexes with the *Ubx* copy, and all three copies are in the same orientation on the chromosome.

The right-most landmark of the left half of the complex is the endpoint of the pbx^1 deletion (Bender et al., 1983b). The pbx^1 mutation has no discernable *cis*-inactivation effects on the abdominal genes. Likewise, all the *iab* mutations appear to complement completely with the mutations in the left half of the complex.

Mutations causing transformations of the abdominal segments have been located on the physical map by probing blots of genomic DNA from mutant flies using DNA fragments from the wild-type walk. *In situ* hybridizations were done to salivary gland chromosomes of several mutant genotypes (see Table 1). We have also cloned the DNA of some of the cytologically normal mutants and have characterized the mutant lesions directly (Fig. 3). Figure 2 and Table 1 show the locations of the different *iab* lesions.

In the next sections, we describe the segmental transformations associated with the DNA lesions. The mutations are discussed in map order, from left to right through the walk. We have crossed all alleles to a deficiency for the complex (*DfP9*) to compare their loss-of-function phenotypes. *DfP9*/+ animals show some segmental transformations in both embryos and adults, because of haplo-insufficiency for the complex. *DfP9*/+ embryos show partial transformation of the eighth setal belt towards the seventh and abnormal posterior spiracles (Fig. 5B). (The setal belt is a band of denticles on the ventral side of each embryonic abdominal segment.) In *DfP9*/+ adult males (Fig. 4), the fifth abdominal segment is partly transformed into the fourth, and the sixth into the fifth. The pigmentation on the fifth tergite is less intense, the trichome density on the sixth tergite increases, and there are occasional bristles on the sixth sternite. Small patches of black pigment also occasionally appear on the fourth tergite, suggesting a transformation of the fourth segment into the fifth. During normal development, the male genitalia undergo a 360-degree rotation, which is only partially completed in *DfP9*/+ males. In addition, the male genitalia are not well fused to the

Figure 3. Maps of clones from mutant libraries.
The map coordinates, indicated with arrows on each of the recombinant phage, correspond to the wild-type map (Figs. 1 and 2). Deletions are indicated by the dotted lines flanked by vertical bars. Insertions are shown with triangles. Chromosomal rearrangement breaks are represented by heavy vertical bars. Restriction sites missing because of polymorphisms are shown in parentheses; each additional restriction site due to polymorphism is marked with an asterisk. The scale is in kilobases.


Gypsy
lab-2 Kuhn
lab-2 C26
DNA from 29A-C
Dp P10
Df P10
DNA from 89C1-2
MX2
abdA
lab-4,5 DB
Mcp 1
DNA from 90E
lab-5 C7
lab-7 D14
Delta 88
InUab 1
tuh-3
DNA from bxd

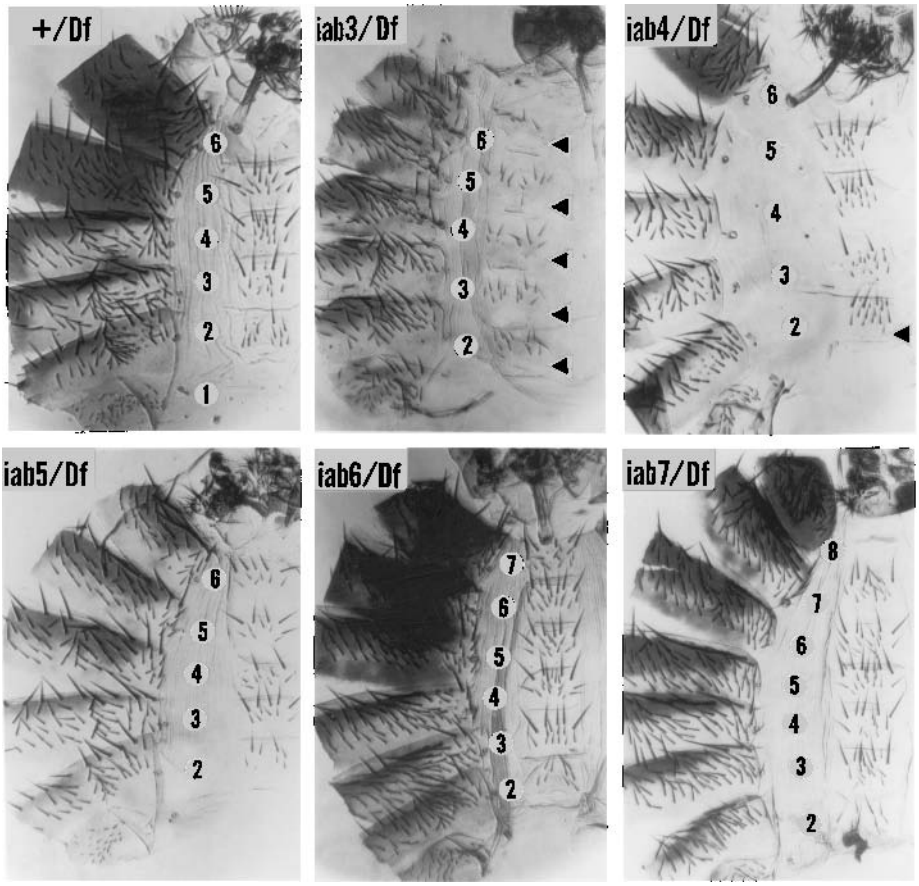


Figure 4. Adult transformations of *iab* mutations.
Photographs of whole mounts of adult male abdominal cuticle. Adult abdomens were cut along the dorsal midline, opened, and flattened on a slide (see Experimental Procedures). The detailed anatomy of the cuticle of the abdominal segments is described in the first section of the results. Each picture shows half an abdomen, and each has the segments numbered. To the left of the numbers are the tergites, and to the right are the sternites. *+/Df: S485/DfP9* (*S485* is the right-most chromosomal rearrangement shown on the map in Fig. 2 and does not cause any segmental transformation). This hemizygous male looks almost like wild type. Subtle haplo-insufficiency effects include occasional bristles on the sixth sternite and patchy pigmentation on the anterior fourth tergite. Note that the seventh and eighth segments give no tergite or sternite in the such adult males. *iab-3/Df: iab-3^{277} Mcp/DfP9* note Wheeler's organs (*arrows*), which appear on sternites 3 through 6, and pigment loss on the fifth and sixth tergites. There are also a few large bristles on the first tergite (partial *Uab* transformation). *iab-4/Df: iab-4^{302}/DfP9* note partial pigment loss on the fifth tergite and absence of Wheeler's organ on the second sternite (*arrow*). *iab-5/Df: iab-5^{Vno}/DfP9* note extra pigment on the fourth tergite, pigment loss on the fifth and sixth tergites, and bristles on the sixth sternite. *iab-6/Df: iab-6^{SGA62}/DfP9* note large seventh tergite, and sixth and seventh sternites with bristles. *iab-7/Df: iab-7^{D6}/DfP9* note eighth tergite and sternite, partial loss of pigment on the fifth, sixth, and seventh tergites and bristles on the sixth and seventh sternites.

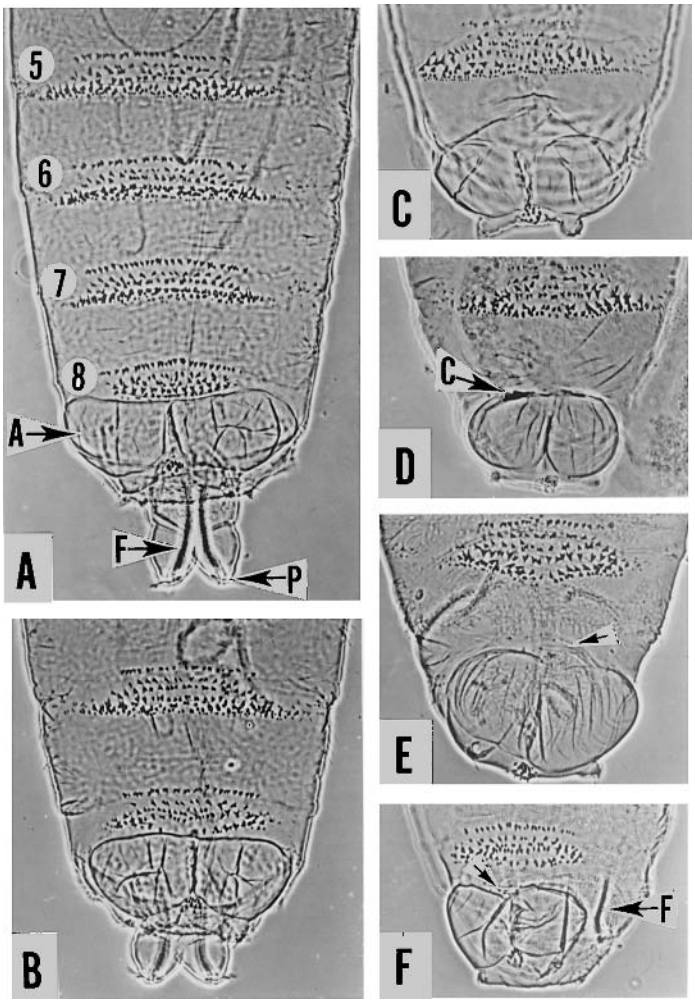


Figure 5. Larval transformations of *iab*-7 and *iab-8* alleles.
The larvae were mounted as described in Experimental Procedures. (A) Wild type, Canton S first larval instar showing the fifth through eighth abdominal segments. Unlike the more anterior segments, the denticles of the eighth setal belt are arranged in rows of equal length. Posterior to the eighth abdominal denticle belt are the anal pads (A), the posterior spiracles (P) and filzkörper (F). (B) +/*DfP9* hemizygote. The eighth ventral setal belt partially resembles the seventh because the anterior row is no longer as broad as the posterior row. The anal pads are normal, but the protuberances that house the filzkörper are reduced. (C) *iab*-7^{D14}/*P9*. The eighth setal belt resembles the seventh or the sixth. The anal pads are normal but posterior spiracles and filzkörper are absent. (D) *iab*-7^{D16}/*P9*. The transformations are similar to those in C, but chitinous plates (C) appear posterior to the eighth setal belt. Occasional ninth row denticles are also seen in this genotype. (E) *iab*-7^{65}/*P9*. The transformations are again similar to those seen in C, but there is a group of small denticles (ninth setal belt) posterior to the eighth setal belt (*arrow*). (F) *iab*-8^{Tab}/*P9*. The transformation of the eighth setal belt is not as extreme as the *iab*-7 mutants shown in C, D, and E. The anal pads are normal, but the posterior spiracles and filzkörper (F) are abnormal. Behind the eighth setal belt is a rudimentary ninth setal belt (*arrow*).

posterior edge of the sixth abdominal tergite. Hemizygous adults also show occasional loss of gonads and occasional duplications of structures derived from the genital disc; both sexes are nearly completely sterile.

The *iab-2* Region

The *iab*-2 mutations are the first group in the abdominal half of the bithorax complex. The left-most landmark of this region is $abdA^{S3}$, an inversion with a break point at about position +26 kb (Fig. 2). This mutation is homozygous viable and causes a partial transformation of the second abdominal segment into the first. $iab\text{-}2^{Kuhn}$ (Kuhn et al., 1981b) is due to the insertion of a gypsy transposable element (Modelell et al., 1983) at position +27 kb (Figs. 2 and 3). This mutation is also homozygous viable and causes a similar weak transformation of the second abdominal segment into the first. Other *iab-2* alleles are embryonic lethals; in *iab-2/Df* embryos (*Df* refers to *DfP9*, unless otherwise specified), abdominal segments 2 through 8 are partly transformed into copies of the first (Morata et al., 1983). *T(2;3)P10* (Lewis, 1978) is a translocation breaking at about +35 kb (this break is probably within the +35 kb homeo box). Two alleles were recovered as revertants of *Hab;* $iab\text{-}2^{C51}$ is an inversion breaking at position +43 kb, and $iab\text{-}2^{C53}$ is an apparent point mutation (the dominant *Hab* mutation may also be a true point mutant). Another apparent point mutation, $iab\text{-}2^{D24}$, was recovered by its failure to complement an *iab-5* allele (see below). The rearrangement break points of $abdA^{MX1}$ (Sanchez-Herrero et al., 1985) and $abdA^{S2}$ (Tiong et al., 1985), are located between position +50 and +56 kb (Fig. 2). Two *iab-2* alleles are associated with small deletions; both have been recloned (Figs. 2 and 3). $iab\text{-}2^{C26}$ was isolated as a partial revertant of *Mcp*; it has a deletion of 1.4 kb at position +35 kb (this deletion probably removes the M repeat but not the homeo box at +35 kb). The $abdA^{MX2}$ mutation (Sanchez-Herrero et al., 1985) has a 3 kb deletion around +57 kb (Figs. 2 and 3). The positions of the *iab-2* break points define the DNA between position +26 and +56 kb as the minimum extent of the *iab-2* region.

The *iab-3* Region

The *iab-3* group includes two alleles, Uab^4 and $iab\text{-}3^{277}$. Both are associated with rearrangements, with break points at +60 and +64, respectively (Fig. 2). The $iab\text{-}3^{277}$ allele is a partial revertant of *Mcp*. The phenotype of Uab^4 has been described (Lewis, 1978). *iab-3/Df* adults of either allele have normal second abdominal segments, but their third, fourth, fifth, and sixth abdominal segments are transformed towards the second. The Wheeler's organ, which normally appears only on the second abdominal sternite, now appears partially formed on the third through sixth sternites (Fig. 4). These *iab-3/Df* animals also lack gonads in both sexes. The gonad loss might be due to *cis*-inactivation of $iab\text{-}4^{+}$ by the *iab-3* mutation (see below).

Uab^4 also has a dominant *Uab* (*Ultraabdominal*) phenotype; the first abdominal segment is partly transformed towards the second or third (large bristles on the first tergite). The other *iab-3* allele, $iab\text{-}3^{277}$, shows no dominant *Uab* phenotype. But

iab-3^{277}/Df animals show a weak *Uab* phenotype, with a partial transformation of the first abdominal segment towards the second or third (Fig. 4). Thus both *iab-3* mutations may cause misexpression of the *iab-2^{+}* or *iab-3^{+}* functions in the first abdominal segment.

The *iab-4* Region

In the region from +76 to +86 kb, there are four chromosomal rearrangements with *iab-4* phenotypes (Fig. 2). *iab-4/Df* adults of any allele have normal second and third abdominal segments, but the fourth abdominal segment is transformed into the third. In *iab-4^{302}/Df* or *iab-4^{45}/Df* males, the band of pigment found on the fourth abdominal tergite does not reach the lateral edge of the tergite as it does in wild-type males (Fig. 4). The pigmentation now resembles that found on a normal third abdominal tergite. Even the black pigmentation of the male fifth abdominal segment does not reach the lateral edge of the tergite; this suggests a partial transformation of the fifth abdominal segment to the fourth or third (Fig. 4). The two other chromosomal rearrangements mapping in this region were isolated as partial revertants of *Mcp.* Their transformations are more difficult to score on the tergite, because their remaining *Mcp* phenotype leaves some pigment on the fourth abdominal segment.

iab-4/Df flies lack gonads. The origin of the somatic gonadal tissue is unclear. Gehring et al. (1976) place the gonadal mesoderm anterior and ventral to the primordia of the genital disc. The ventral position of the somatic gonadal tissue in the fate map makes it difficult to assign it to a particular segment. However, the loss of gonads in *iab-4* mutants, but not in *iab-5* through *iab-8* mutants, suggests they they originate in the fourth abdominal segment.

iab-4^{302}/Df and *iab-4^{45}/Df* animals show loss of the Wheeler's organ on the second sternite (Fig. 4), which suggests a transformation of the second abdominal segment into the third or fourth. This suggests that *iab-4* mutations can cause misexpression of the *iab-3^{+}* function in the second abdominal segment.

The *iab-5* Region; *Mcp* Revertants

We have many *iab-5* alleles; the majority are revertants of the *Mcp* mutation. *Mcp* is a spontaneous dominant mutation that causes a posterior transformation of the fourth abdominal segment into the fifth. Normal males have pigmented fifth and sixth abdominal segments; *Mcp* males also have pigment on the fourth abdominal segment. The DNA from the *Mcp* lesion has been cloned and shows a 3 kb deletion at position of +95 kb (Figs. 2 and 3). It should be noted that no other spontaneous mutations in the complex are deletions.

Three revertants of *Mcp* (*iab-5^{75}, iab-5^{C1}*, and *iab-5^{105}* are caused by chromosomal rearrangement break points localized between positions +103 and +110 kb (Fig. 2). They all cause partial loss of pigmentation on the fourth abdominal segment in the heterozygous males, relative to *Mcp*/+ males. *iab-5/Df* flies of all three alleles show a transformation of the fifth and sixth abdominal segment to the fourth. There is almost

no pigment on the male fifth and sixth tergites, and the sixth sternite is transformed into the fifth. In males and females, the trichome density increases on both the fifth and sixth tergites, indicating a similar transformation.

iab-4,5^{DB} is a revertant of *Mcp* that deletes DNA from position +85 to +113 kb and thus spans the *Mcp* lesion (Figs. 2 and 3). It is homozygous viable but sterile. Homozygous males show a transformation of the fourth and fifth abdominal segments into the third. They have no pigment on the fifth abdominal segment. In addition, the stripes of pigment on the fourth and fifth tergites do not reach the lateral edges of the tergites. The *iab-4,5^{DB}* homozygotes also lack gonads. Thus it appears that this deletion removes both the *iab-4^{+}* and *iab-5^{+}* functions.

iab-5^{C7} (Lewis, 1981b) is a partial revertant of *Mcp* associated with an insertional translocation of DNA from region 90E of chromosome 3R into the complex at +124 kb (Figs. 2 and 3). The inserted piece is at least 10 kb. The *iab-5^{C7}* insertion has been separated from the *Mcp* lesion by recombination. *iab-5^{C7}/Df* animals show a transformation of the sixth and fifth abdominal segment into the fourth. There is also a transformation of the seventh segment towards the fourth, in that an unpigmented seventh tergite and a seventh sternite with bristles both appear in the adult male. Thus *iab-5^{C7}* shows more transformation of the seventh and sixth abdominal segments than *iab-4,5^{DB}* or the other *Mcp* revertants.

Other *iab-5* Alleles

The *iab-5^{301}* mutation is associated with a rearrangement that breaks in the *iab-5* region; in addition, there is apparently a deletion of about 9 kb at between +95 and +104 (Fig. 2). *iab-5^{301}/Df* animals have their fifth abdominal segment transformed into the fourth or third, and the sixth partly transformed to the fifth. Such males lose almost all their male-specific pigmentation on the fifth tergite. In addition, the sixth tergite is partially converted to the fifth, as judged by the pattern of trichomes. In *iab-5^{301}/+* males, there is a weak dominant patchy pigmentation on the fourth tergite.

Vno and *Camel* are two mutations that map in the *iab-5* region and that were initially noticed because of their dominant phenotypes. *Vno* causes a gap in the second longitudinal vein of the wing and is associated with an insertion of the chromosomal region from 94A to 96F into 89E at about position +110 kb (Fig. 2). The insertion at 89E has been separated by recombination from the deletion at 94A–96F. The dominant wing phenotype segregates with the deletion and thus appears to be unrelated to any change in the bithorax complex. *Vno* resembles *iab-5^{301}* both in the recessive transformations of the fifth segment towards fourth, and in the slight dominant transformation of the fourth to the fifth (Fig. 4). *Vno* has a stronger recessive transformation of the sixth segment to the fifth or fourth than does *iab-5^{301}*.

Camel/+ heterozygotes have a longitudinal furrow on the notum (the dorsal part of the second thoracic segment); the mutation is associated with an inversion that breaks at about +116 kb (Fig. 2). Heterozygotes also show a dominant partial *iab-5* phenotype; *Camel/+* males lack pigment on the fifth tergite and have a few bristles

on the sixth sternite. *Camel/Df* animals resemble *iab-5^{C7}/Df* in the transformations of the fifth, sixth, and seventh segments towards the fourth. In *Camel/Df* animals, the longitudinal furrow on the notum extends into the abdominal segments and causes a dorsal fusion problem in the tergites.

The *iab-6* Region

We have mapped four DNA lesions from position +126 to +142 kb that cause transformation of the sixth abdominal segment to the fifth (Fig. 2). *SGA62* (Awad et al., 1981) has a chromosomal inversion that breaks the bithorax DNA at about +136 kb. *SGA62/+* flies have dominant tumor-like growths on the back of the head that are composed of tissue similar to that of the sixth tergite. *iab-6^{Spth}* results from another chromosomal rearrangement with a dominant phenotype similar to *Camel* (longitudinal furrow on the notum). The *iab-6^{Spth}* break point is in the same restriction fragment as the *SGA62* break point. Both *SGA62* and *iab-6^{Spth}* show a dominant haplo-insufficiency phenotype of partial transformation of the sixth segment towards the fifth. In heterozygous males, the sixth tergite is bigger than normal, and the sixth sternite has a few bristles. Occasionally *iab-6/+* males also have a very thin seventh tergite. (The seventh segment gives no external cuticle in adult males unless this segment is transformed to a more anterior state.) When over *DfP9*, these two chromosomal rearrangements show a very strong transformation of the sixth and seventh abdominal segments to the fifth, and a partial transformation of the fifth to the fourth (Fig. 4). Males have a complete seventh abdominal segment including a sternite. In both sexes, the fifth and sixth tergites are covered with trichomes, both the sixth and seventh sternites in the male have a full set of bristles, and the male fifth tergite shows patchy loss of pigment. Embryos of *SGA62/DfP9* and *iab-6^{Spth}/DfP9* are indistinguishable from *DfP9/+* embryos; there is no apparent affect on the eighth denticle belt or the more posterior structures. We have also localized two *iab-6* alleles from Sanchez-Herrero et al. (1985). *AbdB^{MX1}* and *AbdB^{MX2}* are two chromosomal rearrangements (Table 1); both are viable over *DfP9*, with recessive phenotypes similar to those of *iab-6^{Spth}* or *SGA62*. *AbdB^{MX1}* maps at position +127 kb, and *Abdb^{MX2}* at +141 kb, to the left and right, respectively, of *SGA62* and *iab-6^{Spth}* (Fig. 2).

The *iab-7* Region

We have isolated eight mutations we call *iab-7* alleles. All show a dominant, haplo-insufficient phenotype in which both sexes are partially or completely sterile, and males have a thin seventh tergite. As mentioned above, the *iab-7^{+}* function presumably suppresses the development of the seventh abdominal segment in adult males. All *iab-7* alleles are lethal over *DfP9*, or nearly lethal, and *iab-7/Df* embryos show a strong transformation of the eighth setal belt to the seventh. These embryos also lack posterior spiracles and filzkörper (Fig. 5C, Sanchez-Herrero et al., 1985; Tiong et al., 1985), which are also thought to come from the eighth abdominal segment (Denell and Frederick, 1983). In four of the mutants (*iab-7^{65}*, *iab-7^{D3}*, *iab-7^{D16}*, and

iab-7^{297}), there are a few extra denticles of a ninth setal belt (Fig. 5E), which are also seen in *iab-8/Df* hemizygotes (Fig. 5F). In three of these mutants (*iab-7*^{D3}, *iab-7*^{D16}, and *iab-7*^{297}), tiny chitinous plates appear anterior to the anal pads (Fig. 5D). These plates may be rudiments of head mouth hooks that normally appear in the head (Lewis, 1978); it is not clear how to explain their appearance in terms of segmental transformations. The anal pads, which are thought to derive from the tenth abdominal segment (Denell and Frederick, 1983), are intact in all these genotypes. Thus, there are three groups of *iab-7* alleles distinguishable by their embryonic transformations (in order of severity: *D6*, *D14*, *D15*, and *380*; *65*; and *D3*, *D16*, and *297*); these groups can also be distinguished by their complementation patterns (see below). Tiong et al. (1985) have described three similar phenotypic types for their *AbdB* alleles.

The *iab-7*^{D6} and *iab-7*^{D14} alleles occasionally survive over *DfP9* as adults. These hemizygotes show nearly complete transformation of fifth, sixth, and seventh segments towards fourth or third, as judged by pigmentation, trichome density and sternite pattern. The males also have a partial eighth abdominal segment, genitalia that are not completely normal, and complete analia (Fig. 4, Sanchez-Herrero et al., 1985; Tiong et al., 1985). The females have seven abdominal segments and a normal anal tuft, but lack genitalia. These transformations are consistent with the suggestion of Nothiger et al. (1977) that female genitalia are derived from the eighth abdominal segment, and male genitalia from the ninth.

All of the *iab-7* alleles are cytologically normal, except for the *iab-7*^{65} allele that is associated with a translocation break point at about +165 kb (Fig. 2). This break is different from the other alleles in its pattern of complementation, interacting more strongly with *iab-8* alleles than with *iab-6* alleles (see below). The *iab-7*^{D14} allele has a 0.4 kb deletion at position 157 kb (Fig. 3). The remaining *iab-7* mutants appear to be point mutations.

The *iab-8* Region

There are three mutants, *Uab*^{1}, *Tab*, and *tuh-3*, which affect the development of the eighth and maybe the ninth segments in the embryo as well as the genitalia and analia in the adult. All three have dominant phenotypes (see below) by which they were first recognized. In *Uab*^{1}*/Df* or *Tab/Df* embryos, the eighth setal belt is partly transformed to the seventh, the posterior spiracles and filzkörper are reduced but not gone, and a ninth setal belt appears as a small group of denticles behind the eighth setal belt (Fig. 5F). There are no chitinous plates like those found in some *iab-7* mutants. *Uab*^{1}*/Df* and *tuh-3/Df* animals survive as adults; they lack internal and external genitalia and analia in both sexes, but they have normal second through seventh abdominal segments.

Uab^{1} shows a dominant transformation of first abdominal tergite to second or third (Lewis, 1978). It is also inseparable from a *bxd* phenotype, and it was suggested (Lewis, 1981a) that *Uab*^{1} was an inversion between *bxd* and *iab-8*. We identified a chromosomal break in the *bxd* region and used a probe next to the *bxd* break to clone the break point fusion fragment. This fusion fragment hybridizes to both the

bxd and the *iab-8* regions, thus confirming the prediction. The Uab^1 break in the *iab-8* region maps at position +185 kb (Figs. 2 and 3). *Tab* was induced on a double mutant chromosome, *Mcp Sab. Sab*, which puts pigment on the male third abdominal segment, was induced on *Mcp*, and it extends the dominant effect of *Mcp* onto the third abdominal segment (Sakonju, 1984). We have not found any lesion for *Sab. Mcp Sab Tab/+* heterozygotes have stripes of tissue on the second thoracic segment, probably like tissue from the sixth tergite (Celniker and Lewis, 1984). *Mcp Sab Tab/+* flies are sterile and have a thin seventh tergite in males. The *Tab* inversion breaks at position +188 kb (Fig. 2).

Flies with the tumorous head mutation, *tuh-3*, have one of two phenotypes, depending on the allele of an X chromosome locus called *tuh-1*. Stocks containing a $tuh\text{-}1^h$ allele show a dominant expression of head tumors composed of genital tissue (Gardner and Woolf, 1949; Kuhn et al., 1981a). In the presence of the $tuh\text{-}1^g$ allele, *tuh-3* shows a recessive phenotype of loss of genitalia in both sexes (Kuhn et al., 1981a). The fact that *tuh-3* arose spontaneously and that it interacts with an unlinked locus, *tuh-1*, suggested that *tuh-3* might be caused by the insertion of a transposable element. We have cloned the DNA from position +200 kb in *tuh-3* flies, and it contains a moderately repeated 7 kb element that we presume is responsible for both phenotypes (Figs. 2 and 3). The restriction map of this insert does not correspond to any other known transposon, and we have named this new element "*Delta 88*" (GM, 1966).

Deficiencies

We have mapped several deficiency endpoints in the abdominal region of the bithorax complex. Figure 2 shows five deficiencies originating from the left half of the complex that progressively remove DNA from *iab-2* to *iab-5* (see Table 1 for their cytology). All of them die over *DfP9*. This is expected because *DfP10*, *DfP2*, *DfP13* and $DfUbx^{109}$ are *Ubx* deficiencies, and all five (including $Dfbxd^{121}$) remove or inactivate the *iab-2* region.

We have also mapped the endpoints of two deficiencies originating from the right of the complex. *DfC4* was isolated as a revertant of *Mcp* and extends to position +137 kb (Fig. 2). *DfSX1* extends to position +35 kb (Fig. 2). Like *DfP9*, *DfC4* and *DfSX1* are sterile in heterozygotes, and male heterozygotes have rotated genitalia.

Finally, we have localized the right endpoint of *DfP9*, our standard deficiency for the whole bithorax complex. *DfP9* extends to position +227 kb (Fig. 2). Sanchez-Herrero et al. (1985) describe a lethal complementation group (*lrb*) uncovered by *DfP9* that does not cause any segmental transformation in embryos. Since *lrb* maps to the right of their mutations affecting abdominal segmentation, we expect that the right end of our chromosomal walk includes at least part of *lrb* and covers the whole bithorax complex. We have also localized a chromosomal rearrangement break, *T(2;3)S485*, to approximately position +215 kb (Fig. 2). Since *S485/DfP9* is viable and does not show any segmental transformation other than that seen in *DfP9/+* (Fig. 4), we presume that this break is beyond the complex.

Complementation Studies

Most of the abdominal mutations described above cause transformations in more than one segment. This can be explained if particular products are expressed in more than one segment (Lewis, 1978). In addition, some classes of mutations may *cis*-inactivate regions defined by other classes of mutations, as has been described for the left half of the complex. To test whether *cis*-inactivation (or polarity) exists in the abdominal region, we have carried out a complementation analysis with a number of *iab* mutants. We used primarily chromosomal rearrangements for the complementation studies, because such breaks cause the most extreme segmental transformations. It is possible that some alleles are actually double mutants, so we have repeated most complementation tests with two or more alleles of each mutant class. Many alleles have haplo-insufficient phenotypes; we have tried to distinguish these transformations from any loss-of-function in a complementation test. Reciprocal crosses were done for some pairs of mutations, and we saw a few cases where the two mating schemes gave differences. We presume this is due to undefined modifiers in some stocks. We attempted to control for such effects by conducting parallel crosses with the background chromosomes for the mutant alleles, when such were available.

iab-2,3* and *4

We used *T(2;3)P10* as the *iab-2* allele in our matrix of crosses. Any non-*iab-2* mutant over *T(2;3)P10* survives to adulthood. In $iab\text{-}2^{P10}/iab\text{-}3^{277}$ *Mcp* flies, the first and second abdominal segments look normal, but the third, fourth, fifth, and sixth segments are partially transformed towards the second. A partial Wheeler's organ is found on the third sternite, and progressively less well formed ones are on the fourth, fifth, and sixth sternites (Fig. 6A); all transformations are less extreme than in $iab\text{-}3^{277}$ *Mcp/DfP9* (Fig. 4). Thus $iab\text{-}3^{+}$ appears partially *cis*-inactivated by *iab-2* mutations, but not vice versa. $iab\text{-}2^{P10}/iab\text{-}4^{302}$ adults have normal first, second, and third abdominal segments, but there are no gonads in either sex. Thus the $iab\text{-}4^{+}$ region is *cis*-inactivated by the *iab-2* break, but not vice versa. Similarly, $iab\text{-}3^{277}Mcp/iab\text{-}4^{302}$ animals also lack gonads, but have a normal third abdominal segment. $iab\text{-}2^{P10}/iab\text{-}3^{277}Mcp$ heterozygotes have rudimentary gonads, which suggests that there is only partial polarity of the *iab-2* or the *iab-3* breaks on the *iab-4* region.

The *iab-2,3* and *4* breaks do not dramatically *cis*-inactivate the region of DNA further to the right of *iab-4*. The genotypes $iab\text{-}2^{P10}/iab\text{-}5^{Vno}$, $iab\text{-}3^{277}Mcp/iab\text{-}5^{Vno}$, and $iab\text{-}4^{302}/iab\text{-}5^{Vno}$ all look like *Vno/+*. However, several *iab-2* alleles (*P10*, *C51*, *C26*, and *D24* were tested) showed partial failure of complementation with $iab\text{-}5^{C7}$; male heterozygotes show partial pigment loss on the fifth tergite, bristles on the sixth sternite, and extra trichomes on the sixth tergite (Fig. 6B). Indeed, the $iab\text{-}2^{D24}$ allele was recovered by its failure to complement $iab\text{-}5^{C7}$. None of the $iab\text{-}2^{P10}$, $iab\text{-}3^{277}$, or $iab\text{-}4^{302}$ mutations over the $iab\text{-}6^{SGA62}$, $iab\text{-}7^{65}$, *or* $iab\text{-}8^{Uab1}$ mutations show phenotypes different from $iab\text{-}6^{SGA62}/+$, $iab\text{-}7^{65}/+$ or $iab\text{-}8^{Uab1}/+$.

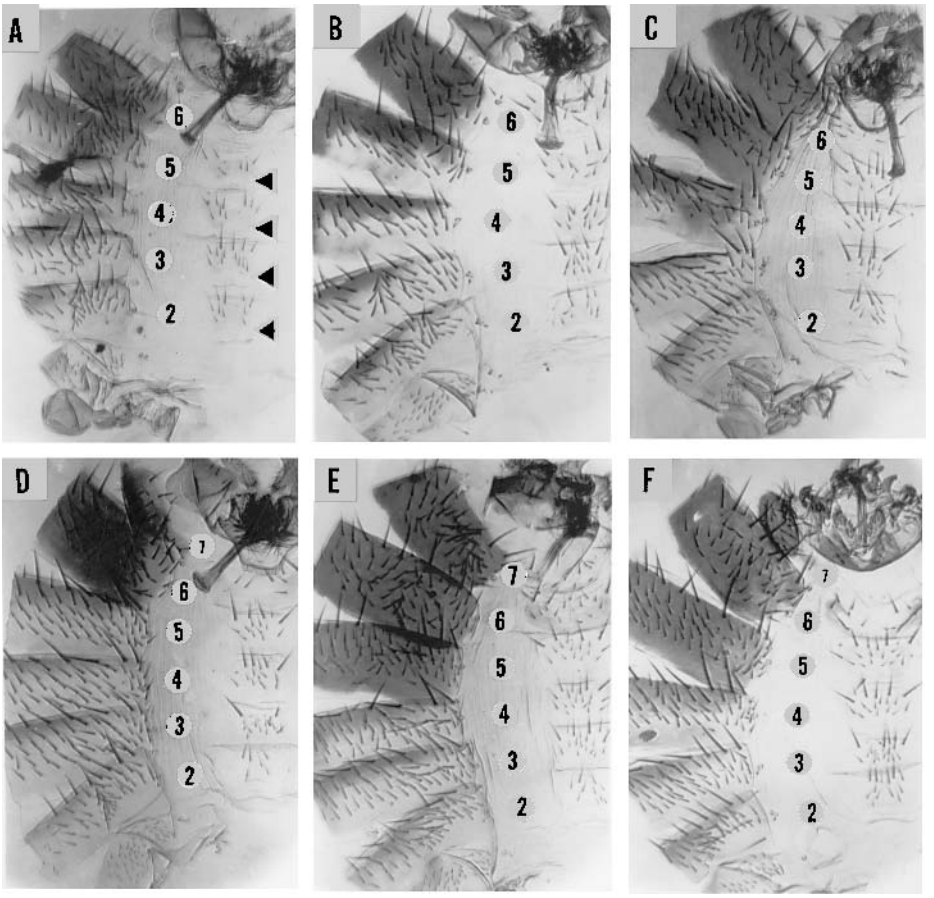


Figure 6. Complementation tests.
Photographs of whole mounts of adult male abdominal cuticles as in Fig. 4. (A) *iab-2*^{P10}/*iab-3*^{277} *Mcp*. Note partial Wheeler's organs on sternites 3, 4, and 5 (arrows). (B) *iab-2*^{C26} *Mcp*/*iab-5*^{C7}. Note partial loss of pigment on fifth tergite and bristles on sixth sternite. (C) *iab-5*^{Vno}/*iab-6*^{SGA62}. Note bristles on sixth sternite and tiny seventh tergite. (D) *iab-4,5*^{DB}/*iab-7*^{D14}. Note loss of pigment on fifth tergite, bristles on sixth sternite, and small seventh tergite. (E) *iab-6*^{SGA62}/*iab-7*^{D14}. Note full seventh tergite, and sixth and seventh sternites with bristles. (F) *iab-6*^{SGA62}/*iab-7*^{65}. Note bristles on sixth sternite, and small seventh tergite.

iab-5,6, and 7

The complementation among *iab-5,6*, and 7 alleles is confused by overlap in the domains of expression of different *iab* products. The *iab-7*$^{+}$ product may be expressed in the fifth and sixth segments (Regulski et al., 1985). Since the *iab-5*$^{+}$ and *iab-6*$^{+}$ products are presumably also expressed in these segments, it is difficult to decide which product is lost when the fifth or sixth segments are transformed.

In *iab-5^{Vno}/iab-6^{SGA62}* males, there is a strong transformation of the sixth segment to fifth (Fig. 6C). The sixth tergite is covered with trichomes, and the sixth sternite is like the fifth with a full set of bristles. In addition, a small pigmented seventh tergite appears. These results are consistent with *cis*-inactivation of *iab-6^{+}* by *iab-5^{Vno}*. There is a similar pattern of transformations in the sixth and seventh segments in *iab-4,5^{DB}/iab-6^{SGA62}* heterozygotes, and in addition, partial loss of pigment on the fifth tergite (not shown).

In heterozygotes between *iab-4,5^{Db}* and *iab-7^{D14}* (Fig. 6D), there is a strong transformation of the fifth segment to the fourth or the third (loss of pigment in male and change in trichome density on the fifth tergite), a partial transformation of the sixth to the fifth (extra trichomes on the sixth tergite and bristles on the male sternite), and a partial transformation of the seventh to the sixth (small seventh tergite in the male). These effects could be due to inactivation of *iab-7^{+}* in the *iab-4,5^{DB}* chromosome, or by inactivation of *iab-5^{+}* on the *iab-7^{D14}* chromosome, or both.

In *iab-6^{SGA62}/iab-7^{D14}* males (Fig. 6E) we observe a partial transformation of the sixth tergite and sternite towards the fifth, and males show a seventh tergite and sternite that look like the normal sixth. Again, the transformations could be due to *cis*-inactivation of *iab-6^{+}* by *D14*, or of *iab-7^{+}* by *SGA62*, or both. A similar pattern of transformations is seen in heterozygotes between *iab-6^{SGA62}* and any of the apparent point *iab*-7 mutations.

The *iab-7^{65}* break also shows failure of complementation with *iab-6^{SGA62}*. In males, the sixth tergite and sternite are partially transformed towards the fifth, and a small seventh tergite appears (Fig. 6F). These transformations are weaker than those in *iab-6^{SGA62}/iab-7^{D14}* animals, but they are clearly stronger than in *SGA62/+* animals.

Breaks anywhere between *iab-2* and *iab-6* appear to complement completely with the *iab-8* alleles, *Uab^{1}* and *Tab*. Likewise, the *iab*-7 alleles *D6* (apparent point) and *D14* (small deletion) both complement completely with *Uab^{1}* and *Tab*. By these tests, *iab-8* appears to be a separate complementation group. However, the *iab-7^{65}* break point allele and three *iab*-7 apparent point mutations (*D3*, *D16*, and *297*) fail to complement with the *iab-8* alleles *Uab^{1}* and *Tab.* Such *iab-7/iab-8* heterozygotes lack genitalia and analia. These *iab*-7 alleles are the ones that give the most extreme embryonic phenotypes (see above).

Interactions across *iab-5*

We have paid special attention to complementation in the *iab-5* region, since it apparently contradicts reports that the abdominal region can be divided into two separate complementation groups (Sanchez-Herrero et al., 1985; Tiong et al., 1985). There are three indications that the *iab-2* and *iab*-7 regions cannot be completely separated. First, the direct complementation tests mentioned above show interactions with *iab-5* alleles from both directions. Partial rightward polarity on *iab-5* is seen in *iab-2/iab-5^{C7}* males (Fig. 6B). Failure of complementation is seen in *iab-4,5^{DB}/iab-7^{D14}*, where there is transformation of the fifth to fourth abdominal segment (Fig. 6D). No interaction was seen between the *iab-2* alleles and *iab-5^{Vno}*, but any fifth to fourth

transformation in these tests may be masked by the dominant partial fourth to fifth transformation (*Mcp*-like) seen in *iab-5*^{Vno}/+.

Second, it seems possible to affect the dominant misexpression of the *iab-5* region in *Mcp* by lesions mapping to its left and right. There are seven revertants of *Mcp* with lesions in the *iab-5* region, but partial revertants of *Mcp* include lesions spanning most of the abdominal region. These include *iab-2*^{C26}, *iab-3*^{277}, *iab-4*^{166}, *iab-4*^{125}, *iab-7*^{297} *iab-7*^{380}, and *DfC4*.

Finally, *Dfbxd*^{121}*/Mcp DfC4* males show a strong transformation of the fifth and sixth abdominal segments into the fourth or third (Fig. 7). In addition, these flies show dorsal fusion problems on all the tergites, and they typically have brown material filling the tracheal tubes beneath the spiracles in the fourth, fifth, and sixth segments. The tergite fusion and spiracle phenotypes cannot be easily interpreted in terms of segmental transformations. They may be malformations of the cuticle caused by segmental transformations of internal tissues. The *iab-5* region is still present on both chromosomes, but its expression seems to be affected by both deficiencies. These phenotypes are not seen in *Dfbxd*^{121}*/Mcp* animals.

Discussion

The whole bithorax complex is now cloned, and the complete walk covers 400 kb. The abdominal region is very large; the left-most mutation in the *iab-2* region maps at position +25 kb and the right-most lesion in the *iab-8* region lies 175 kb distal (Fig. 2). As predicted by Lewis (1978), there is a locus for each abdominal segment, and they lie on the physical map in the same order as the segments on the body of the fly (see Fig. 2). This linear order might be related to the evolution of these genes, or it may be required by the way they function.

Dominant Mutations

There are several dominant mutations that apparently express specific *iab* genes in inappropriate segments; they can be grouped into two classes. One class puts tissue from an abdominal segment onto a distant part of the fly. Examples of this type of mutation include *SGA62*, which puts sixth abdominal tissue on the head; *Tab*, which puts stripes of abdominal tissue on the notum (second thoracic segment); and *tuh-3*, which puts genital tissue in the head. These effects could be due to the fusion of bithorax sequences to a promoter specific for the thorax or head.

The second class of dominant mutations is more subtle; they lead to the misexpression of bithorax genes in neighboring segments. *Hab* is thought to misexpress the *iab-2* function in the third thoracic and first abdominal segments (Lewis, 1978). We have not been able to map *Hab* by whole-genome Southerns, but two revertants of *Hab* are *iab-2* alleles.

There are several types of *Uab* mutations that transform the first abdominal segment into the second or third. The *Uab* phenotype may arise from the production of the *iab-2* or *3* gene product in the first abdominal segment. A peculiar *Uab* effect is seen

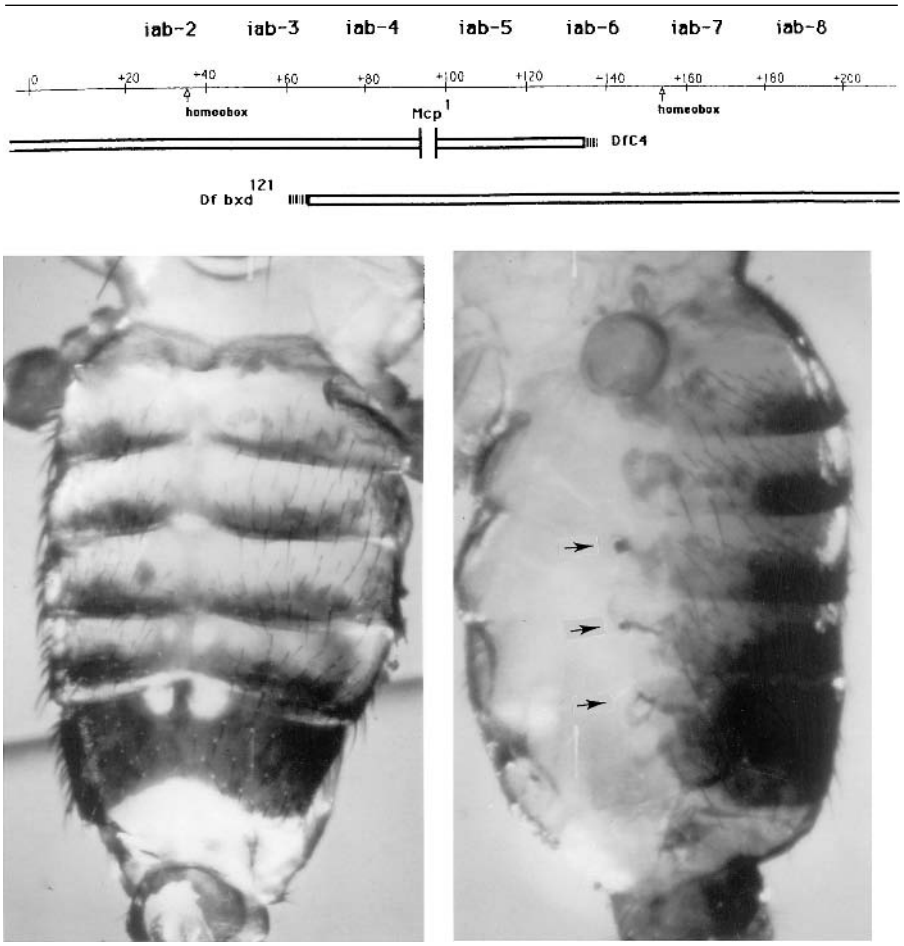


Figure 7. Polarity of nonoverlapping deficiencies.
Two views are shown of a *Df bxd*^{121}*/Mcp DfC4* male. The diagram shows the extents of the deficiencies (double lines indicate extent of DNA remaining). (*Left*) the incomplete fusion of half-tergites in the second through the sixth segments and the loss of pigment on the fifth tergite. (*Right*) the abnormal spiracles of the fourth, fifth, and sixth segments (*arrows*).

in *iab-2*^{Kuhn}. *iab-2*^{Kuhn} flies contain the insertion of a gypsy transposable element in the *iab-2* gene. Gypsy element insertions have been found in nearly all Drosophila mutations suppressed by the second-site suppressor, *suppressor of Hairy-wing (su(Hw))* (Modolell et al., 1983). The recessive *iab-2*^{Kuhn} phenotype can also be suppressed in the presence of *su(Hw)*, but these suppressed flies now show a partial A1 to A2 transformation. Suppression of *iab-2*^{Kuhn} may lead to the overexpression of *iab-2* and thus its expression in the first abdominal segment. *Uab*^{4} is associated with a break

just to the right of the *iab-2* lesions, and the *iab-3*^{277} break also shows a slight *Uab* phenotype when over *DfP9* (Fig. 4). Both breaks may also cause overexpression of *iab-2*$^{+}$. The *Uab*^{1} inversion breaks in both the *bxd* and *iab-8* regions. We do not understand how either lesion could result in a *Uab* phenotype.

The *iab-4*^{302} and *iab-4*^{45} mutations in some genotypes show transformation of the second abdominal segment into the third, by absence of the Wheeler's organ on the second sternite (Fig. 4). This may indicate a misexpression of the *iab-3* function. This transformation is strong in *iab-4/Df*, or in heterozygotes with *iab-2* or *iab-3* alleles. Thus, the balance between the different *iab* products may be important for proper development of the abdominal segments.

Mcp causes the transformation of the fourth abdominal segment into the fifth, and it is thought to express the *iab-5*$^{+}$ function in the fourth segment. Heterozygotes of *iab-5*^{Vno} or other *iab-5* breaks have their fourth segment partially transformed into the fifth. This appears paradoxical, but this transformation could be due to a slight overproduction of certain subfunctions within *iab-5*$^{+}$, or to production of *iab-6*$^{+}$ in the fourth segment.

These dominant effects of *iab* lesions seem surprisingly frequent, and they usually appear in the two segments anterior to the segment most affected by the *iab* loss of function. This could reflect some interaction among the various *iab* products to maintain balanced expression.

DNA Domains

There are two reasons why most abdominal mutations could affect multiple segments; single gene products may be required in a number of segments, and single lesions may *cis*-inactivate a number of genes. An analysis of embryonic effects of hemizygous partial duplications of the complex has shown (Lewis, 1978) that the various bithorax genes are expressed in a linear progression along the body axis. The most anterior segment with *iab-2*$^{+}$ expression, for example, appears to be the second abdominal segment, but *iab-2*$^{+}$ seems to be expressed also in the third through the seventh or eighth abdominal segments. Thus *iab-2* mutations could affect all these segments. This model makes no prediction about complementation among various *iab* alleles.

In fact, the complementation pattern is complex; it is summarized in Fig. 8. The DNA region required for *iab-2*$^{+}$ function is defined by mutant lesions extending from +24 to +58 kb; the *iab-3* breaks beyond +58 kb do not affect *iab-2*$^{+}$. The *iab-3*$^{+}$ function is mostly inactivated by lethal *iab-2* lesions. In other words, one chromosome must have the DNA from about +35 to about +70 kb intact to complement an *iab-3* mutation on the other chromosome. *iab-4*$^{+}$ is partially inactivated by both *iab-2* and *iab-3* lesions, so that +35 to about +90 kb must be intact for complete *iab-4*$^{+}$ function.

The *iab-5* region is defined by lesions spanning about +93 to +124 kb. However, there is weak failure of complementation with *iab-2*, *iab-3*, and *iab-4* breaks, and stronger failure of complementation with the *iab-6* breaks and all the *iab-7* alleles except *iab-7*^{65}. Thus, the DNA from +35 to at least +158 kb must be uninterrupted to supply full *iab-5*$^{+}$ function.

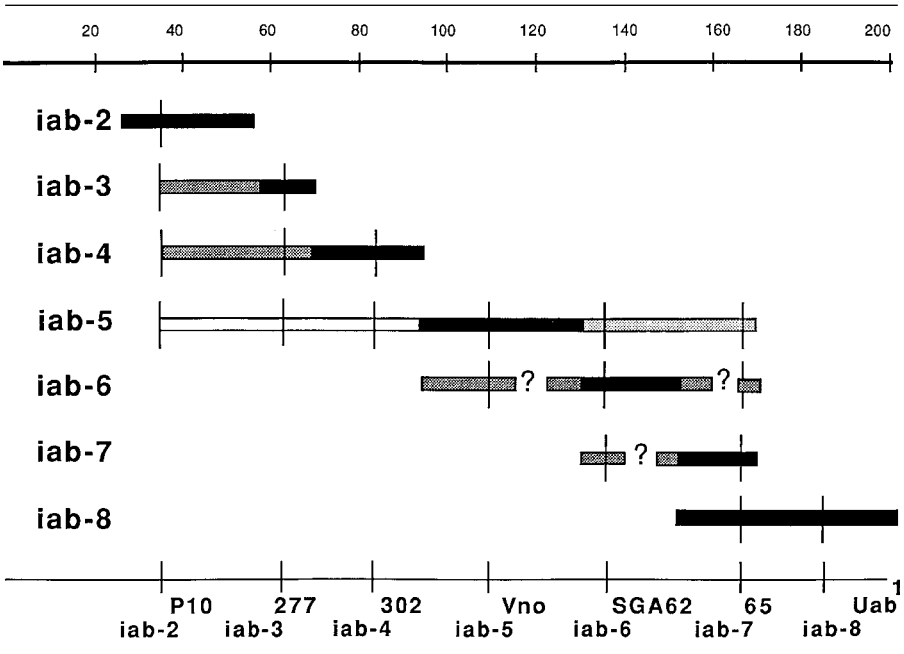


Figure 8. Domains of DNA required to make each *iab* function.
The thin line on top of the figure represents the DNA map marked in kilobases, as in Fig. 2. The horizontal bars show the DNA regions that must be continuous on one chromosome to complement the relevant *iab* break on the other chromosome. The tested break points are indicated along the bottom. Solid bars indicate complete lack of complementation; hatched or open bars indicate intermediate or weak inactivation. The question marks in the *iab-6* and *iab-7* bars indicate uncertainty about the direction of polarity between *iab-5* and *iab-6*, and between *iab-6* and *iab-7* (see text).

The *iab-6* mutations fail to complement with *iab-5* and *iab-7* alleles (including *iab-7*^{65}). It is difficult to decide whether the *iab-5* and *iab-7* regions need be intact for *iab-6*$^{+}$ function. The *iab-5/iab-6* heterozygote partially transforms both the fifth and sixth segments; the phenotype could be explained by inactivation of *iab-5* or *iab-6* (see above). Likewise, it is difficult to assign a direction to the *cis*-inactivation between *iab-6* and *iab-7*.

The *iab-8* mutations fail to complement with the single *iab-7* break and with three *iab-7* points, but they do complement with other *iab-7* points. It is difficult to interpret the polarity of point mutations when we don't know the molecular reasons for the polarity; the analysis would be helped by additional *iab-7* breaks. But at least the DNA from +163 to about +188 kb is required for *iab-8*$^{+}$ function.

Recently, Sanchez-Herrero et al. (1985) and Tiong et al. (1985) have suggested that the abdominal region contains only two complementation groups, *abdA* and *AbdB*. Mutations in *abdA* would transform the second, third, and fourth abdominal segments into the first; mutations in *AbdB* would transform the fifth, sixth, seventh, and eighth

abdominal segments into the fourth abdominal segment. Their analysis is based on the isolation of lethal mutants. We have mapped three of their lethal *abdA* mutants, and they are like our *iab-2* alleles in map position and phenotype. Their lethal *AbdB* alleles are like our *iab-7* alleles in phenotype, although we have not found DNA lesions in the lethal *AbdB* alleles examined so far. (Two viable *AbdB* alleles are *iab-6*-type breaks). The *iab-3,4,5,6*, and *8* alleles are viable, and thus they are mostly missed by a screen for lethal alleles. We also see complete complementation between *iab-2* (*abdA*) and *iab-7* (*AbdB*) alleles, but the analysis of complementation with the *iab-3,4,5*, and *6* alleles shows that separation of the region into two discrete complementation groups is an oversimplification. The complementation is usually partial, and the patterns of partial polarity defined by these complementation tests link the whole abdominal region into one continuous unit.

Analogy to the Left Half, and Model

The left and right halves of the bithorax complex seem similar in their genetic organization. The *bxd* and *pbx* regions must be next to the *Ubx* region to be properly expressed. Likewise, the *iab-3,4* and *5* regions have to be adjacent to the *iab-2* region, and the *iab-5,6*, and *8* regions close to the *iab-7* region. There is complementation dependent on chromosome pairing (transvection) in the left half of the complex. Transvection has also been demonstrated in the abdominal region between $iab\text{-}7^{D14}$ and $iab\text{-}4,5^{DB}$ (Lewis and Duncan, unpublished results). These genetic similarities must reflect similar molecular mechanisms for expression of the genes in the two halves of the complex.

In the accompanying paper, Regulski et al. (1985) describe the presence of two homeo box homologies in the abdominal region. The homeo boxes are short sequences (180 bp) that are conserved at the 3′ ends of the *Ubx*, the *Antp*, and *ftz* transcription units (Scott et al., 1984; McGinnis et al., 1984). In these other genes, the conserved sequences are part of longer open reading frames, and the predicted amino acid sequence is more conserved than the nucleotide sequence. One of the abdominal homeo box homologies is located at position +35 kb in the *iab-2* region and the other one at position +155 kb in the *iab-7* region (Fig. 2). The transcription of the abdominal homeo boxes should be from right to left, as judged by their orientation relative to the *Ubx* copy.

The *iab-2* mutations that interrupt the continuity of the DNA from position +35 to +60 kb are lethal when over a deficiency, and the dead embryos have their second through eighth abdominal setal belts transformed toward the first. Transcription studies by S. Sakonju (personal communication) and isolation of cDNAs (F. Karch, unpublished; S. Sakonju, personal communication), indicate that this region contains at least one transcriptional unit that spans the lethal *iab-2* chromosomal rearrangements. As in *Antp* and *Ubx* cDNAs, the *iab-2* homeo box is located at the 3′ end of the *iab-2* transcription unit. These results suggest that the *iab-2* transcription unit generates a protein product containing the *iab-2* homeo box and that this product is required in all the posterior segments.

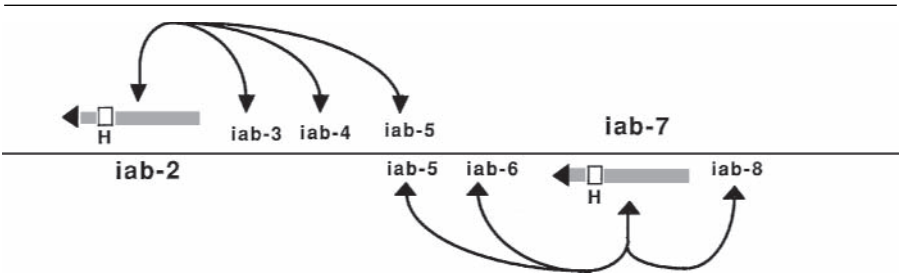


Figure 9. Model for *cis* interactions.
The *iab-3,4,5,6*, and *8* regions might contain *cis*-acting regulatory regions or encode RNA transcripts that interact with either the *iab-2* or the *iab-7* regions, as indicated by the double-headed arrows. The horizontal arrows in the *iab-2* and *iab-7* regions represent RNA transcripts that contain homeo box homologies and presumably encode protein products. The partial polarities of *iab-2* and *iab-7* mutations on the *iab*-3, 4, 5, 6, and *8* regions suggest that the latter regions could encode additional products that act independently.

To make proper third and fourth abdominal segments, the DNA must be continuous from +35 kb to about +70 or +90 kb, respectively. The *cis* interaction of the *iab-3* and *4* regions with *iab-2* is diagrammed in Fig. 9. There are several possible molecular models for this interaction, which are not mutually exclusive. Some or all of the *iab-3* and *iab-4* transcripts could initiate in the +60 to +90 kb region and could be transcribed and spliced so that they contain the *iab-2* homeo box. Alternatively, the products of the *iab-3* and *iab-4* regions could be RNA molecules that are joined onto separate transcripts from the *iab-2* region, which modulate initiation, or which guide a particular splicing pathway of *iab-2* transcripts. A third possibility is that the *iab-3* and *iab-4* regions contain *cis*-acting control regions, perhaps segment-specific "enhancers."

We can invoke similar models to explain the failure of complementation between *iab-7* mutations and *iab-5*, *iab-6*, or *iab-8* (Fig. 9).

The interactions diagrammed in Fig. 9 can explain much of the *cis*-inactivation in the abdominal region, but it is not clear why the *cis*-inactivation is usually only partial. It is possible that an individual region like *iab-5* could interact with both *iab-2* and *iab-7*, so that the loss of either would still leave some *iab-5* function. Alternatively, a region like *iab-5* may encode multiple functions, only some of which are subject to inactivation by *iab-2* or *iab-7* mutations. It will be instructive to examine an *iab-2 iab-7* double mutant chromosome, to see what, if any, abdominal functions remain.

EXPERIMENTAL PROCEDURES

Chromosomal Walking and Whole Genome Southerns

The preparation of probes, the screening of the phage libraries, the growth of phage stocks, the analysis of the recombinant phages by heteroduplex and restriction digests

and the purification of fly DNA for whole-genome Southerns are described in detail in Bender et al. (1983a).

Construction of Recombinant Phage Libraries from Mutants

DNA from adult mutant flies was partially digested with Eco RI and ligated into purified arms of the lambda vector Sep 6 (Davis et al., 1980) or EMBL4 (Frischauf et al., 1983). For three libraries (*iab-2*^{Kuhn}, *Uab*^{1}, and *tuh-3*), we ligated partial Sau 3A digests into the Bam HI sites of EMBL4. The phage were packaged *in vitro* (Hohn, 1979) and were screened for homology to the relevant regions of the Canton S bithorax walk (Bender et al., 1983b).

In situ Hybridization

In situ hybridization was performed essentially as described by Pardue and Gall (1975; see also Spierer et al., 1983). All the probes were labeled with [^{125}I]dCTP.

Mounting Embryos

Embryos were dechorionated, fixed, and cleared according to the procedure of Van der Meer (1977).

Mounting the Abdominal Cuticle

Adult abdominal cuticles were mounted as described by Duncan (1982). Flies were preserved for at least 1 night in a solution of 1 part glycerol in 3 parts 95% ethanol. Abdomens were pulled out, split mid-dorsally with a razor blade, and incubated for 30 min in 10% KOH to digest the soft internal tissue. The remaining cuticle was transferred to a slide and arranged with forceps so that the hemitergites were spread out. They were squashed under coverslips and placed on a slide warmer at 45°C for 3 h. The abdomens were then washed, dehydrated in n-propanol, and mounted in Euparal "Vert" (BDH Chemicals).

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TRANSABDOMINAL, A DOMINANT MUTANT OF THE BITHORAX COMPLEX, PRODUCES A SEXUALLY DIMORPHIC SEGMENTAL TRANSFORMATION IN *DROSOPHILA*

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***Transabdominal (Tab)*, a dominant mutation in the Bithorax Complex (BX-C) of *Drosophila*, creates a sexually dimorphic pattern of segmental transformation that has complete penetrance and expressivity. Specific regions within the notum of the second thoracic segment (T2) are transformed into abdominal-like cuticle; thus, the *Tab*/+ notum has sets of short stripes that are black in males and only bordered with black in females. Also, *Tab*/+ abdominal tergites, A1–A6, inclusive, have small patches of A7-like tergite cuticle. *Tab* is inseparable from an 89E/90D inversion, whose DNA breakpoint in 89E is at +188 kb in the *infra-abdominal-8 (iab-8)* region of the BX-C. When probed with a pupal cDNA from the *iab*-7 region, labeling above background was not detected in wild-type wing discs but was detected in, and confined to, the notal region of *Tab*/+ wing discs. The *Tab*/+ phenotype is assumed to result from *cis*-overexpression of *iab*-7 in localized regions of segments T2-A6, inclusive.**

[*Key Words:* Bithorax Complex; *cis*-regulation; homeotic loci; *Drosophila*; imaginal discs; sexual dimorphism]
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There is now a considerable body of evidence suggesting that *Drosophila* embryonic development is controlled by a heirarchy of sequentially activated genes. The spatial coordinates of the egg are determined early in oogenesis by the maternal-effect genes (Nüsslein-Volhard, 1979), followed by activation or derepression of two types of zygotic genes: (1) segmentation genes, including the gap, segment-polarity, and

pair-rule genes that control the number and polarity of the segments (Nüsslein-Volhard and Wieschaus, 1980), and (2) homeotic genes that control segmental identity. The majority of the latter genes appear to reside in two major gene complexes, the Antennapedia Complex (ANT-C) (Kaufman et al., 1980; Scott et al., 1983; Garber et al., 1983) and the Bithorax Complex (BX-C) (Lewis, 1978). The derepression of these latter two complexes seems to be under the control of a number of *trans*-regulatory genes such as *Polycomb* (*Pc*) (Lewis, 1978) and *extra sexcombs* (*esc*) (Struhl, 1981).

The BX-C contains the genes necessary for the development of the third thoracic segment (T3) and at least the first eight abdominal segments (Lewis, 1978). The complex has been cloned and covers over 300 kb of DNA (Bender et al., 1983a; Karch et al., 1985). Studies of the effects in the adult fly of recessive loss-of-function and dominant gain-of-function mutants have shown that the BX-C genes accomplish intersegmental transformations in which a given segment, or portion thereof, achieves a level of development that is one step more advanced than that of the immediately anterior segment. Thus, in the case of the abdominal segments that are of primary concern in this paper, *iab-7*$^+$ controls a transformation of the seventh abdominal segment, A7, from a sixth abdominal level of development, LA6, to a seventh level, LA7. Similarly, in adults *iab-8*$^+$ controls a transformation of A8 from LA7 to LA8. In keeping with these interpretations of the wild-type functions of *iab-7* and *iab-8* are the effects of recessive loss-of-function mutants. Thus, *iab-7* mutants have as their primary effect in the adult the following transformation: In the case of the homozygous *iab-7* mutant female, a large tergite resembling that of A6 (or A5) arises in place of the normally rudimentary A7 tergite of that sex; in the case of the *iab-7* homozygous male, a large seventh tergite resembling that of A6 (or A5) arises, even though the male normally lacks an A7 tergite. Recessive loss-of-function mutants of *iab-8* show as their primary effect in adults, reduction or absence of genitalia as the result of defects in the genital discs which are derived from segments A8, A9, and A10. Initial descriptions of the adult and ventral embryonic *iab-7* and *iab-8* recessive phenotypes are described in Karch et al. (1985) and a further characterization of the dorsal and ventral embryonic phenotypes is given below. [Sanchez-Herrero et al. (1985) and Tiong et al. (1985) have described a group of mutants they call *Abd-B* mutants, some of which are like *iab-7* mutants and some like *iab-8* mutants.] Moreover, not only have DNA regions been identified within the BX-C that correspond to each such segmental transformation from T3 to A8, inclusive, but also the order of such regions in the chromosome parallels the order of the segments along the body axis. In addition to recessive loss-of-function mutants, there is a much rarer class of dominant gain-of-function mutants, such as *Contrabithorax (Cbx)*, which tend to result in transformations of a given segment toward an immediately posterior one.

Still more rare are segmental transformations that span many segments; namely, *SGA-62*, which has rudiments of an A6-like tergite in the head region (Awad et al., 1981), and *tumorous head* (*tuh*), which has either rudiments of tergite-like tissue (Postlethwaite et al., 1972) or of genitalia-like tissue in the head region (Kuhn et al.,

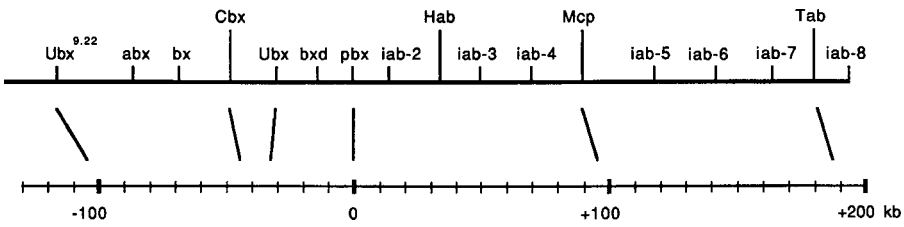

Figure 1. A correlation of the genetic and molecular maps of the BX-C. The upper line represents the genetic map. The lowest row of symbols is loss-of-function mutants, and the top row is dominant gain-of-function mutants. (*Ubx*), *Ultrabithorax*; (*abx*) *anterobithorax*; (*bx*) *bithorax*; (*Cbx*) *Contrabithorax*; (*bxd*) *bithoraxoid*; (*pbx*) *postbithorax*; (*iab*) *infra-abdominal*; (*Hab*) *Hyperabdominal*; (*Mcp*) *Miscadastral pigmentation*; (*Tab*) *Transabdominal*. The lower line represents the molecular map. The approximate location, in kilobases (kb), of a few key loci is indicated in relation to that of *pbx*, which is placed at 0.0 since it was the start of the DNA walk (Bender et al., 1983a).

1981), depending upon complex genetic background factors. *Transabdominal* (*Tab*), which we describe in this paper, is unique among such mutants. It not only transforms specific regions of the thorax into sexually dimorphic abdominal-like cuticle, but in doing so has complete penetrance and expressivity, whereas *SGA-62* and *tuh* tend to have poor penetrance and poor expressivity. We present cytogenetic and molecular evidence that this pattern results from a *cis*-overexpression of *iab*-7 in many segments anterior to A7 and in a pattern-restricted (possibly clonal) manner.

RESULTS

The current status of the map of the BX-C and references for mutant symbols is shown in Fig. 1. *Transabdominal (Tab)* is a dominant mutant that alters the cuticular pattern of the notum of T2 (Celniker and Lewis, 1984) and male tergites, A1–A5, inclusive, and female tergites A1–A6, inclusive. *Tab* was induced with X-rays on a p^p*Mcp Sab* chromosome and is associated with a small inversion, *In(3R)Tab*, having one breakpoint within the BX-C in 89E and the other outside the BX-C in 90D. The mutant is 100% penetrant and has complete expressivity of the thoracic component of the phenotype.

Dominant Phenotype of *Tab*

The wild-type adult thorax is light tan in color and is covered with very tiny hairs or trichomes. The dorsal surface, or notum, has in addition regular rows of small bristles, or microchaetes, and 15 pairs of larger bristles or macrochaetes (e.g., see Bryant 1978). In *Tab*/+ flies (Fig. 2) the notum bears two sets of longitudinal stripes that are partially devoid of trichomes (Fig. 3). In *Tab*/+ males both sets of stripes are solidly pigmented black. On the other hand, in *Tab*/+ females the inner set is lighter in color than the rest of the notum and devoid of black pigment, while the outer set is bordered with black pigment along the edge nearest the midline. The inner set of stripes in

Figure 2. Thoracic pigmentation pattern of *Tab*. A photograph of adult wild-type and *Tab*/+ flies. (*Left to right*) Wild-type (Canton S) male; *Tab*/+ male showing two sets of solidly black-pigmented notal stripes, the inner set extending from the neck region posteriorly for one-half the length of the notum, and the outer set extending two-thirds the length of the notum and ending in a vortex lateral to the dorsocentral region; and a *Tab*/+ female showing identically located sets of stripes as in the male, but with the inner set devoid of black pigment and the outer set only bordered with black pigment along the edge nearest the midline.

some *Tab*/+ females has a small patch of black pigment at the posterior margin. In both sexes, the posterior end of the outer set of stripes forms a raised vortex with whirled microchaetes. The outer set of stripes is complex in that it is interrupted in the middle; alternatively, that set can be described as consisting of two short stripes with the more posterior one bearing the raised vortex. The notal stripes presumably correspond to the cuticle of tergite A7, or A6, since in wild-type and *Tab*/+ animals only those tergites are largely devoid of trichomes (Fig. 4).

In the wild-type female the tergites are light tan in color and bear a band of black pigment along the posterior margin. In the wild-type male, tergites A1–A4, inclusive, resemble those of the female, while tergites A5 and A6 are completely covered with black pigment. Since the *Tab* chromosome contains two other dominant mutations, *Mcp* and *Sab*, which cause A3 and A4 to be partially transformed toward A5, the A3 and A4 tergites tend to be pigmented black in *Mcp Sab Tab*/+ males. However, *Dp(3;1)68; Mcp Sab Tab*/+ males (*Dp-68* is a duplication that includes the entire BX-C) occasionally show virtually normal male-type pigmentation on A4 and A5 tergites. Since such males retain the strongly black-pigmented notal stripes, it is unlikely that *Mcp* and *Sab* are playing any role in determining the pattern of sexual dimorphism in the *Tab* thorax. Because the inversion associated with *Tab*

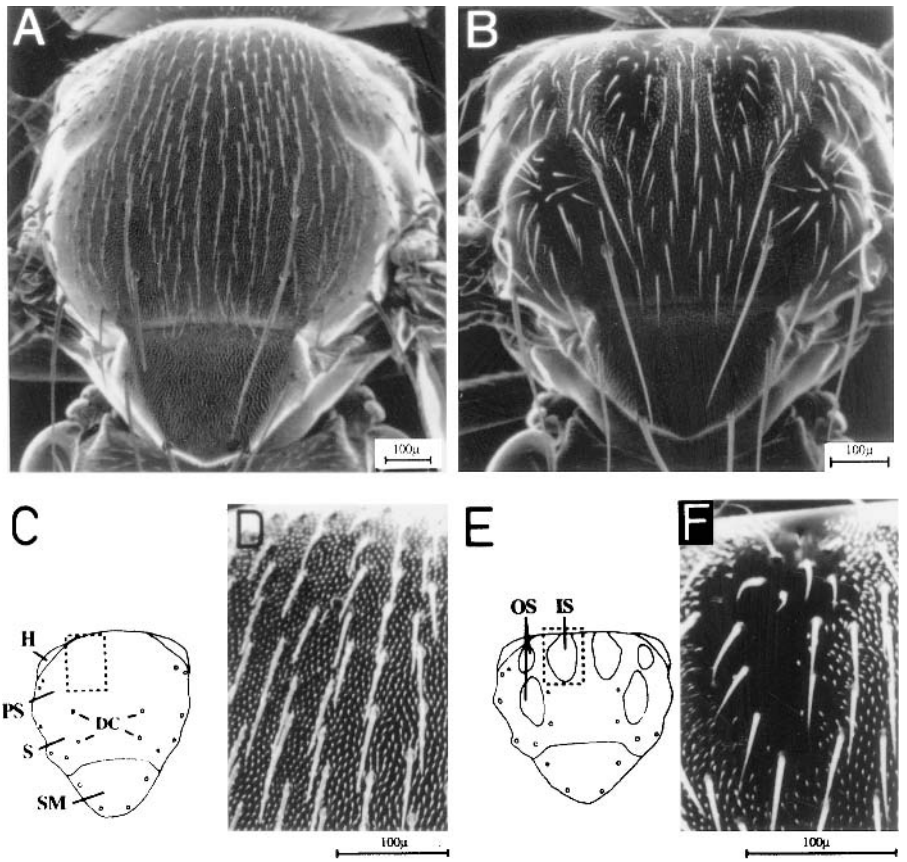

Figure 3. Adult thoracic cuticular phenotype of *Tab*. Scanning electron micrographs and diagrams of the dorsal surface of the notum from wild-type and *Tab* mutant flies. (A) The wild-type notum shows a uniform distribution of microchaetes and trichomes. (B) The *Tab*/+ notum shows two sets of stripes on either side of the midline; the microchaetes in these stripes are abnormally oriented. (C) A diagram of the wild-type notum shown in *A*. (H) Humerus; (PS) prescutum; (S) scutum; (SM) scutellum; (DC) dorsocentral bristles. The small open circles represent the sites of macrochaetes (for identification see, e.g., Bryant 1975). (D) An enlargement of the portion of the prescutum outlined by the hatched line in diagram C to show the trichome pattern. (E) A diagram of the *Tab* notum shown in B. The elliptically shaped regions outlined in the diagram represent the two sets of *Tab* stripes; namely OS (outer set) and IS (inner set). (F) An enlargement of the portion of the prescutum outlined by the hatched line in diagram E to show the absence of trichomes and the abnormal microchaete pattern.

strongly reduces recombination in its vicinity, removal of *Mcp* and *Sab* from the *Tab* chromosome by crossing over is not practical.

Tab/+ animals show small trichomeless patches in the tergites of A1–A5, inclusive (Fig. 5). Since the A6 tergite is devoid of trichomes in wild-type as well as in *Tab*/+ animals, except along the anterior and lateral edges of that tergite, *Tab*/+ patches in

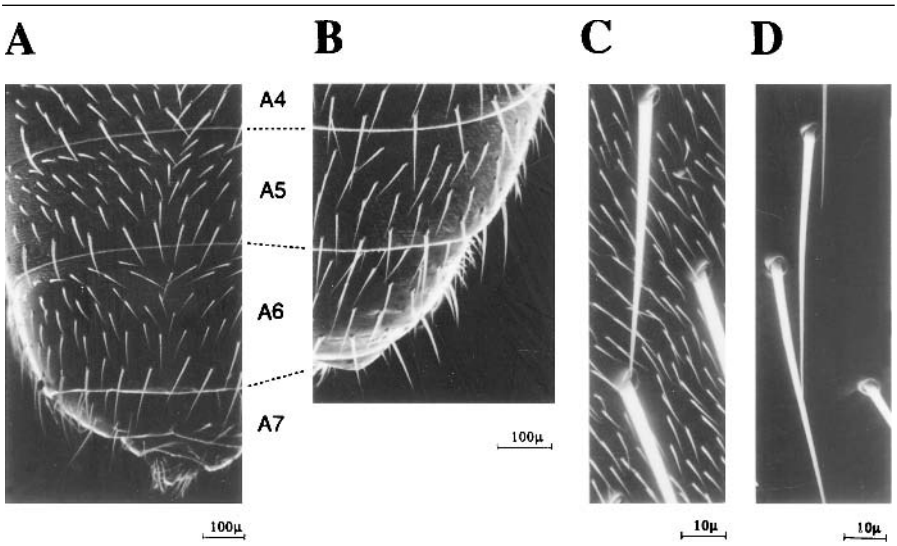

Figure 4. Cuticular *iab-7* and wild-type phenotypes of adult abdominal segments. Scanning electron micrographs of a portion of the dorsal surface or tergite of the male adult abdomen. (A) Heterozygous *iab-7*^{D6}/+ (the + chromosome also carries the TMl balancer see Lindsley and Grell 1968) male showing portions of the tergites of abdominal segments A4–A7 inclusive. The pattern of bristles and trichomes on A4–A6 is similar to the corresponding patterns of wild-type (see B, C, and D). The rudimentary tergite on A7 has patches of microchaetes, but no trichomes. (B) Wild-type (Canton S) male showing portions of A4, A5, and A6 tergites. There is a single row of macrochaetes along the posterior edge of each tergite and three or four rows of microchaetes on A5 and A6. Trichomes are present on A4 and A5 but absent in the central area of A6. (C) Portion of wild-type A5 tergite enlarged to show the presence and pattern of trichomes. (D) Portion of wild-type A6 tergite enlarged to show the absence of trichomes.

A6 are only readily identifiable in females on the basis of pigmentation. Thus, in such females small black-pigmented trichomeless patches of presumptive A7-like cuticle are occasionally seen in A6.

The frequency of trichomeless patches has been determined omitting the tergite of A6 (and also that of A1, since the latter is smaller and frequently tends to be damaged in preparing whole mounts of abdomens). Among six *Tab*/+ males and six *Tab*/+ females scored over tergites A2–A5, inclusive, a total of 35 and 46 trichomeless patches, respectively, were found among the 24 male tergites and 24 female tergites scored, or a mean of 1.5 and 1.9 patches per tergite, respectively; the number of tergites lacking such patches was three and the number having two or more patches was 31.

In *Tab*/+ animals the lateral edges of tergites A1–A6, inclusive, show variable degrees of erosion of the cuticle. This erosion appears to be a transformation toward an A7-like tergite inasmuch as that tergite is normally reduced in size compared with that of A6.

Although *Tab*/+ females lay eggs and the males have motile sperm, both sexes are sterile, or virtually so. The sterility is rescued by adding duplications of the BX-C,

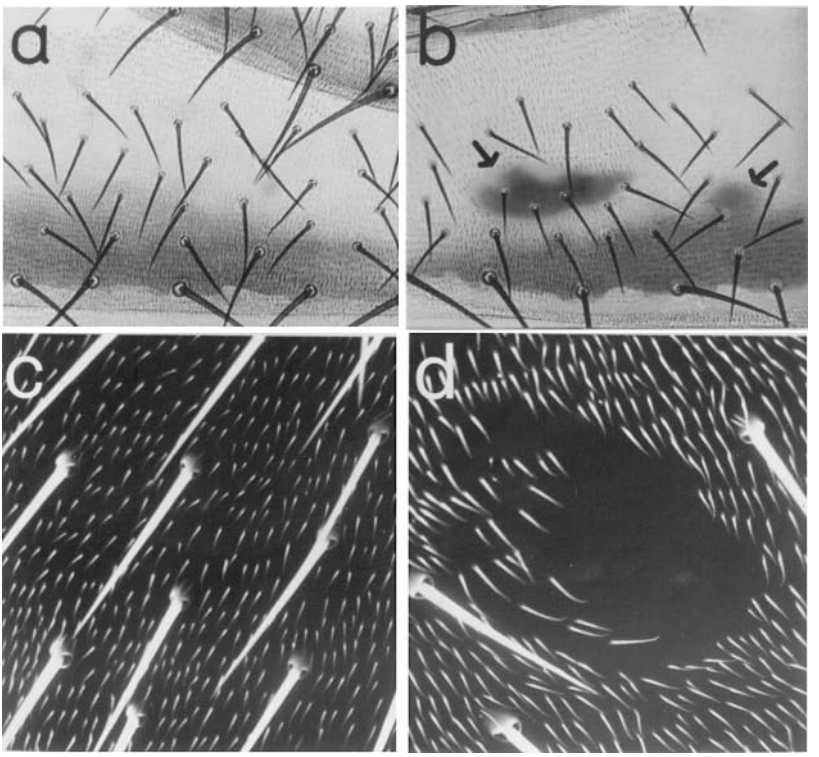

Figure 5. Abdominal cuticular phenotype of *Tab*. (a) Portion of an A3 hemitergite from a wild-type (Canton S) female. A band of black pigment identifies the posterior one-third of the tergite. (b) Portion of an A3 hemitergite from a *Tab*/+ female showing (*arrows*) two patches of cuticle that are pigmented black and devoid of trichomes. (c) SEM of a portion of the third hemitergite from a wild-type (Canton S) male showing the wild-type trichome pattern. (d) SEM of a portion of the third hemitergite of a *Tab*/+ male showing a patch of abdominal tissue devoid of trichomes.

such as *Dp(3;1)68*. Although such duplications suppress the dominant sterility of *Tab*/+, they do not suppress its abnormal morphology in the cuticle of the thorax and abdomen.

Recessive Phenotype of *Tab*

A recessive *Tab* phenotype can be detected in adults that are heterozygotes for *Tab* and loss-of-function mutants of *iab-8*. Two such mutants, *Ultrabdominal* ^{1} (*Uab* ^{1}) and *tumorous-head* ^{3} (*tuh* ^{3}) were first recognized because of their dominant phenotypes and only later found to be recessive mutants of *iab-8*. (For a description of the molecular basis of *Uab* ^{1} and *tuh*, see Karch et al., 1985.) *Tab/iab-8*^{Uab1} and *Tab/iab-8*$^{tuh-3}$ heterozygotes both survive as adults. They lack internal and external genitalia and analia in both sexes; in males, lack of genital ducts results in their having

unattached testes which then fail to undergo coiling. When *Tab* is heterozygous with weak *iab*-7 mutants (*D6*, *D14*, *D15*) there is a rotation of genitalia in the males that suggests a weak *cis*-inactivation of *iab-8* in the *iab*-7 mutant chromosome. When *Tab* is heterozygous with *iab*-7^{65} both sexes lack genitalia. *iab*-7^{65} is possibly a very weak *iab*-7 mutant with a strong *cis*-inactivation of *iab-8* (Karch et al. 1985), but since, in fact, it is an extreme loss of function of *iab-8* it is more likely an *iab-8* than an *iab*-7 mutant (Fig. 6*D*). *iab*-7^{65} is associated with a translocation with breakpoints in 89E and 41. A DNA breakpoint was located within the BX-C at +165 kb, which places it in a region as yet poorly characterized that extends from the distal portion of *iab*-7 to the proximal region of *iab-8* (Karch et al., 1985).

Tab/Df-P14 heterozygotes (where *Df-P14* is a deficiency for 90C–91A) are lethal in the late embryonic stage. They lack any obvious cuticular transformations, whereas heterozygotes involving *Tab* and deficiencies for either the entire BX-C (*Df-P9*) or the distal region (*Df-C4*) thereof are not only lethal in the embryonic stage but also show a strong cuticular transformation of A8 toward A7 (Karch et al. 1985). Thus, as shown in Fig. 6C, *Tab/Df-P9* embryos have dorsal triangles in the posterior region of A7; transformation of dorsal hairs of anterior A8 toward the pattern in A7, abnormal posterior spiracles, and a rudimentary ninth row of denticles. This row presumably corresponds to the anterior row of denticles found in the ventral setal bands of segments A2–A8, inclusive, of wild type, and from the work of Szabad et al. (1979), it probably belongs to the posterior compartment of the segment ahead. Hence, the ninth row is shown as belonging to posterior A8. Although such animals have the ventral setal band of A8 partially transformed toward that of A7, that band is also transformed to about the same extent in the control animals, *Df-P9/+* (Fig. 6B), indicating that the *iab-8* function, like most other genes in the complex, is not entirely haplo-sufficient.

The recessive lethal *Tab* phenotype seen in A8 of *Tab* homozygotes and hemizygotes is also found in heterozygotes between *Tab* and strong *iab*-7 mutants (*297, 380, D3*, and *D16*). Such heterozygotes are lethal in the late embryonic stage and closely resemble *Tab/Df-P9* animals, i.e., they have a strong transformation of A8 toward A7, and a ninth setal band consisting of only a single row of denticles.

In embryonic and larval stages the *iab* transformations behave for the most part in a parasegmental (Martinez-Arias and Lawrence, 1985) rather than segmental manner. Correspondences between segments and parasegments are shown in Figure 6 for A6, A7, and A8, the segments of primary concern. In the embryo, a loss of function in *iab-8* can be said to transform parasegment 13 (PS13) toward PS12; however, the presence of the ninth row of denticles in posterior A8 without a transformation of the remaining portion of the ventral setal band in the ninth segment indicates that the anterior part of PS14 transforms toward the corresponding part of PS13; hence the latter can be better described as part of a segmental transformation of A8 toward A7. It seems likely that there are genes for controlling the partial suppression of A9 and A10, but they have not yet been located, nor have their segmental or parasegmental transformations been determined.

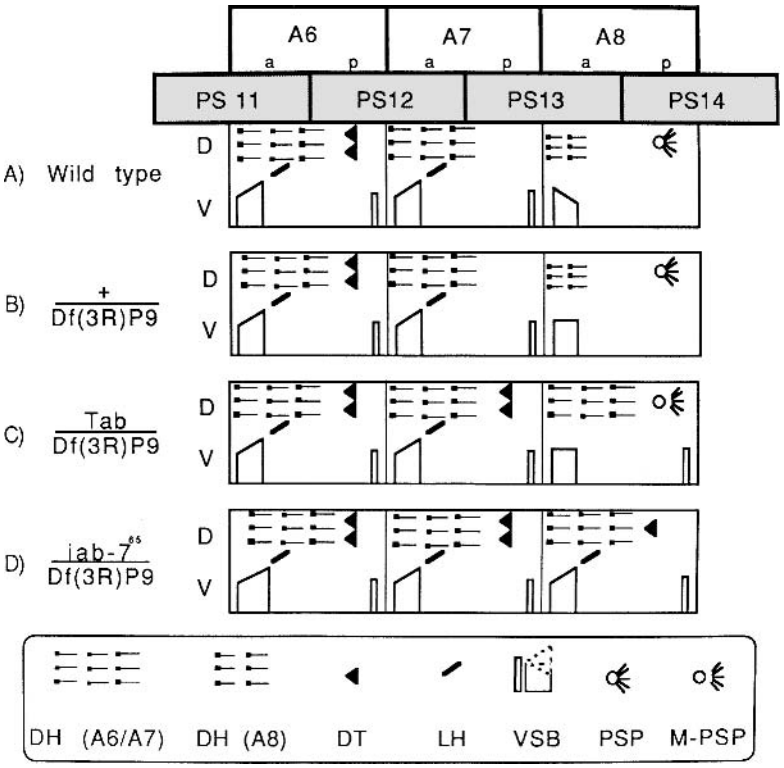

Figure 6. Diagram of wild-type and BX-C mutant phenotypes detectable in abdominal segments A6, A7, and A8 of the first-instar larva. Dorsal and ventral aspects of abdominal segments A6, A7, and A8 are schematically presented. All drawings are based on whole mounts of animals examined with phase-contrast microscopy. (DH) dorsal hairs; (DT) dorsal triangles; (LH) lateral hair; (VSB) ventral setal band; (PSP) posterior spiracle; (M-PSP) malformed posterior spiracle; (a) anterior; (p) posterior; (D) dorsal; (V) ventral. Omitted are abdominal segments A9 and A10 and structures from abdominal segment A8, whose segmental location is unclear, namely, the filzkorper, the caudal sense organs, dorsal hairs associated with the posterior spiracle, and dorsal hairs posterior to the posterior spiracle. (A) Wild type. (B) Wild-type hemizygote showing similarity to wild-type homozygote, except that the VSB of A8 is broader. (C) *Tab* hemizygote showing dorsal transformation of pA7 to pA6 and aA8 to aA7; the transformation of A8 to A7 does not include the lateral hair; ventrally aA8 appears similar to aA8 of the wild-type hemizygote, but the beginning of a ninth ventral setal band appears in pA8; the spiracles are farther apart and their terminal hairs are misplaced. (D) *iab-7*^{65} hemizygote showing the complete transformation of pA7 to pA6, aA8 to aA7, and pA8 to apparently an intermediate state between pA6 or pA7. The ventral aspects of all of the above phenotypes are figured in Karch et al. (1985).

Revertants of *Tab*

To analyze which end of the inversion associated with *Tab* might be responsible for the dominant *Tab* phenotype, we have induced revertants of the dominant *Tab* phenotype with X-rays. Roughly 80,000 *Tab* chromosomes were irradiated at a dose of 4000 r

Table 1 Cytological and molecular (DNA) analysis of *Tab* revertants.

Class	Mutant	Screen	Cytology[a]	Position[b]
I	R96	1	Tp(3)80; 89E	170–173
I	R100	1	T(2;3)37EF/38A;89E	183–184
I	R107	1	In(3R)89E;90A±	185–187
I	R111	1	T(Y;3)89E	170–180
I	R114	1	T(1;3;4)20;89E;101	183–184
I	R175	1	T(1;3)20;89E	
I	R185	1	T(2;3)50D;89E	
II	R89	1	T(Y;3)89E	
II	R82	2	Tp(3)64EF-65A;89E	
III	R5[c]	1	NNR[d]	
III	R10	2	NNR	
III	R75	2	NNR	

[a] Revertants of the dominant *Tab* phenotype were X-ray induced on the chromosome, *In(3R)Tab*, and have not been separated from this inversion
[b] Refers to the composite restriction map of the bithorax complex (Bender et al., 1983; Karch et al., 1985)
[c] Recovered as a gonadal mosaic
[d] No new rearrangement detectable

units and 12 complete revertants of the dominant *Tab* phenotype were recovered. Eight of the 12 revertants are associated with a new cytologically visible rearrangement superimposed on the original *Tab* inversion (Table 1). In every case the proximal junction (89E) of the inversion was rearranged rather than the distal junction (90D).

When hemizygous, each of the revertants shows a stronger transformation of the eighth ventral setal band toward that of the seventh than does the *Tab* hemizygote (*Tab/Df-P9*). *Tab* revertants fall into three classes based on their phenotypes when hemizygous. Class I revertants (*R96, R100, R107, R111, R114, R175,* and *R185*) when hemizygous show abnormal posterior spiracles and a rudimentary ninth ventral setal band similar to that of *Tab* hemizygotes (data not shown). Class I revertants correspond to extreme *iab-8* mutants. Class II revertants (*R89* and *R82*) when hemizygous, have A7 transformed toward A5 or A6, lack posterior spiracles and filzkorper, and have no ninth ventral setal band. Class III (*R5*, *R10*, and *R75*) when hemizygous, have in addition to the effects seen in the case of Class II, chitinized plates located just posterior to the ventral setal bands of A8 (data not shown); such plates, which were first seen in *Df-P9* homozygotes, are believed to represent rudiments of the mandibular hooks (Lewis, 1978). Class II and Class III *Tab* revertants have embryonic phenotypes that characterize them as weak and strong *iab*-7 mutants, respectively, as defined in Karch et al. (1985).

DNA Analysis of *Tab* and Revertants

To localize the *Tab* breakpoint within the BX-C we have used DNA fragments isolated from recombinant DNA clones representative of wild-type BX-C to probe blots of restriction endonuclease-digested genomic DNA from wild-type and from *Tab*/+

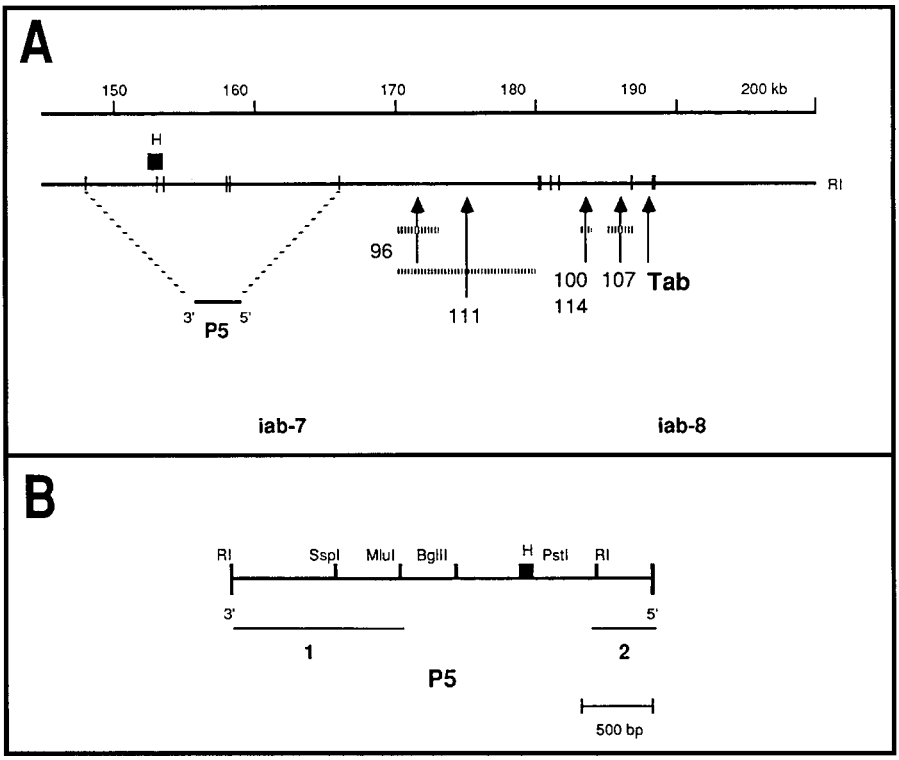

Figure 7. DNA maps of mutant lesions, of the P5 cDNA, and the restriction map of P5 cDNA. (A) The upper horizontal line represents the right end of the BX-C DNA walk. The coordinates show the distance in kilobases from the starting point of the BX-C DNA walk (see Bender et al. 1983a; Karch et al. 1985). The middle line shows the wild-type (Canton S) composite DNA map for the *Eco*RI restriction enzyme sites. The vertical arrows indicate the location of breakpoints of chromosomal rearrangements. Hatched horizontal lines indicate the amount of uncertainty in the position of those breakpoints as identified by DNA blot hybridization. The dotted line indicates the span of genomic DNA sequence which shows homology to the *iab-7* cDNAs by DNA blot hybridization. The exact boundaries of the *iab-7* and *iab-8* regions have not yet been determined. (B) Restriction map of P5 cDNA. The restriction enzyme fragments marked 1 and 2 were used to construct probes for DNA blot hybridization studies to determine the orientation of the cDNA relative to wild-type genomic DNA. Fragment 1 was used to construct probes for *in situ* hybridization to sections (see text) and to imaginal discs (see Figs. 8 and 9). (H) Homeo box.

flies. The results show that the breakpoint is located on a 1 kb genomic fragment from +187 to +188 (Fig. 7A). The breakpoint was verified by *in situ* hybridization to salivary gland chromosomes from *Tab*/+ third-instar larvae; i.e., in nuclei in which the *Tab* and wild-type chromosomes were not somatically paired, the genomic fragment from +187 to +188 kb hybridized to the 89E/90D and 90D/89E ends of the *Tab* inversion, but only to the 89E region and not the 90D region of the wild-type chromosome (data not shown).

We have constructed a genomic DNA library from *Tab*/+ flies and isolated and sequenced DNA at the proximal 89E/90D breakpoint of *In(3R)Tab*. This breakpoint is located at 187.6 kb. We have localized the breakpoints of five of the revertants and find they map (see Table 1 and Fig. 7A) just proximally to the proximal *Tab* breakpoint of *In(3R)Tab*, in a region from +170 to +187 kb. The revertant breakpoints span 17 kb of DNA and are distributed within the *iab-7* and *iab-8* regions.

The cytogenetic data on *Tab* revertants are consistent with the *Tab* phenotype being the result of a position effect on the wild-type *iab-7* gene. Therefore we have undertaken a molecular analysis of that gene and its transcripts. Regulski et al. (1985) have identified a homeo box that is located at position +153 kb in the *iab-7* region of the BX-C. Using genomic DNA from +153 kb as a probe, they were able to detect a complex set of transcripts in 6 to 12 h embryonic poly(A)$^+$ RNA ranging in size from 1 to 4.4 kb. On the basis of their results we chose a genomic DNA fragment from +153 to +155 kb as a probe to screen cDNA libraries for *iab-7* cDNA clones. We screened two λgt10 cDNA libraries (Kornberg et al. 1985) that had been constructed from 3 to 12 h embryonic and 5.5- to 7.5-day early pupal poly(A)$^+$ RNA. Approximately 1×10^6 recombinant phage were screened from each library. We obtained seven cDNA clones (five embryonic and two pupal) which have inserts ranging in size from 1.5 kb to 3.0 kb. When the seven cDNA clones are used individually as hybridization probes to the wild-type genomic BX-C DNA, they show homology to restriction fragments spanning 17 kb from +148 to +166. Since the cDNA clones are similar at this level of sensitivity we have used the pupal cDNA clone with the largest insert (clone P5) for a detailed characterization. This cDNA is specific for the *iab-7* region by two criteria: (1) It hybridizes to salivary gland chromosomes only at position 89E, and (2) under high-stringency blot hybridization conditions, it does not hybridize to homeo box-containing sequences from *Ubx* or *iab-2* (data not shown). Employing the synthetic RI restriction sites used to insert the cDNA into λgt10, we subcloned the cDNA into the plasmid pUC 18. The pUC 18 P5 plasmid was used for all further studies.

We assigned a 5′-3′ orientation for *iab-7* cDNA P5 based on three pieces of evidence: the 5′ GC tail (added during the construction of the cDNA library) was identified by sequencing; the cDNA was aligned on the BX-C DNA map using both ends of the cDNA as separate hybridization probes to the genomic sequences; and finally, the orientation of the homeo boxes of *Ubx*, *iab-2*, and *iab-7* was known to be identical by heteroduplex mapping (Karch et al., 1985). Because the cDNA is only 3 kb in length and yet it hybridizes to genomic DNA fragments spanning 17 kb, we can conclude that the *iab-7* gene must contain one or several introns. A restriction map for cDNA P5 is shown in Fig. 7B.

In Situ Hybridization to Imaginal Discs

We have used a 1.2 kb *Mlu*I/*Eco*RI restriction fragment from *iab-7* cDNA P5 (labeled 1, Fig. 7B) which represents the 3′ portion of the transcriptional unit as a probe for *in situ* hybridization to tissue sections from 8 to 24 h *Tab*/+, and

from wild-type embryos and to whole-mounted mass-isolated imaginal discs from *Tab*/+, and from wild-type, third-instar larvae. In agreement with *in situ* hybridization experiments of Regulski et al. (1985), we detect *iab*-7 transcripts in several segments, presumably, A5–A8, in embryos of both *Tab* and wild-type (data not shown). We have not detected hybridization to segments anterior to A4 in *Tab*/+ embryos, but the expression in such segments could be limited to a small region so that a signal could easily have been missed.

Since the notum of T2 is derived from the "wing" discs (dorsal imaginal discs of T2) and since others (e.g., Akam, 1983; Kornberg et al., 1985) have shown that transcripts can be detected in imaginal discs, *Tab*/+ wing discs were analyzed for expression of *iab*-7^{+}. Imaginal discs from wild-type and *Tab*/+ larvae were mass-isolated and probed with ^{3}H-labeled purified DNA fragments from the *iab*-7 cDNA. We have used ^{3}H-labeled hybridization probes rather than ^{35}S-labeled probes to eliminate possible artifacts in labeling of discs (as discussed in Kornberg et al. 1985).

Figure 8 shows the *in situ* hybridization patterns of wing discs from wild-type (*Mc/Mc*) and from *Tab* (*Tab/Mc*) larvae, and genital discs from wild type (Canton S). The wing discs contain the cells that give rise not only to the wing, but also to the notum, postnotum, and several pleural plates on T2 (Fig. 9). Nineteen wing discs from wild-type (Canton S) animals were scored, and no labeling was observed. In one case, label was observed above background but the labeling was uniform over the entire disc. Of 45 unselected wing discs from a *Tab/Mc* mass-isolated disc preparation, 20 showed specific labeling above background; in every case such labeling was confined to the notal region of the wing disc (Fig. 8B,D and Fig. 9B). Twenty had no label above background in the notal or wing regions and presumably included *Mc* homozygotes and any *Tab/Mc* discs that were not oriented properly for detection of the short-ranged ^{3}H disintegrations (Fig. 8A, C). The remaining five were uniformly labeled over the entire disc. The restricted labeling to the notum and not to the wing portion of the disc supports the conclusion that the *Tab* phenotype results from an *iab*-7 RNA or a modified *iab*-7 RNA being abnormally regulated so that it becomes overexpressed in a spatially restricted manner in the thorax.

Figure 8, E and F, shows the *in situ* hybridization patterns of genital discs derived from wild-type flies. The genital discs give rise to the internal genitalia, external genitalia, and analia. Genital discs derived from *Tab*/+ larvae reveal the same patterns of labeling as those from wild-type larvae (data not shown). Two distinct patterns of labeling of genital discs are observed; namely, grains completely covering the disc (Fig. 8E) and grains concentrated in a broad band bordering unlabeled regions of the disc (Fig. 8F). These different patterns of labeling may be the result of differences in male and female genital disc structure (Dubendorfer and Nothiger, 1982) or differences in the sidedness of the discs (Ursprung and Schabtach, 1968). Using electron microscopy to analyze a parasagittal section of a male genital disc, Ursprung and Schabtach (1968) found that half way between the median and lateral tip of the disc the epithelium on one side of the disc is one cell layer thick and on the opposite side is at least two

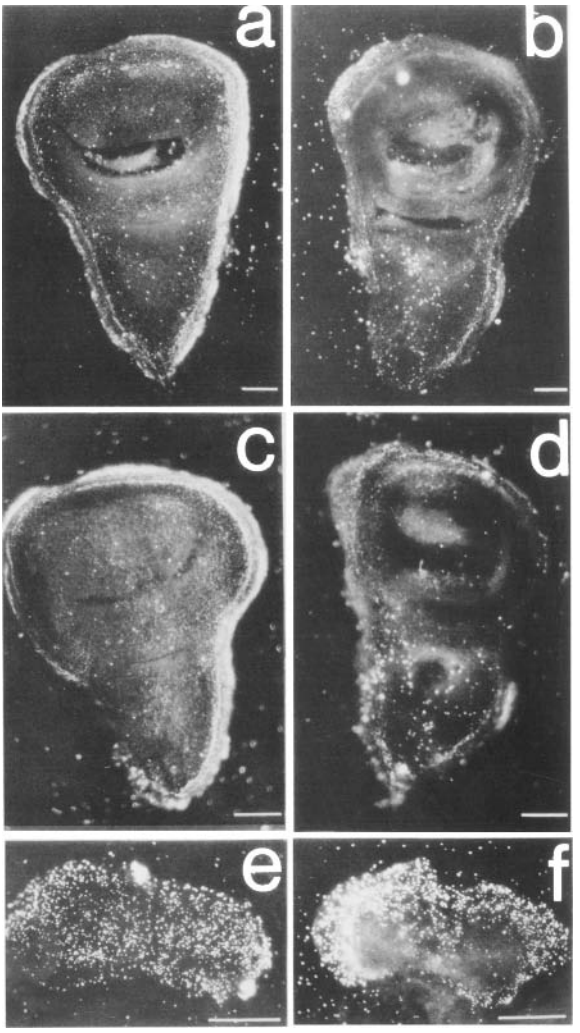

Figure 8. Localization of *iab*-7 transcripts in imaginal discs. Dark-field photomicrographs after autoradiography of imaginal discs hybridized *in situ* with a ^{3}H-labeled probe. (a) An example of a wild-type (*Mc/Mc*) wing disc showing typical grain distribution that is not significantly different from that found in the background. (b) An example of a *Tab* (*Tab/Mc*) wing disc with significant clustering of grains in the notal but not the wing region of the disc. (c) Another example of a typical wild-type (*Mc/Mc*) wing disc. (d) Another example of the typical pattern of labeling of a *Tab/Mc* disc with significant labeling again clustered over the notal but not the wing region. (e) Wild-type (Canton S) genital disc with dense distribution of grains over the entire disc. (f) Wild-type (Canton S) genital disc with grains densely clustered primarily at the edge of the disc. Bar, 0.02 mm.

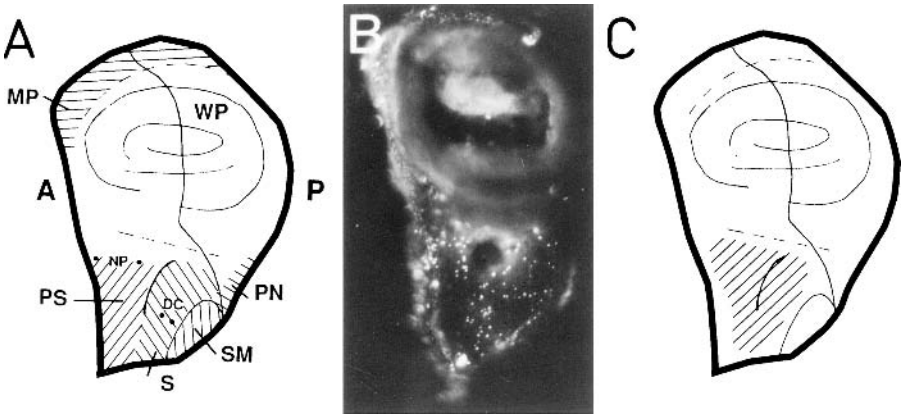

Figure 9. Localization of *iab*-7 transcripts in *Tab* wing discs in relation to the wing disc fate map. (A) Fate map of the wing disc (after Bryant 1975). The regions of the wing disc which give rise to the thoracic as opposed to wing tissue are marked with hatched lines. The positions of two sets of bristles are shown by the filled circles. (A) Anterior; (P) posterior; (DC) dorsocentral bristles (cf. Fig. 3); (MP) mesopleura; (NP) notopleural bristles; (PN) postnotum; (PS) prescutum; (S) scutum; (SM) scutellum; (WP) wing pouch. (*B*) *Tab/Mc* wing disc (enlargement of Fig. 8B) showing grain clustering over the notal but not the wing region. (C) Composite diagram of a *Tab/Mc* wing disc. The hatched lines within the notal portion of the disc indicate the common area over which grains are found to be clustered in *Tab/Mc* wing discs.

layers thick. In the latter case, the thickness may be great enough to prevent most ^{3}H disintegrations from being detected, or nonuniform *iab*-7 expression may be involved.

DISCUSSION

Dominant Phenotype of *Tab*

In *Tab*/+ animals abdominal tergite-like tissue in the notum appears to replace thoracic tissue rather than being additional to it. Apparently, a group of notal cells, possibly clonal in origin, takes on characteristics of tergite tissue. In the thorax of *Tab*/+ animals, the notal stripes are assumed to represent A6-like or A7-like tergite cuticle. Since the abdominal patches that arise in A1–A6, inclusive, can be most simply interpreted as resulting from a transformation to an A7 level of development in the dorsal ectoderm of each segment from T2–A6, inclusive, we conclude that the notal stripes are A7-like rather than A6-like. The aggregation of transformed A7-like tissue in well-defined stripes in the *Tab*/+ thorax may reflect a clonal origin of the transformed tissue; if nonclonal in origin, then the striping pattern may be related to the tendency of dissociated imaginal disc cells with different developmental fates to sort out and reaggregate in accord with those fates (Garcia-Bellido and Lewis, 1976). Whether *Tab* is active in dorsal T3 is indeterminate since in Diptera the notum of T3 is all but obliterated.

Roseland and Schneiderman (1979) find that the bulk of each hemitergite arises from a nest of histoblast cells that number 15–16 at the start of pupal life. Madhavan and Madhavan (1980) find that these cells undergo eight divisions before differentiating into a hemitergite and estimate that the entire tergite contains approximately 9000 cells. The size of the patches of A7-like tergite cuticle in *Tab*/+ abdomens suggests that perhaps only one cell, or a few, of the initial nest are involved in producing each patch.

The notum of T2 arises from a pair of wing discs, each of which arises from a small cluster of cells that undergo some nine divisions to produce approximately 22,000 cells (Ripoll, 1972). Thus, the ratio of the number of notal cells to the number in one of the major tergites (namely A2–A5) is very approximately 2.5. This differential between cell number in the wing disc versus the number in the histoblast nest possibly accounts for the finding that the area of the two sets of *Tab* stripes combined is much larger than that occupied by the average amount of *iab*-7-like tissue that arises in patches on a given tergite of the abdomen.

There is a striking precedence for such differential growth rates of the same type of tissue in the case of another dominant mutant in the complex, *Cbx*, which produces a haltere-wing by transforming the wing toward haltere. As Morata and Garcia-Bellido (1976) have shown, more haltere tissue can be found in the haltere-wing of T2 than is ever found in the haltere in its normal location in T3. In this case ectopic overexpression of Ubx^{+} in T2 is assumed to be responsible for the transformation of wing tissue to haltere tissue (T2 toward T3), and a higher rate of cell proliferation in wing than in haltere discs is assumed to account for the excessive amount of haltere tissue in the haltere–wing of T2.

We do not understand why the stripes arise at right angles to the abdominal tergites and why there are two sets of stripes instead of one. The invariance of the notal pattern may be related to the tendency of cells of different fates to sort out and then aggregate in accord with those fates (Garcia-Bellido and Lewis, 1976). The presence on the *Tab* notum of two sets of stripes (or actually three sets if the outer divided stripe represents separate units of the pattern) of tergite-like tissue instead of a single strip of such tissue may be indicative of different groups of tergite cells which in the environment of the notal cells of the imaginal disc are set apart as two (or three) separate islands of cells that ultimately become the stripes.

An explanation is needed for the striking sexual dimorphism in the striped pattern of the *Tab*/+ thorax if only A7 cuticle is involved. Animals that have only one dose of BX-C have rudimentary A7 tergites that are solidly pigmented black; whereas the A7 tergite in females, although reduced in size compared with that of A6, still contains a small light tan area as well as a black-pigmented area. Thus, A7, as well as A5 and A6, is sexually dimorphic. Therefore, we assume that the genetic controls that establish sexual dimorphism in the A5, A6, and A7 tergites of wild-type and *Tab*/+ animals are operative in T2, just as they are operative in other thoracic regions such as T1, where the leg bears a sexcomb in males only.

We cannot completely rule out the possibility that other genes of the BX-C besides *iab-7*$^+$ must also be ectopically expressed in T2 before the *Tab* pattern of striping can be expressed. Direct probing of the wing discs with probes derived from such regions of the complex has not been carried out. Although our *Tab* revertant analyses have failed to uncover deletions or inactivations of BX-C genes other than those involving the *iab-7* and *iab-8* regions, the number of revertants obtained is too few to exclude the possibility that an inactivation of *iab-2*, for example, would lead to a loss of the striping pattern.

Recessive Phenotype of *Tab*

The recessive phenotype of *Tab* is a complex one. In addition to a recessive lethal effect from the inversion *Tab* breakpoint in 90D, which appears to be without any detectable segmental transformations, there is a recessive lethal in 89E, which appears from an analysis of embryonic phenotypes to be a weak to moderate inactivation of the *iab-8* function.

Cis-Regulatory Action of *Tab*

A combination of cytogenetic and molecular results indicates that the dominant functional component of the *Tab* phenotype represents a highly unusual case of overexpression of a BX-C gene. Overexpression of BX-C gene functions is known to occur by *trans*-regulation, as in the case of such mutants as *Pc* and *esc*, and by *cis*-regulation, either as *cis*-overexpression (Lewis 1985) or, as in cases such as *Cbx*, by dominant gain-of-function mutants. In the latter case, proof that *Cbx* acts in *cis* to cause strong overexpression of *Ubx*$^+$ (in T2) was obtained by deriving the double mutant, *Cbx Ubx*, as a crossover (Lewis, 1955). Although the association of the *Tab* mutant with the *Tab* inversion, *In(3R)Tab*, virtually precludes any attempt to derive the analogous double-mutant crossover (i.e., *Tab* combined with an *iab-7* loss-of-function mutant), the results of analyzing *Tab* revertants strongly suggests that the observed overexpression of *iab-7*$^+$ in the *Tab* mutant is a *cis* effect. The overexpression of *iab-7* appears to be limited to only a fraction of the cells making up the notum or a tergite, in the sense that only stripes of A7-like cuticle appear on the notum and only patches of such cuticle appear on the tergites of A1–A6, inclusive.

In the *Tab* mutation the coding sequence of the *iab-7* gene appears not to be altered; instead, the small inversion associated with *Tab* presumably results in changes in the upstream region of *iab-7* that somehow cause that gene to be ectopically overexpressed in *cis*. Preliminary studies show that the *iab-7* gene has multiple transcripts with stage-specific differential splice patterns (S. Celniker and E. Lewis, unpublished.). The conditions that are necessary and sufficient for such overexpression remain to be determined, One possibility is that the *Tab* mutation results in altering only one or two of the stage-specific transcripts.

Analysis of revertants of the dominant phenotype of *Tab* requires an understanding of how the BX-C is organized in the *In(3R)Tab* chromosome. The proximal 89E/90D juncture of that inversion brings the *iab-7* region, and possibly a portion of the *iab-8* regions as well, next to sequences in 90D; as a result a portion at least of the *iab-8* region has been removed from *iab-7* and brought next to 90D sequences at the distal 89E/90D junction. The 12 revertants that disrupt the proximal junction bring about reversion of *Tab* by two mechanisms; one group (Class II and Class III) made up of five revertants, inactivate the *iab-7* function, and therefore presumably prevent that function from being overexpressed; the other group (Class I) made up of seven revertants, disrupts the proximal junction in such a way that the abnormal *cis*-regulation of *iab-7*$^{+}$ is abolished without disturbing the normal *cis*-regulation of that gene. As expected, both classes of revertants retain a recessive loss-of-function of *iab-8* that was present in the original *In(3R)Tab* chromosome from which, of course, the revertants were derived; in seven of the revertants the new breakpoint resulted in a more extreme loss of *iab-8* function than that present in the original *Tab* chromosome.

Tissue Expression of *iab-7*

The results of probing imaginal discs of *Tab*/+ for *iab-7* transcript expression support the foregoing interpretation of the dominant *Tab* phenotype. In these experiments the genital discs provided a built-in control. Such discs are believed to be derived from segments A8 and A9, which give rise to internal and external genitalia, and A10, which gives rise to analia. Since wild-type, as well as *Tab*/+, genital discs gave excellent hybridization with the *iab-7* probe, *iab-7* is evidently active at least in segments A8 and A9, as well as in A7. Such hybridization would be in accord with the rule that once a gene of the BX-C becomes derepressed it tends to remain derepressed in more posterior segments. This rule has been verified for the distribution of *Ubx* transcripts in embryonic tissues, except that in wild type the extent of expression of those transcripts in more posterior segments tends to diminish (Akam 1983; White and Wilcox 1984; Beachy et al. 1985). In the absence of genes in the abdominal portion of the complex, the extent of *Ubx* transcript expression does not diminish posteriorly, at least through segment A8 (parasegment 13) (Struhl and White 1985). The rule has also been verified for *iab-7* itself in embryonic tissues by Regulski et al. (1985), using an *iab-7* homeo box-containing genomic DNA fragment, and by us, using a DNA fragment that does not contain the homeo box, from an *iab-7* cDNA.

CONCLUSIONS

1. The *Tab* phenotype is the result of misregulation of the *iab-7* gene. Thus, molecular studies indicate that *iab-7* transcripts are detectable in the notal region of wing discs of *Tab*/+ but not wild-type wing discs. That both *Tab*/+ and wild-type animals show strong expression of *iab-7* transcripts in genital discs indicates that *Tab* retains the normal expression of *iab-7* in the terminal regions of the body at the same time that it causes ectopic expression

of those transcripts in a highly constant and highly specific pattern in the thorax and a variable patch-like expression in the abdominal tergites, A1–A6, inclusive.

2. The mechanism by which *Tab* causes misregulation of *iab*-7 is not known but might involve: (1) Alteration in *cis*-regulatory sequences upstream to the *iab*-7 gene as the result of the 89E/90D inversion junction in that region; or (2) the juxtaposition of *iab*-7 to *cis*-regulatory sequences from 90D, which in turn create the new ectopic tissue distribution of transcripts.
3. Mutants of the *Tab* type provide useful tools for studying ectopically expressed transformations of cells and tissues, especially when expressed in regions such as the notum of T2 that have differentially high rates of cellular proliferation.

EXPERIMENTAL PROCEDURES

Mutant Descriptions

Descriptions of standard mutants used in this paper can be found in Lindsley and Grell (1968) or more recent BX-C mutants in Lewis (1978), except for the following: *iab*-7 alleles *D3*, *D6*, *D14*, *D15*, *D16*, *297*, *380*, and *65* (Karch et al., 1985); *Sab* (Sakonju et al., 1984); *Uab* (Lewis 1981); *Dp(3:3)P5* (Duncan and Lewis, 1982); Dp*(3:1)68* (Lewis, 1985); TM12 (carrying multiple inversions, *Sb*, *Tb*, and *Dp(3)462);Tb* (Auerbach cited in Craymer, 1980).

Induction and Recovery of Revertants

Revertants of the dominant mutant *Tab* were obtained from two different genetic screens. Males p^p *Mcp Sab Tab/bxd Mcp Sab Mc* were irradiated with 4000 r. In screen 1 (Table 1) they were mated to homozygous sbd^2 *bxd* females. Each putative revertant was then crossed to *Sb Dp(3;3)P5/Df(3)P9* to establish a stock. In screen 2 the irradiated males were mated to homozygous *Dp(3:1)68;Cbx Ubx* females, that were also homozygous for recessive markers, y^2 (*yellow-2*) and gl^3 (*glass-3*).The presence of *Dp(3;1)68* allows homozygous *Ubx* to survive and allowed revertant to be recovered that might otherwise have been sterile from loss of function in *iab*-7 and/or *iab-8*.

Cytology

Tab revertant/TM6, *Hu Tb* males (where *Tb* is a dominant larval marker) were mated to sbd^2 *bxd* females and non-*Tb* (*Tab/*sbd^2 *bxd*) larvae were selected for polytene chromosome analysis. Haplosterile revertants were maintained in stock by balancing over a TM12 chromosome. Males of such stocks were mated to wild-type (Canton S) or sbd^2 *bxd* females and non-*Tb* larvae were selected for salivary gland chromosome analysis.

Isolation and Purification of DNA

Purification of fly DNA followed the procedure of Bender et al. (1983b). Phage DNA and plasmid DNA were isolated as described by Snyder et al. (1982) and Fyrberg et al. (1980), respectively.

DNA Labeling and Hybridizations

^{32}P-labeled nick-translated probes were synthesized according to the method of Mullins et al. (1981). Hybridization reactions for whole genome Southerns contained 1×10^6 dpm/ml and were performed as described by Rozek and Davidson (1983).

Construction of the Recombinant Phage Library

DNA from adult *Tab* flies was partially digested with *Mbo*I and sized, and the fractions containing DNA fragments 10–15 kb in length were ligated into purified arms of the lambda vector J1 (Davis et al., 1980). The phage were subsequently packaged *in vitro* according to the method of Hohn and Murray (1977).

Screening cDNA libraries

Two λgt10 cDNA libraries, 3- to 12-h embryos and 5.5- to 7.5-day pupae, kindly provided by L. Kauvar (Poole et al., 1985), were screened by plaque hybridization according to the method of Benton and Davis (1977).

Scanning Electron Microscopy

Adult flies were briefly etherized and mounted on a specimen holder using metallic nail polish. The specimen holder with the flies was immediately placed in an ETEC Autoscan SEM. The flies were photographed operating the SEM between 10 and 20 kv.

Preparation of Embryos for Cuticular Analysis

For temporary preparations late embryos and first-instar larvae were mounted in a drop of lactic acid/ethanol (9:1), a coverslip was added, and the slide was left overnight on a 42°C warming plate. For permanent preparations embryos were dechorinated, fixed, and cleared according to the procedure of Van der Meer (1977).

Preparation of Adults for Cuticular Analysis

Adult flies were preserved, cleared, and mounted as described by Duncan (1982).

In Situ Hybridization

In situ hybridizations to polytene chromosomes were performed according to Pardue and Gall (1975). Probes were labeled with ^{35}S or ^{3}H. Tissue sections from embryos and *in situ* hybridizations were performed according to Hafen et al. (1983) without dechorionating the embryos. Probes were labeled with ^{3}H.

Whole-Mount *In Situ* Hybridization in Discs

Mass-isolated discs from a *Tab/Mc* stock were prepared according to Eugene and Fristrom (1976). The *Mc* chromosome probably carries a duplication for the distal end of the complex and covers the dominant sterility of *Tab*. The *Tab/Mc* stock produces *Tab* homozygotes which are embryonic lethal and *Mc* homozygotes which are pupal lethal. We verified that the ratio of *Tab/Mc* to *Mc/Mc* larvae was approximately 2:1. As a result only 67% of the animals and therefore of the discs are expected to be of genotype *Tab/Mc*. Whole-mounted *in situ* hybridizations were performed essentially as described by Kornberg et al. (1985) with the following modification of the method of preparing the probes. DNA fragments were purified by low-melting-temperature agarose (Rozek and Davidson 1983) and an aliquot was nick-translated with Polymerase 1 and five different concentrations of bovine pancreas DNase 1 (2–16 ng/μg) for 2 h at 14°C in the presence of 100 μCi of [^{32}P]dCTP. The products were sized and the reaction that gave fragments in the range of 35–150 bp was repeated using 100 μCi of each of two [^{3}H]dNTPs. Autoradiographic exposures were for 2–4 weeks.

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MOLECULAR BASIS OF TRANSABDOMINAL—A SEXUALLY DIMORPHIC MUTANT OF THE BITHORAX COMPLEX OF *DROSOPHILA*

(cis regulation/macromutation/homeotic/Abdominal B/evolution)

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Contributed by E. B. Lewis, November 5, 1992

Transabdominal (*Tab*) is a dominant gain-of-function mutation that results in islands of sexually dimorphic abdominal cuticle in the dorsal thorax of the adult fly. This phenotype has complete penetrance and constant expressivity, and we show that it results from ectopic expression of ABD-BII, one of two proteins derived from the Abdominal B (*Abd-B*) domain of the bithorax complex (BX-C) and one that is normally expressed only in terminal portions of the abdomen. In *Tab*/+ animals ABD-BII is ectopically expressed in the relevant imaginal "wing" disc as three islands of cells whose location on the fate map corresponds to the three islands of transformed cuticle in each half of the adult thorax. *Tab* is associated with an inseparable inversion bringing sequences in 90E next to sequences in the transcription unit encoding ABD-BII in 89E. That 90E sequences drive ectopic expression of ABD-BII is indicated by our finding that such sequences in a *P*-element transformant express the reporter gene's product (β-galactosidase) in the same three islands of wing disc cells. On morphological grounds, the transformed islands in the adult thorax correspond to subsets of muscle attachment cells. Ectopic expression of a homeodomain protein thus creates a unique and invariant pattern of sexual dimorphism.

Conspicuous sexual dimorphism in wild-type *Drosophila melanogaster* is largely confined to the posterior abdomen, where the male has solidly black tergites on

Figure 1. Sexual dimorphism in the thorax of *Tab*/+ male and female compared with wild type. (*Left* to *Right*) *Tab*/+ male with three solidly black-pigmented islands on each half of T2, wild-type male with uniformly tan-colored T2 (the wild-type female being identical), *Tab*/+ female with islands in T2 that are only black banded.

the fifth abdominal (A5) and A6 segments and lacks tergites on A7 and A8, while the female has only black-banded tergites on A5–A8 inclusive. In *Tab*/+ animals, the same strikingly dimorphic pattern of pigmentation also occurs on the dorsal thorax (T2) in the form of islands of abdominal-like cuticle that are solidly black pigmented in the male and only partially banded with black in the female (Fig. 1).

In wild type, the ability to differentiate properly the abdominal body segments depends on the expression of the homeotic genes of the bithorax complex (BX-C) [1]. Development of A5 and the more posterior segments of the abdomen is specified by the *Abd-B* domain, one of three domains of the BX-C—the other two being Ultrabithorax (*Ubx*) and abdominal A (*abd-A*) (ref. [2]; reviewed in ref. [3]). The *Abd-B* domain encodes two proteins, ABD-BI and ABD-BII [4]. They share the same homeodomain, a 60-amino acid helix-turn-helix motif that mediates DNA-binding interactions and is also found in other proteins that regulate development (reviewed in ref. [5]). In wild-type embryos, the ABD-B proteins have spatial distributions that are restricted to the posterior body regions [4]. Later in development *Abd-B* is expressed in the imaginal disc that gives rise to the adult internal and external genitalia [6].

To determine what role the *Abd-B* domain plays in bringing about the *Tab*/+ phenotype, we have studied the pattern of protein and transcript expression in the larval wing discs and in embryos of *Tab*/+ animals. Here we provide evidence that

ABD-BII is able to direct differentiation of abdominal tissues in T2 in the absence of other abdominal genes of the BX-C.

MATERIALS AND METHODS

Materials

We were provided with gifts of materials as follows: a monoclonal anti-UBX (Ultrabithorax) antibody was from R. Mann and D. Hogness (Department of Biochemistry, Stanford University; originally made by R. White and M. Wilcox), monoclonal anti-ABD-A (abdominal A) antibody was from I. Duncan (Department of Biology, Washington University, St. Louis), and the B14 *P*-element strain was from R. Kolodkin and C. Goodman (Department of Molecular and Cellular Biology, University of California, Berkeley).

Immunohistochemistry

Wing discs were dissected from third-instar larvae in 1 × TBS (0.1 M Tris/1.29 M NaCl/0.05 M KCl/0.01 M EGTA, pH 8.0)/0.1% Triton X-100 and fixed for 5 min in 2% EM-grade formaldehyde (Polysciences). The discs were serially incubated with a monoclonal anti-ABD-B antibody (1A2E9) [4], biotinylated anti-immunoglobulin antibody (Bio-Rad), and avidin DH-biotinylated antihorseradish peroxidase H complex (Vector Laboratories). Discs were then stained with the peroxidase substrate diaminobenzidine tetrahydrochloride (1 mg/ml) (Polysciences) and 0.01% hydrogen peroxidase. Embryos (3–15 h old) were fixed and protein was localized as described [4].

In Situ Hybridization and Tissue Localization

Wing discs were dissected from third-instar larvae in 1× phosphate-buffered saline (PBS) and fixed three times: first in 4% paraformaldehyde in 1 × PBS for 5 min at room temperature and 15 min at 4°C, second in 4% paraformaldehyde/1 × PBS/0.6% Triton X-100 (PBT) for 5 min at room temperature, and third after treatment with proteinase K (10 mg/ml in PBT) for 3 min in 4% paraformaldehyde/0.2% glutaraldehyde in PBS for 15 min at room temperature. Hybridization conditions were as described [7]. Embryos were prepared and hybridization conditions were as described [7]. Antisense single-stranded probes labeled with digoxigenin were generated by PCR. Transcripts encoding ABD-BI (also called class A transcripts) and ABD-BII (class B) were detected by PCR-generated anti-sense probes 5013 and 5005, respectively. Their position on the genomic map relative to the *Abd-B* cDNAs is shown in Fig. 2. Probe 5013 was generated by using a 1327-bp *Bss*HII genomic restriction fragment from +157 on the BX-C walk and the primer 5′-TACTCGAGTTCTGCCCC-3′. Probe 5005 was generated by using a 1145-bp *Spe* I/*Sca* I genomic restriction fragment from +187 on the BX-C walk and the

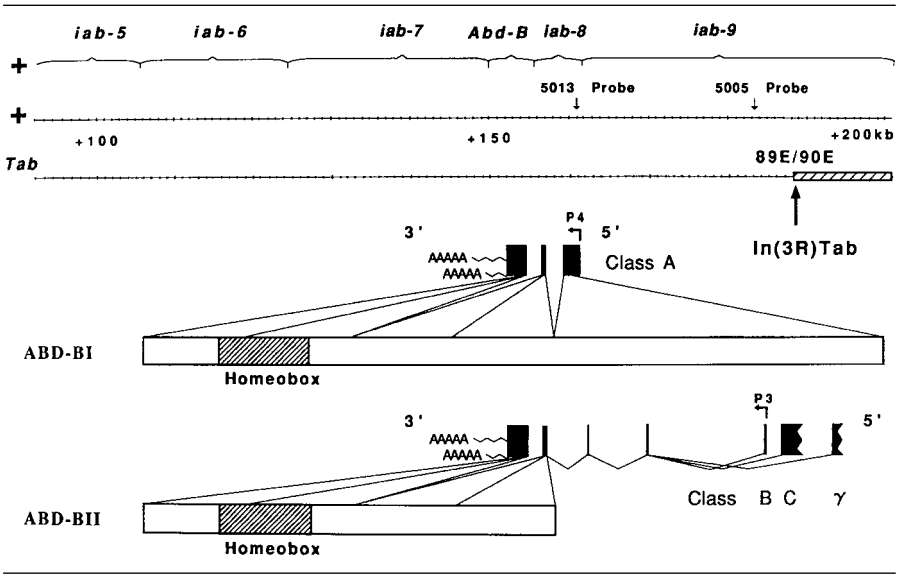

Figure 2. Organization of wild-type and *In(3R)Tab* genomic DNA and representative homeobox-containing cDNAs from the *Abd-B* domain [4, 8, 9]. The *Abd-B* domain extends approximately from +100 to +200 kb of the BX-C walk [10]. The *iab-5–iab-9* regions are delineated by brackets [11]. Boldfaced arrow points to *Tab* breakpoint. *Tab* is associated with a small inversion with breakpoints in 89E and 90E of the salivary gland chromosomes [6]. Four classes of *Abd-B* transcripts are designated A, B, C, and γ [12]. Class A transcripts encode ABD-BI and class B, C, and γ transcripts encode ABD-BII. Transcription start sites for classes A and B are marked with bent arrows and are designated P4 and P3, respectively. Molecular breakpoint in 89E is located in a 5′ nontranslated exon of class C and 4315 bp from the transcription start site P3 for class B.

primer 5′-CCGCTGATTAATTCAAG-3′. Discs and embryos were photographed with Nomarski optics.

5-Bromo-4-chloro-3-indolyl β-D-galactoside staining of discs from the B14 line was as described [13].

RESULTS

In *Tab*/+ wing discs, we detect ABD-B protein principally in three regions whose location in the fate map of the disc correlates remarkably well with the three islands of abdominal-like cuticle in each half of the adult T2 (Fig. 3A). We have confirmed by *in situ* hybridization that such discs express the transcript encoding ABD-BII protein (Fig. 3B). We fail to detect transcripts encoding ABD-BI in these discs, nor do we detect either UBX or ABD-A protein (data not shown). [ABD-BI and ABD-BII are also known as *Abd-B* m and *Abd-B* r, respectively [15].] We conclude that ectopic expression of ABD-BII is responsible for the *Tab*/+ transformation in T2.

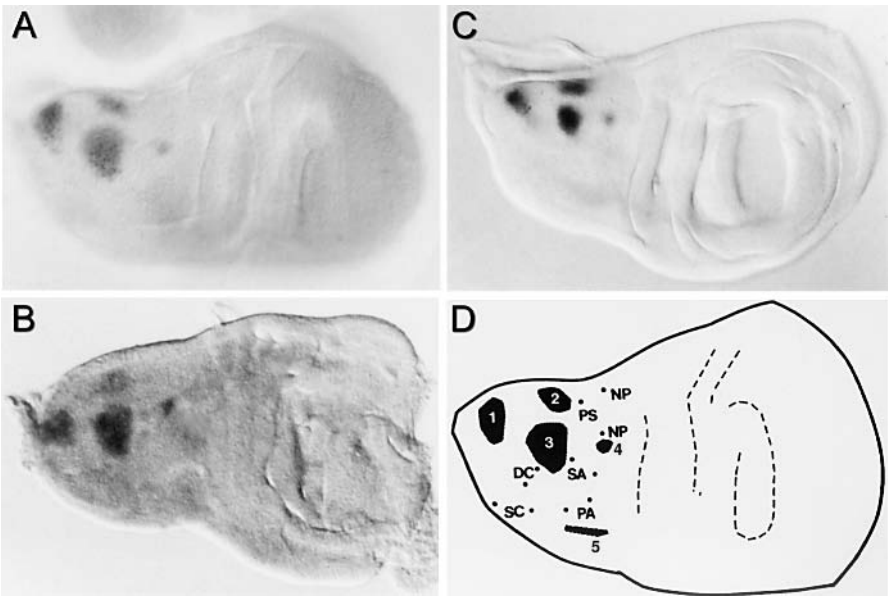

Figure 3. Comparison of ABD-B protein and transcript expression patterns in *Tab*/+ wing discs with the pattern of β-galactosidase expression in a disc from a *P*-element transformant. (A) ABD-B protein in a *Tab*/+ disc. (B) RNA encoding ABD-BII in a *Tab*/+ disc. Since the ABD-B antibody is directed against an epitope common to both ABD-BI and ABD-BII proteins, it has been necessary to determine the distribution of transcripts encoding these proteins in order to identify the ectopically expressed one(s). (C) β-Galactosidase staining in a disc from *P*-element transformant B14. (D) Diagram of the wing disc in *A* superimposed on the fate map of the wild-type disc [modified from Bryant [14]]; ABD-B-expressing regions of the disc are shown shaded and numbered. Location of presumptive bristle primordia in the wing disc is shown by solid circles. Dashed lines outline the region of the disc that gives rise to the wing tissue. Two small islands, numbered 4 and 5, of ABD-B protein expression fail to have a discernible effect on the cuticle of T2. (NP), notopleural bristle; (PS), presutural bristle; (SA), supraalar bristle; (DC), dorsocentral bristle; (SC), scutellar bristle; (PA), postalar bristle.

That enhancer-like elements in 90E drive ectopic expression of ABD-B in *Tab*/+ discs is made likely by results of studying a germ-line transformant (B14) having a *P*-element inserted in 90E. In an otherwise wild-type background, the reporter gene in this case, β-galactosidase, is expressed in the same three islands of wing disc cells (Fig. 3C) that in *Tab*/+ discs express ABD-B protein and transcripts encoding ABD-BII.

In T2 of *Tab*/+ animals, the position of the sexually dimorphic cuticle coincides with the position of subsets of muscle attachment sites (Fig. 4). We presume that the cells in such sites secrete cuticle as well as having a muscle attachment function. If so, the cuticular transformation in T2 of *Tab*/+ is a homeotic one in which ectopic

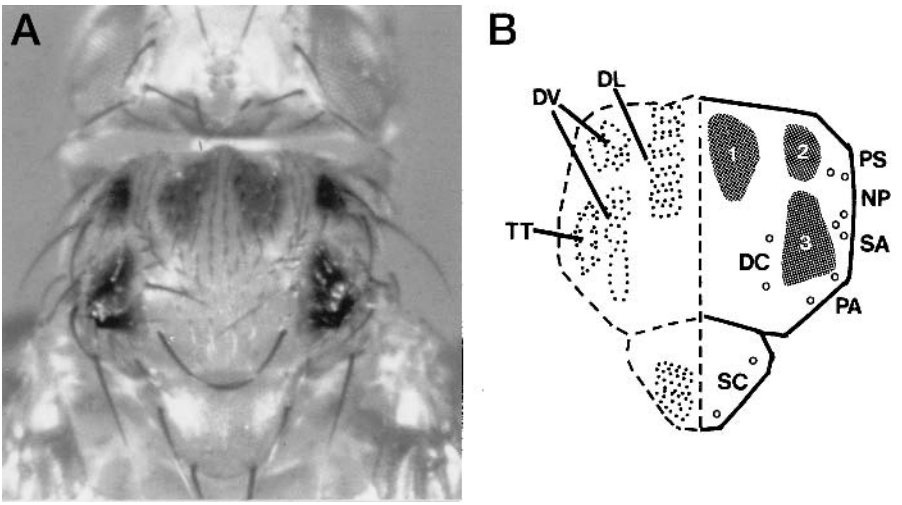

Figure 4. Comparison of location of islands of abdominal-like cuticle in dorsal T2 of *Tab*/+ with the location of subsets of thoracic muscle attachment sites in dorsal T2 of wild type. (A) *Tab*/+ male thorax. (B) Diagram of T2 as shown in A. On the right half, the islands are numbered in agreement with Fig. 3D and small open circles represent sites of macrochetes: on the left half, thoracic muscle insertion sites immediately underlying the cuticle are enclosed in dotted lines [modified from Miller [16]. (DC), dorsocentral bristle; (DL), dorsal longitudinal muscle; (DV), dorsoventral muscle: (NP), notopleural bristle; (PA), postalar bristle; (PS), presutural bristle; (SA). supraalar bristle; (SC), scutellar bristle; (TT), tergotrochanteral muscle.

expression of ABD-BII causes thoracic-type cuticle to be replaced by abdominal-like cuticle typical of the terminal half of the abdomen.

Indirect evidence suggests that *Tab*/+ animals are globally expressing ABD-B protein and transcripts for ABD-BII in subsets of muscle attachment cells throughout the animal. Thus, such expression is seen in groups of ectodermal cells in virtually all segments of the *Tab*/+ embryo (Fig. 5). At a somewhat later stage, muscles from between adjacent sets of these cells, suggesting that the cells are at least involved in the muscle attachment process, if they are not indeed muscle attachment cells. Consistent with ABD-BII being expressed in subsets of ectodermal cells in the embryonic abdomen, *Tab*/+ flies have on abdominal segments A2–A5 inclusive patches of cuticle that are homeotically transformed toward segments posterior to A5 [6].

DISCUSSION

Our results indicate that the ABD-BII protein shares with each of the other homeodomain proteins of the BX-C—namely, UBX, ABD-A, and ABD-BI—the ability to program development of abdominal cuticle, presumably by turning on one or more downstream target genes. Since only ABD-BII is expressed in the wing discs of *Tab*/+ animals, yet T2 has abdominal characteristics normally developed under

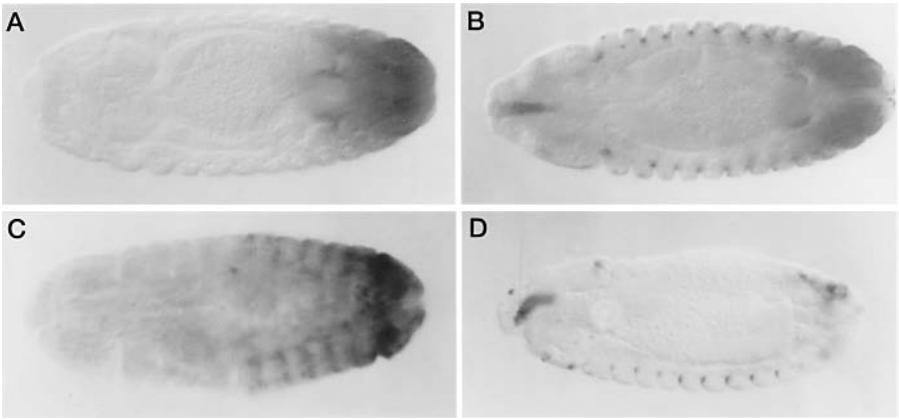

Figure 5. ABD-B protein and *Abd-B* transcript expression in wild-type and *Tab*/+ heterozygous embryos that are 10 h old. (A) Wild type. ABD-B proteins are expressed in parasegment 10 (PS10)-PS15 inclusive and in visceral mesoderm surrounding the hindgut [4, 15, 17]. (Embryonic parasegments are one-half segment offset from the adult segments—e.g., PS10 comprises posterior A4 and anterior A5 [18].) (B) *Tab*/+. Superimposed on the ABD-B protein pattern in *A*, ectopic expression of that protein is observed in PS8 and PS9, the gnathal region that gives rise to the pharynx and in a laterally localized pattern repeated in each abdominal segment. (C) *Tab*/+. Transcripts encoding ABD-BI are restricted to the ectoderm of PS8–PS13. Since *Tab* was induced on a chromosome containing the double mutant Miscadestral pigmentation (*Mcp*) [1] and Super-abdominal (*Sap*) [19]. transcripts encoding ABD-BI show an extension of their wild-type embryonic expression pattern [9, 20] into PS8 and PS9. (D) *Tab*/+. Transcripts encoding ABD-BII follow the same ABD-B protein expression in the gnathal region of the head and in restricted regions in the remaining segments from PS4 to PS15. Anterior is to the left. (A–C) Dorsal views. (D) Lateral view.

control of ABD-BI, we conclude that ABD-BII shares with ABD-BI the ability to activate genes required for developing the sexually dimorphic pattern of the posterior abdomen. This sharing of similar functions by homeotic gene products is known for the development of other structures, such as formation of the longitudinal tracheal trunks [1] and suppression of chitinized plates [21].

In some cases, the BX-C proteins appear to have unique, as well as shared, functions—e.g., UBX under control of the bithoraxoid (*bxd*) *cis*-regulatory region suppresses ventral pits in the abdominal segments [1]. Similarly, ABD-BII appears to have at least one unique function—programming development of genitalia. Thus, animals lacking this protein—namely, homozygotes for the infraabdominal 9 mutant *iab-9*^{1065}—occasionally survive as adults and lack internal and external genitalia [11]. Heterozygotes for *Tab* and such *iab-9* mutants also lack genitalia [6], as is expected since the *Tab* breakpoint in 89E interrupts the transcription unit encoding ABD-BII (Fig. 2). The *iab-8*^{D14} mutant of Duncan is a 396 bp deletion spanning the promoter of the transcription unit encoding ABD-BI [11, 12]. Homozygotes for this mutant survive, although rarely, and possess genitalia, showing that ABD-BI protein is not required for their development.

In spite of the ability of ABD-BII to promote development of genitalia in the terminal abdominal regions, no trace of such development is visible in T2 of *Tab*/+ adults. However, for such development to occur it may be necessary for ABD-BII to be expressed in conjunction with one or more proteins that are only expressed in the terminal body regions [see reviews by Lipshitz [22] and St. Johnson and Nusslein-Volhard [23]]. We would predict that protein(s) of the latter types would have to be ectopically expressed along with ectopic expression of ABD-BII before genitalia could develop in T2 of *Tab*/+ animals. [Two BX-C mutants, tumorous head (*tu-h*) [24] and SGA-62 [25], are reported to express rudiments of genitalia ectopically but only in the head region and with incomplete penetrance and variable expressivity.]

Unlike most gain-of-function mutants, *Tab* has complete penetrance and constant expressivity. The archetypal example of a dominant, homeotic, gain-of-function mutant is Antennapedia (*Antp*), Although some alleles have complete penetrance, even the most extreme allele, $Antp^{Yu}$, has variable expressivity; that is, only rarely are both antennae of an $Antp^{Yu}$/+ animal transformed toward a full leg. In the BX-C, Haltere mimic (*Hm*) and Miscadestral pigmentation (*Mcp*) do have complete penetrance and constant expressivity and represent striking gain-of-function mutations. However, the gain of function extends only to one segment anterior to that in which the function is normally expressed, whereas the *Tab* transformation is expressed in anterior T2, which is 9 segments anterior to A9 where ABD-BII is normally first expressed.

Evolutionary Considerations

Tab is remarkable among dominant homeotic mutants in a number of ways. It results in an extension of sexually dimorphic traits into the dorsal thorax of the adult, where it could clearly play a role in the sexual behavior of the organism and therefore might under some circumstances have a selective advantage.

Tab represents a dominant gain-of-function mutant that alters the *cis* regulation of a gene encoding ABD-BII, one of two proteins of the *Abd-B* domain of the BX-C. In doing so, *Tab* does not appear to alter the ABD-BII protein itself but, instead, radically alters where that protein is expressed, at the same time eliminating its expression in the terminal abdomen. A macromutation will generally, as in the case of *Tab*, represent loss of an "old" gene function and gain of a "new" one. Since the old function will seldom be a dispensable one, such a mutant will tend to be lethal when homozygous and can be maintained in a population only over a chromosome containing the wild-type allele. If a duplication for the wild-type allele is already present in the species at the time the mutation to a new function arises, then obviously the resultant mutant can immediately become viable as a homozygote. Otherwise, a duplication containing one copy of the mutant allele and one copy of the wild-type allele would need to arise later and could do so by a number of mechanisms, such as an illegitimate recombination event between the mutant gene in one chromosome and the wild-type allele in the homologous chromosome.

Altering the *cis*-regulation of a gene, such as occurs in the *Tab* mutant, has been proposed as a mechanism for producing major changes in the structure and function

of higher organisms [26]. The *Tab* mutant is an unusually relevant example in which a change in *cis*-regulation of a homeodomain-containing protein dramatically alters cell fate and in doing so creates an unusual pattern of sexual dimorphism in the *Drosophila* thorax.

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Genetics

SEQUENCE ANALYSIS OF THE *CIS*-REGULATORY REGIONS OF THE BITHORAX COMPLEX OF *DROSOPHILA*

(Ultrabithorax/abdominal-A/Abdominal-B/Markov chain/cis regulation)

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Contributed by Edward B. Lewis, June 19, 1995

The bithorax complex (BX-C) of *Drosophila*, one of two complexes that act as master regulators of the body plan of the fly, has now been entirely sequenced and comprises ≈315,000 bp, only 1.4% of which codes for protein. Analysis of this sequence reveals significantly overrepresented DNA motifs of unknown, as well as known, functions in the non-protein-coding portion of the sequence. The following types of motifs in that portion are analyzed: (i) concatamers of mono-, di-, and trinucleotides; (ii) tightly clustered hexanucleotides (spaced ≤5 bases apart); (iii) direct and reverse repeats longer than 20 bp; and (iv) a number of motifs known from biochemical studies to play a role in the regulation of the BX-C. The hexanucleotide AGATAC is remarkably overrepresented and is surmised to play a role in chromosome pairing. The positions of sites of highly overrepresented motifs are plotted for those that occur at more than five sites in the sequence, when <0.5 case is expected. Expected values are based on a third-order Markov chain, which is the optimal order for representing the BXCALL sequence.

The bithorax complex (BX-C) is a set of master control genes that programs the development of the abdomen and a portion of the thorax of the fruit fly (reviewed in refs. [1 and 2]). The complex consists of three homeobox-containing genes—Ultrabithorax (*Ubx*), abdominal-A (*abd-A*), and Abdominal-B (*Abd-B*)—and 12 *cis*-regulatory regions. The anterobithorax (*abx*), bithorax (*bx*), and postbithorax (*pbx*)

regions function in the wild type to promote development of the third thoracic segment into a haltere-bearing, instead of a wing-bearing, segment. The remaining nine regions, *bxd* and *iab-2–iab-9*, inclusive, determine the pattern of differentiation of abdominal segments A1–A9, respectively.

The complex has been sequenced and comprises ~315,000 bases. The methods used in sequencing it and an analysis of its open reading frames are described in the preceding paper [3]. The sequence itself is highly unusual in that >98% of it fails to code for protein. The challenge is to identify in the noncoding fraction DNA sequence motifs that are involved in *cis* regulation of the complex, such as activators or repressors of transcriptional initiation, enhancers or silencers of transcription, and motifs involved in such processes as DNA replication, splicing, chromatin activation and deactivation, and chromosome pairing. Also expected are binding sites for trans-acting repressor proteins of the Polycomb (*Pc*) family type (reviewed in ref. [4]) and *trans*-acting activator proteins of the trithorax (*trx*) type (reviewed in ref. [5]). Finally, an unknown fraction of the noncoding sequence may consist of spacer DNA that is needed to establish correct boundaries for proper expression of motifs, especially those that function in a clustered fashion.

This paper reports a preliminary analysis of DNA motifs that for the most part are highly overrepresented, singly and/or in a clustered manner. In some cases, biochemical and developmental studies have already identified motifs that have functional significance; hence their degree of abundance becomes of interest. In other cases, there are sequences so overrepresented as to suggest that they also have functional importance. The latter cases, if verified by molecular and developmental studies, will extend the known repertoire of motifs thought to regulate the complex. Increasingly, as sequence analysis becomes available for noncoding regions of the complex in other organisms, such as *Drosophila virilis*, the degree of sequence conservation of motifs between species will help identify those that are functionally indispensable.

MATERIALS AND METHODS

The BX-C is located in the 89E region of the salivary gland chromosomes. A total of 338,324 bp from that region have now been entirely sequenced and designated SEQ89E [3] (GenBank accession no. U31961). At each end of SEQ89E are sequences of putative genes that appear to be functionally unrelated to the BX-C. We have therefore deleted such genes to generate a sequence of 314,895 bp, designated BXCALL, that is expected to include the entire BX-C. All base-pair positions in this paper refer to SEQ89E.

Since the sequence of base pairs in noncoding as well as in coding regions of eukaryotic DNA is known to be nonrandom, Markov chain theory has been adopted to represent such sequences (reviewed in ref. [6]). We find that a third-order Markov chain (TMC) best describes the sequence, based on the Bayesian independence criterion (BIC) [7]. Thus, for all orders tested from the zeroth—identical with base

pair frequencies generated independently—to the fifth, the BIC is at a minimum for the third order (TMC) (data not shown).

The TMC assumes that the probability of occurrence of a given base in a sequence is not independent of the base that precedes it but instead is conditional upon the probability of obtaining the three bases that immediately precede it. As an example, the probability of obtaining AGATAC in BXCALL is the probability of obtaining the trinucleotide AGA multiplied by the three conditional probabilities of obtaining the three bases which follow it. An estimate of the conditional probability of obtaining a T following the trinucleotide AGA can be obtained as the ratio of the total number (n) of AGAT tetranucleotides observed in the entire sequence (BXCALL) to the total number of AGA trinucleotides in that sequence; similarly, for the A which follows GAT, the estimate becomes the ratio of the number of GATA tetranucleotides to the number of GAT trinucleotides, and for the C that follows ATA the estimate is the ratio of the number of ATAC tetranucleotides to the number of ATA trinucleotides. The expression for the probability (P) of obtaining the sequence AGATAC is

$$P(\text{AGATAC}) = P(\text{AGA})[P(\text{T/AGA}) \times P(\text{A/GAT}) \times P(\text{C/ATA})],$$

which can be estimated as

$$(n_{\text{AGAT}} \times n_{\text{GATA}} \times n_{\text{ATAC}})/(n_{\text{GAT}} \times n_{\text{ATA}}).$$

To derive expected numbers of occurrences of a given clustered sequence, when a space is allowed between members of the cluster, a pseudorandom method is adopted. We used the observed frequency of a given clustered sequence in 100 pseudorandom control sequences based on the TMC to estimate its expected frequency in BXCALL. The program MAKE_RANDOM_DNA (devised by D.R.M.) was used to generate such control sequences. It sequentially assigns bases by using a pseudorandom number generator and the Markov transition weightings derived from BXCALL.

RESULTS

DNA Motifs of Unknown Function

We have identified (Fig. 1) significantly overrepresented concatamers of mono-, di-, and trinucleotides for which the TMC expectation in every case is <0.5 (Table 1). Overrepresented clusters of two or more hexanucleotides (spaced ≤ 5 nt apart) have been identified with the aid of the CORES program (devised by D.R.M.). Excluding rotational derivatives, we have identified six such clusters (Fig. 1) on the basis that for each cluster at least six cases occur in BXCALL, and the TMC expectation, in every case, is <0.5 case (Table 2). If the spacing between members of the cluster is allowed to increase and/or the number of observed cases in BXCALL is <6, many more types of hexanucleotides are significantly overrepresented; however, since the TMC expectation exceeds 1, they are not plotted in Fig. 1.

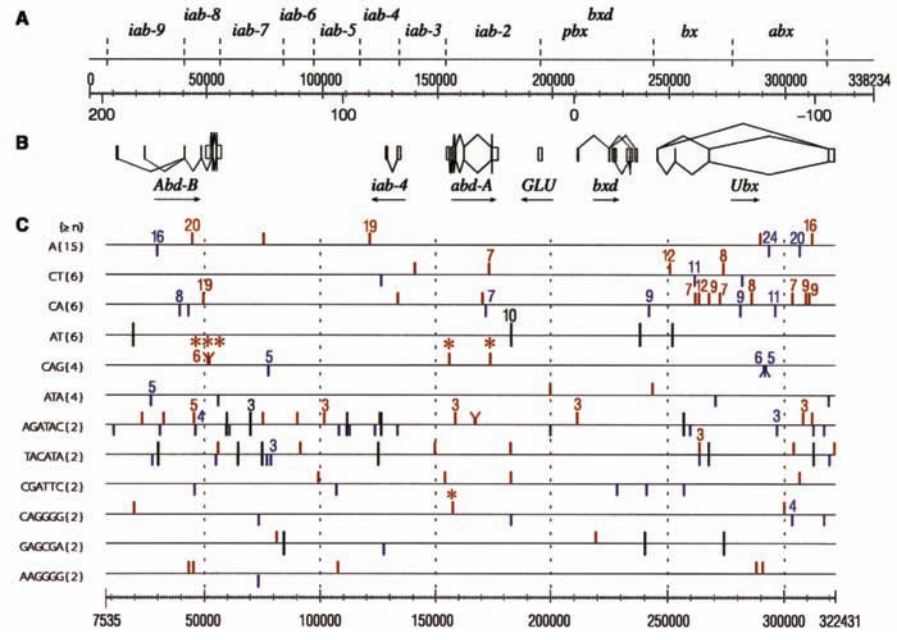

Figure 1. Correlation of molecular and genetic maps of the BX-C. (A) Boundaries of *cis*-regulatory regions delineated by hatched vertical lines are only approximately known. Numbers below the line refer to the map positions in kilobases. The bases numbered 1 and 313,000 correspond approximately to +204 kb and −100 kb, respectively, on the BX-C walk [8, 9]. (B) The transcription map is based on intron-exon mapping, cDNA, and computer analysis. Transcription units include homeobox-containing *Abd-B, abd-A*, and *Ubx*; the noncoding *bxd* and *iab-4*; and the predicted glucose transporter-like gene [3]. Arrows indicate the direction of transcription. (C) Distribution of DNA sequence motifs. Motifs observed four or more times in BXCALL yet having a TMC expectation of 0.5 or less. The number above each plotted position refers to the number of repeats of the concatamer at that location. The position of concatamers of a given DNA motif (m) are shown in red; that of its complement (m′), in blue; and that of concatamers of both m and m′, in green.

A limited study has been made of repeats in BXCALL. Direct repeats were identified in two ways, using the REPEAT program of the Genetics Computer Group [41] or the BLAST program [42], which compares the sequence with itself. Reverse repeats were analyzed with the STEMLOOP program of the Genetics Computer Group or the BLAST program. Since the maximum length of a perfect repeat found in performing these programs on 10 of the TMC-generated sequences of BXCALL was 18 bp, we have searched only for perfect repeats of 20 or more. Only three such direct repeats were found in BXCALL after we excluded the long repeats of mono-, di-, tri-, and hexanucleotides already considered in Fig. 1. These three repeats map within the *Abd-B* domain: one of 110 bp at positions 68,556 and 68,656; one of 31 bp at 26,013 and 26,045; and one of 24 bp at 31,458 and 31,482. Only two reverse

Table 1 Comparison of observed (O) and expected (E) numbers of mono-, di-, and trinucleotide concatamers in BXCALL.

Motif	O	E-M[a]	E-I[b]	P^c
$(A)_{15}$	5	0.182	0.004	$< 10^{-5}$
$(T)_{15}$	3	0.178	0.004	$< 10^{-3}$
$(CT)_6$	4	0.022	0.018	$< 10^{-8}$
$(AG)_6$	3	0.007	0.016	$< 10^{-7}$
$(CA)_6$	14	0.063	0.018	$< 10^{-15}$
$(TG)_6$	6	0.057	0.016	$< 10^{-10}$
$(AT)_6$	4	0.214	0.140	$< 10^{-4}$
$(CAG)_4$	5	0.104	0.008	$< 10^{-7}$
$(CTG)_4$	4	0.064	0.008	$< 10^{-6}$
$(ATA)_4$	2	0.204	0.127	0.018
$(TAT)_4$	4	0.213	0.125	$< 10^{-4}$

[a] Expectation based on the TMC method
[b] Expectation based on the independence of the single base frequencies
[c] Probability that the observed (O) number or a larger number exceeds E-M based on the cumulative Poisson distribution

Table 2 Comparison of observed (O) and expected (E) numbers of motifs of unknown function in BXCALL.

Motif[a]	O	E-M[b]	E-I[c]	P^d
AGATAC	138	44.790	99.433	$<10^{-15}$
GTATCT	121	39.511	98.913	$<10^{-15}$
$(AGATAC)_{n\geq 2}$	31	0.12	0.752	$<10^{-15}$
TACATA	141	78.894	141.348	$<10^{-9}$
TATGTA	126	74.622	137.954	$<10^{-7}$
$(TACATA)_{n\geq 2}$	19	0.41	1.494	$<10^{-15}$
CGATTC	76	52.151	71.107	0.001
GAATCG	101	58.007	69.764	$<10^{-6}$
$(CGATTC)_2$	9	0.23	0.378	$<10^{-11}$
CAGGGG	57	48.357	36.433	NS
CCCCTG	61	52.667	36.652	NS
$(CAGGGG)_{n\geq 2}$	7	0.21	0.096	$<10^{-8}$
GAGCGA	80	56.262	49.077	0.002
TCGCTC	77	65.773	50.984	NS
$(GAGCGA)_2$	6	0.29	0.191	$<10^{-6}$
AAGGGG	83	66.317	48.024	0.027
CCCCTT	93	72.793	52.102	0.013
$(AAGGGG)_2$	6	0.40	0.191	$<10^{-5}$

[a] Each group of three lines shows a motif (m), its complement (m′), $(m)_2$ = m#m, m#m′, m′#m, and m′#m′, where # = spacing of ≤5 nt
[b] Expectation based on the TMC method
[c] Expectation based on independence of the single base frequencies
[d] Probability that the observed (O) number or a larger number exceeds E-M based on the cumulative Poisson distribution. NS, not significant ($P > 0.05$)

repeats of 20 bp or more were identified, one of 24 bp at positions 246,197 and 246,138 and one of 21 bp at positions 50,785 and 57,889.

Motifs Derived from Consensus DNA/Protein-Binding Sites

Activation and/or repression of the BX-C genes (reviewed in ref. [10]) has been inferred from analyses of mutants of the gap genes giant (*gt*), hunchback (*hb*), tailless (*tll*), knirps (*kni*) and Kruppel (*Kr*); the pair-rule gene fushi-tarazu (*ftz*); homeotic genes such as caudal (*cad*), *Ubx*, *abd-A*, and *Abd-B*; and the pairing-related gene zeste (*z*).

We have deduced consensus DNA-binding motifs from *in vitro* footprinting studies for the protein products of *gt* [11], *hb* [12, 13], *kni* [14], and *Kr* [12–15]. For the remaining genes in Table 3 we have used published consensus DNA-binding motifs as follows: *tll* [14], *ftz* [16], *cad* [17], *z* [18], and from a biochemical approach for the protein products of *Ubx* [19] and *Abd-B* [20]. Table 3 summarizes the result of analyzing such consensus motifs. Contiguous oligomers of such motifs were insufficient in number to warrant plotting in Fig. 1. When ≤ 5 nt separates such motifs, certain of them are found to be significantly overrepresented; however, since the Markov expectation exceeds one in such cases their positions are not plotted in Fig. 1.

We also analyzed the octanucleotide, ATTTGCAT, to which the mammalian proteins OCT1 and OCT2 bind (reviewed in ref. [21]). In *in vitro* cell studies, ATTTGCAT acts as a binding site for UBX and ABD-BII proteins [22]. BXCALL has 78 sites for this octanucleotide, which exceeds the TMC expected number of 32.2 at a highly significant level ($<10^{-12}$) by a cumulative Poisson test.

DISCUSSION

DNA Motifs of Unknown Function

Long runs of the mononucleotide A (or its complement, T) are highly overrepresented in BXCALL (Fig. 1), as judged by the TMC expectation. The observed excessive number of runs of six or more dinucleotides of the type CA or its inverse, TG, are unlikely to be due to chance. Runs of six or more tend to occur in the introns of *Ubx*, *abd-A*, and *Abd-B* transcription units. This correlation with transcription units is consistent with the theory that such runs generate negative supercoiling during transcription [23]. Runs of CT, or its inverse, AG, are also significantly overrepresented. Runs of three or more of the rotational derivative GA have been proposed to be involved in determining chromatin structure [24] and also act as enhancers of transcription when bound by the transcriptional activator known as GAGA factor [25].

Repeats of four or more CAG trinucleotides occur in the coding regions of *abd-A* and *Abd-B*. Such repeats are responsible for the long runs of glutamine in the ABD-B proteins [26] and the ABD-A proteins [27]. Repeats of four or more CTG (the

Table 3 Comparison of observed (O) and expected (E) numbers of DNA motifs of known function in BXCALL.

Gene	Binding domain	Consensus-binding site[a]	O	E-M[b]	E-I[c]	P^d
Gap genes						
giant	bZip	WHWWRAYYGH	358	355.492	299.829	NS
		DCRRTYWWDW	355	354.037	299.897	NS
		$(WHWWRAYYGH)_{n=2}$	9	9.08	6.853	NS
hunchback	Zinc finger	CNYAAAAA	178	168.908	71.310	NS
		TTTTTRNG	185	161.999	68.360	0.042
		$(CNYAAAAA)_{n\geq 2}$	7	2.00	0.373	0.005
knirps	Steroid receptor	WWMTRRRHC	269	286.591	322.288	NS
		GDYYYAKWW	251	280.970	324.945	NS
		$(WWMTRRRHC)_{n=2}$	4	6.29	7.982	NS
Kruppel	Zinc finger	GGGTKAA	28	30.942	34.313	NS
		TTMACCC	41	33.094	36.142	NS
		$(GGGTKAA)_{n=2}$	1	0.11	0.095	NS
tailless	Steroid receptor	AAATTAA	171	138.840	57.709	0.005
		TTAATTT	180	143.756	57.258	0.002
		$(AAATTAA)_{n=2}$	6	1.50	0.252	0.004
Pair-rule gene						
fushi-tarazu	Homeo-domain	CCATTA	118	137.600	72.666	NS
		TAATGG	113	134.599	67.267	NS
		$(CCATTA)_{n=2}$	1	1.39	0.378	NS
Homeotic genes						
Abdominal-B	Homeo-domain	TTTATKRC	119	62.687	35.205	$<10^{-9}$
		GYMATAAA	108	63.991	34.615	$<10^{-6}$
		$(TTTATKRC)_{n=2}$	3	0.27	0.093	0.003
caudal	Homeo-domain	TTTATG	331	225.627	137.593	$<10^{-10}$
		CATAAA	343	226.846	141.718	$<10^{-12}$
		$(TTTATG)_{n\geq 2}$	19	4.20	1.494	$<10^{-6}$
Ubx	Homeo-domain	$(TAA)_4$	1	0.218	0.123	NS
		$(TTA)_4$	3	0.229	0.122	0.002
		TTAATGG	50	52.217	28.334	NS
		CCATTAA	54	53.072	29.668	NS
		$(TTAATGG)_{n=2}$	0	0.17	0.064	NS
Pairing-related gene						
zeste	—	YGAGYG	289	225.494	201.173	$<10^{-4}$
		CRCTCR	289	231.912	210.598	$<10^{-3}$
		$(YGAGYG)_{n\geq 2}$	14	4.08	3.262	$<10^{-4}$

[a] K = G or T; M = A or C; R = G or A; Y = C or T; N = A, G, C, or T; W = A or T; H = A, C, or T; D = A, G, or T
For each motif (m) and its complement (m′), $(m)_{n=2}$: m#m, m#m′, m′#m, and m′#m′, where # = spacing of ≤5 nt

[b] Expectation based on the TMC method

[c] Expectation based on the independence of the single base frequencies

[d] P = Probability that the observed (O) number or a larger number exceeds E-M based on the cumulative Poisson distribution
NS, not significant ($P > 0.05$)

complement of CAG) trinucleotides occur in the noncoding region of the *Ubx* and *Abd-B* domains.

The most striking of the concatamers of hexanucleotides is that of AGATAC. Hogness et al. [28] reported AGATAC as a consensus sequence in the *Ubx* domain and suggested that it might serve as a binding site for "coupling proteins" that would bring distant *cis*-regulatory regions closer together, specifically the *bxd* and *abx/bx* regions.

Known Motifs Within the BX-C

In constructing Table 3, we have relied on consensus DNA-binding motifs that were deduced largely from *in vitro* footprinting studies. These sites are not only tentative, they are frequently degenerate and they may not be the ones used *in vivo.* Among the gap genes none except *tll* are significantly overrepresented or underrepresented in BXCALL as single motifs. Nevertheless, a function for a single Kr-binding sequence is indicated based on a mutational analysis. Specifically, two independent Hyperabdominal mutants, *Hab* and Hab^2, involve the same mutated base pair (G to A) in a Kr-binding site, GGGTGAA, located at 172,672 in the *iab-2* region of the *abd-A* domain [29]. These mutations are postulated to prevent binding of the Kr protein, thereby preventing the repression by that protein of *abd-A* function. The resultant overexpression of *abd-A* leads to a four-legged fly (T3 being transformed toward A1) [30].

The distribution of DNA-binding motifs for the protein products of the *Abd-B* gene is particularly interesting. TTTAT(G/T)(G/A)C, an ABD-B consensus DNA-binding site, is found at four sites clustered between 43,400 and 43,821 that are located 5.8 kb 5′ from the start of transcription for the mRNA that encodes ABD-BI. This number of potential binding sites and their spacing is similar to findings for autoregulatory elements of several other homeotic genes [16, 31–33].

Of special interest are clusters of the consensus-binding sequence, YGAGYG (Y = T or C), of the zeste (*z*) gene product. The protein product of the wild-type *z* gene is assumed to facilitate pairing of homologous chromosome regions, whether located in *cis* or in *trans* [18]. Loss of function mutants of the *z* gene suppress transvection (or pairing-dependent complementation) within the BX-C (34, 35, 43), the decapentaplegic gene [36], and the eyes-absent gene [37]. Clusters of YGAGYG are not plotted in Fig. 1, since several are expected to occur by chance owing to the degeneracy of the hexanucleotide. Clusters of AGATAC have also been suggested to be involved in pairing, as already mentioned. It may be of interest that YGAGYG and AGATAC, when reduced to their purine (R)/pyrimidine (Y) hexanucleotides, YRRRYR and RRRYRY, respectively, are derivatives of one another.

The BX-C has been postulated to derive from a common ancestral sequence that tandemly duplicated and then diverged by mutation to acquire new functions [38]. This postulate is consistent with the finding that the BX-C genes have their homeobox sequences highly conserved [39, 40]. Whether the *cis*-regulatory regions will also turn out to have sequence similarities suggesting a duplication origin cannot

be answered at present and will need much more extensive analysis. As a start, the distribution of significantly clustered sequences in these regions as enumerated in Fig. 1 may be viewed as an attempt to develop a "signature" for each of the *cis*-regulatory regions of the complex. It should be stressed that sequence analysis of the entire BX-C, as begun in this paper, will become a powerful approach to understanding regulation of the BX-C when coupled with biochemical and developmental approaches and ultimately with a comparative sequence analysis of the homologous genes in other organisms.

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SPLITS IN FRUITFLY *HOX* GENE COMPLEXES

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SIR—The homeotic genes are strikingly conserved between invertebrates and vertebrates. There is conservation, not only of the homeobox sequences, but also of their colinear order on the chromosome. The genes are found in clusters or "complexes", and are arranged on the chromosome in the order of their function along the anteroposterior body axis (for review, see ref. [1]). In the fruitfly *Drosophila melanogaster*, the homeotic genes are split into two separate clusters, the Antennapedia complex (ANT-C) and the Bithorax complex (BX-C), which direct development of the anterior and posterior segments, respectively. We show that in *Drosophila virilis*, a closely related species[2], the homeotic genes are also in two clusters, but the split occurs within the BX-C. The existence of two independent splits in the *Drosophila* lineage suggests that these flies lack the molecular constraint responsible for the ordered clusters in other animals.

In *D. virilis*, an *Ultrabithorax* mutation was recovered and found to be associated with a T(Y;2), which breaks chromosome 2 at map position 27F (according to the map of Gubenko and Evgen'ev[3]). The chromosome also carries an inversion between positions 24E and 25G (see Fig. 1). To determine at which site the *Ubx* gene resides, we isolated two phage clones from a *D. virilis* library that carry the 5′ *Ubx* exon. *In situ* hybridization to *D. virilis* chromosomes revealed that the *Ubx* region resides at

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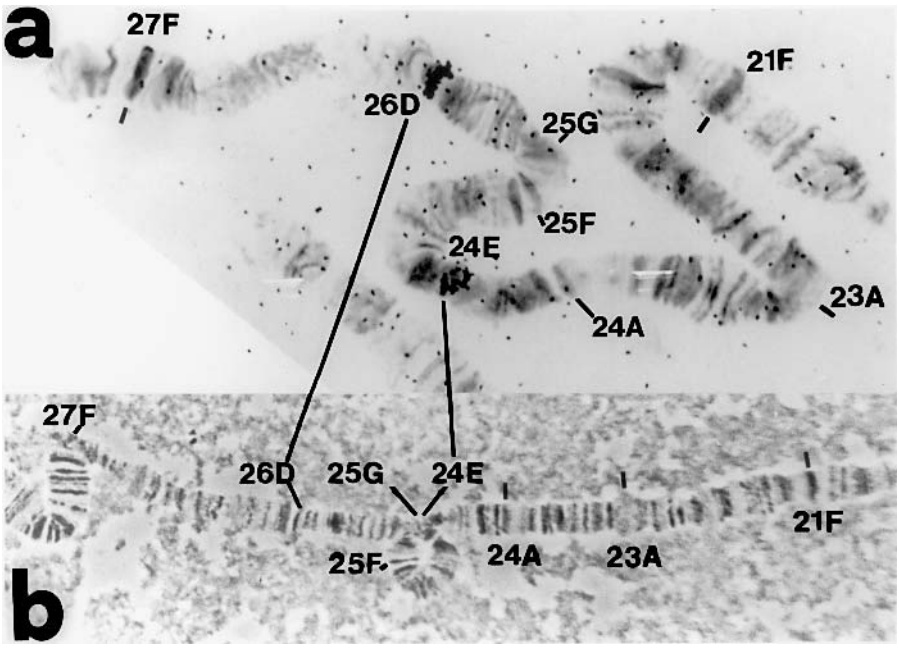

Figure 1. Polytene chromosomes from salivary glands of *D. virilis*. *(a) In situ* hybridization (bright field, ×93) of the *Ubx* and *Abd-B* clones on a salivary gland chromosome from a wild-type larva. Chromosome 2 is shown. A few relevant sites are indicated. Grain clusters are visible at 26D and 24E. (*b*) Squash from a larva heterozygous for the T(Y;2) chromosome associated with a *Ubx* mutation (phase contrast, ×23). The same cytological sites shown in *a* are indicated. The translocation point is at 27F. The inversion between 24E and 25G carried by the T(Y;2) chromosome is visible as an inversion loop with the wild-type chromosome. Preparation of the chromosomal squashes and *in situ* hybridization using probes labelled with tritium (*Ubx* and *Abd-B*) were performed as previously described[12]. Screening of phage libraries was at 55 °C in 6 × SSPE and the washes at 55°C in 2 × SSPE. The *Ubx* probe used to screen the *D. virilis* library was the 3108 subclone spanning −31 to −34 kb[13]. The *Abd-B* probe was a *Pst*I-*Pvu*II restriction fragment from the cDNA pAB713 (ref. [14]). This fragment covers the coding region just downstream from the CAX repeat through to the first third of the homeobox. The *abd-A* probe was a *Bam*HI-*Sal*I fragment from the phage iab-2^{C26}, which covers the genomic region from +34 to +36.5 kb[15].

24E, a constricted region of the second chromosome (see Fig. 1). This is one of the sites broken in the T(Y;2) mentioned above.

During studies of the *Abd-B* domain of the BX-C, we recovered *D. virilis* genomic clones containing the DNA flanking the *Abd-B* homeobox. We performed *in situ* hybridization on *D. virilis* chromosomes. Instead of hybridizing to 24E, as anticipated, the phages hybridized at 26D, a more proximal site on the second chromosome, indicating that the BX-C in *D. virilis* is split (see Fig. 1).

The *abd-A* gene lies in the middle of the BX-C in *D. melanogaster*, between *Ubx* and *Abd-B*. We have cloned an *abd-A D. virilis* phage, and it hybridized to 26D. Thus, the *D. virilis* copy of the BX-C is split between the *Ubx* and *abd-A* transcription units.

We wondered whether the *D. virilis* homologue of the Antennapedia complex lies at 24E, at 26D, or at a third site. We obtained two fragments of genomic DNA of the *D. virilis Antp* gene covering both the P1 promoter and the homeobox exon[4]. Both probes labelled the 24E constriction, at a position indistinguishable from that of the *Ubx* probe (not shown). In *D. melanogaster*, the *zen* gene lies in the middle of the Antennapedia complex[5]. Using the *D. virilis* homologue of the *zen* gene[6] as a probe, we also find hybridization at 24E, indicating that there is a continuous cluster between *zen* and *Ubx* (not shown).

It has long been known that the homeotic genes of the ANT-C and BX-C are split in *D. melanogaster*. Furthermore, extensive genetic studies have shown that the genes of the BX-C need not be together in a continuous complex for proper function (for review see ref. [7]). Our data demonstrate that in *D. virilis*, the integrity of at least part of the BX-C is not important. Mutations have been isolated in the flour beetle, *Tribolium castaneum*, which correspond to *D. melanogaster* mutations of both the Antennapedia and Bithorax complexes. These mutations all map to a single cluster[8], suggesting that some insects mirror the vertebrate case. The colocalization of the *Antp* and *Ubx* genes in *D. virilis* shows that these genes were together in the common ancestor of *D. virilis* and *D. melanogaster*. Similarly, *Ubx, abd-A*, and *Abd-B* were together in the ancestor, as they are today in *D. melanogaster*. Thus, the ancestral drosophilid had a single cluster, like the current arrangement in *Tribolium*.

Perhaps the break-up in the *Hox* cluster in *Drosophila* is related to its unusual mechanism of segmentation. *Tribolium*, like other short- or intermediate-germband insects, form segments in a temporal order, from anterior to posterior[9]. In long-germband insects like *Drosophila*, the embryo is subdivided into segments all at the same time[10]. It has been suggested that the linear order of the ancestral *Hox* complex is mechanistically linked to the temporal order in the specification of body segments[11]. *Drosophila* has no apparent temporal order of segmentation or of *Hox* gene expression, and thus may be freed from the major selective pressure to maintain these genes in a single complex.

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EVOLUTION OF THE HOMEOBOX COMPLEX IN THE DIPTERA

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The homeobox-complex (HOX-C) programs much of the basic body plan and forms, in most metazoans, one continuous cluster with the order of genes paralleling their order of expression along the body axis [1–5]. Here we compare the HOX-C organization of several *Drosophila* species and of *Anopheles gambiae*, a representative of lower Dipterans. We develop a model to account for the evolution of the HOX-C in the Diptera.

In *D. melanogaster*, the HOX-C is split into the Antennapedia complex (ANT-C) [6] and the bithorax complex (BX-C) [7, 8]. In *D. virilis*, a split is found between *Ubx* and *abd-A* [9], which corresponds to a separation of approximately 3–4 Mb, based on estimates in *D. melanogaster.*

Recently, a third species, *D. pseudoobscura*, has been sequenced [10] and aligned to *D. melanogaster* [11]. In *D. pseudoobscura*, the split occurs between *Antp* and *Ubx*. Thus, *D. pseudoobscura* is more similar to *D. melanogaster* than to *D. virilis*, which is consistent with the phylogeny of these species [12]. In contrast, *D. repleta* [13], like *D. virilis*, carries the split between *Ubx* and *abd-A*, which is consistent with the two species being closely related [12]. This breakdown of the contiguity of the HOX-C in four *Drosophila* species is presumably a relatively recent event, as in *A. gambiae* the HOX-C has remained intact [14, 15].

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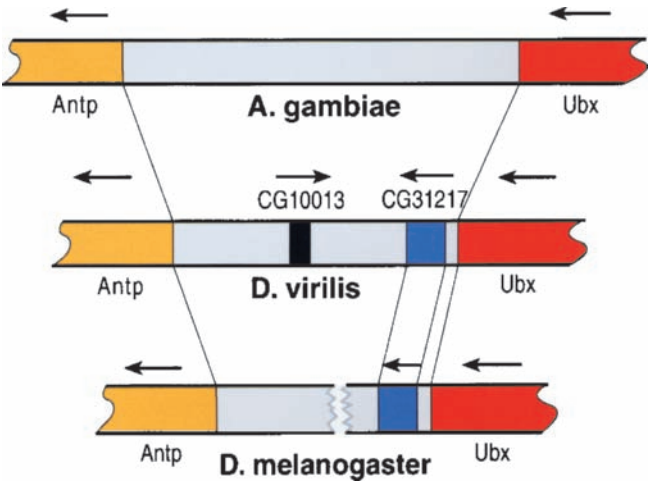


Figure 1. Species comparison of the region between *Antp* and *Ubx*. The stretch from the end of the last exon of *Ubx* to the beginning of the first exon of *Antp* is shaded in gray. The length of this region is 275 kb in *A. gambiae* and 190 kb in *D. virilis.* The length of the homologous region in *D. melanogaster* is 143 kb, excluding the 9.6 Mb represented by the gap. *CG31217* is a newly defined gene that was formerly considered to be two separate genes [17]. *CG10013* is either absent from *A. gambiae* or poorly conserved compared to the *Drosophila* genera.

Our sequencing of the *Antp* and *Ubx* genes and the intervening region in *D. virilis*, comprising 308,092 base pairs (bp) (AY333070) and including the previously sequenced *Antp* gene [16], confirms that there is no split between *Antp* and *Ubx* (Fig. 1). However, we find that between *Antp* and *Ubx* a gene, *CG31217*, is inserted immediately downstream of *Ubx*. In *D. melanogaster*, *CG31217* is located adjacent to *Ubx* and immediately downstream of *CG31217* is the breakpoint of the split.

A. gambiae lacks any obvious protein-coding genes in the interval between *Antp* and *Ubx* and the homolog of *CG31217* is located distant from the HOX-C on the same chromosome arm, in a 16.2 Mb sequence scaffold (AAAB01008987).

Our results suggest a model for the evolution of the HOX-C of the higher Diptera (Fig. 2). At the base of the Dipterans, an ancestor is assumed with the primordial HOX-C, from which two evolutionary branches can be inferred. One branch led to *A. gambiae*, and the other, after insertion of *CG31217*, to a postulated "vir-mel" chromosome, which subsequently led to the chromosome organization in *D. virilis* and to *D. melanogaster.*

In the *D. virilis* lineage, an inversion, Inv(pre-vir), gave rise to a "pre-vir" chromosome, and insertion of a second gene, *CG10013*, led to the present configuration in

D. virilis. In Fig. 2 we arbitrarily show the inversion occurring first. In *D. melanogaster*, *CG10013* is remote from both *Antp* and *Ubx*.

In the lineage leading to *D. melanogaster*, the split between *Ubx* and *Antp* is postulated to be an inversion, Inv(pre-mel) that upon an additional inversion Inv(mel) could give rise to the standard gene order. These two events placed *Ubx* and *CG31217* next to a *chaperonin-containing gamma gene (Cctg)* [17], and *Antp* next to a *sorbitol dehydrogenase I (Sodh)* gene (AE001572).

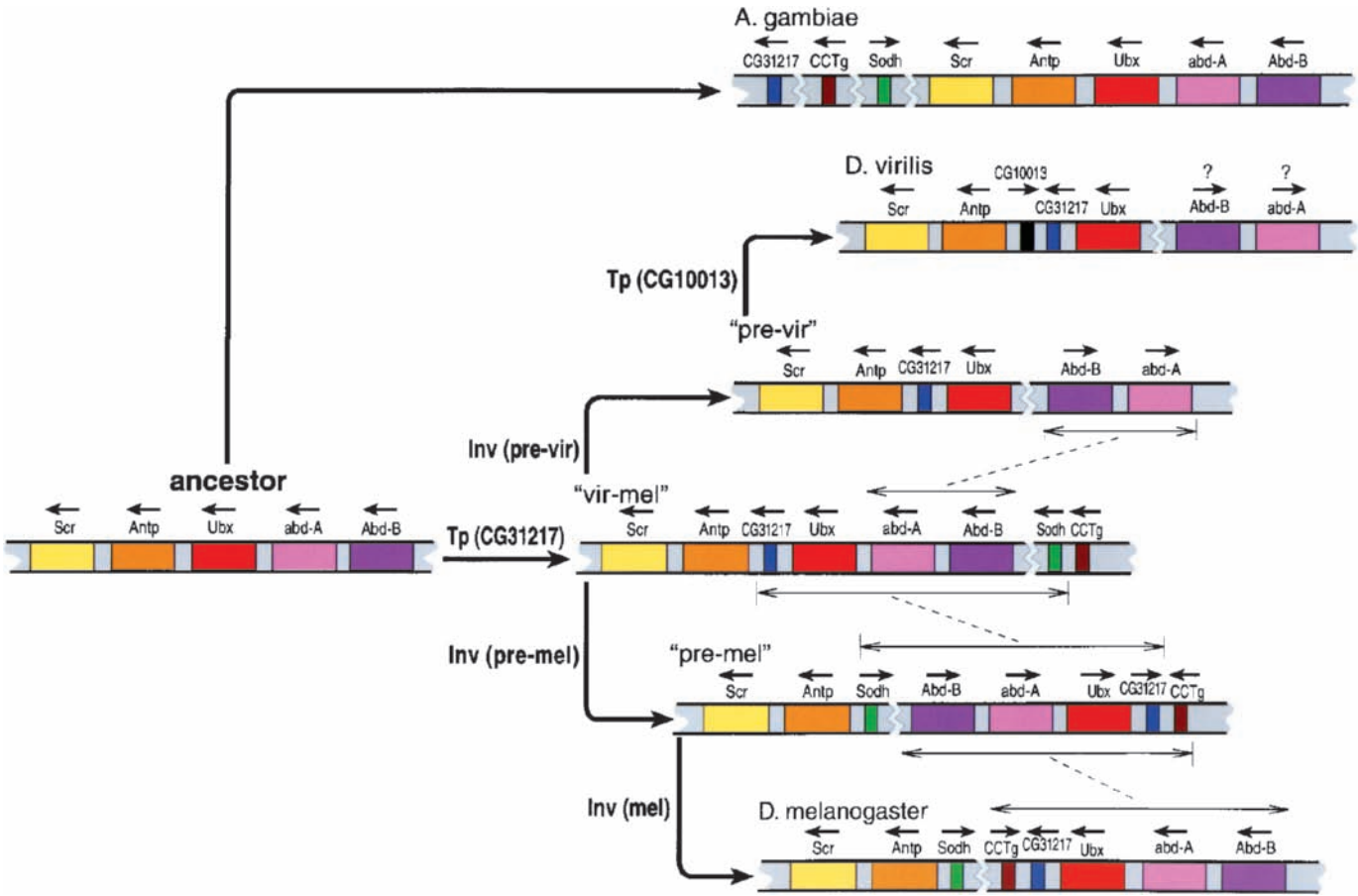
The simplest assumption is that *Sodh* and *Cctg* were closely linked in "vir-mel" and that the inversion had one breakpoint between them (Fig. 2). However, in *A. gambiae* these well conserved genes are located distant from each other and from the HOX-C.

Interestingly, *Inversion(3R)Payne*, which is widespread among wild *D. melanogaster* populations, shows a gene order similar to "pre-mel", [13,18]. Moreover, the closely related *D. simulans* contains the only other known chromosome that is similar to "pre-mel." The major cytological difference between *D. simulans* and *D. melanogaster* is a large inversion with breakpoints in sections 84B and 92C [19]. The 84B breakpoint is adjacent to, or identical with, the one that separated *Antp* and *Ubx*, whereas the 92C breakpoint is distal to the BX-C.

Several hypotheses have been invoked to explain the persistence of the HOX-C as a single cluster. A common view [8], holds that the *cis*-regulatory regions between adjacent HOX genes are bifunctional. In this scenario, splitting can occur only if preceded or accompanied by a duplication of the enhancer.

The persistence of an intact and colinear HOX-C over hundreds of millions of years suggests that its organization is advantageous. Thus, any rearrangement that splits the HOX-C will generally become fixed only if it confers a selective advantage. Such a rearrangement is evidently a rare event. Possibly it has occurred in *Drosophila* for a combination of reasons: the extreme fecundity and short life cycle of *Drosophila*, the fact that paracentric inversions do not reduce fertility when heterozygous, which is due to the elimination of dicentric chromatids that arise from single crossovers within the inverted regions [20], the rarity of crossing over in the *Drosophila* male, and, finally, the possibility of a high frequency of transposon-mediated inversions, as has been found for *D. buzzatii* [21].

Figure 2. A model for the evolution of the HOX-C in Diptera. A hypothetical ancestor of the Diptera contains a colinear HOX-C. Only five genes are shown here, *Sex-combs reduced (Scr), Antp Ubx, abd-A* and *Abd-B. A gambiae* is presumably derived from the ancestor without alteration of the HOX-C. The hypothetical chromosome "vir-mel" has arisen by transposition (Tp) of the gene *CG31217* between the *Antp* and *Ubx* genes. To account for additional genes present in the *Drosophila* lineage, "vir-mel" is postulated to have contained the *Sodh* gene closely linked to the *Cctg* gene. The "pre-vir" chromosome arises by a postulated inversion, Inv(pre-vir). *D. virilis* differs from pre-vir in having a Tp of the gene *CG10013* between *Antp* and *Ubx* (as shown in Fig. 1). In the *D. melanogaster* lineage, a precursor chromosome, "pre-mel" is assumed to have arisen by an inversion, Inv(pre-mel), with breakpoints proximal to *CG31217* and between the *Sodh* and *Cctg* genes of "vir-mel". For the modern *D. melanogaster* gene arrangement, a second inversion Inv(mel) is postulated that included the BX-C and extended just beyond *Cctg.*


A. gambiae
CG31217 CCTg Sodh Scr Antp Ubx abd-A Abd-B
D. virilis
CG10013
Scr Antp CG31217 Ubx ? Abd-B ? abd-A
Tp (CG10013)
"pre-vir"
Scr Antp CG31217 Ubx Abd-B abd-A
Inv (pre-vir)
ancestor
Scr Antp Ubx abd-A Abd-B
Tp (CG31217)
"vir-mel"
Scr Antp CG31217 Ubx abd-A Abd-B Sodh CCTg
Inv (pre-mel)
"pre-mel"
Scr Antp Sodh Abd-B abd-A Ubx CG31217 CCTg
Inv (mel)
D. melanogaster
Scr Antp Sodh CCTg CG31217 Ubx abd-A Abd-B

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SECTION IV: RADIATION AND CANCER

LEWIS AND THE SOMATIC EFFECTS OF IONIZING RADIATION

GENETIC AND SOMATIC EFFECTS OF IONIZING RADIATION

In 1927, H. J. Muller proved that ionizing radiation causes genetic mutations (Muller, 1927). Muller found that, in X-irradiated Drosophila, the mutation rate was almost 200-fold that seen in control, unirradiated flies. The spectrum of mutations obtained ranged from lethals to visibles, recessives to dominants, "invisible" lesions to chromosomal rearrangements. Similar effects were seen when either sperm or oocytes were irradiated.

At the time that he published this landmark paper, the relationship between the dose of radiation and the mutation rate was unclear. Subsequently, Muller and others went on to show that there is a direct proportionality between the dose of X-rays and the frequency of induced mutations (see, e.g., Muller, 1928; Hanson and Heys, 1929; Oliver, 1930). Furthermore, neither the type of radiation—ranging from X- to gamma-rays—nor the time distribution of treatment altered the linear relationship between dose and mutation rate. Subsequently, it was shown that low doses of X-rays (25, 50, or 100 r*), given in small doses (2.5 r per day) over prolonged periods

*Note that the units in use at that time are defined as follows: The Roentgen (r) = the intensity of X- or gamma-radiation that produces in one cubic centimeter of dry air, ions carrying one electrostatic unit of charge of the same type (positive or negative), which amounts to 1.61×10^{12} ion pairs per gram of air, or 2.08×10^{9} ion pairs per cubic centimeter of air. The rad is the unit of absorbed dose of ionizing radiation where one rad = the energy of absorption of 100 ergs per gram of irradiated material. The unit currently in use, the Gray (Gy) = 100 rads. The rem—Roentgen equivalent man—is a normalized unit dose of ionizing radiation that gives the same biological effect as one Roentgen (r) of X-rays. It is calculated by multiplying the number of rads by the relative biological effectiveness (RBE) for the particular type of ionizing radiation in producing the specific biological damage. The unit currently in use, the Sievert (Sv) = 100 rem.

(21 days), produced the same effects as these doses when administered all at once (Uphoff and Stern, 1949). These authors were led to conclude that: "it appears that irradiation at low doses, administered at low intensity, induces mutations in Drosophila sperm. There is no threshold below which radiation fails to induce mutations."

Shortly after Wilhelm Roentgen's discovery of X-rays in 1895, it was understood that there is an association between ionizing radiation and cancer. However, much of the data that accumulated over the next half century were anecdotal or poorly analyzed (for an excellent review, see Doll, 1995). In his 1927 paper, Muller had speculated that the "effect of X-rays, in occasionally producing cancer, may also be associated with their action in producing mutations". In 1930, the McCombs proposed that "cancer is due primarily to mutation in a somatic cell . . . It is a well-known fact, genetically, that mutations experimentally can be speeded up tremendously by exposure to stimulating amounts of radiation and the X-ray effect . . . Perhaps the frequently observed 'skin-cancers' in Roentgenologists are due to such mutations occurring from stimulating exposure to the X-ray effect" (McCombs and McCombs, 1930). Muller subsequently extended his 1927 hypothesis: "A . . . case can . . . be made . . . that the cancers produced by radiation are examples of its mutation-producing action . . . [G]ene mutations occasionally occur spontaneously in somatic cells . . . [and] irradiation enormously increases the frequency of these somatic mutations. . . . If then, the mutational concept of 'spontaneous' cancers and other diseases of indefinite proliferation . . . is correct, it is but a logical step to conclude that the carcinomas, sarcomas and leukemias arising after irradiation represent mutations induced by the latter. The . . . study of the manner and conditions of mutation produced by irradiation should, if this conception is right, be of some value in relation to the problem of cancer production" (Muller, 1937).

The genetic and somatic effects of ionizing radiation—particularly at low doses—came to the forefront during the Cold War.

THE POLITICAL AND SOCIAL CONTEXT

The mid-1950s represented the height of the Cold War and of nuclear weapons testing. Subsequent to the Hiroshima and Nagasaki atom bomb attacks in 1945, both the United States and the USSR had further developed both fission- and fusion-based weapons, all of which were being tested in the atmosphere. The radioactive fallout from these tests could, potentially, result in both genetic and somatic effects, although an accurate estimate of such effects was absent.

In 1955, Muller addressed the need for accurate quantitative estimates of potential genetic damage from test explosions (Muller, 1955): "To calculate the genetic damage caused in this country [USA] by all the nuclear tests to date . . . we will provisionally take the U.S. Atomic Energy Commission's published estimate of 0.1r as the average for each American . . . This amount seems minute, but we must multiply it by 160 million, representing the population. It is curious that the product that we then obtain,

16 million 'man r's', is the same as that obtained when we take 100r, assuming this to be not far from the average dose received by all Hiroshima survivors, and multiply it by 160,000, the approximate number of those survivors. Hence the number of harmful mutations that will be inherited by our descendants as a result of all test explosions turns out to be not far from the number among the Japanese as a result of the Hiroshima explosion. This number of mutations is certainly in the tens of thousands at least . . . and it will mean, in the end, several times this number of hampered lives. Yet, far more than at Hiroshima, the effects will be so scattered, in this case not only in time but also in space and separated by many more individuals who have mutations of natural origin only, that, as a group, the effects will be completely lost to sight. That is, their connection with the radiation will not be traceable. It is, nevertheless, true that each individual casualty, although concealed, must be regarded as a significant evil, which we have no right to dismiss lightly . . . In order to decide whether a continuance of the tests is justified, it is necessary first to admit the damage and then to weigh our estimate of it against the potential benefits to be derived from the tests or, rather against the probable damage that would follow from the alternative policy." (Muller, 1955, p. 838)

Three points are worth noting here. First, Muller was emphasizing the need for accurate risk estimates. Second, he was not advocating either for or against the tests, but rather was emphasizing the need for the public and its representatives, to make informed decisions after assessing the risks. Finally, and curiously, despite his arguments decades earlier that radiation would have somatic effects, including the induction of cancers, he made no mention at all of this type of risk, focusing instead exclusively on genetic effects.

In his 1954 presidential address to the Pacific Division of the American Association for the Advancement of Science (AAAS), A. H. Sturtevant too considered the potential genetic effects of ionizing radiation (Sturtevant, 1954). Beyond that, he had commented: "gene mutations, induced in an exposed individual, also constitute a hazard to that individual—especially in an increase in the probability of the development of malignant growths, perhaps years after exposure. There is, in fact, no clearly safe dosage—all high-energy radiation, even of low intensity and brief duration, must be considered as potentially dangerous to the exposed individual."

Sturtevant had been "disturbed" that Admiral Lewis Strauss, Chairman of the Atomic Energy Commission of the United States, had stated on 31 March 1954 that the United States and Russian atomic weapons tests had resulted in a "small increase in natural 'background' radiation in some localities within the continental United States . . . far below the levels which could be harmful in any way to human beings". Sturtevant went on to emphasize that "every geneticist familiar with the facts knows that any level whatever is certain to be at least genetically harmful to human beings when it is applied to most or all the inhabitants of the earth."

As in the case of Muller, Sturtevant did "not wish to be understood as arguing that the benefits ultimately to be derived from atomic explosions are outweighed by the

biological damage they do. It may be that the possible gains are worth the calculated risk. But it must be remembered that the risk is one to which the entire human race, present and future, is being subjected. I regret that an official in a position of such responsibility should have stated that there is no biological hazard from low doses of high-energy irradiation".

A few months later, in January 1955, Sturtevant again focused on possible genetic effects of high-energy radiation, this time attempting to estimate the risks from radioactive fallout derived from nuclear weapons tests (Sturtevant, 1955). He used numbers from AEC reports for an "average" locality, in the United States in September 1954 to calculate that the increase in radiation due to fallout would "be expected to give one deleterious mutation per 100,000 germ cells per generation . . . This means that, if the increase in irradiation due to fallout continues at the estimated present rate, it will lead to the functioning of about 78 mutated germ cells every year in the United States; and, if the same level of irradiation occurs in the rest of the world, of about 1800 per year in the population of the world. These will go on rising at this rate, year after year, as long as the irradiation continues and the number of births stays in this same range . . . It may still seem that these numbers are too small to be seriously considered, but . . . I have made every effort to be conservative; the numbers given should be considered minimal ones—the true value could possibly be 100 times greater . . . "

Concerns about somatic effects were absent from reports in the mid-fifties on the biological effects of radiation by the National Academy of Sciences in the USA and of the Medical Research Council in the UK. However, both organizations recognized the need for assessment of somatic effects, and large-scale studies were initiated: the Life Span Study of Hiroshima and Nagasaki survivors in the United States (Beebe et al., 1971; Beebe, 1979); and studies of the quantitative relationship between X-ray dose and the incidence of leukemia among ankylosing spondylitis patients who had been given radiotherapy (Court Brown and Doll, 1957).

LEUKEMIA AND IONIZING RADIATION

Lewis, as one of Sturtevant's colleagues in the Biology Division at Caltech, was familiar with the ongoing scientific and public policy debate regarding the effects of nuclear fallout and the need for accurate risk estimates. He became interested in the somatic effects of ionizing radiation when he realized—in a discussion of nuclear weapons testing at a Caltech Athenaeum faculty lunch table—that some of the physicists present assumed that a threshold would be operating. Lewis knew that Muller had pointed out as early as 1927 that cancer following radiation exposure was likely to result from somatic mutation. Because the effects of ionizing radiation in producing genetic mutations were linear without a threshold, Lewis became curious about quantitative analyses of somatic effects. In late 1955 he decided to "see what was known".

This was the beginning of an almost two-decade long detour into the somatic effects of low-dose ionizing radiation. Lewis' landmark paper in this area, published

in 1957, in many ways was to studies of the somatic effects of ionizing radiation, what his 1978 paper on the bithorax complex was to developmental biology (Lewis, 1978). As will be seen below, however, unlike his developmental biology research, the scientific and political community did not embrace his results on ionizing radiation with open arms. Rather, this era in Lewis' career was marked by politically motivated public and private attacks on his motivations, methods and expertise.

In his 1957 paper Lewis made the first risk estimates of leukemia incidence among Japanese A-bomb survivors in relation to the estimated doses to which they would have been exposed. George Beadle, then Chair of the Biology Division at Caltech, was an advisor to the Atomic Energy Commission (AEC) and requested that Lewis be given access to the data that had been collected on leukemia cases occurring in Hiroshima and Nagasaki in the years following the bombing. Using these data, Lewis arrived at a best estimate of the absolute risk of leukemia as being one to two cases per million persons per rem per year (Lewis, 1957). He went on to show that this estimate was compatible with the risk of leukemia that had already been calculated for patients treated with spinal irradiation for an arthritic disease, ankylosing spondylitis (Court Brown and Doll, 1956). Furthermore he calculated a similar risk from data for a population of children who had been irradiated for thymic enlargement as infants (Simpson et al., 1955).

Lewis then focused on radiologists who, as a group, had been reported to have a higher percentage of deaths from leukemia than other physicians who were not radiologists. He obtained a set of data on the deaths of practicing radiologists listed in the American Medical Dictionary for the period 1938 through 1952 and corrected for age distribution. He then calculated an expected death rate from leukemia for radiologists if they had received no occupational exposure (3.4) and compared this with the observed death rate (17). From the Poisson distribution, the probability of such an observed number given the expected number was less than one in a million. By estimating the occupational exposure of radiologists at between 3 and 30 rad per year, Lewis calculated the probability of leukemia per rad of accumulated dose per annum for radiologists as likely to be between 0.7 and 7×10^{-6}. This range was fully consistent with his best estimates of $1–2 \times 10^{-6}$ per rad per year based on the A-bomb survivors, ankylosing spondylitis and thymic enlargement patients. He was to return to radiologists in a much more extensive analysis several years later (Lewis, 1963b).

Lewis addressed the implications of his results with respect to the linearity of the dose–response curve for induction of leukemia: "This is presumptive evidence that the relationship between incidence of induced leukemia and dose of radiation is either linear or approximately linear. A striking feature of the Japanese data . . . is that the incidence of leukemia in . . . the zone with a calculated average 'air' dose of 50 rem—is significantly higher than in . . . the 'control' zone . . . Thus these data provide no evidence for a threshold dose for the induction of leukemia. Moreover, chronic irradiation at a relatively low dose rate (perhaps 0.1 rad per day or less) appears to induce leukemia in radiologists at a rate per rad which is comparable to

that observed for the Japanese survivors. This finding also fails to support the concept of a threshold dose below which leukemia will not develop." It should be noted that Lewis was very careful in the wording of his conclusions, stating that his evidence was "presumptive" of a linear relationship and emphasizing that his data "provide no evidence" for a threshold dose, rather than stating that they prove that there is no threshold. As described below, he was equally careful in his public testimony regarding the conclusions that could be drawn from his data analysis.

Lewis knew from the Court Brown and Doll study of 1956 that the risk of leukemia increases with the age of the person exposed. Therefore, he was careful to stress: "It is likely that there will be individual differences in susceptibility to radiation-induced leukemia as well as to 'spontaneous' leukemia. The indication of a linear relationship between dose of radiation and incidence of leukemia implies that there are some individuals in whom a single radiation-induced event (perhaps a gene mutation) suffices to produce leukemia. There may, however, be other individuals in whom two or more such events would be required before leukemia would be manifested. Thus the values of the probability of leukemia per individual per rad per year that have been derived here apply to the 'average' individual in a given population, but do not necessarily apply equally to each and every individual in that population."

Lewis' absolute risk estimate of one to two cases of leukemia per million persons per rem per year has held up remarkably well. Subsequent studies have shown that the absolute risk is 1.0–2.0 for Hiroshima A-bomb survivors over 10 years of age at the time of the explosion (depending on the RBE of fast neutrons; if the RBE = 1.0 then the risk is 2.0 while, if the RBE = 5.0, then the risk is 1.0) and 1.6–2.6 for children under 10 years of age at the time of the explosion (again depending on whether the RBE = 1.0 or 5.0); 1.3 for ankylosing spondylitis patients, ages 15–55 inclusive; 1.2 for menorrhagia patients, ages 20–55 inclusive; 3.0 for thymus-irradiated infants; and 3.4 for scalp-irradiated children with tinea capitis, ages 3–12 years (BEIR I, 1972). More recently, revised dose estimates for A-bomb survivors have led to higher risk estimates than in BEIR I (BEIR V, 1990).

Also relevant to Lewis' risk estimate had been an important case-control study by the Oxford epidemiologist, Alice Stewart and colleagues (Stewart et al., 1956). They found that children who had been irradiated with doses that were unlikely to have exceeded one rad (and usually even less) had a higher relative risk per rad of leukemia than unirradiated controls. This suggested that the fetus is extraordinarily sensitive to the X-ray induction of such cancers. Although he cited Stewart's study in his 1957 paper, Lewis had thought it premature to stress the results because they depended upon recall by the mother of whether she had received radiation exposure at the time of the relevant pregnancy. Subsequently Brian MacMahon published a study that was completely free of possible recall inaccuracies by matching death records against relevant hospital records (MacMahon, 1962). This study confirmed the very high relative risk per rad that Stewart had found. Since A-bomb survivors who had been irradiated in utero did not shown an elevated risk of childhood cancers, the Stewart and MacMahon studies were challenged (Jablon and Kato, 1970). However,

it should be noted that the high peak of childhood cancers that is observed in Western countries, was not present in Japan at that time. Therefore, failure to see an increased risk was likely due to the fact that radiation increases cancer in proportion to the latter's natural incidence, which is many times higher in the West than in Japan.

In the final section of his 1957 paper, Lewis turned to a consideration of radioactive fallout. At that time it was assumed by the AEC and the NAS committee on pathological effects of radiation that only bone cancer would result from the strontium fission products—Sr^{90} and the shorter-lived Sr^{89}—which concentrate in skeletal tissue. Furthermore, these organizations had assumed that a dose of 2,000 rad would be needed before bone cancer would be expected. Lewis pointed out that radiostrontium could result in an increased risk of leukemia by irradiating the bone marrow. Using the permissible level of Sr^{90} that the National Academy of Sciences (NAS) had recommended for the population at large in 1956—a concentration for the total body of 0.1 microcuries or '0.1 MPC'—Lewis calculated that the dose rate to leucocyte-producing cells would be expected to be 0.1–0.2 rad. Using his earlier estimate of the probability of leukemia of 1×10^{-6} per individual per rad to bone marrow per year, he calculated a 5–10% expected increase in leukemia incidence in the United States from a constantly maintained level of 0.1 MPC of Sr^{90}. This estimate was to be cited repeatedly in the public debate of Lewis' data, described below.

POLITICAL FALLOUT

Lewis' motivation for carrying out the leukemia studies was to provide the best estimates of risk, hence enabling other scientists as well as public officials to make educated decisions. However, the debate on nuclear weapons tests was already highly polarized and any study was subject to attack from one or both poles.

This was recognized by the editor of Science, whose editorial in the 17 May 1957 issue that carried Lewis' article, focused on that paper and was headed 'Loaded Dice' (DuShane, 1957). Graham DuShane wrote: "Unfortunately, though doubtless inevitably, the question of the effects of radiation upon human health has become a subject for partisan political debate and has become linked with questions of national power and prestige. Thus the great debate about the effects of radiation cannot readily be conducted in the dispassionate atmosphere of scientific discussion. In this issue of Science, E. B. Lewis has taken an important step in bringing us the kind of information we need to conduct such a discussion." DuShane then elaborated on some of the history of the debate before returning to Lewis' paper: "E. B. Lewis shows that there is a direct linear relationship between the dose of radiation and the occurrence of leukemia, a fatal disease characterized by increases in the number of white blood cells. The meaning of such findings is that any amount of radiation takes its toll of the population and any increase takes a greater toll. Thanks to Lewis it is now possible to calculate—within narrow limits—how many deaths from leukemia will result in any population from any increase in fallout or other source of radiation. And for the individual it is possible to calculate the probability of death from leukemia

as a result of any particular dose of radiation. We are approaching the point at which it will be possible to make the phrase 'calculated risk' for radiation mean something a good deal more precise than the 'best guess'. It is apparent that the atomic dice are loaded. The percentages are against us and we ought not play unless we must assure other victories."

The first political use to which Lewis' data were put was in the "Declaration of Conscience" written by Albert Schweitzer (Nobel Peace Prize Laureate, 1952) and issued by the Nobel Prize Committee on 24 April 1957. The declaration challenged the need for nuclear warfare and testing. Lewis had sent a draft of his manuscript to other Caltech faculty members, including Harrison Brown (Professor of Geochemistry), for critical input in November 1956. Brown brought Lewis' data to the attention of Schweitzer, who was in the process of drafting the declaration. One of the reasons that Schweitzer gave for concern—derived no doubt from Lewis' draft—was that "internal" radiation from accumulated radioactive elements from nuclear fallout causes "mainly serious blood diseases . . . It is unfortunately very probable that internal radiation affecting the bone marrow and lasting for years will have the same effect [as external radiation], particularly since the radiation goes from the bone tissue to the bone marrow. As I have said, the radioactive elements are by preference stored in the bone tissue."

Subsequent to publication of the Science paper, attacks on Lewis and his data occurred in both the scientific and the popular press. Publicly, for example, Lewis' credentials and data were challenged on NBC's 'Meet the Press' program on 26 May 1957 by none other than Admiral Lewis L. Strauss, Chair of the AEC, the very person whose claims that radiation levels from fallout were "far below the levels which could be harmful in any way to human beings" had disturbed Sturtevant in 1954. (Lewis was not alone: Linus Pauling's scientific credentials were also challenged by Strauss.)

The relevant segment of the program is reproduced here verbatim to give the reader a flavor for the style of the challenge:

MARTIN AGRONSKY (NBC NEWS, INTERVIEWER): Admiral Strauss you spoke of the report of [the National Academy of Sciences] last year. Just this week in Science magazine which as you know is a very reputable scientific magazine. . .

STRAUSS: Yes sir.

AGRONSKY: . . . one of our most distinguished professors of biology, Dr. E. B. Lewis of the California Institute of Technology, writes that there will be a 5 to 10% increase in the spontaneous incidence of leukemia—cancer of the bloodstream—if the population were to reach and maintain a body level of strontium-90, which is precipitated by the explosion of H-bombs, amounting to only one tenth of the maximum safe limit of concentration set by the AEC. That's a pretty terrifying statement. For one thing it is a charge that your estimate of the safe level of strontium precipitation is wrong by a ten-to-one ratio. Do you think it is?

STRAUSS: No. I don't want to get into an argument between scientists, Mr. Agronsky, for the very simple reason that I do not have enough information self-generated to qualify me to enter

such a debate. But your question, as being a lengthy one, I'm going to take the liberty of reading a brief comment on it. This is a comment on Dr. Lewis's article. (Reading statement:) On the basis of estimates of the probability of production of leukemia from high doses of radiation and on the assumption that the natural incidence of leukemia is due to natural sources of radiation, Dr. Lewis speculates that each roentgen of radiation dose received by an individual contributes 2×10^{-6} to the probability of his contracting leukemia in any subsequent year. Now, if his hypothesis were correct, this would mean that an individual receiving a dose of 10 roentgens would have two chances in 100,000 of contracting leukemia in any subsequent year and one chance in 1,000 of contracting leukemia in the next fifty years. I call your attention to the fact that that is based on a dose of 10 roentgens. The amount of radiation that we have received in the United States from all the tests to date and that a man would receive throughout his normal reproductive lifetime if tests continued at this rate, would be one tenth of one roentgen.

NED BROOKES (NBC, MODERATOR): Admiral, is that something from the [Atomic Energy] Commission that you have been reading?

STRAUSS: I've been reading a Commission release. Let me say one thing further. Quoting Lewis it should be mentioned that he is not a specialist in leukemia, that it is too early to say what acceptance his hypothesis will receive among scientists who are students of leukemia, but his hypothesis should rather be used as an illustration of the kind and degree of risk which might conceivably be involved if a radiation dose as high as 10 roentgens were received.

AGRONSKY: You're saying he is wrong and you are right, sir?

STRAUSS: No. No. I say I think the chances are that he is wrong and I believe the chances are that the advisory commission on biology and medicine of the Atomic Energy Commission is right.

Lewis was invited to testify before the Congressional Joint Committee on Atomic Energy's hearings on "The Nature of Radioactive Fallout and its Effects on Man" chaired by Representative Chet Holifield of California in late May and early June of 1957. Lewis appeared before the committee on June 3. He presented a graphical summary of the incidences of induced leukemia among A-bomb survivors, radiologists, X-rayed infants and X-rayed adults. He emphasized that, while he had been asked to confine his remarks to leukemia, "I do not wish to imply that I think that leukemia is the most important effect of radiation on man. I think in fact possibly the genetic effects may be more important. There may also be other malignant diseases that are more important than leukemia with respect to ionizing radiation. However, the reason that I am stressing leukemia today is that we have rather good data and rather good evidence on leukemia as compared to data on other effects on man from ionizing radiation" (Special Subcommittee on Radiation, 1957, p. 956).

Discussing the A-bomb survivor data used in his Science paper, Lewis was careful to acknowledge "that these data have been published by other investigators and collected by a great number of people. The only thing that we do here is to relate incidence

of leukemia to dosage. This has not been done before for the reason that the Atomic Bomb Casualty Commission did not have the doses for so relating the incidence of leukemia" (ibid. p. 957). In summarizing his conclusions, Lewis said: "I want to point out now that one can draw various curves to express these data. I have drawn here a straight line curve which would say that the incidence of leukemia is directly proportional to the dose. I feel that the evidence supports this to some extent in the high-dose region. In the low-dose region here, there is a dashed line, and there are only six individuals on which to say anything. The point here, however, is that in the absence of any other information it seems to me—this is my personal opinion—that the only prudent course is to assume that a straight-line relationship holds here as well as elsewhere in the higher dose region. It may be that there is a threshold—that is, a dose below which leukemia will not develop. However, we can say safely, I think, that if there is a threshold dose it must be below 100 r. The reason for saying that is that in the region below 100 r you would not expect to have gotten the 6 cases of leukemia as a result of chance more than 1 in 50 times . . . If we use this straight line and assume it is a straight line in the low-dose regions—as far as I can see there is little reason to believe that this is not the correct assumption in this region—then we can make some simple calculations . . . I think 0.001 r is a conservative rate per year [for fallout] that one can estimate . . . if we reach this level and maintain it continuously, that leads to 10 as the number of deaths from leukemia per year from fallout sources . . . in terms of our population that is a very minute fraction . . . but after all, it does correspond to somebody" (ibid. pp. 959–960).

In response to a question from Rep. Holifield, Lewis ended his testimony by returning to the threshold issue: "The threshold concept would say that you would not get any leukemia at all until you reach, say 500 r, which in a sense was the assumption when it was thought you could safely accumulate radiation as an occupational worker at a rate of 15 r per year. Now we have more information that says, no, the threshold dose had better be put well below 500 r. I would say we must put it well below 100r; in fact I doubt such a threshold exists. As far as I can see from analogy with phenomena of genetics, which is my field, there is a possible theoretical basis for predicting that there would be no threshold, namely, if leukemia is due to a somatic mutation. That is the interest of geneticists in this disease. However, these calculations do not assume that leukemia is due to a mutation. It does not matter what the leukemia is due to; if this line continues as a straight line to zero then these calculations are valid" (ibid. pp. 960–961).

Others who appeared before the Joint Committee cited Lewis' data, particularly the potential linearity of the dose–response curve and the question of a safe threshold. Several supported his conclusions, but others disagreed with them.

On the political front, a leading opponent of nuclear weapons testing, Linus Pauling of Caltech (Nobel Laureate in Chemistry, 1954), used Lewis' study to extrapolate the harmful effects of ongoing weapons tests to the world population. He cited Lewis' data extensively both in his public statements and in his book 'No More War!' (Pauling, 1958).

In 1958, several publications appeared in the scientific literature whose primary goal was expressly to challenge Lewis' analyses. One of these was written by Niel Wald of the Atomic Bomb Casualty Commission in Hiroshima who questioned "the intrinsic accuracy of the data presented" as well as emphasizing "uncertainties involved in inferring radiation dose from distance alone" (Wald, 1958). A second, by Austin Brues of the Argonne National Laboratory in Illinois, claimed that "data on human leukemogenesis by radiation indicate that a nonlinear relation is more probable" (Brues, 1958). The most detailed scientific attack on Lewis' methods came from biometrician A. W. Kimball of the Oak Ridge National Laboratory in Tennessee, who published an article in the Journal of the National Cancer Institute in 1958 challenging the "statistical reliability of existing data relating human leukemia and ionizing radiation" (Kimball, 1958). Kimball argued that Lewis' data were likely to contain "extraneous" errors resulting, for example, from biases that vary from one experiment to another (e.g., age and weight of subjects), dosimetry errors, sampling bias and truncation errors (e.g., caused by different distributions of ages at death in the irradiated and control populations). Kimball was particularly critical of Lewis' use of the Poisson distribution to calculate confidence limits for the statistical significance of the observed number of leukemia deaths among radiologists (17) when compared with the expected number (3.4). Kimball claimed that the "correct Poisson limits are too narrow, perhaps by a large factor, unless all extraneous variation is negligible . . . Based on the Poisson distribution . . . the test is valid only if there is no extra-Poisson error and if 3.4 is a known constant. In fact the number 3.4 is not without error and any statistical test would have to take this into account. Apparently the number does represent some sort of arbitrary upper limit on the true expected value, but one cannot make probability statements on this basis." (Kimball also pointed out that Lewis had incorrectly claimed 95% confidence limits in his paper, limits that in fact defined a 90% confidence interval. This had derived from an error in the tables that Lewis had used and would not have affected any of Lewis' conclusions.)

Kimball objected to Lewis' use of the χ^2 in testing whether the 10 in 23,060 cases of leukemia among A-bomb survivors in zone C differed significantly from the 26 in 156,400 cases among survivors in zone D. "It may be seen . . . that the chi-square test is actually a t test . . . that is, binomial error has been assumed. The data yield a χ^2 of 5.6 which corresponds to a significance level of about 2 percent, as stated. Suppose now that extra-binomial error contributes twice as much to the total variance as does binomial error, not too unusual for some biological data. In this event the χ^2 would be reduced to 1.9. This corresponds to a significance level of about 24 percent, and one would hesitate to conclude that the observed difference between zones C and D is a real one. The numbers themselves are unimportant, but the possible effect of extra-binomial errors on the tests of significance is of great importance, in view of conclusions reached by some observers."

Sewall Wright, the distinguished geneticist, engaged in an active dialog with Kimball regarding the latter's disagreements with Lewis. In a brief note to Kimball on 16 July 1957, Wright explained: "Lewis' tests are correct (after clearing up his

confusion between 95% and 90% confidence limits) . . . As I see it the conclusions which Lewis draws from his various tests can only be invalidated by questioning whether he has adequately controlled such factors of heterogeneity as differential age incidence and differential completeness of diagnosis, and perhaps others, which might account for the significance of the differences in some way other than by radiation" (Wright, 1957b). Subsequently, Wright expounded on his disagreement with Kimball at greater length: "It seems to me that what you write about the use of χ^2 . . . would practically rule out the use of this method except in rigorously controlled laboratory data. I think that it has a very useful place in analyzing uncontrolled data provided that the interpretation is expressed properly (as I think was done by Lewis) . . . I feel that your use of the phrase 'extraneous error', while mathematically correct, tends to lead the mind into an unprofitable and misleading channel. One may be inhibited by the likelihood of heterogeneity (which makes no difference if sampling is random) rather than led to ask whether or not the observations are taken in a way that gives bunches of necessarily similar results, a matter that can perhaps be investigated . . . It seemed to me that Lewis' analysis, while not constituting proof, made a sufficiently strong case for the linear hypothesis to make it necessary for some one to make laboratory experiments on a sufficiently large scale, to settle this very important question . . . I think the thing that impressed me most about Lewis' analysis was that essentially the same rate seemed to come out without any straining from such exceedingly different kinds of radiation exposure. If leukemia depends on a complex of physiological conditions, it is not clear why a single massive dose (Japanese data), an accumulation of minute doses over a period of years (radiologists) and intermediate situations (spondylitis, thymus) should all be equivalent at the same total dose." (Wright, 1957a).

Rep. Holifield summed up the political issues at stake in an article in the 3 August 1957 issue of Saturday Review. He first summarized the scientific data, including Lewis' "very recent study [which] says that even natural radioactivity accounts for a certain percentage of deaths from blood cancer, and that even a small fraction of the natural dose adds to those deaths" (p. 35). Then he went on to consider some of the political issues: "I believe from our hearings that the Atomic Energy Commission approach to the hazards from bomb test fall-out seems to add up to a party line—'play it down'. As custodian of official information, the AEC has the urgent responsibility to communicate the facts to the public . . . Tardy release of information is bad enough in itself. But there is something worse. That is the selective use and release of information to favor a political position . . . The prestige and resources of the AEC have been lent to the fulfillment of partisan purposes . . . Who evaluates the risk? . . . I see here a conflict of interests [on the part of the AEC]. Is it prudent to ask the same agency both to develop bombs and evaluate the risks of fall-out? . . . How can we ensure that the President is getting the best scientific evaluation of the problem?" (p. 37).

Suppression of information to suit partisan purposes was not restricted to the AEC. For example, it has recently become apparent that the distinguished British epidemiologist, Richard Doll, had in 1955 reached very similar conclusions to those that Lewis

would reach in 1957 regarding the leukemogenic effects of thermonuclear explosions (see prefatory note to Doll, 1996). Unlike Lewis, Doll did not have access to the information needed in order to estimate the dose received by Hiroshima survivors. He therefore based his calculations on published data for American radiologists (e.g., March, 1944) and irradiated ankylosing spondylitis patients (later published as Court Brown and Doll, 1957). He concluded "that (1) the effect [of ionizing radiation from fallout] is proportional to the total dosage irrespective of the length of time over which the dose is administered, and that (2) there is no threshold dose below which no effect is produced" (pp. 4–5).

Doll's May 1955 memo to the Medical Research Council in the U.K. remained unknown and unpublished until 1996 because "he was advised against publication on the grounds that it was too speculative." This reflects—perhaps better even than the above description of the attacks on Lewis, or Holifield's comments—the effects of the political climate of the time on free scientific discourse.

THYROID RADIATION DOSES FROM WEAPONS FALLOUT

Subsequent to the Joint Committee hearings, Lewis was asked to serve, starting in 1958, on the National Advisory Committee on Radiation, which reported to the Surgeon General rather than to the AEC. In 1959, this committee recommended that the U.S. Public Health Service rather than the AEC be given authority for advising on the hazards of nuclear radiation.

That same year Lewis was to publish his second study in the field of radiation effects (Lewis, 1959). The study was prompted by the A-bomb tests in Nevada and the claim of the AEC that fallout from those tests would produce doses far below background levels. Lewis pointed out the special hazard to the thyroid of infants and young children in the United States from radioiodine (chiefly I^{131}) released by the weapons tests in Nevada. Studies in the late 1950s had shown that radioiodine is concentrated in the milk of cows feeding on grass contaminated from fallout, and that the infant thyroid, averaging only two grams, absorbs all of the beta radiation dose from that isotope. As a result the infant thyroid that weighs 1–2 g receives 10–20 times the dose that an average 20 g adult thyroid receives after ingesting the same amount of the isotope. Lewis thus estimated, for the first time, that the radiation dose to the thyroid of U.S. infants from fallout was, on average, one or two times background.

Lewis pointed out, in a footnote that, with the cessation of atmospheric nuclear weapons testing in November 1958, human thyroid levels of radioiodine should decline exponentially and, by January 1959 there should no longer be any appreciable contamination of milk with radioiodine from fallout. He went on to say: "the level of radioiodine in cow's milk may well be the most important index of short-term environmental contamination with fission products whether from weapons tests or other sources." Over a quarter century later, the accident at the nuclear power station at Chernobyl, Ukraine in 1986 released large amounts of radioiodine into the atmosphere over Northern Europe, particularly Belarus. A subsequent study showed a

significant increase in thyroid cancer among individuals who were infants or children at the time of the accident, and were likely to have ingested contaminated cow's milk (Astakhova et al., 1998). Lewis reviews the Chernobyl incident in his paper written in honor of the 100th anniversary of the Curies' discovery of radium (Lewis, 1998), which is included in this collection.

BEST ESTIMATES OF RISK: LEUKEMIA IN POPULATIONS EXPOSED TO LOW DOSES OF RADIATION

Lewis had emphasized in his 1957 paper, as well as in his subsequent testimony before the joint congressional subcommittee, that there were uncertainties in the shape of the dose–response curve for induction of cancer by radiation because of limited data in the low-dose region. Over the next 15 years, he was to return to analyses of populations that had received low doses of radiation, in order to reassess in greater depth whether they show an elevated risk of cancer.

In 1963, Lewis made use of an important finding by Court Brown and Doll that the risk of chronic lymphatic leukemia (CLL) was not elevated in their group of irradiated arthritic patients (Court Brown and Doll, 1957). He used this finding to assess whether the elevated risk of leukemia in radiologists is due to better diagnosis of the disease in this group or to their exposure to ionizing radiation (Lewis, 1963b). To do so he studied a group of American Board-certified radiologists who had died at age 35–74 during the period 1948–1961. He obtained 425 death certificates for this group and showed that, not only was their death rate from leukemia higher than that of U.S. white males, but none of the deaths was the result of CLL. This last point was particularly telling since CLL is one of the commonest types of leukemia in older age groups. Thus, differential diagnosis could be ruled out as the cause of the excess leukemia experienced by the radiologist group. This was because "if radiation . . . is responsible for a rise in the death rates for leukemia in a given population, the death rate for CLL in that population should rise little if at all. On the other hand, if diagnosis is responsible, the death rate for CLL should rise proportionately, since diagnosis ought not to affect classification by histological cell type differentially" (Lewis, 1963b).

Lewis' 1963 radiologist study was the first to find that low doses of ionizing radiation induce multiple myeloma (MM)—a leukemia of the antibody-producing cells. Five cases were observed versus 1.01 expected for U.S. white males, after adjustment for age and year. Medical specialists in the United States, such as radiologists, enjoy a 'healthy worker effect' in comparison to U.S. white males. Thus the P value of 0.004 that Lewis calculated is conservative. Incidence of MM is very rare until age 60, after which it rises very rapidly. Thus the failure of epidemiological studies up to that time to implicate MM upon radiation exposure can be attributed to the fact that those studies involved populations that were, on average, much younger than the radiologist group. In Japan, mortality from MM has been shown to be significantly increased among A-bomb survivors as that group has aged (Shimizu et al., 1990). Particularly

relevant in this regard is the fact that MM is much rarer in Japan and Asia than in the U.S. and Europe. Those authors conclude: "A significantly increased risk also occurs in cancers of the lung, breast and urinary tract as well as multiple myeloma . . . On a site-specific basis, the proportion of radiation-induced cancers ranges from 6% for stomach cancer to 33% for multiple myeloma". MM has also shown a statistically significant increase among persons irradiated for ankylosing spondylitis as that group has aged (e.g., Weiss et al., 1994) while, among British radiologists, MM has been found to be elevated but not approaching statistical significance (Berrington et al., 2001).

In a later study, Lewis found that American board-certified dermatologists and urologists experienced a statistically significant increase in mortality from leukemia (Lewis, 1970). Lewis approximated the age composition for dermatologists but not for urologists; however several approaches indicated that the excess of leukemia in these specialties was significant. Tellingly, as in his 1963 analysis of radiologists, no cases were specifically CLL. Why the elevated risk among these specialists? Probably because, in the United States during the first part of the Twentieth Century, dermatologists had been, almost universally, using X-rays to treat skin conditions such as acne and fungal infections. During the same period, U.S. urologists had X-ray machines in their offices to carry out, for example, fluoroscopy of kidney stones. In that sense urologists were in fact more like radiologists than dermatologists, who did not conduct fluoroscopy. A big factor in the exposure of specialists who had X-ray machines in their offices may well have been the nature of shielding of the tubes. The manufacturer had to shield them so that the stray radiation would not exceed the permissible limits, which were, at that time, set much higher than they are now.

In a 1971 letter to Science, Lewis wrote regarding the excess in leukemia cases among patients who had received I^{131} radiation therapy to treat hyperthyroidism (Lewis, 1971). He was commenting on a paper claiming that the increase in leukemia was due to the disease rather than to the radiation (Saenger et al., 1968, 1971). Because that study found an increase in leukemia incidence, not only in the I^{131} treated patients, but in a surgically treated control group, the authors had concluded that hyperthyroidism predisposes to leukemia. Lewis reexamined the data and found that the excessive death rate from leukemia was largely confined to patients who were over 50 years old at the time of I^{131} therapy. When the incidence of radiation-inducible leukemia was measured in these patients, there was a significant increase in leukemia. In contrast, the leukemia incidence for the surgically treated patients in the same age range was not significantly elevated.

Hyperthyroidism patients treated with I^{131} provide a unique opportunity to determine the leukemia risk (and possibly risks for other cancers besides thyroid cancer) at well-defined, extremely low dose rates and low total dose. (Such patients receive a relatively low total dose to their bone marrow—estimated to be 5–15 rads—over a period of some 10 days following ingestion of the therapeutic dose.) I^{131} treatment for hyperthyroidism is also unique in continuing to be a widely used therapy, often preferred for older patients with the disease, for whom surgery carries considerable risk. At present the I^{131} treated patients represent the largest practically available

population that can be followed to detect whether there is a leukemogenic effect of low doses of radiation.

ESTIMATES OF LUNG CANCER RISK: THE BATELLE BEAGLES

Lewis' goal in all of the papers discussed in this section of the book was to make the best estimates of cancer risks rather than to estimate the maximum risks that might be involved. An interesting—and until now largely inaccessible—final example included here is Lewis' analysis of lung cancer induced in beagle dogs exposed to plutonium by inhalation (Lewis, 1976). This report was written by Lewis as an appendix to a report on the "Health Effects of Alpha-Emitting Particles in the Respiratory Tract" of an *ad hoc* committee on "hot particles" of which he was a member.

The analysis was prepared by the National Academy of Sciences/National Research Council at the request of the Environmental Protection Agency to evaluate charges that "radioactive material deposited in tissues of the body as high specific activity particles might be a greater health hazard than the same source distributed more homogeneously. This has been referred to as the 'hot particle problem'." In particular, it had been hypothesized that "the intense and highly localized dose from inhaled insoluble plutonium particles larger than a specified size causes greater tissue damage, and is therefore more carcinogenic, than more uniformly-delivered radiation" (Tamplin and Cochran, 1974). The National Resources Defense Council had, on this basis, petitioned the EPA for a 115,000-fold reduction in standards governing exposure to alpha-emitting hot particles.

Data were already available on lung cancer risk from inhalation of plutonium by beagle dogs. Lewis' analysis therefore assessed the lung tumor mortality rate in 40 beagles that had been exposed to known doses of radioactive plutonium (^{239}P) by inhalation. This was done relative to the estimated number of hot particles deposited in their lungs. Lewis concluded: (1) that the generalized alpha radiation from the plutonium in the dogs' lungs was "sufficient to account for all of the lung cancer deaths" and (2) "if there is a hot particle effect . . . the risk of a lung cancer death per particle is orders of magnitude smaller than they [Cochran and Tamplin] estimated and could well be so small that the contribution from any hot particle effect to the total lung cancer mortality is negligible". Thus the ad hoc committee was led to conclude that the "experimental evidence suggests that the carcinogenic response is more a function of the *amount* [committee's emphasis] of radioactivity in the lung than of its distribution". The NRDC petition was not supported, and the EPA standard was not changed.

SUMMARY

Lewis' detour into somatic effects of ionizing radiation was driven by the imperative to provide objective and accurate data to the scientific community and the public. The public attention and the politically motivated attacks that accompanied his radiation

studies, haunted Lewis, who preferred the solitude of his laboratory to the limelight. Following the radiation literature became a hobby for Lewis, which he pursued in his "spare" time. In retrospect, the two decades during which he made his major contributions in the field of radiation effects overlapped with the Drosophila studies that led to his famous model for the genetic control of development. During this same period, Lewis had sole responsibility for lecturing Caltech undergraduates on genetics as well as teaching the genetics laboratory course; he ran the Drosophila stock center at Caltech that provided genetic stocks to laboratories world-wide; he served on the National Advisory Committee on Radiation as well as Secretary, Vice President and then President of the Genetics Society of America; and he and his wife Pam raised their children. Science for Lewis was never a part-time occupation: it was an obsession. That is why, until shortly before his death at the age of 86, he continued to spend the better part of every day in his laboratory continuing his 70-year long dialog with the laws of heredity and development.

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LEUKEMIA AND IONIZING RADIATION

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Quantitative estimates of the genetic effects of ionizing radiation on human beings have been carried out by a number of investigators [1–3]. Estimates of this kind involve extrapolating from induced mutation rates in such organisms as *Drosophila* and mice. Quantitative estimates of the somatic, or "direct," effects of radiation must also be attempted if the biological hazards of ionizing radiation are to be fully assessed. In the case of direct effects, it is particularly difficult to extrapolate from results with lower organisms, and it becomes important to have data on man himself.

It is the purpose of this article to examine the evidence for the induction of leukemia in man by ionizing radiation. Although ionizing radiation has been implicated in the production of other human malignancies, such as bone tumors [4] and thyroid carcinoma [5, 6], only the data on induction of leukemia seem sufficiently extensive to warrant a study at this time of the quantitative relationship between incidence of the disease and dose of radiation. Evidence bearing on this relationship is drawn from studies of leukemia among four groups of individuals: (i) survivors of atomic bomb radiation in Japan; (ii) patients irradiated for ankylosing spondylitis; (iii) children irradiated as infants for thymic enlargement; and (iv) radiologists. An estimate of the probability of developing leukemia per unit dose of radiation [7] per time unit is derived for each of these groups. This probability of radiation-induced leukemia is discussed and its application to a specific example of a possible radiation hazard—namely, radiostrontium—is outlined. Certain properties of the disease, relevant to the radiation studies, are presented first.

DESCRIPTION OF THE DISEASE

Leukemia is a malignant disease in which the leucocytes undergo a more or less unrestricted proliferation. The "acute" form of leukemia differs from the "chronic" form, not only in being usually of shorter duration, but also in being a more severe disease with a higher percentage of immature white blood cells in the circulating blood. Another classification of the leukemias is based on the type of white blood cell predominating in the marrow or in the circulating blood. The two most common of these types are known as "granulotytic" (or myelogenous) and "lymphocytic" (or lymphatic). The presumption is that the granulocytic type arises in the red bone marrow. The lymphocytic type is thought to arise in the lymphatic elements of the blood-forming system (thymus, spleen, and other lymph glands), although the marrow is not excluded as a source for this type.

SPONTANEOUS INCIDENCE OF LEUKEMIA

In 1947, Sacks and Seeman [8] reported that the recorded death rate from leukemia had increased steadily from 1900 to 1944 and at an accelerated rate after 1930. The death rate has continued to increase [9]. By 1954, the crude mortality rate for leukemia among the US white population had reached 68 per million individuals per year [10] compared with 42 per million in 1940 [11]. The male and female crude death rates in that population were 79 and 58 per million per year, respectively, in 1954 [10]. The observed increase in death rate from this disease may be partly due to improvements in diagnosis. Other factors may also be responsible, such as the increased exposure of the population to ionizing radiations employed in medicine and dentistry, as was recently discussed by Dameshek and Gunz [12].

MacMahon and Clark [13] have recently studied the spontaneous incidence of the common forms of leukemia. They have attempted to determine the total number of valid cases diagnosed among residents of the borough of Brooklyn from 1943 to 1952, inclusive. In this study the overall ratio of acute to chronic forms among the white population was nearly 1/1 (726/732), but there were marked differences in the incidence of these two forms with respect to age at time of diagnosis, as is shown in Table 1 [14]. The ratio of granulocytic to lymphocytic types in the Brooklyn study was 1.6/1 (512/318).

LEUKEMIA IN HIROSHIMA AND NAGASAKI

Studies of the incidence of leukemia among survivors of the atomic bomb bursts over Hiroshima and Nagasaki have established that ionizing radiations induce leukemia in man [15–17]. Table 2 summarizes the incidence of leukemia in terms of four concentric zones about the hypocenter (the point on the ground under the aerial burst). This table includes only those cases of leukemia which were (i) diagnosed during the period January 1948 to September 1955, inclusive; (ii) resident in the city at the time of diagnosis (Hiroshima) or at the time of death (Nagasaki); and

Table 1 The spontaneous incidence of leukemia for the white population of Brooklyn, N.Y., 1943–52, according to chronicity. Data of MacMahon and Clark [13].

Age	Percentage in age interval	Incidence per million per year[a]		
		Acute	Chronic	Total
0–9	15.3	48	1	49
10–19	13.5	24	2	26
20–29	16.5	12	6	18
30–39	16.5	20	14	34
40–49	14.8	22	28	50
50–59	11.8	44	64	108
60–69	7.6	58	133	191
70+	3.9	59	182	241

[a]The incidence of "subacute" and unknown types of leukemia has been allocated to the observed incidences for the acute and chronic forms in the proportions in which the latter were diagnosed at each age interval [14].

(iii) considered by several criteria to be valid cases of the disease [18]. For each zone, the estimate of the number of exposed survivors resident in Hiroshima as of October 1950 [17] has been combined with the corresponding number for Nagasaki [15] to obtain a combined population estimate for both cities.

Lange et al. [16] have studied the pattern of types of leukemia in the exposed and unexposed populations of Hiroshima and Nagasaki. They conclude that radiation induces the same pathological types that are found spontaneously and, as far as can be judged by the limited data, induces them in roughly the same relative proportions that occur spontaneously. This is especially evident in the case of chronic lymphocytic leukemia, which is rare in both the exposed and unexposed Japanese populations, whereas it is the most common form of leukemia after age 50 in the United States [13]. Lange et al. found no marked influence of sex or age on the incidence of leukemia among the exposed populations. However, they point out that, for a number of reasons, the data are not very satisfactory for assessing the incidence in individuals under 5 years of age [19].

The published accounts of leukemia in Hiroshima and Nagasaki have not contained estimates of the doses received by the bomb survivors. Recently, however, distance-dose curves for these cities have been published [20]. These curves give, for each city,

Table 2 Incidence of leukemia among the combined exposed populations of Hiroshima and Nagasaki by distance from the hypocenter (January 1948–September 1955).

Zone	Distance from hypocenter(m)	Estimated population of exposed survivors (Oct. 1950)	Number of confirmed cases of leukemia	Percentage of leukemia
A	0–999	1,870	18	0.96
B	1000–1499	13,730	41	0.30
C	1500–1999	23,060	10	0.043
D	2000 and over	156,060	26	0.017

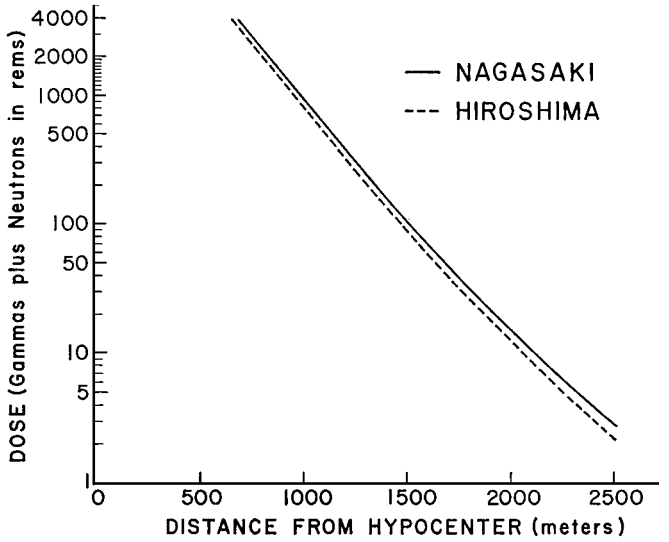

Figure 1. Distance–dose curves for atomic bomb blasts at Nagasaki and Hiroshima.

the relationship between the slant distance from the burst and the "air" (unshielded) dose of gamma rays and of neutrons. From this information, curves have been constructed (Fig. 1) showing the relation between distance from the hypocenter and the combined "air" dose from gamma rays and neutrons (Fig. 1) in rem [7]. In computing the latter dose, it has been assumed that the relative biological effectiveness (RBE) of neutrons for inducing human leukemia is 1.7. This value is chosen since Dunning has recently stated that "for generalized whole-body effectiveness it is thought that 1.7 is a reasonable representative value for neutrons from a nuclear detonation" [21]. This is believed to be a conservative estimate for the RBE, since Upton et al. [22] found that the RBE for induction of leukemia in mice by fast neutrons is somewhat lower than this.

In the absence of precise knowledge of the distribution of survivors within the different zones about the hypocenter, it is conservative to take the mean "air" dose for a zone as the average "air" dose received by survivors in that zone. The zone from 0 m to 999 m, which is designated here as zone A, is a special case, however, since there was heavy mortality near its center. The mean dose for this zone has been computed for the portion of this zone extending from 850 m to 999 m. The majority of leukemia cases in zone A occurred in this latter region [15]; moreover, two (among five) cases at a distance closer than 850 meters had the type of shielding specified, and in each case it was listed as heavy [18]. Since the doses for the two cities are slightly different at a given distance from the hypocenter, the average value of these two doses is used without correcting for differences in population size. In this way, a dose of

Table 3 Incidence of leukemia per year among the combined exposed populations of Hiroshima and Nagasaki (January 1948 to September 1955) in relation to dose of radiation (gammas plus neutrons).

Zone	Average maximum dose (rem)	Incidence of leukemia per million per year	Incidence of radiation-induced leukemia per million per year	Probability of leukemia per individual per rem per year
A	1300	1200	1179	0.9×10^{-6}
B	500	390	369	0.7×10^{-6}
C	50	56	35	0.7×10^{-6}
D	5	21		

about 1300 rem is arrived at for zone A. For zones B (1,000–1,499 m) and C (1,500–1,999 m), the mean doses are approximately 500 rem and 50 rem, respectively. At 2,000 m the dose has fallen to 14 rem and by 2,500 m to less than 5 rem. Since the majority of the population in zone D (from 2,000 m on) was beyond 2,500 m, the average dose is under 5 rem and is thus so low that zone D can be treated as if it were a "control" zone.

The relation of dose, estimated as described in the preceding paragraph, to the incidence of leukemia per year, based on the combined Hiroshima and Nagasaki data, is shown in Table 3. The incidence per year in the "control" zone, D, is subtracted from the incidence in each of the other zones to obtain the "incidence of induced leukemia per year" in these zones. The values of the incidence of induced leukemia are likely to be minimum ones, since, as Lange *et al.* have noted, "some cases of leukemia have undoubtedly been missed and other cases have been omitted because of lack of adequate material to confirm the diagnosis" [16]. The incidences of induced leukemia in zones A, B, and C have been divided by the respective mean "air" doses in rem, derived in the preceding paragraph, to give estimates for the probability of induced leukemia for these zones. The values for this probability are seen to range from 0.7×10^{-8} to 0.9×10^{-6} per individual per rem per year. These are minimum estimates of the probability of induced leukemia, since the survivors were shielded in varying degrees from the "air" doses, calculated in the preceding paragraph. The shielding of survivors has two major components: (i) the body's own shielding of its blood-forming tissues by the surrounding bone and soft tissues; and (ii) external shielding by buildings or other shelters. A shielding factor of 2 is believed to be a conservative one for correcting for both of these components—the true factor might be at least 4 [23]. The "best" estimate for the probability of induced leukemia from these data is, therefore, taken as approximately twice the aforementioned minimum estimates of 2×10^{-6} per individual per rem per year (of the 7.75-year period). A rough range for this probability is 0.7×10^{-6} to 4×10^{-6}.

LEUKEMIA AND ANKYLOSING SPONDYLITIS

Court Brown and Doll [24] and others [25, 26] have investigated the incidence of leukemia among patients treated with X-rays for ankylosing spondylitis—a hereditary

Table 4 Incidence of leukemia among ankylosing spondylitis patients receiving different doses of radiation (X-rays). (Data from Court Brown and Doll [24].)

Maximum dose to spinal marrow (r)	Estimated average maximum dose (r)	Number of males developing leukemia	Crude incidence per million males per year	Incidence of radiation-induced leukemia per million males per year	Probability of leukemia per individual per r (to spinal marrow) per year
0			50		
Under 500		2	220	170	
500–999	750	8	410	360	0.5×10^{-6}
1000–1499	1250	8	420	370	0.3×10^{-6}
1500–1999	1750	8	1130	1080	0.6×10^{-6}
2000–2749	2375	6	1300	1250	0.5×10^{-6}
2750 or more		5	1760		

disease of the spine. Among 11,287 male patients irradiated during the period from 1935 to 1954, inclusive, 37 cases of leukemia were discovered. The average period of follow-up of these patients was "just under five years" [24]. The distribution of cases by amount of treatment, measured as maximum dose in roentgens [7] to the spinal marrow, is shown in Table 4. A highly significant increase in the incidence of leukemia is apparent among those receiving the heavier treatments.

Court Brown and Doll have estimated the expected incidence of leukemia in a comparable group of unirradiated normal males as 50 cases per million individuals per year. Subtraction of this expected incidence from the observed incidence of leukemia per year in the irradiated patients gives an estimate of the incidence of radiation-induced leukemia per year. This calculation has been carried out for each of the groupings of leukemia cases according to amount of treatment. For each such grouping between 500 and 2,750 r, an average maximum dose to the spinal marrow is taken as the mid-point of the dose range (e.g., for leukemia cases developing after treatments ranging from 500 to 999 r, 750 r is taken as the average dose). By dividing the calculated incidence of radiation-induced leukemia for each of the four groupings of this kind (column 5, Table 4) by the respective average maximum dose (column 2, Table 4), a set of four minimum estimates of the probability of leukemia per individual per roentgen (to the spinal marrow) per year is obtained. These latter estimates are seen to range from 0.3×10^{-6} to 0.6×10^{-6} per individual per roentgen per year (column 6 of Table 4). It seems likely that the absorbed dose to the entire red-marrow system would be lower than the stated doses to the spinal marrow by a factor of at least 2 or 3. Therefore, it is estimated that the probability of leukemia ranges from about 0.6×10^{-6} to 2×10^{-6} per individual per rad (to the red-marrow system) per year.

LEUKEMIA AND THYMIC ENLARGEMENT

Simpson et al. [6] have traced a series of 1,400 individuals who had been irradiated as infants for an enlarged thymus condition. The average period of follow-up appears to have been about 15 years. As a "control" 1,795 unirradiated siblings were also

traced. In the irradiated group there were seven confirmed cases of leukemia (and one unconfirmed case), while there was none in the control group. The calculated number of cases of leukemia that would have been expected in a sample of comparable size and age from the general population was 0.6. The difference between this expectation and the observed number of cases (seven) is statistically significant (P less than 0.01).

In the majority of the 1,400 infants, the radiation (X-rays) had been more or less restricted to the chest region. It was estimated that the "air" dose to the thymus region was more than 200 r ("the great majority being less than 600 r") in 57% of the treated individuals and under 200 r in the remainder. The average absorbed dose to the entire lymphatic system is roughly estimated as 100–300 rad. On the basis of these dose estimates, the probability of leukemia ranges from 1×10^{-6} $(6.4/15 \times 1400 \times 300)$ to 3×10^{-6} per rad (to the lymphatic system) per individual per year. The number of cases (seven) on which this estimate is based is, of course, small. The 95% confidence interval for an observation of seven when the frequency is as low as in the present case lies between 3.3 and 13.2 [27]. Therefore, the probability of leukemia in the thymic enlargement group may well range from 0.4×10^{-6} to 6×10^{-6} per rad (to the lymphatic system) per individual per year.

LEUKEMIA AMONG RADIOLOGISTS

March [28, 29] and others [30, 31] have called attention to the fact that among physicians the percentage of deaths from leukemia is much higher for radiologists than for physicians who are not radiologists. The percentage of deaths that are due to leukemia can be a misleading statistic, however, since it is sensitive to differences in age distribution between the groups of individuals being compared. Such differences are marked in the case of radiologists, on the one hand, and all physicians, on the other, as is discussed later. To assess the radiation factor in the leukemia among radiologists, it first becomes necessary to estimate (i) the death rate from leukemia among radiologists, (the number of deaths per total number of living radiologists), and (ii) the expected death rate from leukemia among radiologists if they had received no occupational exposure to radiation.

The study of mortality among medical specialists by Dublin and Spiegelman [32] has been used here as a guide in computing the afore-mentioned rates and as source of data for the years 1938–1942, inclusive. The latter data and additional data for the years 1943–1952, inclusive, are summarized in Table 5. The term *radiologist* is restricted here, following Dublin and Spiegelman, to those physicians who were listed in editions of the *American Medical Directory* [33] as limiting their practice to radiology (and roentgenology). Since only deaths occurring at ages 35–74 years, inclusive, were included in the mortality study for 1938–1942, the same practice is adopted here for the supplementary 10-year period.

In order to estimate the mean annual population of radiologists at ages 35–74 years, during the entire 15-year period from 1938 to 1952, the age distribution of

Table 5 Deaths and death rates from leukemia among radiologists, at ages 35–74 years, by 5-year periods from 1938 to 1952, inclusive.

Period	Estimated number of radiologists at mid-point of period	Number of death from leukemia	Observed death rates per million per year	Expected death rates per million per year	Incidence of radiation-induced death per million per year
1938–1942	1451.5	5	690	101	589
1943–1947	(1850.7)[a]	6	650	(121)[a]	429
1948–1952	2250.0	6	530	141	389
(1938–1952)	(1850.7)[a]	17	610	(121)[a]	489

[a]The arithmetic average of the values for the 1938–1942 and 1948–1952 periods.

radiologists is required. This age distribution for the year 1940 was computed by Dublin and Spiegelman from the 1940 edition of the *American Medical Directory* and is shown in Table 6. The age distribution for a similar group of radiologists in 1950, also shown in Table 6, has been computed [34] by reference to the 1950 edition of this directory. The 1940 age distribution was based on a total of 1595 radiologists of whom 1451.5 (91.0%) can be inferred to have been at ages 35–74 years inclusive [32]. The 1950 age distribution was based on a total of 2443 radiologists of whom 2250 (92.1%) are calculated to have been at ages 35–74 years, inclusive, as of 1 July 1950. The mean number of radiologists (at ages 35–74) per year from 1938–1952, inclusive, is roughly approximated as 1850.7, which is the average of the number of such radiologists in 1940 and the corresponding number in 1950.

Deaths from leukemia occurring at ages 35–74 years, inclusive, among radiologists have been located in several ways with the results shown in Table 5. For the period from 1938 to 1942, inclusive, five such deaths are recorded by Dublin and Spiegelman. For the period from 1943 to 1948, inclusive, the carefully documented studies of March [28, 29] record eight such deaths. For the remaining 4-year period from 1949 to 1952, inclusive, four additional deaths from leukemia have been located by reference to death notices in a medical journal [35]. Thus, a minimum of 17 deaths

Table 6 Age distribution of radiologists in 1940 (*32*) and 1950.

	Percentage distribution as of	
Age	1940	1950 (1 July)
Under 35	8.3	6.1
35–44	31.3	38.9
45–54	33.8	26.6
55–64	19.8	18.7
65–74	6.1	7.9
75 and over	0.7	1.8
Total	100.0	100.0

Table 7 United States white male death rates from leukemia per million per year.

	Period	
Age	1939–1941	1949–1951
25–34	18	26
35–44	29	34
45–54	55	68
55–64	104	149
65–74	154	276
75+	181	385

from leukemia has been located among radiologists who died between the ages of 35 and 74 years during the 15-year period from 1938 to 1952. The upper and lower 95% confidence limits for this observation of 17 deaths are 25.5 and 10.8 deaths, respectively [27]. Thus, a likely range of values for the average death rate from leukemia among radiologists at ages 35–74 years is 390 ($10.8/15 \times 1{,}850.7$) to 920 ($25.5/15 \times 1{,}850.7$) deaths per million per year, and the "best" estimate is 610 ($17/15 \times 1{,}850.7$) deaths per million per year (of the 1938–1952 period).

The expected death rate from leukemia among radiologists, if they had received no occupational exposure to radiation, is estimated by first calculating the death rate they would have experienced if subject to U.S. white male death rates from leukemia. This calculation has been made for a 3-year period from 1939 to 1941 by first computing [36] the mean annual age-specific U.S. white male death rates from leukemia (Table 7) and then applying them to the 1940 age distribution of radiologists (Table 6), restricting the computation to the 35–74 year age interval. The resultant expected death rate for the latter age interval is 63 deaths per million per year.

The same type of calculation has been carried out for a 3-year period from 1949 to 1951 by computing [36] the appropriate age-specific death rates for that period (Table 7) and applying them to the 1950 age distribution of radiologists. The resultant expected death rate is 88 deaths per million per year.

The average of the rates for the 1939–1941 and 1949–1951 periods is 76 deaths per million per year. The latter rate should roughly approximate the mean annual death rate from leukemia which radiologists would have experienced during the 1938–1952 period if they had been subject to U.S. white male death rates for this disease. The observed death rate for this period was 610 deaths per million per year (Table 5), which is eight times the expected rate, just calculated.

It is possible, however, that reasons other than radiation exposure may account for the high death rate from leukemia among radiologists. For example, leukemia might be more likely to be diagnosed among radiologists than among the group of all U.S. white males. To correct for such possibilities as this, the expected death rate of 76 deaths from leukemia per million per year, calculated in the preceding paragraph, is multiplied by a correction factor of 1.6. This factor is the ratio

of the observed number of deaths from leukemia among physicians who were nonradiologists to the expected number of deaths calculated on the assumption that such physicians were subject to U.S. white male age-specific death rates for leukemia.

This factor of 1.6 has been inferred from data for the 1938–1942 period given by Dublin and Spiegelman [32] and is applied throughout the entire 15-year period from 1938 to 1952 to give the expected death rates shown in Table 5. It is a conservative factor in the sense that it is possible that the increased death rate from leukemia among physicians who are nonradiologists is partly due to exposure of some of them to ionizing radiation [31]. Thus, 121 (1.6×75.5) deaths per million per year is probably a conservative estimate of the expected death rate from leukemia among radiologists in the 1938–1952 period, if they had received no exposure to radiation.

The expected death rate from leukemia, just calculated, would be expected to yield 3.4 ($15 \times 1850.7 \times 121 \times 10^{-6}$) deaths among radiologists during the 1938–1952 period. It is appropriate at this point to compare this with the observed number—namely, 17 deaths (Table 5). The probability of observing 17 or more when the expected number is 3.4 is readily obtained from the Poisson distribution and is found to be less than 1×10^{-6}. Hence, the observed value exceeds the expected value at a statistically highly significant level.

The difference between the expected death rate from leukemia calculated on the assumption of no occupational exposure to radiation and the observed death rate is designated the "incidence of radiation-induced leukemia," L. For a stationary population chronically irradiated at a constant dose rate, D, the incidence. L, can be approximated if it is assumed that the probability of leukemia per rad of accumulated dose per year, P_L, is a constant for all age groups in the population and for all values of the accumulated dose. On these assumptions, a stationary population exposed for a mean number of years, E, to the dose rate, D, will have an incidence of radiation-induced leukemia that can be expressed as follows:

$$L = (D) \cdot (E) \cdot (P_L)$$

To estimate the value of E, it is assumed that occupational exposure of radiologists starts at age 25 and ends at age 65. The value of E for individuals who were at ages 35–74 in 1940 can then be approximated from the age distribution of radiologists for that year (Table 6) and is found to be 24.7 years. The corresponding value of E approximated from the 1950 distribution (Table 6) is 24.1 years. The average of these two values, 24.4 years, is used as the value of E for the population of radiologists who were at ages 35–74 years in the 1938–1952 period. The "best" estimate of L is 489 deaths per million per year, (Table 5), and a likely range of values for L is 270 to 800 deaths per million per year, based on the 95% confidence limits for the observed death rate of 610 deaths per million per year. For reasons discussed later, the value of D is estimated to lie between 3 and 30 rad per year.

The "best" estimate for the range of values of P_L is then given by the expression

$$P_L(\text{likely range}) = \frac{489}{24.4 \times (3 \text{ to } 30)} = (0.7 \text{ to } 7) \times 10^{-6} \text{ per individual per rad per year}$$

A broader range, based on the confidence limits for L, is $(0.4 \text{ to } 11) \times 10^{-6}$ per individual per rad per year.

Since the dose rate, D, in the foregoing discussion, represents the average absorbed dose rate to the leucocyte-producing system, it is likely to be lower by at least a factor of 2 than the "air" dose rate to which radiologists were exposed. The recommended maximum dose rate (in air) for such workers was set at 0.2 r per day in 1931 by the U.S. National Committee on Radiological Protection; this rate was reduced to 0.1 r per day in 1936 and to 0.05 r per day in 1949. Although some radiologists may well have exceeded the recommended dose rates, it seems unlikely that the average dose rate for all radiologists in the group under consideration would have exceeded the permissible limits set in 1931. Thus 30 rad per year has been taken as an upper limit for the absorbed dose to the leucocyte-producing system. The lower limit for D has arbitrarily been taken as one-tenth of this or 3 rad per year.

This estimate that D might be much less than 30 rad per year is somewhat at variance with the following conclusions from a recent study of longevity among radiologists [37]. "In comparison with non-exposed physicians the shortening of life of radiologists is 5.2 years or 11% of the adult life span (after 20 years). If extrapolation from the animal data . . . is permissible, this would be expected to result from chronic whole body exposure to about 1.5 LD_{50} dose or possibly 1000 roentgens. Although this exposure was partial body and possibly less effective, it seems unlikely that the equivalent whole body exposures differed from the above value by a factor greater than 2 or 3. Consequently it appears that, within these limits at least, extrapolation from short-lived animals to man may be made with some confidence on the basis of per cent life-shortening per unit dose."

The shortening of life by 5.2 years just cited is based on the observation that during the period 1930–1954 the difference between the mean age at death of physicians estimated to have had "no known contact with radiation" and the mean age at death of radiologists was 5.2 years [37]. It can be calculated [38], however, that a difference of at least 6 years would be expected in this case solely as the result of differences in age distribution (as of 1940 or 1950) between radiologists, on the one hand, and all physicians, on the other. That is, radiologists may have a slightly longer life-span than physicians as a whole. Moreover, for the 1938–1942 period, Dublin and Spiegelman showed that, after appropriate adjustment for differences in age distribution, the total death rate from all causes was lower for radiologists than it was for all physicians combined; however, this rate was slightly higher for radiologists than it was for all specialists combined. Thus, either a chronic whole-body exposure of 1,000 r, does not have a marked effect on longevity or, more probably, radiologists have *averaged* much less than this as a life-time absorbed dose.

DISCUSSION

Table 8 summarizes the various estimates of the probability of leukemia derived from the four sets of data reviewed here. For acute whole-body irradiation, the "best" estimate of this probability will be taken as 2×10^{-6} per individual per rad per year. This value is based on the studies of leukemia among survivors of atomic bomb radiation. For acute partial-body irradiation, the available data are conveniently discussed in terms of a probability of leukemia "of bone-marrow origin" (ankylosing spondylitis patients) or a probability of leukemia "of lymphatic origin" (thymic enlargement patients).

As has already been noted, granulocytic and lymphocytic leukemias may have bone-marrow and lymphatic origins, respectively. Since these two types of leukemia constitute the majority of all leukemias and occur in proportions which are, for present purposes, roughly equal, it is assumed that the "best" estimate of the probability of leukemia of bone-marrow origin is one-half of that for all leukemia, or 1×10^{-6} per individual per rad to the red marrow per year. Similarly, the "best" estimate of the probability of leukemia of lymphatic origin is taken as 1×10^{-6} per individual per rad to the lymphatic system per year.

These estimates fall within the range of values calculated for either the ankylosing spondylitis patients or the thymic enlargement patients. Moreover, there is some evidence that leukemia following irradiation of the spinal marrow is primarily granulocytic [26]. Whether lymphocytic leukemia predominates in the thymic enlargement series [6] is uncertain on two grounds: (i) it is difficult to differentiate granulocytic and lymphocytic types in infants and children; and (ii) some irradiation of bone marrow would, in any case, be expected in this series of patients [39]. Finally, the "best" estimate of the probability of leukemia following chronic whole-body irradiation is taken as identical with that for acute whole-body irradiation—namely, 2×10^{-6} per individual per rad (of accumulated dose) per year. This value is seen to be close to the lower limit of the range of values deduced for radiologists.

Simpson et al. [6] and Court Brown and Doll [24] point out that their studies lack a control in the form of an unirradiated series of patients. Thus, the possibility is not excluded that thymic enlargement and ankylosing spondylitis predispose toward leukemia. However, a comparison of the various estimated ranges for the probability of leukemia (Table 8) suggests that patients with the afore-mentioned conditions are no more prone to develop leukemia than are radiologists or the Japanese survivors.

Presently available determinations of the incidence of induced leukemia per year are based on average follow-up periods that are comparatively short in terms of the normal human life-span. Thus, the probability of leukemia per individual per rad per year may not be constant for an indefinite period beyond the initial time of irradiation. By choosing the lower limit for the probability of leukemia at about 0.7×10^{-6} per individual per rad per year, it is felt that adequate account is taken of the possibility that the incidence of leukemia per year following an acute dose of radiation may, as some have suggested on the basis of the data from Hiroshima [37], reach a peak followed

Table 8 Summary of the estimates of the probability of radiation-induced leukemia per individual per rad per year.

Source of estimate	Type of radiation	Region irradiated	Types of leukemia produced	Probability of leukemia of specified type per individual per rad (or rem) to region irradiated per year		
				Estimated range		
				Lower limit	Upper limit	"Best estimate"
Atom-bomb survivors	Gamma rays plus neutrons	Whole body	All	0.7×10^{-6}	3×10^{-6}	2×10^{-6}
Ankylosing spondylitis patients	X-rays	Spine	Granulocytic(Only?)	0.6×10^{-6}	2×10^{-6}	1×10^{-6}
Thymic enlargement patients	X-rays	Chest	Lymphocytic(Only?)	0.4×10^{-6}	6×10^{-6}	1×10^{-6}
Radiologists	X-rays, radium, etc.	Partial to whole body	All (?)	0.4×10^{-6}	11×10^{-6}	2×10^{-6}
Spontaneous incidence of leukemia (Brooklyn, NY.)	All natural background sources	Whole body	All (?)		10×10^{-6}	2×10^{-6}

by a steady decline. It is noteworthy, however, that Court Brown and Doll have concluded, from an analysis of 108 cases of leukemia among the exposed populations of Hiroshima and Nagasaki, that "the data provide no evidence of a sharp peak in incidence at any particular period after the explosion nor any clear indication that the incidence had yet begun to diminish by the end of the ninth year" [40].

The probability of leukemia per individual per rad per year is nearly constant over a rather wide range of doses in the case of the Japanese survivors (Table 3) and in the case of ankylosing spondylitis patients (Table 4). This is presumptive evidence that the relationship between incidence of induced leukemia and dose of radiation is either linear or approximately linear. A striking feature of the Japanese data shown in Table 2 is that the incidence of leukemia in zone C—the zone with a calculated average "air" dose of 50 rem—is significantly higher than in zone D, the "control" zone ($P = 0.02$, by the Chi-square test). Thus, these data provide no evidence for a threshold dose for the induction of leukemia. Moreover, chronic irradiation at a relatively low dose rate (perhaps 0.1 rad per day or less) appears to induce leukemia in radiologists at a rate per rad which is comparable to that observed for the Japanese survivors. This finding also fails to support the concept of a threshold dose below which leukemia will not develop.

A linear relationship between the incidence of leukemia and dose of radiation, which is suggested by the available data for man, may have its explanation in a somatic mutation hypothesis [41]. Thus, radiation-induced leukemia may result from a somatic gene mutation, presumably occurring in one of the precursor cells destined to give rise to mature leucocytes. Such a mutation might cause the cell, or its descendants, to acquire an unregulated growth habit or to release, or to respond to, viruslike or hormonal agents—to mention only a few of many possibilities. Thus, the somatic mutation hypothesis and other hypotheses for the origin of radiation-induced malignancies [42] are by no means mutually exclusive. Gene mutation has long been known to show a linear relationship with respect to dose of ionizing radiation from studies with *Drosophila*. This linearity has been extended by Spencer and Stern [43] to doses of 50 and 25 r. Gene mutation is also known to be directly proportional to the accumulated dose of radiation, even when the radiation is chronically administered at a relatively low dose rate, as in the studies of Uphoff and Stern [44].

The concept of somatic mutation is also helpful in attempting to explain the long period of time which sometimes intervenes between irradiation and onset of leukemia. Thus, it may be that some of the precursor cells of leucocytes lie quiescent for years before they are brought into leucocyte production. A somatic mutation in such a cell might, therefore, be long delayed in producing its effect.

In leukemia of "spontaneous" origin, there is also likely to be a somatic mutation component which would be attributable to "spontaneous" mutation in the somatic cells. In addition, there is likely to be a "hereditary" component in spontaneous leukemia—that is, the presence of defective genes (dominant or recessive) which are transmitted through the germ line and which result in, or predispose toward

the development of, leukemia. It is well known from the work of MacDowell and associates [45] that the pronounced differences among certain strains of mice in susceptibility to leukemia have a genetic basis. In man, there is evidence for familial factors in leukemia from the work of Videbaek [46] and others, but the type of inheritance involved is not clear [47]. It should be noted that cases of leukemia which arise somatically—for example, those which are radiation-induced—will tend to obscure the analysis of the hereditary component in leukemia [48].

It is likely that there will be individual differences in susceptibility to radiation-induced leukemia as well as to "spontaneous" leukemia. The indication of a linear relationship between dose of radiation and incidence of leukemia implies that there are some individuals in whom a single radiation-induced event (perhaps a gene mutation) suffices to produce leukemia. There may, however, be other individuals in whom two or more such events would be required before leukemia would be manifested. Thus, the values of the probability of leukemia per individual per rad per year that have been derived here apply to the "average" individual in a given population, but do not necessarily apply equally to each and every individual in that population.

SPONTANEOUS LEUKEMIA AND NATURAL BACKGROUND RADIATION

The possibility that a portion of the "spontaneous" incidence of leukemia may be due to radiation from natural background sources is briefly considered. For this purpose, the same type of approximation procedures, employed for assessing radiation-induced leukemia among radiologists is applied to the data of MacMahon and Clark on the spontaneous incidence of leukemia in the white population in the borough of Brooklyn (Table 1). Thus, the incidence of leukemia, L_B, that would be attributable to irradiation of that population from natural background sources can be approximated by assuming that it is a product of the following three quantities: (i) a constant dose rate, D_n, from all natural background sources; (ii) the mean age, E_B, of the Brooklyn population, which is equivalent to the mean number of years exposed to D_n; and (iii) the probability of leukemia, P_L per individual per rad per year. The value of D_n is not known but probably is in the range of 0.1–0.2 rad per year [49]. The value of E_B can be readily approximated from the age distribution (Table 1) of the Brooklyn population and is about 33.7 years. The value of P_L is chosen as the "best" estimate from the afore-described radiation studies, namely 2×10^{-6} per individual per rad per year. Thus, L_B can be estimated as 7–13 cases per million per year. The observed total spontaneous incidence in this study was 64.4 cases per million per year [13]. Thus, possibly 10–20% of the "spontaneous" incidence of leukemia in this Brooklyn population is attributable to ionizing radiation from natural background sources.

A maximum value for the probability of radiation-induced leukemia may also be inferred from the Brooklyn data. The calculation of such a value is based on the incidence of acute leukemia, since in this form of the disease the time of onset and time of diagnosis probably nearly coincide, while in chronic leukemia some years

may elapse between these two times. The observed incidence of acute leukemia has a minimum value of 12 per million per year which occurs in the 20–29 age group (Table 1). By assuming that individuals in that age group had an average accumulated dose of not less than 2.5 rad (0.1 rad per year for 25 years) and by further assuming, as an artifice, that all of the acute leukemia in that age group was due to radiation, the probability of acute leukemia may be estimated to have an upper limit of 5×10^{-6} ($12 \times 10^{-6}/2.5$) per individual per rad per year. Since the overall ratio of acute to chronic forms was about 1/1 in the Brooklyn data, it may be inferred that the maximum value, or upper limit (Table 8), of the probability of leukemia (acute and chronic) is about 10×10^{-6} per individual per rad per year.

APPLICATION TO RADIOSTRONTIUM EXPOSURE

The foregoing estimates of the probability of radiation-induced leukemia have been attempted in order to have some basis for assessing direct effects of ionizing radiations on human populations. An example of the application of these estimates to a man-made radiation exposure—namely, that from radiostrontium (Sr^{89} and Sr^{90})—is briefly discussed [50].

The maximum permissible concentration (MPC) of Sr^{90} has been set at 1 microcurie for the total body *for workers with radioisotopes* [51]. A level of 1 microcurie of Sr^{90} per 1,000 g of calcium (the mass of calcium in the average adult individual) has been designated as 1 "MPC" unit of Sr^{90} [52]. Various estimates are at hand for the level of radiostrontium that is being accumulated in the human body as the result of past testing of atomic weapons [53]. The present discussion is restricted to examination of the following recent suggestion for a permissible level (presumably of Sr^{90}) *for the population at large* [54]. "There seems no reason to hesitate to allow a universal human strontium (very similar chemically to calcium) burden of 1/10 of the permissible, yielding 20 rep in a lifetime, since this dose falls close to the range of values for natural radiation background. Visible changes in the skeleton have been reported only after hundreds of reps were accumulated and tumors only after 1500 or more."

A body level of 0.1 MPC is expected to irradiate skeletal tissue at a dose rate of about 0.25 rad per year, on the assumption of uniform distribution of Sr^{90} throughout that tissue. Because of the limited range in tissue of the beta particles emitted in the decay of Sr^{90} and of its daughter element, Y^{90}, the leucocyte-producing cells may receive somewhat less than this dose rate, depending on the exact location of such cells with respect to the surrounding calcium of the bone. This reduction factor, of perhaps 2, tends to be offset by the fact that ingested Sr^{90} is not uniformly distributed throughout the skeletal tissue but appears instead to be concentrated in regions more actively concerned with red-marrow formation [55]. The dose rate to the leucocyte-producing cells is estimated as 0.1–0.2 rad per year for a body level of 0.1 MPC of Sr^{90}. This irradiation will be largely restricted to the skeletal tissue, since (i) the radiation from the decay of Sr^{90} is exclusively of the beta type and (ii) 70% of the Sr in the body

is estimated to lie in the skeletal tissue, [51]. Hence, leukemia induced by Sr^{90} would be expected to be largely of bone-marrow origin [56].

The problem of assessing the incidence of Sr^{90}-induced leukemia from a constantly maintained level of Sr^{90} is essentially identical with that dealt with here for determining the component of the spontaneous incidence of leukemia owing to natural background radiation. Thus, the incidence of Sr^{90}-induced leukemia in a stationary population maintaining a constant level of 0.1 MPC of Sr^{90} is considered to be the product of (i) a dose rate of 0.1–0.2 rad per year to red bone marrow; (ii) a mean age for the stationary population of 31.7 years, which is that expected from the age distribution of the total U.S. white population as of 1 July 1955 [57]; and (iii) a probability of leukemia of bone marrow origin of 1×10^{-6} per individual per rad to bone marrow per year. This computation gives an incidence of three to six cases of Sr^{90}-induced leukemia per million per year. For a population of 1.6×10^{8} individuals, the current population of the United States, the expected number of cases of leukemia induced by a constantly maintained level of 0.1 MPC of Sr^{90} would thus be about 500–1,000 per year. The range for this estimate is a factor of about 3, giving 150–3,000 cases per year. Currently (1954), there are about 10,500 deaths from leukemia per year in the U.S. population [10]. Thus, if Sr^{90} induces leukemia of bone-marrow origin at the same rate (per rad) as X-rays and radiations from atomic bombs, then a constantly maintained level of 0.1 MPC of Sr^{90} would be expected to increase the present incidence of leukemia (in the United States) by about 5–10%.

SUMMARY

Leukemia in man can be induced by ionizing radiations and also occurs spontaneously. For the "average" individual in a population, the probability of developing radiation-induced leukemia is estimated to be 2×10^{-6} per rad (unit of absorbed dose of radiation) per year. The available data from four independent sources make it likely that this estimate is valid within a factor of about 3, giving a range from 0.7×10^{-6} to 6×10^{-6} per rad per year. It is pointed out that 10–20% of the spontaneous incidence of leukemia (Brooklyn, 1943–1952) may result from radiation from natural background sources. It is estimated that a 5–10% increase in the current spontaneous incidence of leukemia would occur if the population were to reach and maintain a body level of Sr^{90} amounting to one-tenth of the "maximum permissible concentration."

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[35] The procedure was to obtain a list of deaths of members of one of the leading radiological societies and to search for the cause of death in the death notices of the *Journal of the American Medical Association*. In the period 1949–1952, inclusive, only some 70% of all such deaths were found to have a cause of death listed. In order to have a conservative estimate of the incidence of leukemia, no correction for

this defect is attempted. The four deaths from leukemia in the 1949–1952 period have the following volume and page locations in the afore-mentioned journal: **144**, 407; **147**, 1065; **148**, 218; **151**, 488.
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Addendum: The confidence limits used in this paper are the 95% asymmetrical limits and they therefore define a 90% confidence interval.

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THYROID RADIATION DOSES FROM FALLOUT

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Absorbed doses of radiation from fallout, when averaged over yearly periods and over large numbers of individuals, have usually been assumed to be well below the corresponding doses from natural background sources. Although this assumption may be valid for most body organs, it is unlikely to have been valid for the thyroid glands of the average infant and child in the United States. The purpose of this note is to point out that for the last few years such thyroids probably have received an annual radiation dose (chiefly, from the beta-rays of radioiodine) that is approximately equal to the annual dose which they receive from natural background radiations.

The radioiodines in fallout (chiefly, iodine-131, with a half-life of 8.05 days) pose a special hazard to infants and children. This arises in part because radioiodine tends to concentrate in fresh cow's milk[1, 2]—a major item in the diet of young people. Upon ingesting milk contaminated with radioiodine, human beings are expected to concentrate the isotope in their thyroids. Moreover, for the same amount of iodine-131 orally ingested, the average infant receives, according to estimates by Halnan and Pochin,[3] about 18 times the thyroid dose that the average adult

[1] See review of cattle radioiodine studies by C. L. Comar, and R. H. Wasserman, in *Progress in Nuclear Energy, Ser. VI* (London: Pergamon Press, 1956), pp. 184–196.

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receives.[4] This 18-fold increase results from the smaller size and possibly somewhat higher iodine uptake[3,5] of the infant thyroid, and from the almost complete absorption of the iodine-131 beta-rays even within the small infant gland. Finally, there is evidence that the thyroid gland of infants and children is especially sensitive to radiation-induced carcinogenesis.[6]

Estimates of fallout doses to thyroids of infants and young children can be made by considering measurements of radioiodine activity levels in milk. Recently, Campbell et al.[7] have published such data for milk samples taken once a month at a sampling station in each of five metropolitan milksheds in the United States. The radioiodine activities of such samples fluctuated widely with time and with locality. For a 1-year period ending June, 1958, the overall average for the five milksheds was 150 μμc per liter. From a more recent report,[8] it may be calculated that for a 16-month period ending September, 1958, the corresponding average had declined to 122 μμc per liter. In order to have an estimate for this 16-month period that takes into account the higher population density in the eastern United States and that avoids giving too much weight to extreme fluctuations from less densely populated areas (namely, from a low of 41 μμc per liter for a milkshed in the far West to a high of 219 μμc per liter for one in the Middle West), the US average for this period is taken as that of a milkshed in the East (New York City). The latter average was 64 μμc per liter, or roughly one half the average for the five milksheds combined.

Continued ingestion of milk contaminated at an average iodine-131 level, L (in μμc per liter), is expected to give at equilibrium a thyroid dose rate R (in rads per year), which can be expressed as follows:

$$R = \frac{0.039 \times L \times C \times U}{M}$$

where C is the average daily consumption of fresh milk in liters; U is the fraction of ingested radioiodine taken up by the thyroid; and M is the thyroid mass in grams.[9]

[4]It is this fact which seems to have been overlooked in evaluating fallout doses of radioiodine. However, the importance of this fact was recognized in the case of the Windscale reactor accident in England; see, e.g., Burch, P. R. J., *Nature*, **183**, 515 (1959).

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Table 1 Evaluation of average thyroid dose rates (R) by age of individual for a 16-month period commencing June, 1957. The estimated range of the average values is shown in parentheses.

Age of Individual	L = Average Milk Iodine-131 Levels, μμc per liter	C = Average Rate of Fresh Milk Consumption (liters per day)	U = Fraction of Radioiodine Taken Up by Thyroid	M = Average Thyroid Mass, (g)	R = Thyroidal Dose Rate (rads per year) $= \frac{0.039 \times L \times C \times U}{M}$
0–1	60 (35–200)[a]	0.3 (0.2–0.6)	0.3 (0.2–0.5)	1.9[b]	0.11 (0.03–1.2)[c]
1–2	60 (35–200)	0.7 (0.5–0.9)	0.3 (0.2–0.5)	2.5	0.20 (0.05–1.4)
2–3	60 (35–200)	0.7 (0.5–0.9)	0.3 (0.2–0.5)	3.4	0.14 (0.04–1.0)
3–5	60 (35–200)	0.7 (0.5–0.9)	0.3 (0.2–0.5)	5.1	0.10 (0.03–0.7)
5–10	60 (35–200)	0.7 (0.5–0.9)	0.3 (0.2–0.5)	8.6	0.06 (0.02–0.4)

[a]Range of average values for five milksheds, corrected to time of milk ingestion
[b]Range of values not known
[c]Range calculated by compounding ranges shown in preceding columns

Rough estimates of the values of these parameters for average infants and children in the United States are shown in Table 1.

Values of L are expected to be lower than the aforementioned observed iodine-131 levels in milk by a decay factor of about 0.9: that is, the elapsed time between collection of the milk sample at the sampling station (the arbitrary reference point for calculating radioiodine activities[7]) and human ingestion is presumably one to two days. This gives a value of L of 60 μμc per liter for the average U.S. value and a range of 35–200 μμc per liter for the five milksheds. Estimates of the values of C are taken as 0.3 liter per day for the first year of life, and 0.7 liter per day thereafter up to age ten.[10] These values are subject to considerable uncertainty and are based on the consideration that the range of values of C is expected to be between one pint and one quart per day, except during the first year of life when fresh milk consumption may well be lower owing to the widespread use of evaporated milk in infant feeding programs.[11] On the basis of measurements on normal infants and children by Oliner et al.,[5] U is taken to be 0.3 (however, Halnan and Pochin[3] estimate a value of 0.45 and some earlier studies summarized by Oliner et al.[5] gave values below 0.3). Estimates of the values of M are based on measurements compiled by Boyd.[12]

From Table 1, it can be seen that the calculated values of R for average infants and children in the United States during the 16-month period commencing June, 1957, depend upon age and vary from 0.06–0.2 rad per year. It should be noted that actual

[10]A recent survey of 312 children under the age of six in suburban Long Island by H. H. Neumann, *Arch. Pediat.*, **74**, 456 (1957), gave a mean value of 840 ml of fluid cow's milk consumed per day with the lower third of the group averaging 647 cc per day and the upper third averaging 1,005 cc per day.

[11]Since evaporated milk will tend to be several months old before it is consumed, its iodine-131 activity should be virtually nil.

[12]Boyd, E., in *Handbook of Biological Data*, ed. by W. S. Specter (Philadelphia: W. B. Saunders, 1956).

thyroid dose rates during this period may have been slightly higher than the calculated values of *R*, since there must also have been a dose contribution from the shorter-lived radioiodines in fallout[13] and from inhaled radioiodine.[14] For each age group the estimated range in values of *R* (shown in parentheses in Table 1) is considerable, depending chiefly upon observed geographical variation in milk radioiodine levels and upon uncertainties as to the true average values for rate of milk consumption and radioiodine uptake. On an individual basis the range in values of *R* would be somewhat greater than that shown in Table 1, since there are expected to be greater individual variations in the values of *L*, *C*, *U*, and *M*. Since the monthly variation in milk radioiodine levels was observed[7] to range from lows of 0 to highs (in two of the milksheds) of over 900 μμc per liter, thyroidal dose rates to infants and children consuming such milk probably fluctuated widely on a short-term basis. For example, on a weekly basis, such dose rates would be expected to have ranged from 0 to about 0.1 rad per week during the 16-month period under consideration. Moreover, dose rates to different parts of the thyroid gland may fluctuate considerably on a short-term basis, owing to the possibility of non-uniform distribution of the radioiodine, as discussed by Oddie.[15]

Prior to June, 1957, data on milk levels of radioiodine activity are not available. However, cattle and human (largely adult) thyroid radioactivity measurements have been related to nuclear testing patterns for part of the year 1954 and for the years 1955 and 1956.[2,16] In addition, the number and timing of such tests during 1957–1958 have been reported in relation to observed iodine-131 levels in milk.[7] On the basis of such information, it appears that for several years prior to 1957 annual thyroid doses to the average infant and child in the United States may have been similar to those calculated above for the 16-month period commencing June, 1957.

Natural background radiations are believed to contribute an absorbed dose to soft tissues of about 0.1 rad per year.[17] From the above account it appears that for the last few years the radioiodines in fallout have contributed an annual dose to the thyroids of average infants and children in the United States that is similar to, or in some circumstances greater than, the annual dose to such organs from natural background radiations.[18]

[13]Dunning, G. M., *Nucleonics*, **14**, No. 2, 38 (1956).

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[15]Oddie, T. H., *Brit. J. Radiol.*, **24**, 333 (1951).

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[17]Spiers, F. W., *Brit. J. Radiol.*, **29**, 409 (1956).

[18]It should be noted that with the cessation of nuclear weapons testing in November, 1958, human thyroid levels of radioiodine should decline exponentially and by January, 1959, there should no longer have been any appreciable contamination of milk with radioiodine from past weapons tests. The level of radioiodine in cow's milk may well be the most important index of short-term environmental contamination with fission products whether from weapons tests or other sources [see discussion in *Safety Aspects of Nuclear Reactors*, ed. C. R. McCullough (New York: D. Van Nostrand Co., 1957), 28].

SUMMARY

On the basis of published data on iodine-131 levels in cow's milk during a 16-month period ending September, 1958, the thyroid glands of average infants and children in the United States are estimated to have experienced over the last few years annual doses of about 0.1 rad to 0.2 rad from the radioiodine in fallout. These doses are roughly one to two times the annual dose to such organs from natural background radiation. For a number of reasons, individual thyroid dose rates from fallout are expected to show wide deviations from the average rate.

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LEUKEMIA, MULTIPLE MYELOMA, AND APLASTIC ANEMIA IN AMERICAN RADIOLOGISTS

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A survey of 425 death certificates of radiologists dying between the ages of 35 and 74 during the years 1948–1961 reveals a statistically highly significant excess of deaths from leukemia, multiple myeloma, and aplastic anemia. That this excess is due to radiation exposure (or to some factor acting in a similar manner), rather than to an artifact of diagnosis is suggested by the absence of deaths ascribed to chronic lymphatic leukemia.

It has never been excluded that the excessive number of deaths from leukemia in American radiologists [1–3] is largely or wholly an artifacts of diagnosis. For example, leukemia might have the same probability of occurrence in a radiologist as in a member of the general male population, but have a higher probability of being accurately diagnosed in a radiologist owing to such factors as his more ready access to medical facilities. A way of testing such a possibility became evident when Court Brown and Doll [4] discovered that one form of the disease, namely, chronic lymphatic leukemia (CLL), is apparently either not induced by ionizing radiation, or is much less readily induced than are the forms of this disease in which other types of cells are affected. Their finding was based on leukemia deaths arising in a group of adult British males who had received X-ray therapy for an arthritic condition (ankylosing spondylitis). Among 28 such deaths only one was reported as due to CLL; yet, as will be discussed below, this type of leukemia is one of the commonest forms of the disease in adult white males. More recently, Pochin [5] cites only one death reported as being

due to lymphatic leukemia (subchronic) among 17 deaths from leukemia arising in a group of adults who had received radioiodine therapy for hyperthyroidism.

Evidently, if radiation (or some other agent acting in a similar manner) is responsible for a rise in the death rates for leukemia, in a given population, the death rate for chronic lymphatic leukemia in that population should rise little, if at all. On the other hand, if diagnosis is responsible, the death rate for CLL should rise proportionately, since diagnosis ought not to affect classification by histological cell type differentially.

This report presents the principal findings of a study designed to answer three interrelated questions. (i) Do excessive numbers of deaths from leukemia continue to occur in American radiologists in recent years? (ii) If so, does the number of deaths from CLL occur in accord with expectation based on radiation or on diagnosis as the responsible factor? (iii) Do excessive numbers of deaths occur in this group from diseases related to leukemia?

To assess the significance of an observed number of deaths from a given cause it is first necessary to determine the composition, with respect to age and size, of the living population that produces such deaths, and then to compute the number of deaths expected in that population had it been subject to the mortality of some standard reference population. The living population of radiologists chosen for study is restricted to those physicians who are listed in the biennial editions of the *Directory of Medical Specialists* (DMS) [6] as being certified by the American Board of Radiology. Punched cards showing name, year of Board-certification, and year of birth were prepared for all such individuals residing in the continental United States and having entries in the 1950 and 1960 editions of these directories [7]. Similar cards, showing also year of death, were prepared for Board-certified radiologists who were known to have died in the study period and whose names appeared in one or more of the DMS editions spanning the years 1948–1960, inclusive. The resulting deck of cards, after elimination of duplicates and of cards bearing female names, provided the basic data for computing the composition, with respect to age, of the living male population as of July 1 of each year of the 14-year period, and for each year of age

Table 1 The percentage composition, according to age, of the population of male radiologists for selected years. The estimated total number of living radiologists between the ages of 35 and 74, inclusive, for these years were: (1950) 2542.0; (1955) 3353.0; (1960) 4571.5 (7).

Age	1 July 1950	1 July 1955	1 July 1960
35–39	27.0	22.8	26.8
40–44	21.2	24.3	19.9
45–49	15.6	17.8	18.6
50–54	11.3	12.1	13.3
55–59	10.3	8.4	8.8
60–64	7.4	6.9	5.6
65–69	5.0	4.8	4.3
70–74	2.2	2.8	2.8

from 35 to 74, inclusive [8]. The results, by 5-year age groups, for representative years, 1950, 1955, and 1960, are shown in Table 1. The estimated number of male radiologists aged 35–74 years, inclusive, increased from 2,167 in 1948 to 4,713.5 in 1961; for the entire 14-year period the number of man years at risk at these ages was 47,348.

The U.S. white male population was the standard chosen for the present study. It is the ultimate population from which the radiologists are drawn and it is the only relevant population for which sufficient data are available to calculate death rates for the rather rare diseases here under study.

Death rates for leukemia and related diseases categories in the U.S. white male population were computed for each 5-year age group between 35 and 74, inclusive, and for as many years of the study period as the data permitted [9] (Fig. 1).

The population of deceased radiologists was identified by two methods; by scanning the death notices in the *Journal of the American Medical Association* for any reference to radiology, and by matching all the names of radiologists entered in the 1950 or 1954 DMS editions with all such names in the 1960 edition [10]. The first method yielded the names of 426 individuals who were listed as Board-certified radiologists in one or more of the DMS editions from 1948 to 1960, inclusive, and whose deaths could be substantiated by means of death certificate sources as having occurred between the ages of 35 and 74, inclusive. The second method yielded the names of two more such individuals; for one, the name appeared in the death notices of the Journal without mention of radiology; for the other, no death notice could be found but a death certificate was located.

Certified copies of death certificates were obtained for all but three of the 428 deaths located in the population under study [11]. Selection of the main cause of death from the group of causes usually listed on the death certificate was then made by following the international rules for coding the cause of death [12].

In Table 2, the observed number of deaths for which the main cause was leukemia or a related disease is compared with the corresponding expected numbers calculated by applying the death rates (with respect to specific age and year of death) in the U.S. white male population for the disease category in question to the number of living radiologists at risk [13]. Also shown are values of the "mortality ratio," defined in the usual way as the ratio of the observed number of deaths from a given cause to the number expected had the population at risk been subject to the mortality experience of the standard [14].

It is evident that the observed 12 deaths from leukemia greatly exceeds the expected number of 4.02. The probability of observing 12 or more deaths under these circumstances can be obtained from the Poisson distribution and has a value of 0.001. The mortality ratio for leukemia is 3.0 (12 : 4.02); however, the limits for such a ratio are wide. The 95% Poisson confidence limits for the 12 observed deaths are 6.2 and 21.0 deaths; it follows that the true mortality ratio for leukemia is not likely to be lower than 1.5 (6.2 : 4.02) nor higher than 5.2 (21.0 : 4.02).

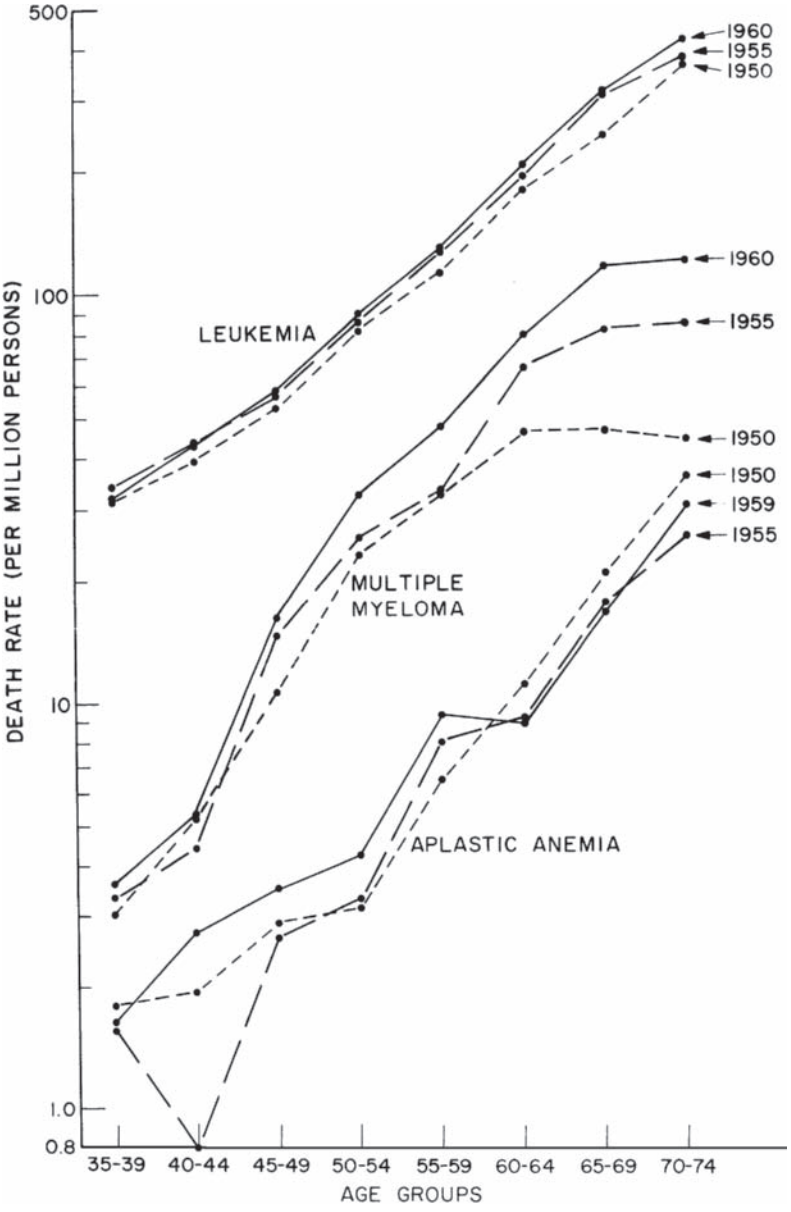

Figure 1. Death rates, with respect to specific age and cause, in the U.S. white male population (deaths per million living persons per year) for selected years of the study period (semi-log plot) [11]. Death rates for aplastic anemia for each age group below age 50, and for multiple myeloma for each group below age 40, are based upon 20, or fewer, deaths.

Table 2 Mortality among radiologists: deaths attributed to cancers of the lymphatic and blood-forming tissues and from aplastic anemia. Only deaths occurring between the ages of 35 and 74, inclusive, in the 14-year period, 1948–1961, are included [12–14, 18].

International code rubric	Principal disease	Number of deaths			Mortality ratio	95%
		Observed	Expected	*P**	(M.R.)	Confidence M.R.
200	Lymphosarcoma	4[a]	2.4	>.05	1.7	0.5–4.3
201	Hodgkin's disease	1	1.6	>.05	0.6	0.02–3.5
202, 205	Lymphoblastoma	1	0.38	>.05	2.6	0.07–14.6
203	Multiple myeloma	5	1.01	.004	5.0	1.6–11.6
204	Leukemia	12	4.02	.001	3.0	1.5–5.2
292.4	Aplastic anemia	4	0.23	.0001	17.0	4.7–44.5

*Probability that the observed number of deaths, or a larger number, would occur by chance
[a] Induded two deaths from lymphosarcoma, one from reticulum cell sarcoma, and one from malignant lymphoma

It becomes important, as already noted, to compare observed and expected numbers of deaths from chronic lymphatic leukemia in the present study population. On the basis of the death rates for lymphatic leukemia in the United States white male population for the years 1949 [15], 1951, and 1956 [9], the only years for which data are available, roughly 4.4 deaths among the 12 leukemia deaths in the population under study are expected to be of the lymphatic type [16]. Although death rates for CLL as such are not tabulated, it is known from studies of MacMahon and Clarke [17] that among adult white males (in Brooklyn) the vast majority of cases of lymphatic leukemia are of the chronic rather than of the acute type. Among the 12 deaths from leukemia in the present study, only one was reported as being due to the lymphatic type and this case was further specified (on the death certificate) to be acute rather than chronic. The failure of any of these 12 deaths to be ascribed to CLL suggests that radiation exposure rather than diagnosis is the principal factor responsible for the excessive number of deaths from leukemia in this population [18]. It is, of course, possible that some other factor (or factors) which acts in the same manner as radiation, is responsible.

For certain lymphomas such as Hodgkin's disease, lymphosarcoma, and lymphoblastoma, the observed numbers of deaths in the population under study occur in reasonable agreement with the expected numbers (Table 2). On the other hand, for both multiple myeloma and aplastic anemia the observed numbers of deaths exceed the expected numbers at levels which, statistically, are highly significant [19].

The excessive number of deaths from aplastic anemia in radiologists (4 observed as opposed to 0.2 expected) parallels a similar finding in the study of Court Brown and Doll [4]; namely, that an excessive number of deaths from aplastic anemia (12 observed as opposed to 0.3 expected) occurred among patients receiving large doses of X-rays to the spine. Although aplastic anemia is not classified as a cancer [12], some cases of the disease are believed to be identical with aleukemic leukemia—a form of leukemia in which there is an excessive number of white cells in the bone marrow but not in the peripheral blood. Upon review of the 12 deaths ascribed to aplastic anemia

in their series. Court Brown and Doll concluded that one-half had been certain or probable cases of aleukemic leukemia.

The excessive number of deaths from multiple myeloma (5 observed as opposed to 1.01 expected) in the population under study has no parallel in other studies of irradiated adults with one exception [20]. That there may be a real association between radiation and an increased risk of multiple myeloma is supported by the similarity of this disease to aleukemic leukemia. Thus, multiple myeloma is characterized by an excessive proliferation of immature plasma cells in the bone marrow without an excessive count of these cells in the peripheral blood; hence, it is sometimes classified as an aleukemic phase of plasma cell leukemia [21].

It is important to consider why, if radiation increases the risk of multiple myeloma, other studies of irradiated groups have not detected such an increase. Reports of multiple myeloma being a cause of death in individuals under the age of 30 are so rare [22] that only studies of adult individuals are likely to yield significant numbers of cases of this disease. Studies of atom-bomb survivors [23] fail to mention multiple myeloma.

However, deaths from this disease are so rarely reported in Japan [24], that few or no deaths would be expected among such survivors even if their death rate from this disease were many times the normal Japanese rate. The failure of Court Brown and Doll [4] to mention this disease in their series of spondylitic patients may or may not represent a real discrepancy with the present findings; it can be inferred that roughly only four or five deaths from multiple myeloma would be expected in their spondylitic series if the age-specific death rates for this disease had increased to the same proportionate extent as did the age-specific death rates for leukemia in that series [25].

Although an association between radiation exposure and increased risk of multiple myeloma is suggested by the present study, it is not established. Additional studies are needed of the incidence of multiple myeloma in other groups of irradiated adults, such as hyperthyroid patients who have received radioiodine therapy [5]. It would also be of value to conduct retrospective studies, along the lines of the ingenious ones of Faber and of Stewart et al. [26]; that is, the histories of exposure to radiation experienced by multiple myeloma patients would be compared with such histories obtained from various "control" groups such as CLL patients [27].

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[7] When year of birth was not stated, it was readily found in the case of every male radiologist by reference to editions of the *American Medical Directory* (American Medical Association, Chicago).

[8] Dates of births were punched to the nearest half year for the decreased group. For the living population it was necessary to assume that a birth was equally likely to have occurred in the first as in the second half of a year. This accounts for the presence of "half-individuals" (Table 1). Any errors due to wrong assignment to sex are likely to be negligible since only 2.8% of the names of Board-certified radiologists in the 1950 and 1960 editions of the *Directory of Medical Specialist* are estimated to be of females.

[9] Death rates were computed by dividing the number of deaths in each 5-year age group (obtained from *Vital Statistics of the United States*, U.S. Govt. Printing Office. Washington, D.C. 1948–59) by the latest mid-year census estimates of the living population of U.S. white males (obtained from *Current Population Reports*, Series P-25, Nos. 98, 246, and 265, US Bureau of the Census, Washington D.C.). The National Vital Statistics Division of the U.S. Public Health Service kindly provided the following unpublished data: deaths of U.S. white males (by 5-year age groups) from leukemia for 1960–1961, from multiple myeloma for 1949–1961, from aplastic anemia for 1950 and for 1953–1959; and deaths of U.S. white males (by 10-year age groups) from lymphatic leukemia for 1951 and 1956.

[10] A third method was used, but yielded no additional names. This involved checking the death notices in unpublished annual bulletins of the American College of Radiology, the majority of whose members are Board-certified radiologists. It remains possible that a few deaths occurred that were not detected by any of the three methods.

[11] In two cases, death certificates were unobtainable because the place of death could not be traced; in the third case, death occurred outside the United States Among the 425 death certificates obtained, four were for deaths occurring outside the continental United States; in none of these four cases, however, was death ascribed to causes under discussion in this report.

[12] *Manual of the International Statistical Classification of Disease. Injuries and Causes of Death*. Fifth, Sixth and Seventh Revisions. (World Health Organization, Geneva, 1938, 1948, 1955).

[13] In this computation the terms of an 8×14 matrix of white male death rates (eight 5-year age groups for 14 years of the study period) were identically multiplied by the terms of a similar matrix of the numbers of living radiologists, and then summed over all 112 products. Death rates were calculated for leukemia for the years 1948–1961 inclusive and for multiple myeloma for the years 1949–1961, inclusive. For all other diseases shown in Table 2, the necessary data were lacking for 1948 and for 1960–1961. For these two time periods, death rates for such diseases were assumed to be the same as the corresponding rates for 1949 and for 1959, respectively. The over-all systematic error introduced by using such substituted rates in computing the expected number of deaths of radiologists is believed to be, at the most, a 3% under- or over-estimation of the true expected numbers.

[14] The value of the mortality ratio for deaths from all causes in the present study population is 0.8. This agrees well with Dublin and Spiegelman's [2] findings for the years 1938–1942, that the class of full-time medical specialists, and the subclasses, thereof, including radiologists, each enjoyed lower death rates from all causes of death combined than did the class of all physicians (or of all white males). (See also reference [3]). For the present study population, it should be remembered that the value of the mortality ratio for any given cause of death is expected to be less than 1.0 if the radiologists were to enjoy as low a mortality rate for that cause as they do for all causes of death combined.

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[16] In this computation the 1949 rates were applied to the years 1948–1949, the 1951 rates to the years 1950–1954 and the 1956 rates to the years 1955–1961.

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[18] For four additional deaths in the study population, the death certificates reported leukemia as a contributory rather than as the underlying or main cause of death; among these four, one was listed as lymphatic (chronicity unspecified) and the other as CLL. With these four deaths added to the 12 valid deaths, the ratio stands at one death with mention of CLL to 15 deaths without mention of this type. For still another death in the study population, the Journal death notice listed the cause as "myelogenous leukemia"; however, a certified copy of the death certificate failed to mention leukemia and ascribed death in this case to another cause.

[19] An analysis (in preparation) of the distribution of deaths in the study population from leukemia, multiple myeloma, and aplastic anemia by age at death and year of death shows (i) that the observed number of deaths in each age group tends to exceed the expected number to the same proportionate extent that the observed total number of deaths in all age groups exceeds the corresponding expected

total number, and (ii) that there is no tendency for the death rates for leukemia and multiple myeloma to decline during the 14-year period; however, for aplastic anemia, all four observed deaths occurred between 1948 and 1953, inclusive.

[20] W. Pohl [*Med. Klin. Munich* **55**, 1839 (1960)] reports three cases of multiple myeloma among German medical technicians with long histories of occupational exposure to radiation: however, the statistical significance of such a finding cannot be assessed because no information was provided on composition, in terms of size and age, of the living population of technicians from which the there cases were drawn.

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[25] In this computation it was assumed that for each 5-year age group in which leukemia deaths occurred in the Court Brown and Doll study, the ratio of leukemia deaths to multiple myeloma deaths would be the same as the corresponding ratios for British males for the years 1950-1952, the only years for which published data (see reference *24*) are available.

[26] M. Faber, in *Transaction of the 6th Congress of the European Society of Haematology* (Karger, New York, 1957), p. S-211; A. Stewart, W. Pennybacker, and R. Barber, *Brit. Med. J.* **II**, 882 (1962).

[27] I thank R. Giesen, M. Hershey and S. Hillyard and especially my wife for technical assistance. I thank F. Lawler for writing the programs used to calculate the age composition of, and the expected numbers of deaths in, the study population. This work was supported in part by an institutional grant from the American Cancer Society (IN-39).

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IONIZING RADIATION AND TUMOR PRODUCTION

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Ionizing radiation has long been a promising agent for studying the mechanism of cancer induction. The dose of radiation delivered to a given tissue can usually be determined with much greater precision than can the dose of a chemical agent. By varying the radiation dose, or dose rate, while holding other factors constant, and then measuring the yield of malignant conditions, the investigator can hope to quantitate the kinetics of the cancer induction process. To be sure, there are many obstacles. For example, to assay for malignancy after exposure of somatic cells to radiation is much more difficult than to assay for chromosomal or gene damage after exposure of germ cells to radiation. Doses as low as several hundred rads, when delivered to the organism acutely, may not only kill large numbers of somatic cells but may disrupt the immunological and hormonal systems in such a way as to modify the yield of malignancies in a complex manner.

I would like to review the current status of our knowledge of dose-response relationships for radiation carcinogenesis in man, and to present some new data on cancer of the blood-forming organs in physicians. At the outset, it should be stressed that one cannot expect with data on human beings to have the degree of precision which can be achieved with data based on animal experiments. In addition, because of inherent differences in such factors as life-span or susceptibility of tissues to cancer induction, it will often be difficult to extrapolate from animal studies to man. Moreover, the detection of malignancies, especially at low doses of radiation, often requires careful examination of thousands of individuals over long time periods. This

can sometimes be done more easily and more economically with human beings than with animals. Finally, the baselines for kinetic studies are the spontaneous frequencies of specific types of cancer. These frequencies are often known with a greater degree of accuracy for human than for animal populations.

REVIEW OF DOSE–RESPONSE RELATIONSHIPS

Leukemia

Populations Receiving Relatively High Doses of Radiation

Court Brown and Doll (1957) were the first to point out that if the relationship between incidence of induced leukemia and radiation dose is linear, then, on the basis of their data, 1 rad to the spinal marrow increases the incidence of this disease by about one case per million persons per year; in other words, the probability of leukemia per rad to the spinal marrow per year is about 1×10^{-6}. This estimate, however, applied to adult males who suffered from ankylosing spondylitis and who received fractionated, partial body irradiations yielding total mean spinal marrow doses of 400 rads or more. Nevertheless, an estimate of the corresponding probability of leukemia, derived from a population of Japanese atomic bomb explosion survivors, turned out to be in good agreement, probably within a factor of two or three, with that from the spondylitic series (Lewis, 1957). Furthermore, the incidence of leukemia in persons in the zone from 1,500 to 2,000 m from the hypocenter was significantly higher, statistically, than in those in the "control" zone lying beyond 2,000 m. However, the average absorbed dose of those in the 1,500–2,000 m zone was unlikely to have been more than about 50 rems. These conclusions were deduced from an analysis of the combined data of Hiroshima and Nagasaki for the period January 1948 to September 1955 (Lewis, 1957).

A review will not be given here of numerous other studies of leukemia risks after relatively heavy exposures of children and adults to ionizing radiation. A particularly comprehensive review is that of Wald et al. (1962). One of the few studies of any magnitude which failed to demonstrate a significant increase in leukemia following heavy exposure to radiation is a recent prospective study by Hutchison (1968) of women who had received radiation therapy for cervical cancer and who were followed in a group of clinics scattered throughout Europe and the United States. Yet, the observed number of cases was small (four), and Hutchison concluded that "for patients at risk in the interval four to eight years after exposure, a fivefold increased risk could be excluded, but a fourfold increase remained possible."

Populations Receiving Relatively Low Doses of Radiation

That absorbed doses well below 50 rads might suffice to induce leukemia first became evident from results of case-control studies of Stewart et al. (1958) for children in England and Wales who had been irradiated in utero. Their findings were fully confirmed by MacMahon (1962), who analyzed a population of 734,243 children in the northeastern United States for the years 1947–1954. From an analysis of all

such retrospective and prospective studies reported by 1961, including those which failed to find a positive association with exposure, MacMahon (1962) showed that the results of every study were compatible with a relative risk factor of 1.4 for the influence of maternal abdominal irradiation on subsequent development of leukemia in the child during the first 10 years of life. Most estimates would place the averaged absorbed dose to the fetus from such irradiation at 1 or 2 rads.

In a three-state study of childhood leukemias, Graham et al. (1966) not only found relative leukemia risk factors for *in utero* irradiations similar to those found in MacMahon's study, but as the result of inquiry into parental preconceptional exposures. Graham and colleagues also discovered an elevated leukemia risk in children whose mothers had received this latter type of exposure. This latter finding, which is compatible with the hypothesis of Stewart (1961) that some childhood leukemias arise as the result of prezygotic mutational or nondisjunctional events, may prove to be of great significance for understanding the etiology of leukemia. It is reminiscent of the elevated risk of mongolism associated with preconceptional maternal irradiation detected in the recent studies of Sigler et al. (1965) and of Uchida et al. (1968), but not, however, in the studies of Carter et al. (1961) or of Schull and Neel (1962).

That the relatively low absorbed doses received by adults in diagnostic irradiation of the trunk (chest and abdomen) may be leukemogenic is suggested by the results of a British study (Stewart et al., 1962), which confirmed and extended earlier results of a Danish study (Faber, 1957; Faber et al., 1958). Both groups of investigators exploited a finding which was most clearly evident in the series of irradiated spondylitic patients studied by Court-Brown (sic) and Doll (1957), namely, that the incidence of chronic lymphocytic leukemia was not markedly increased, if at all, whereas, the incidence of other common forms of adult leukemia, primarily the acute and chronic granulocytic types, was dramatically increased. Faber and coworkers and Stewart and her colleagues therefore compared the histories of diagnostic and therapeutic irradiations given to patients with these latter radiation types of leukemia and to patients with lymphocytic leukemia (or, in the Stewart series, lymphocytic leukemia and lymphosarcoma). Other control groups were also used. Both therapeutic and diagnostic procedures were more common in the radiation histories of the acute and granulocytic series of patients than in the lymphocytic series of patients or in other control groups. Stewart and her colleagues, on the basis of a study of leukemia patients older than 20 years selected during the years 1958–1960, concluded that "about 8% of leukemias other than the lymphatic leukemias were caused by diagnostic X-rays, and a further 3.6% by therapeutic X-rays." Gunz and Atkinson (1964) failed to find evidence associating higher leukemia risk with prior diagnostic radiation exposures in a similar study made in New Zealand; however, their results are still compatible with the risks found in the Danish and British series.

In a recent report of a prospective study of leukemia arising after radioiodine and/or surgical therapy for hyperthyroidism, Saenger et al. (1968) found a significantly higher mortality from leukemia than would have been expected on the basis of mortality rates from this disease in the general population. These investigators are inclined to favor the hypothesis that hyperthyroidism per se increases the risk of

leukemia since a higher mortality rate from leukemia was found in both ^{131}I-treated patients and those treated surgically. Judgment must be withheld until the pattern of frequencies of other neoplasms related to leukemia, such as lymphosarcoma, has been reported in this series of patients.

The earlier studies of Pochin (1960) and of Werner et al. (1961) called attention to the excess of cases of the radiation type following radioiodine therapy for hyperthyroidism. This excess may be caused, in part at least, by an artifact arising from the exclusion of cases prevalent in the patients at the time of irradiation. The prevalent cases, or those present at the time of therapy, tended, of course, to be of the chronic types; there is, therefore, a deficit of chronic lymphocytic types in the incident cases, or those which arise after therapy.

Some Factors Affecting the Dose–Response Relationship

It was noted some years ago that the probability of leukemia per individual per rad may not be constant for an indefinite period beyond the initial time of irradiation (Lewis, 1957). MacMahon (1962) estimates that the elevated risk of leukemia peaks at 5–7 years after *in utero* irradiation and may be exhausted by age 8. There is evidence that the increased risk of leukemia in older individuals extends over longer periods of time. i.e., up to 15 years and perhaps longer in the case of Japanese survivors of the atomic bomb explosion (Bizzozero et al., 1966), but the risk probably declines after 10–15 years, as judged by the experience of both the Japanese survivors and the irradiated spondylitic patients (Court Brown and Doll, 1965).

It was also pointed out some years ago (Lewis, 1957) that there are likely to be individual differences in susceptibility to radiation-induced leukemia, just as there are to spontaneous leukemia. "The indication of a linear relationship between dose of radiation and incidence of leukemia implies that there are some individuals in whom a single radiation-induced event (perhaps a gene mutation) suffices to produce leukemia. There may, however, be other individuals in whom two or more such events would be required before leukemia would be manifested. Thus, the values of the probability of leukemia per individual per rad per year . . . apply to the average individual in a given population, but do not necessarily apply equally to each and every individual in that population" (Lewis, 1957). That the age of the individual may be an important variable is evident from several studies. Doll (1962) analyzed the observed relationships between age and sensitivity to radiation-induced leukemia in spondylitic patients and atom bomb survivors. He showed that sensitivity to radiation-induced leukemogenesis tends to have an age dependence like that seen in the natural incidence of the disease.

The probability of leukemia per rad per year appears to be higher, perhaps by a factor of 10–20, following irradiation of a fetus (assuming an average dose from pelvimetry of 1–2 rads) than following irradiation of children, for example. However, as already noted, the duration of the elevated risk following the time of irradiation is evidently less, by a factor of at least two, for a fetus than it is for children or adults. The well-known childhood peak in leukemia mortality (approximately 70 deaths per million per year between ages 3 and 5 in white children) (Fraumeni and Miller, 1967)

may be a reflection of the special sensitivity of the fetus to whatever leukemogenic factors are involved in determining the natural incidence of the disease. High fetal sensitivity to radiation-induced leukemogenesis may therefore be another example of Doll's rule.

Other Types of Cancer

Simpson et al. (1955) compared children given partial body irradiation in infancy for an enlarged thymus condition with untreated siblings. This study provided the first evidence that doses of only a few hundred rads applied in infancy suffice to increase significantly the incidence not only of leukemia but also of thyroid tumors and benign bone tumors. Further follow-up of these children and of others has been done by Pifer et al. (1963), and more recently by Hempelmann et al. (1967). The risk of leukemia from such irradiation appears to have disappeared by age 20; however, the risk of developing other malignancies or benign tumors has tended to remain elevated beyond this age. Hempelmann (1968), in a review of a variety of groups of children receiving various kinds of thyroid exposure to ionizing radiation, concluded that if there is a threshold dose for the induction of thyroid nodules, it is below 20 rads. Risk estimates for thyroid carcinoma, however, are based on so few cases that they are to be regarded as provisional. Beach and Dolphin (1962) estimate that the risk is one or two cases per million persons per rad yer year averaged over a 20-year period following irradiation in infancy.

In the aforementioned studies of Stewart et al. (1958) and of MacMahon (1962), irradiation of the fetus was shown to be associated with an elevated risk of the child developing not only leukemia, but a variety of other childhood cancers as well. The relative risk factor for all such other cancers combined was 1.4, or the same as that found for leukemia (MacMahon, 1962).

Three recent studies indicate that the incidence of breast cancer and of lung cancer (Wanebo et al., 1968a, 1968b) and of thyroid cancer (Wood et al., 1969) are significantly elevated in Japanese atom bomb survivors who were within zones of heavy irradiation (0–1,500 m from the hypocenter).

Court Brown and Doll (1965) have reported a further follow-up of their series of irradiated spondylitic patients; they found significantly elevated death rates, not only from leukemia, but also from other cancers of organs in or near the radiation fields. For a period of 10–15 years following irradiation, the leukemia death rates in these patients averaged roughly 10 times the expected rates before declining to nearly normal levels. For at least 20 years after irradiation, death rates from other cancers of heavily irradiated organs have gradually increased to a level between two and three times the normal death rates.

There is increasing evidence that radiation exposure is associated with proportionately higher mortality rates from leukemia than from other neoplasms of the lymphatic and hematopoietic systems (LHS neoplasms). LHS neoplasms are defined here as those diseases which are given the code rubrics 200 through 205 in the Sixth and Seventh Revisions of the *Manual of the International Statistical Classification of Disease, Injuries,*

and Causes of Death (1948, 1957). These neoplasms are comprised of lymphosarcoma (200), Hodgkin's disease (201), lymphoma (202), multiple myeloma (203), leukemia (204), and mycosis fungoides (205).

Court Brown and Doll (1965) showed that the ratio of observed to expected deaths from leukemia in patients therapeutically irradiated for spondylitis was approximately nine (based on 60 deaths occurring over a 20-year follow-up period) compared to a ratio of approximately two (based on 11 deaths) for observed to expected deaths from other LHS neoplasms. Studies of Japanese survivors of atomic bomb radiations show that the prevalence of LHS neoplasms other than leukemia, as measured at autopsy, is increased in heavily exposed survivors, as compared to that in lightly exposed or unexposed control groups (Anderson and Ishida, 1964). However, the increase in prevalence is proportionately much less for such neoplasms than it is for leukemia. The studies of American radiologists discussed below are consistent with these findings. However, mortality from leukemia is relatively much less in radiologists than it is in the heavily exposed Japanese or spondylitic populations.

CHRONIC OCCUPATIONAL EXPOSURE TO RADIATION

Groups of healthy individuals who receive relatively low doses of radiation from chronic occupational exposure provide an excellent opportunity to assess cancer risks in the sense that such individuals should experience less cellular destruction and less disturbance to their hormonal and immunological systems than individuals who receive radiation for therapeutic reasons. Radiologists and other medical specialists exposed to radiation provide such occupational groups and the class of all medical specialists not using radiation, or using it on a limited basis, provides one kind of control group. Moreover, for the parent population, namely U.S. white males, death rates by cause are well defined. The principal difficulties in studying occupationally exposed groups stem from uncertainties about the actual absorbed dose levels. In the future, the frequency of chromosomal rearrangements scored in marrow and peripheral blood by standardized methods may serve as a valuable, if crude, biological dosimeter for assessing the accumulative exposure dose (see Court Brown et al., 1965).

Dublin and Spiegelman (1948) showed that for the years 1938–1942, age-specific mortality rates for physicians listed as specialists in radiology were lower than the corresponding rates for all physicians but somewhat higher than the corresponding rates for the class of all medical specialists. Seltser and Sartwell (1965) studied a longer time period and compared members of a radiological society with members of two other major specialty societies, one of pathologists and one of ophthalmologists and otolaryngologists. They found that mortality rates were highest in the radiologists; however, they noted that the opthalmologists and otolaryngologists appear to have exceptionally low mortality rates.

That American radiologists appeared to experience more deaths from leukemia than would be expected was one of the first observations indicating the carcinogenic effect of chronic exposure to ionizing radiation (March, 1944). Dublin and

Spiegelman (1948) were the first to take account of marked differences in age composition between the class of all radiologists and that of specialists. After correcting for such age differences, these investigators showed that leukemia mortality was significantly elevated in radiologists during the 5-year period investigated, 1938–1942.

An excessive number of deaths from leukemia relative to the number expected on the basis of U.S. white male age-specific rates has been shown in radiologists certified by the American Board of Radiology for a more recent time period, namely, the 14-year period from 1948 to 1961 (Lewis, 1963). In this study, it was shown that the excessive mortality from leukemia is unlikely to have been solely the result of better medical diagnosis of the disease in radiologists than in the general population. The operation of such a differential diagnostic factor might have been expected to elevate deaths from the various types of leukemia and from the related LHS neoplasms in the same relative proportions with which these diseases are observed in the general population. Instead, the excessive number of deaths from leukemia in radiologists was confined to leukemias of the radiation type and, although there was an excess of deaths among the lymphomas, the excess was not statistically significant. However, in the case of multiple myeloma, a statistically significant excess of deaths was observed; five deaths from this disease were observed whereas only 1.01 was expected on the basis of U.S. white male rates. Pohl (1960) reported three cases of multiple myeloma occurring among medical personnel after occupational exposure to radiation. The reported fourfold increase in prevalence of multiple myeloma found at autopsy in individuals who had been heavily exposed at Hiroshima appears to be based upon only one case (Anderson and Ishida, 1964).

To what extent can the percentage of all deaths from LHS neoplasms attributed to leukemia be used as an indicator of leukemogenic factors operating on a population? It has seemed desirable to explore this question in other groups of medical specialists who might experience considerable occupational exposure to radiation, although presumably less than that experienced by radiologists. To establish a baseline for such studies, it becomes imperative to determine the pattern of frequency of LHS neoplasms in the class of all medical specialists.

Leukemia and Related Diseases in Physicians

In order to study the pattern of LHS neoplasms in groups of physicians, death certificates were sought for all physicians whose obituary notices in the *Journal of the American Medical Association* reported under cause of death either an LHS neoplasm or certain related diseases, namely the following or their synonyms: polycythemia, myeloid metaplasia, and anemias, including aplastic anemia. Death certificates were also sought for every physician who was listed in the obituary notices as having been a diplomate of the American Boards of Radiology (ABR), Dermatology (ABD), or Urology (ABU).

The limited scope of the present study prevented making a search for death certificates for physicians who were diplomates of American Specialty Boards other than ABR, ABD, and ABU. Previous experience gained in determining causes of deaths

occurring in Board-certified radiologists (Lewis, 1963) showed that some deaths from LHS neoplasms occurred in which the *Journal* notice either failed to mention cause of death or, as in a few cases, ascribed death to an ill-defined cause such as cancer or uremia. Therefore, to obtain a more nearly complete compilation of deaths from LHS neoplasms in other specialty groups, a third procedure was followed. Death certificates were sought for all persons for whom the *Journal* notice specified such specialty boards but either failed to mention cause of death or ascribed death to one of the following ill-defined categories: uremia, sarcoma, carcinoma, or malignancy. For deaths occurring from ages 35–74, there were 1,187 entries of this type in the 17-year period of the study (1948–1964). A search for copies of the death records in these cases yielded 1,169 death certificates, comprising 1,045 for which the *Journal* notice mentioned no cause and 107 for which the cause was listed as cancer and 17 as uremia. This supplementary procedure identified in the other specialty groups 16 leukemia deaths, 16 deaths from lymphomas, one death from aplastic anemia, and none from multiple myeloma.

These procedures for locating death certificates of physicians who died in the 1948 to 1964 period at ages 35–74 are expected to yield virtually complete coverage of deaths from LHS neoplasms in the three specialty boards, ABR, ABD, and ABU. For the remaining specialty boards, it is estimated from experience gained in studying the ABR, ABD, and ABU groups that about five (and not more than 30) more deaths from LHS neoplasms may have occurred. Although the *Journal* notice sometimes reports the immediate rather than the underlying cause of death, this occurs rarely in the case of LHS neoplasms, so far as can be judged by the ABR, ABD, and ABU groups.

A few deaths were traced for which the *Journal* notice failed to mention certification by a specialty board. Such deaths were located by tracing the names of specialists listed in the 1950 edition of the *Directory of Medical Specialists* but omitted from one or more later editions. Editions of this *Directory* were also used as the source for validating the information on specialty contained in the *Journal* notice.

The follow-up of the class of all physicians not listed as diplomates of American Specialty Boards is the least complete, since no effort was made to locate death certificates in those instances in which no cause or an ill-defined cause of death was reported in the obituary notices. Since seven of 79 deaths from LHS neoplasms in the ABR, ABD, and ABU groups had either no cause or an ill-defined cause cited in the *Journal* notice, it may be roughly estimated that the coverage of deaths from LHS neoplasms in all physicians other than Board-certified specialists is roughly 90%, if a proportionate degree of reporting of cause in *Journal* notices occurred in such physicians.

Simple tabulations of the number of deaths from specific LHS neoplasms would be meaningless for comparative purposes, since the different study populations do not have the same composition with respect to age, and since the degree of ascertainment of deaths varies in the different groups as noted above. Moreover, the actual age composition is not known with sufficient accuracy to justify calculation of death rates for either nonspecialty or specialty groups, except for Board-certified radiologists for

the years 1948–1961 (Lewis, 1963). It is therefore necessary to make age- and year-specific adjustments before making any comparisons of populations. It is believed that the following procedure adequately achieves these adjustments. An expected number of deaths from leukemia, for example, was calculated by assuming that the group of physicians would experience the same age- and year-specific proportions of deaths from this cause among all deaths from LHS neoplasms as would all U.S. white males. That is, for each of the eight 5-year age intervals from 35 to 74 years, and for each of the 17 years of the study period, such proportions were calculated. In other words, the number of leukemia deaths (International code rubric 204) in U.S. white males divided by the number of all deaths ascribed to LHS neoplasms in such males (code rubics 200 through 205) were calculated from published sources of data. The resultant 8×17 matrix was then identically multiplied by a corresponding matrix containing in each cell the observed number of deaths from all LHS neoplasms in the population of physicians under study. Summation of all cells yielded the expected number of deaths from leukemia for the study period. A comparison of observed with expected numbers of deaths calculated in this way is shown in Table 1. In making similar calculations for multiple myeloma, unpublished data on deaths from this cause in U.S. white males for years 1949 through 1962 were made available by the National Center for Health Statistics. Deaths from multiple myeloma for the year 1949 were assumed applicable to the year 1948, those for 1962 were assumed applicable to the years 1963 and 1964.

It is evident that observed percentages of deaths from the three major groupings of LHS neoplasms, i.e., leukemia (code rubric 204), multiple myeloma (203), and other lymphomas (200 to 202), are not greatly different from expected percentages. (No deaths from the rare mycosis fungoides [code rubric 205] were recorded in any of the series). In both the class of specialists and the class of physicians not belonging to a

Table 1 Comparisons of observed and expected numbers of deaths from LHS neoplasms in specified groups of physicians who died at ages 35–74 during the 17-year period, 1948 to 1964[a].

Study Population		Number of deaths							
		Leukemia		Multiple myeloma		Lymphomas[b]		All LHS neoplasms	
		No.	%	No.	%	No.	%	No.	%
American Board specialists	Observed	89	49.7	23	12.9	67	37.4	179	100.0
	Expected	77.0	43.0	20.1	11.2	81.9	45.8	179.0	100.0
All other physicians (M.Ds)	Observed	187	51.0	43	11.7	137	37.3	367	100.0
	Expected	163.6	44.6	42.3	11.5	161.1	43.9	367.0	100.0

[a]Expected numbers of deaths from a stated cause are relative, being based upon the number of deaths that would have occurred had the physician group experienced the same age-specific and year-specific proportions of deaths from that cause among deaths from all LHS neoplasms as did the U.S. white male population. The estimated percentage of follow-up of all deaths that may have occurred from LHS neoplasms is 97% for the American Board specialists and 90% for the group of all other U.S. physicians

[b]Lymphosarcoma, Hodgkin's disease, and lymphoma

Abbreviation: LHS, lymphatic and hematopoietic systems

Table 2 Observed numbers of deaths of physicians who were diplomates of specified American specialty boards and for whom the underlying cause of death as stated on the death certificate was one of the specified hematological diseases[a].

Underlying cause of death	American Board of Radiology	American Board of Dermatology	American Board of Urology
Leukemia	13(5)[b]	6(2)	7
Multiple myeloma	5	1	1
Lymphomas[c]	7	0	1
Aplastic anemia	5	0	1
Polycythemia vera	0	1(1)	0
Total	30(5)	8(3)	10

[a]The tabulation includes only deaths occurring at ages 35–74 for the 17-year period, 1948–1964
[b]Numbers in parentheses indicate additional deaths which are nonvalid for statistical purposes since the specified hematological disease was listed on the death certificate as a contributory, rather than as the underlying cause of death
[c] Lymphosarcoma, Hodgkin's disease, and lymphoma

specialty there were proportionately more deaths from leukemia than expected. The presumption that this reflects occupational exposure to radiation can be tested readily, however, only for the specialists.

The excess number of deaths from leukemia in the class of all American Board specialists can be attributed, in part at least, to an excess of deaths from this cause in the three major specialty groups ABR, ABD, and ABU. The breakdown of LHS neoplasms, as well as of the two related diseases aplastic anemia and polycythemia vera, in these specialty groups is shown in Table 2.

In the case of ABR specialists, it was shown (Lewis, 1963) that the observed numbers of deaths from leukemia and multiple myeloma for the years 1948–1961 exceeded the expected numbers at statistically significant levels, U.S. white male age- and year-specific death rates were used as a basis for calculation. On the basis of preliminary estimates of the age composition of the living population of dermatologists at risk during each year of the 1948–1964 study period, the observed number of deaths from leukemia in this group significantly exceeds the expected number calculated on the basis of U.S. white male age-specific death rates for this disease. Judgment must be withheld on whether the death rate for leukemia is significantly elevated in the case of urologists, however, until the age composition of the living population at risk is evaluated.

It is of interest that Dublin and Spiegelman (1948), in a study of mortality rates during the 5-year period 1938–1942, called attention to a possibly elevated risk of death from leukemia in a population of full-time specialists in dermatology. These investigators did not single out urologists as a separate group of specialists and therefore their study did not present estimates of risk in such individuals.

A clear indication of excessive mortality from leukemia, relative to mortality from all causes of death, is evident for dermatologists and urologists, as well as radiologists. This is shown in Table 3, where the observed number of deaths from leukemia in each 5-year age group is compared with an expected number calculated on the assumption

Table 3 Comparison of observed and expected numbers of deaths from leukemia occurring at ages 35–74 in a 17-year period from 1948–1964 among diplomates of specified American specialty boards[a].

	American Board of Radiology			American Board of Dermatology			American Board of Urology		
		Leukemia deaths			Leukemia deaths			Leukemia deaths	
Age interval (yr.)	All deaths[b]	Obs.	Exp.	All deaths[b]	Obs.	Exp.	All deaths[b]	Obs.	Exp.
35–39	17	1	0.21	5	2	0.06	4	2	0.05
40–44	26	1	0.25	13	1	0.14	13	1	0.14
45–49	50	1	0.39	16	0	0.14	19	1	0.16
50–54	54	1	0.39	17	0	0.13	27	1	0.21
55–59	102	2 (1)[c]	0.70	30	0	0.22	39	0	0.28
60–61	78	2 (1)	0.55	37	1	0.28	50	1	0.38
65–69	105	2 (2)	0.76	25	1	0.19	48	0	0.36
70–74	98	3 (1)	0.66	33	1 (2)	0.24	46	1	0.33
Totals	530	13 (5)	3.91	176	6 (2)	1.40	246	7	1.91

[a]Expected deaths are those which would have occurred had the same age- and year-specific proportions of deaths from leukemia among deaths from all causes which characterized the U.S. white male population been applicable to the specialist population

[b]This column contains the observed number of deaths in the specialty group from all causes, including leukemia

[c]The numbers shown in parentheses are additional deaths from leukemia which are nonvalid for statistical purposes since leukemia was listed on the death certificate as a contributory, rather than as the underlying cause of death

Abbreviations: Obs., observed; Exp., expected

that the specialty group experienced the same proportion of deaths from leukemia among deaths from all causes as did US white males. In the ABD group, the ratio of observed deaths from leukemia to expected deaths calculated in this way was 6 : 1.4; in the ABU group, the corresponding ratio was 7 : 1.91.

The 26 observed deaths from leukemia in the ABR, ABD, and ABU groups were ascribed to a variety of types, but chronic lymphocytic leukemia was not mentioned in any case as the underlying cause of death. Two deaths in the ABR group were ascribed simply to "leukemia," one death in the ABD group to "lymphatic leukemia" (chronicity unspecified), and one death in the ABU group to "chronic leukemia" (cell type unspecified). Aside from these possible instances of chronic lymphocytic leukemia, the preponderance of radiation types of leukemia in these specialty groups is supporting evidence for the conclusion that the excessive number of deaths from leukemia in the ABD, ABU, and ABR groups is the result, at least in part, of occupational exposure to ionizing radiation. Finally, additional supporting evidence is provided by the relative rarity of deaths from lymphomas in these specialty groups in relation to deaths from leukemia, as shown in Table 2. Thus, for populations with approximately the same age composition as these specialty groups, the ratio of leukemia to lymphoma deaths is expected to be about 1 to 1 (cf. 43.0% leukemias to 45.8% lymphomas expected in the group of all Board specialists; or 44.6–43.9% in the group of all other physicians) (Table 1). In fact, the ratio was 13 : 7 for radiologists, 6 : 0 for dermatologists, and 7 : 1 for urologists.

SUMMARY

The probability of a tumor per unit dose of radiation per year seems closely dependent on age, so far as can be estimated from a review of studies of radiation-induced carcinogenesis in man. The average value of this probability for leukemia is on the order of one to two cases of this disease per million persons per rad to the marrow per year during childhood and early adult life in white populations (or, apparently, during all ages in Japanese populations). The value, however, is estimated to be 10–20 cases per million per rad per year during fetal stages in white populations. It may be inferred from Doll's analysis (1962) that this value may be reached again, or exceeded, at least in white males, over 60 years. These estimated values of the probability of leukemia are apparently applicable for only 8–10 years if irradiation is given during the fetal stage, but may apply for 10–20 years if irradiation is given at any time after birth.

New data are presented which define the pattern of mortality from LHS neoplasms in groups of medical specialists and other physicians. Three groups of specialists, radiologists, dermatologists, and urologists, have a remarkably aberrant pattern of such neoplasms; i.e., a disproportionately large number of leukemia deaths (and possibly multiple myeloma deaths, as well) occur in relation to deaths from lymphomas. In the case of radiologists and dermatologists, at least, it is likely that this aberrant pattern is the result of occupational exposure to radiation.

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LEUKEMIA, RADIATION, AND HYPERTHYROIDISM

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Saenger et al. (Letters, 19 Mar.) argue that hyperthyroidism per se is responsible for the statistically significant increase in leukemia death rate which they observed in a *combined* group of 18,379 radioiodine-treated (RAI) and 10,731 surgery-treated (SUR) hyperthyroid patients [1]. For the years 1946–1964 this increase amounted to 1.5 times the expected death rate calculated on the basis of the experience of the U.S. general population. I have examined the origins of this increase by comparing observed numbers of leukemia deaths in each patient group with the corresponding expected numbers calculated on the basis of United States age-, sex-, year-specific leukemia death rates [2]. The results show that the excessive leukemia death rate is largely confined (i) to patients who were over 50 years at the time of treatment; (ii) to patients with the acute rather than the chronic form of the disease; and (iii) to the RAI rather than the SUR group. Moreover, in RAI patients there is a statistically significant excess of acute leukemia deaths [2] occurring between ages 50 and 79 (namely, 9 observed versus 3.6 expected; $P = .01$); whereas, in SUR patients, although there is an excess of such deaths between ages 50 and 79 (4 observed versus 2.5 expected), the excess is not statistically significant [3].

The experience of the RAI patients suggests, but of course does not conclusively establish, that doses to bone marrow which are estimated to be 7–13 rads [1] and which are delivered at relatively low rates can induce acute leukemia at a relatively high rate per rad in adults over 50 years. This may not be so surprising as it seems, since not only is acute leukemia the type that predominates after radiation exposure, but Doll

[4] has shown that in a group of adult spondylitic patients in Great Britain radiation-induced leukemia rates (standardized for a constant dose) increase exponentially with age in the same way that spontaneous rates in that country increase with age. Doll's findings and the data of Saenger et al. are in agreement in suggesting that the sensitivity to radiation-induced leukemia of older adults in Western countries may reach or exceed the relatively high sensitivity [5] which characterizes the fetal stage in such countries.

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[2] Based in part on unpublished data on the age-composition of each group supplied by Saenger et al. and unpublished tabulations of acute leukemia deaths (under rubric 204.3) in the United States supplied by the National Center for Health Statistics.
[3] Saenger et al. [1] only attempted a comparison of the leukemia experience of the RAI group with that of the SUR group after adjusting for age by the so-called direct method. This method lacks the power to detect even large differences between the two groups, since the number of deaths from acute leukemia, especially in the SUR group, is too small to calculate meaningful age- and sex-specific rates.
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ANALYSIS OF LUNG TUMOR MORTALITY IN THE BATTELLE BEAGLE LIFESPAN EXPERIMENT*

Forty beagles in the Battelle group between 12 and 43 months of age (mean age = 562 days) were given "single, 10–30 minute inhalation exposures to $^{239}PuO_2$ aerosols via a mask" [52]. Eighteen of the original 40 dogs died with lung tumors as the primary cause of death. Seventeen died of other causes, primarily pulmonary fibrosis, and in nine of these lung tumors were in evidence even though they had not developed to the point of causing death. Finally, five dogs were sacrificed for analysis of tissue distribution of plutonium. Sacrificed animals were asymptomatic for lung tumors and none had lung tumors at autopsy. Details of the experimental procedures and results are given by Park et al. [52].

In order to use the Battelle beagle experiment to test the Cochran and Tamplin Hot Particle Hypothesis it is necessary to assess the lung tumor mortality rate in these beagles in relation to the estimated number of hot particles deposited in their lungs. It must first be noted that the Cochran and Tamplin risk factor of 1/2000 per hot particle is a risk of death from lung cancer. Thus, Cochran and Tamplin used cancer death risks given in the BEIR report [68] to calculate their estimate that 1/1000 is the lung cancer death risk which would result from continuous lung exposure at the current maximum permissible level for workers. Since they went on to calculate that such a risk would be generated by two hot particles with a risk of 1/2000 each, it is

*Prepared for the Committee's use by E. B. Lewis

Table A.II-17 Survival table for the Battelle group of beagles.

(1) x Time in days after inhalation of $^{239}PuO_2$	(2) n_x No. of dogs alive at the start of the day, x	(3) d_x No. of dogs dying of lung cancer during the day, x	(4) P_x Cumulative probability of survival at the end of day, x	(5) m_x[a] Calculated mean number of lethal lung cancers per dog alive at end of day, x
1629	24	1	0.958	0.043
1635	23	1	0.917	0.087
1823	20	1	0.871	0.138
2211	16	1	0.816	0.203
2229	15	1	0.762	0.272
2341	14	1	0.708	0.346
2356	13	1	0.653	0.426
2412	11	1	0.594	0.521
2565	10	1	0.534	0.627
2792	9	1	0.475	0.744
2809	8	1	0.416	0.878
3079	7	1	0.356	1.032
3313	6	1	0.297	1.214
3441	5	1	0.238	1.438
3537	4	1	0.178	1.725
3664	3	1	0.119	2.131
3676	2	1	0.059	2.824
4068	1	1	0.000	—

[a] $m_x = (-)\log_e(P_x)$

clear that the latter figure is a risk of death from lung cancer, as opposed to a risk, for example, that an individual will have an incipient lung cancer developing.

For the purposes of the present analysis, the risk of beagles in the Battelle study dying from lung cancer will be assessed for a risk period extending from the time of the initial exposure to the aerosol to 3,600 days thereafter. At the end of the risk period the animals would be expected to have averaged 11.5 years of age, since the average animal was 562 days of age at the time of initial exposure. Although the mean life span is not known accurately for the normal unirradiated beagle in the Battelle colony, 11.5 years is probably a reasonable estimate.

Two methods will be used to derive the accumulated risk of death from a lung cancer in the Battelle group at 3,600 days postinhalation. The first method involves construction of a survival table and analysis of the cumulative proportion of survivors at 3,600 days. The results are shown in Table A.II-17. Without any assumptions about the nature of the cancer induction process, the estimated cumulative probability of dying from lung cancer, Q_x, at x days after inhalation of the aerosol can be derived from the relationship:

$$Q_x = 1 - P_x \tag{1}$$

where P_x is the cumulative probability of surviving x days after inhalation of the aerosol.

It is evident from Table A.II-17 that for the animal that died of lung cancer at 3,537 days postinhalation, the probability of dying from lung cancer was high enough, 0.82 (1-0.178), to make it likely that the animal could have had more than one primary lethal lung cancer. A lethal lung cancer is defined as one that has developed to the point at which it is capable of causing the animal's death.

The probability of dying from lung cancer is more strictly the probability of dying from at least one lethal lung cancer. Since cancers are expected to arise as rare independent events it is appropriate to use the Poisson distribution to estimate the frequency of multiple primary cancers. It should be noted that the actual number of primary cancers cannot be directly observed since multiple cancer foci may result either from metastases of a single primary cancer or from multiple primary cancers.

The probability of surviving to x days without a lung cancer is given by the first term of the Poisson distribution, e^{-m} or [exp(−m)], where m is the mean number of lethal lung cancers that the average animal in the population would possess at x days. It follows that the cumulative probability of dying from at least one lung cancer at x days is

$$Q_x = 1 - [\exp(-m_x)] \tag{2}$$

Combining Equations 1 and 2 gives

$$m_x = (-)\ \log_e(P_x) \tag{3}$$

Values of m calculated in this way are shown in column 5 of Table A.II-17. For the animal that died at 3,537 days postinhalation the value of m is 1.7. The next death occurred at 3,664 days, for which time the corresponding value of m is 2.1 cancers. At 3,600 days, therefore, the average number of lethal cancers per animal would have been approximately two.

For the purpose of making comparative risk evaluations, it becomes essential to determine also the rate at which the beagles died of lung cancers as a function of the duration of risk; that is, the elapsed time, t, since the initial day of exposure to the radioactive aerosol. For this purpose a life table method of analysis was chosen, since this method has considerable power to dissect the time course of tumor development even when, as in the present case, there are relatively small numbers of animals at risk [69]. The probability of a beagle in the Battelle group dying from lung cancer, q_x, in a given interval (arbitrarily, 100 days in length) is found to be adequately expressed in terms of a simple power function of t, namely:

$$q_x = a(t)^b \tag{4}$$

where a and b are constants. Actually the analysis has been carried out using the more precise relationship,

$$q_x = 1 - \exp[-a(t)^b] \tag{5}$$

where the quantity, $a(t)^b$, is equivalent to m_x of Equation 2 and can be thought of as a rate, R_m, at which lethal lung cancers develop in a given interval. For a sufficiently

small interval, q_x will in fact be equivalent to R_m for all practical purposes, and in the present case it turns out that a choice of an interval of 100 days in length satisfies this condition. By analogy with Equation 2, Equation 5 allows for the contingency that no matter how small the interval in the life table there is a finite chance that more than one lethal lung cancer will develop in that interval.

Briefly, the method of fitting the constants involved use of a computer to generate a life table for each pair of values of a and b to be tested and then to test goodness of fit between observed and expected numbers of lung cancer deaths by the Chi-squared criterion, first grouping such numbers into six successive 800-day intervals. In this way the values of a and b that result in a minimum values of Chi-squared are found to be 9.0×10^{-15} and 3.2, respectively, for t expressed in days. The life table based on these values is shown in Table A.II-18 and the resultant Chi-squared value is 3.8, which for three degrees of freedom is not statistically significant ($P = 0.3$). Even when a finer grouping into 400-day intervals is used, in none of the 12 intervals does the difference between observed and expected numbers of deaths give cause for concern. If Equation 4 is used instead of Equation 5, identical results, including identical values of a and b, are obtained. Substitution of these values of a and b in the right hand side of Equation 4 and integration over the limits of 0–3,600 days gives 1.9 for the mean number of lethal lung cancers per animal at 3,600 days after exposure to the aerosol, which is in good agreement with the number calculated from the survival table (Table A.II-17); namely, two, as shown above.

An approximate upper limit for the mean number of lethal lung cancers at 3,600 days postinhalation has been derived by first estimating an upper limit for the constant b. When b is as high as 4.5, Chi-squared is at a minimum when $a = 3.2 \times 10^{-19}$ but the corresponding value of P has dropped to 0.1; owing to the small numbers involved, such a procedure can only provide a rough estimate of 4.5 as the upper 90% confidence limit for b. When these latter values of a and b are substituted in Equation 4, integration over the limits of 0–3,600 days gives a value of 2.1 tumors. In a similar way an upper limit for the constant a, when b is 3.2, is found to be 1.3×10^{-14}, which yields an estimate of 2.7 lethal lung cancers at 3,600 days. By analogous methods a lower limit for the mean number of lethal lung cancers per animal at that time is 1.2.

If the age of the animal is substituted for t in Equation 4 by adding the mean age of the animals at the time of exposure to the aerosol (namely, 562 days), then the best fitting power of the age is found to be 4.0. The purpose of introducing age, as opposed to duration of risk, is solely to permit comparison of these results with the behavior of other cancer rates. Thus, the natural incidence rates of many types of cancers [70], including lung cancers [71], have been shown to vary also in accord with a power of the age of 4.0 or more. Doll [72] has also shown that radiation-induced leukemia rates in spondylitic patients increased steeply as age at time of irradiation increased and in a manner paralleling the increase in natural leukemia incidence rates with age. It is concluded that, in the case of lung cancer induced by alpha radiation, risk evaluation probably should be based upon the relative risk rather than the absolute risk method. This will be discussed more fully below.

Table A.II-18 Life-table analysis of lung tumor mortality in the Battelle group of beagle dogs.

(1) $\overline{x}$ Mid-point in days of each successive 100-day interval beginning on the day of exposure to $^{239}PuO_2$	(2) I_x No. of dogs alive at the start of interval	(3) w_x No. of dogs dead due to cause other than cancer (No. of dogs with lung cancer which was not the primary cause of death)	(4) s_x No. of dogs sacrificed	(5) d_x No. of dogs dead with primary lung cancer during interval	(6) $[d_x]$ Expected no. of dogs dead with lung cancer [a] $[d_x] = [I'_x]\,[q_x]$ during interval	(7) $[I_x]$ Expected no. of dogs alive at the start of interval [b]	(8) $[I'_x]$ No. of dogs at risk of dying of lung cancer during interval [c]	(9) $[q_x]$ Probability that dog will die of at least one lung cancer during interval [d]	(10) d_x Observed no. of dogs dead with primary lung cancer in 400-day interval	(11) $[d_x]$ Expected no. of dogs dead with lung cancer in 400 day interval
50	40	0	0	0	0.00+	40	40	0.00+		
150	40	0	0	0	0.00+	40.0—	40.0—	0.00+	0	0.0+
250	40	0	0	0	0.00+	40.0—	40.0—	0.00+		
350	40	0	0	0	0.00+	40.0—	40.0—	0.00+		
450	40	0	0	0	0.01+	40.0—	40.0—	0.00+		
550	40	0	0	0	0.02	40.0—	40.0—	0.00+	0	0.1
650	40	0	0	0	0.04	40.0—	40.0—	0.00+		
750	40	0	0	0	0.06	40.0—	40.0—	0.00+		
850	40	1	3	0	0.08	39.9	37.9	0.00		
950	36	2	0	0	0.11	35.8	34.8	0.00	0	0.5
1050	34	0	0	0	0.14	33.7	33.7	0.00		
1150	34	2 (2)	0	0	0.18	33.5	32.5	0.01		
1250	32	1	0	0	0.22	31.4	30.9	0.01		
1350	31	3 (2)	1	0	0.26	30.1	28.1	0.01	0	1.1
1450	27	1	0	0	0.30	25.9	25.4	0.01		
1550	26	1	0	0	0.35	24.6	24.1	0.01		
1650	25	1 (1)	1	2	0.39	23.2	22.2	0.02		
1750	21	1 (1)	0	0	0.43	20.8	20.3	0.02	3	1.9
1850	20	0	0	1	0.49	19.4	19.4	0.03		
1950	19	0	0	0	0.57	18.9	18.9	0.03		

2050	19	3 (2)	0	0	0.59	18.3	16.8	0.04		
2150	16	0	0	0	0.60	14.8	14.8	0.04	4	2.6
2250	16	0	0	2	0.66	14.2	14.2	0.05		
2350	14	1 (1)	0	2	0.70	13.5	13.0	0.05		
2450	11	0	0	1	0.72	12.3	11.8	0.06		
2550	10	0	0	1	0.77	11.1	11.1	0.07	3	3.1
2650	9	0	0	0	0.80	10.3	10.3	0.08		
2750	9	0	0	1	0.83	9.5	9.5	0.09		
2850	8	0	0	1	0.84	8.7	8.7	0.10		
2950	7	0	0	0	0.85	7.8	7.8	0.11	2	3.3
3050	7	0	0	1	0.83	7.0	7.0	0.12		
3150	6	0	0	0	0.81	6.2	6.2	0.13		
3250	6	0	0	0	0.77	5.3	5.3	0.14		
3350	6	0	0	1	0.72	4.6	4.6	0.16	3	2.8
3450	5	0	0	1	0.66	3.9	3.9	0.17		
3550	4	0	0	1	0.60	3.2	3.2	0.19		
3650	3	0	0	2	0.52	2.6	2.6	0.20		
3750	1	0	0	0	0.45	2.1	2.1	0.22	2	1.7
3850	1	0	0	0	0.38	1.6	1.6	0.23		
3950	1	0	0	0	0.31	1.2	1.2	0.25		
4050	1	0	0	1	0.25	0.9	0.9	0.27		
4150	0	0	0	0	0.20	0.7	0.7	0.29	1	0.7
4250	0	0	0	0	0.15	0.5	0.5	0.31		
4350	0	0	0	0	0.11	0.3	0.3	0.33		
4450+[e]	0	0	0	0	0.22	0.2	.....	.....	0	0.2
Total	....	17 (9)	5	18	17.99	.....	.....	.....	18	18.0

[a] Based on model described in text.
[b] Computed by subtracting from the value of $[I_x]$ at the start of the previous interval, the sum of the values of $[d_x]$, w_x and s_x derived from the previous interval.
[c] The quantity $[I'_x]$ for a given interval is approximated by subtracting from the value of $[I_x]$ for that interval one-half the sum of the values of w_x and s_x for that interval.
[d] Derived from expression, $1 - e^{-m}$ where m, the mean number of lethal tumors per animal, is evaluated from the empirically determined expression, $m = 9 \times 10^{-15}(t)^{3.2}$. Values of $[q_x]$ are rounded to two decimal places.
[e] The last of the 40 dogs died at 4068 days post-exposure; however, the table is extended to show the number of expected deaths during subsequent 400-day intervals until at 4400 days the total of all expected numbers thereafter is combined and is 0.2 death as shown in columns 6 and 11.

Table A.II-19 Estimated number of hot particles deposited in the pulmonary regions of the Battelle group of 15 beagles that died between 0 and 3,600 days of lung cancer. (Calculated on the assumption of (1) a log normal frequency distribution with respect to particle size before inhalation of the aerosol; and (2) a constant deposition frequency in the pulmonary regions; that is, any particle is equally likely to reach the pulmonary regions regardless of its size.)

	Type of Aerosol		
	A CMD[b] = 0.5 μm σg[c] = 2.3	B CMD = 0.25 μm σg = 2.1	Weighted means
Number of dogs exposed that died of lung cancer	5	10	
Mean initial lung burden (ILB), μCi	1.01	1.10[a]	1.07
Estimated number of Type 1 hot particles ($\geqslant$ 0.07 pCi)	4.1×10^5	1.8×10^6	1.3×10^6
Estimated number of Type 2 Hot particles ($\geqslant$ 0.6 pCi)	1.4×10^5	2.1×10^5	1.9×10^5

[a]For one of the dogs exposed to aerosol "B" the initial lung burden has not been determined. Therefore, the initial lung burdens and particle number estimates are based upon 10 instead of 11 dogs; the omitted dog died with a lung cancer as cause of death 3,537 days after exposure to the aerosol
[b]CMD = Count median diameter
[c]σg = Geometric standard deviation.
Note: When allowance is made for differential pulmonary deposition (see Figure A.II-2), the numbers of Type 1 and Type 2 particles deposited in the deep lungs are likely to have been higher than those shown in this table

The significance of the beagle findings will be assessed first in relation to the Hot Particle Hypothesis and then in relation to the problem of estimating radiation-induced lung cancer risks in human population groups. It is instructive to use the beagle experience to derive an upper limit for the lung cancer death risk per hot particle and then to compare that risk with the one Cochran and Tamplin derived from skin tumor data in rats. The estimate based on the beagle experience is an upper limit, in the sense that it is based on the arbitrary assumption that the average of two lethal lung cancers per animal at 3,600 days postinhalation results entirely from a hot particle effect. For the animals that died of lung cancer before that time, the mean initial lung burden was 1.07 μCi. As shown in Table A.II-19, the average animal with such a burden is likely to have had deposited in its deep lungs at least 1.3 million Type 1 particles or at least 200,000 Type 2. (Type 1 and Type 2 refer to particles defined by Cochran and Tamplin as having specific activities of 0.07 pCi and 0.6 pCi, respectively.)

It follows that if the beagle experience is used to derive an estimate of the accumulated lung cancer death risk associated with any hot particle effect, then the upper limit for such a risk per Type 1 hot particle is roughly 1.5 per million (2 lethal lung cancers/1,300,000 particles), or one per 100,000 (2/200,000) per Type 2 particle. These hypothetical risks could be one or more orders of magnitude lower, if not zero, if the bulk of the lung cancer risk experienced by the beagles resulted from the generalized alpha irradiation from the total ^{239}Pu activity in their lungs. These risk

estimates based on the beagle experience are thus strikingly lower than the risk of one per 2,000 per hot particle of either Type 1 or Type 2 which Cochran and Tamplin derived on the basis of their analysis of data on skin tumors in rats.

It is especially instructive to assess the induced lung cancer risks experienced by the Battelle beagle group in relation to estimates of lung cancer risks in human beings based upon the experience of occupational groups exposed to alpha radiation. At the outset it should be noted that the BEIR Committee suggested the use of two methods of assessing cancer death risks, including those from lung cancer; namely, an absolute risk and a relative risk method. There were insufficient data to decide between the two methods and the committee therefore calculated risks by both methods.

The BEIR Committee suggested that if the absolute risk method is adopted a risk constant of one lung cancer death per million person-years per rem should be used, this constant to take effect after a 15-year latent period and to remain in effect for either (a) a 30-year period or (b) indefinitely. An estimate can then readily be derived for the accumulated lung cancer risk a person might be expected to acquire by age 70, for example, if he had been continuously exposed over his working life to the maximum permissible occupational level as currently set (15 rem per year to the lung). The accumulated dose over a 48-year work span extending from age 18–65 inclusive amounts to 720 rem (48×15) and the duration over which the risk constant is assumed to apply is either (a) 30 years or (b) 36 years (from age 34, after a 15-year latent period, to the start of age 70). For present purposes the more conservative assumption (b) is desired. The resultant accumulated lung cancer death risk is 0.026 ($1 \times 10^{-6} \times 720 \times 36$). (Strictly speaking, for chronic exposures at a constant dose rate the effective dose is one-half of the total accumulated dose, as shown by Marinelli [73]; however, since the risk constant used in the BEIR Report was derived on the basis of the accumulated rather than the effective dose, it is necessary to use the accumulated dose in applying that risk constant.)

The BEIR Committee suggested that if the relative risk method is used, a value of 0.29% should be adopted for the incremental relative risk per rem. For present purposes, before the relative risk constant can be applied it is necessary to estimate the accumulated lung cancer death risk by age 70 for adult males in the general population. That is, such males constitute the population from which the occupational groups under consideration are expected to be largely drawn; namely, groups that mine or process heavy alpha-emitting elements. From age-specific death rates that have been averaged over the years 1962–1967 and tabulated by Burbank [74], the accumulated lung cancer death risk by age 70 can be estimated from the cumulative proportion of survivors at that age and is found to be 0.036. Such an estimate must be used with caution since it is known that it is markedly influenced by such factors as the smoking habits which characterized different age groups in the population at risk. With this reservation in mind, the accumulated lung cancer death risk at age 70 can be estimated as 0.075 ($0.036 \times 720 \times 0.0029$) for the hypothetical case of continuous occupational exposure of the lungs at the maximum permissible level. It should be emphasized that in applying the relative risk as well as the absolute risk

method the underlying assumption is that of a linear dose–response relationship over the range of exposures being considered. (Again it should be stressed that since the BEIR Committee used the cumulative dose, as opposed to the effective mean dose, to derive the relative risk constant in the case of chronic exposures, it obviously is necessary when applying their estimate of that constant to use the cumulative dose experienced by the population under consideration.)

To recapitulate, 0.026 and 0.075 are estimates based on absolute risk and relative risk methods, respectively, of the accumulated death risk from lung cancer by age 70 for the case of continuous occupational exposure of the lungs at 15 rem per year. As already indicated, the steepness with which lung cancer death rates in the Battelle beagles rose as a function of age strongly suggests that the relative risk estimate is the appropriate one to use in the present context of assessing lung cancer risk from alpha emitters.

The relative risk of 0.075, calculated for humans, will be used as a basis for testing whether the generalized alpha radiation to which the beagle's lungs were exposed can account for the observed lung cancer mortality in those animals. The effective lung dose of alpha radiation which the beagles had accumulated by 3,600 days postinhalation amounted to approximately 51 times[1] the corresponding dose accumulated by age 70 in the hypothetical case of a worker exposed continuously at the occupational maximum permissible level (that is, the dose upon which the estimate of 0.075 is based). Hence on the basis of linear extrapolation (0.075×51) there should have been an average of 3.8 lethal lung cancers per animal at 3,600 days compared to the two previously calculated tumors per animal estimated by the life table method. Since the relative risk constant is itself subject to considerable uncertainty, being based on sets of data for which the calculated values of that constant ranged from 0.0016 to 0.0068 [68], it can be inferred that the expected number of lethal lung cancers for the case of exposure of the human lung could have ranged from 2 to 9. For present purposes it suffices to note that the beagle lung cancer death risk is not markedly different from, and may have been less than, that which would be calculated on the basis of averaging alpha radiation doses over the entire lung.

Finally, it may be of interest to analyze Cochran and Tamplin's original statement that the maximum permissible lung burden (MPLB) should be reduced by a factor of 115,000. This factor, it will be recalled, was derived by dividing the maximum permissible lung burden of ^{239}Pu required to give a dose rate of 15 rem per year (0.016 μCi) by the total activity contained in two of their Type 1 hot particles (0.14 pCi). Their

[1]The mean initial lung burden of the 15 animals that died of lung cancer between 0 and 3,600 days postinhalation was 1.07 μCi, which corresponds to an initial dose rate of 2.05 rad per day. The effective half-life of this activity in the lungs of these beagles averaged 970 days. The total accumulated lung dose at 3,600 days is found to be 2,575 rad, or 25,750 rem if a quality factor of 10 is used for converting rad of alpha radiation to rem. Marinelli [73] has shown that, in determining a linear dose–response relationship, the effective dose is given by the mean accumulated dose, which in the present case is found to be 18,410 rem. For occupational exposure at 15 rem per year for 48 years the total accumulated dose to the lungs is 720 rem, which corresponds to a mean accumulated dose of 360 rem. Hence the ratio of the effective dose to the lungs of the Battelle beagles that died of lung cancer and the effective dose to human lungs from occupational exposure at the maximum permissible level is 51 (18,410/360).

choice of two particles, as already noted, was based on two assumptions: 1) that the lung cancer death risk associated with continuous lung exposure at the rate of 15 rem per year was 1/1,000, and 2) that the lung cancer death risk per hot particle was 1/2,000. The present analysis indicates that the appropriate value for the first of these risks is 1/13 (0.075), rather than 1/1,000; while the value for the second risk is not 1/2,000 but instead has an upper limit of 1.5/1,000,000 per Type 1 and 1/100,000 per Type 2 hot particle. It follows that at least 50,000 Type 1 particles would be required to give the predicted lung cancer death risk of 0.075. That number of particles would constitute a total activity of 0.004 μCi or more and therefore would represent a factor of no less than 1/5, rather than one of 1/115,000, of the maximum permissible occupational level. Nor is the problem changed appreciably with the more recently defined Type 2 particle, since at least 7,500 of such particles would be required, corresponding to a total activity of 0.005 μCi or a factor of no less than 1/4, not 1/115,000. Since all of the lung cancer deaths in the Battelle group of beagles can be accounted for on the basis of the generalized alpha radiation, the actual risks associated with any hot particle effect may be so low as to be negligible when compared to the risk from the generalized alpha radiation.

Radiation standards, as currently applied, are not tied directly to any particular method of calculating risks but instead are set in terms of various absorbed dose levels to the whole body or to critical organs, depending upon the type of population group exposed. In relating such levels to risks, it is appropriate to use the methods outlined in the BEIR Report. Indeed, the relative risk method in the present case may be expected to predict adequately not only lung cancer risks from generalized alpha radiation but also those from insoluble particulates.

SUMMARY

An analysis of lung cancer mortality rates in the Battelle group of beagles indicates that (1) the generalized alpha radiation from the total ^{239}Pu activity in their lungs is sufficient to account for all of the lung cancer deaths which occurred in these animals, and (2) if there is a hot particle effect of the type postulated by Cochran and Tamplin, the risk of a lung cancer death per particle when calculated on the basis of the beagle experience is orders of magnitude smaller than they estimated and could well be so small that the contribution from any hot particle effect to the total lung cancer mortality is negligible. The beagle results also indicate that the relative risk method of assessing risks, as opposed to the absolute risk method, is likely to be the appropriate one for estimating lung cancer risks in human populations exposed to radiation.

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IONIZING RADIATION, CANCER INDUCTION, AND RADIOACTIVE FALLOUT

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I would like to discuss (a) how important discoveries over the last 100 years help to quantify the risk of cancer in populations exposed to man-made sources of ionizing radiation, and (b) how periodic episodes of contamination of the environment with radioactive elements have created public health problems.

IONIZING RADIATION AND MUTATION

Basic research on the fruit fly, Drosophila, has been one of the contributors to our understanding of how ionizing radiation is able to induce cancer. Using this organism, which normally has red eyes, T. H. Morgan (Fig. 1) discovered in 1910 a white-eyed fly and went on to prove that it was the result of a spontaneous gene mutation. Prof. Kuźnicki in his opening lecture at this conference has already stressed the importance of Morgan's work for laying the foundation of modern genetics.

Of particular relevance to the present discussion was the discovery in 1927 by one of Morgan's students, H. J. Muller (Fig. 2), that not only do X-rays induce gene mutations in Drosophila, but they do so in approximately direct linear proportion to the dose.[1] In addition, he commented that the "effect of X-rays, in occasionally producing cancer, may also be associated with their action in producing mutations."[2] More specifically, in 1937 he wrote, "it is but a logical step to conclude that carcinomas, sarcomas, and leukemias arising after irradiation represent mutations induced by the latter."[3]

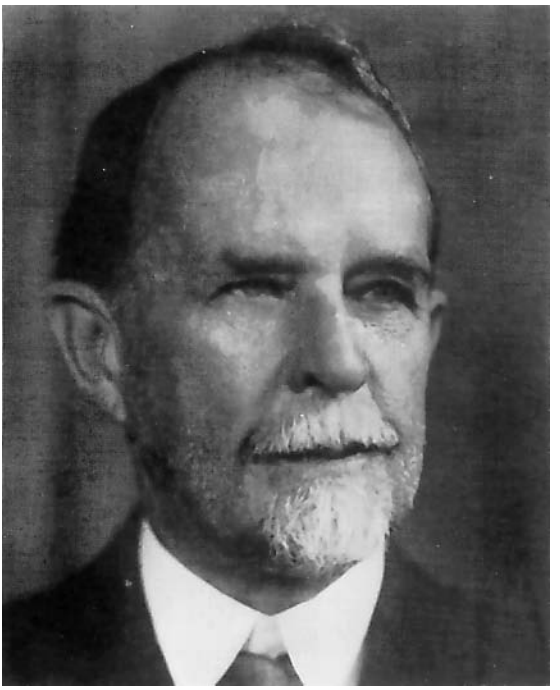

Figure 1. Thomas Hunt Morgan. In 1933 he was awarded the Nobel Prize in Physiology or Medicine "for his discoveries concerning the role played by the chromosomes in heredity." (Courtesy of the Archives, California Institute of Technology.)

It is of historical interest to this Conference that the Joliot-Curies in 1937 offered Muller a temporary position at their Radium Institute in Paris to continue his work on radiation genetics and were eager to have him accept.[4] Muller elected instead to take up a position he had been offered in Edinburgh.

RADIATION UNITS

Before discussing cancer risks associated with ionizing radiation, it is necessary to mention the radical changes in absorbed dose units that have been introduced in recent years. Thus in place of the rad, or 100 ergs per gram, the Gray (Gy) has been adopted and corresponds to 100 rads. In place of the rem (the rad multiplied by the relative biological effectiveness of the radiation) the Sievert (Sv) has been adopted and equals 100 rem. The Gy and Sv are such large units as to be cumbersome to use when discussing risks of low doses of radiation. Therefore, I will use in what follows the centiGray (cGy) which is equal to 0.01 gray or one rad and the centiSievert (cSv) equal to 0.01 Sv or one rem. Air dose measurements are still given in terms of the roentgen (r), corresponding to 2 billion ion pairs per cc of air.

Figure 2. Herman Joseph Muller. In 1946 he was awarded the Nobel Prize in Physiology or Medicine "for the discovery of the production of mutations by means of X-ray irradiation." (Courtesy of Archives of Indiana University.)

DOSE-RATE EFFECTS ON GENE MUTATION IN ANIMALS

Spencer and Stern[5] extended the linear dose response for acute X-irradiation of Drosophila sperm down to, and including, 25 r. Uphoff and Stern[6] found no dose rate effect when the same total dose was chronically delivered, using gamma rays, at a rate of 0.003 r per minute to such sperm. Using mice, the Russells[7] observed a lower mutation rate in spermatogonia when the dose rate was reduced from 90 r per minute to 0.9 r per minute but no further lowering of the mutation rate at dose rates below 0.9 r per minute. Further reduction of the dose rate did, however, have the effect of further lowering the mutation rate in oogonia of mice.[7]

EPIDEMIOLOGY OF CANCER INDUCTION

Doll[8] has recently reviewed 100 years of observations of the hazards of ionizing radiation. For the first 50 years, evidence implicating radiation in the induction of

cancer was largely anecdotal, rather than based on epidemiology. He points out that an increased risk of cancer "came gradually to be accepted as due to one or more somatic mutations, partly as the result of epidemiological observations in the mid 1950s (Court Brown and Doll, 1957; Lewis, 1957)."[9]

Thus, in 1957 Court Brown and Doll[10] reported on a study of 11,287 ankylosing spondylitis patients who had received spinal irradiation with varying doses of X-rays. The incidence of leukemia increased in approximately direct linear proportion to the dose to bone marrow, where the leukemic cells are assumed to originate.

Among survivors of the atomic bomb bursts over Hiroshima and Nagasaki in 1945, a significant increase in the incidence of leukemia was observed[11] as early as 1952. However, estimates of the absorbed doses received by these populations were not made. When distance–dose curves for gamma and neutron exposures at the two cities were published, such estimates were made for persons in each of four concentric zones extending from the hypocenter, or point on the ground under the air bursts.[12] Again, the incidence of leukemia was approximately linearly proportional to the estimated average absorbed dose to bone marrow. On the assumption of a linear, no threshold, dose response, an absolute risk estimate was calculated for the A-bomb survivors and expressed as a probability of one or two cases of leukemia per million persons per cSv per year. This estimate was assumed to "apply to the 'average' individual in a given population, but . . . not necessarily to each and every individual in that population."[13] Approximately the same risk estimate was found to describe the leukemia experience of the spondylitic patients, suggesting that spondylitis does not in itself predispose toward leukemia.

Radiologists provide a group in which to examine the effects of chronic, as opposed to acute, exposure to radiation. American Board-certified radiologists dying during the years 1948–1961 experienced a significantly increased death rate from leukemia (and also aplastic anemia and multiple myeloma).[14] This study ruled out what earlier studies had left open; namely, whether the excess leukemia was simply the result of better diagnosis in radiologists than in other physicians. Thus, only radiation-inducible types of leukemia were significantly elevated, and not chronic lymphocytic leukemia, a type that has not been observed to be elevated in irradiated populations.

Although the accumulated dose to which these radiologists were occupationally exposed is unknown, rough estimates of that dose are possible based on two considerations (a) their overall mortality compared to other physicians and (b) the maximum permissible doses in effect during part of the time they were practicing. A national committee[15] had assumed, based on animal extrapolation, that radiologists who died during the years 1930–1954, inclusive, had been exposed to an average accumulated dose of possibly 1,000 cGy or more. This estimate was based on a report that their average age at death was 5.2 years lower than that of nonexposed physicians. However, such a life-shortening of 5.2 years was shown to be an artifact[12] that arose from a failure of the committee to take into account that the population of radiologists was younger than that of all physicians. Indeed, radiologists who were followed from

1938 to 1952 had a slightly longer life span than that of all physicians, but somewhat shorter than that of all medical specialists, to which radiologists belong.[12] A second consideration is the maximum permissible dose to workers that was first set for the United States in 1931 as 0.2 r per day, and then gradually lowered to 0.05 r per day in 1949. I assumed that the average radiologist was unlikely to have experienced an absorbed dose to marrow that was greater than 30 cGy per year (0.1 cGy per day, or one half the air dose of 0.2 r per day) over an occupational lifetime that averaged 24.4 years.[12]

If the same risk of death from leukemia per year experienced by the spondylitic patients per cGy, and by A-bomb survivors per cSv was experienced by the radiologists then they would have been exposed to an accumulated dose of 5–10 cGy per year. Thus, their average accumulated dose over 24.4 years was probably more like 125–250 cGy rather than 1,000 cGy. Some radiologists may well have received 1,000 cGy or more but on a linear, no threshold, hypothesis it is the accumulated dose over the occupational lifetime of the average radiologist that is relevant.

Epidemiological studies, recently summarized in reports of national[16] and international[17] committees, confirmed that ionizing radiation induces other cancers besides leukemia not only in the spondylitic patients and A-bomb survivors, but in other exposed populations. For a number of such cancers the dose response has also been found to be approximately linear. These reports should be consulted for estimates of the absolute and relative risks per cGy for each type of cancer.

SOMATIC MUTATION AND CANCER

It is now generally accepted that cancer results from somatic mutations in genes which control the normal growth and/or differentiation of cells. Such genes have come to be called tumor-suppressor genes,[18] which function normally to arrest cell division, thus allowing differentiation to progress. When such genes undergo loss-of-function mutations, cell proliferation is restored, creating a potentially cancerous state.

In a pioneering contribution, Knudson[19] invoked somatic, and in some cases germinally derived, mutations in a model to account for the etiology of retinoblastoma. His model is now generally accepted for many cancers and has been verified molecularly for the gene responsible for retinoblastoma as well as for genes involved in several other types of cancers.

On Knudson's model, when a person inherits a loss-of-function mutation in a tumor suppressor gene, cancer may result when one or more of the person's somatic cells acquires a spontaneous or induced loss-of-function mutation in the remaining wild-type allele of that gene. When no inherited mutation is involved, then the model requires that both loss-of-function mutations occur somatically before a cell can result in a cancer. The reason that a linear, as opposed to a quadratic, dose response can exist when two somatic mutations are required suggests that some somatic cells may acquire one of the mutations spontaneously and it is in those cells that a radiation-induced second mutation can result in a potentially cancer-causing cell; or a cell containing

a radiation-induced mutation may undergo a subsequent spontaneous mutation with the same end result.

Still another class of genes, known as proto-oncogenes, are involved in promoting, rather than arresting, normal cell division.[20] When such genes undergo a gain-of-function mutation, the resultant overexpression of the gene and its protein product results in unrestricted cell division. Since such a mutation is expected to be dominant, only one mutation is required, resulting in a linear dose response.

THE SPECIAL CASE OF FETAL IRRADIATION

Pioneering studies of Alice Stewart and colleagues[21] and of Brian MacMahon[22] found an increased risk of cancers, including leukemia, in children who had been irradiated in the fetal stage as the result of diagnostic radiography of the mother. Doll and Wakeford[23] have critically reviewed these and other such studies and have concluded that irradiation of the fetus with a dose of the order of one cGy increases the risk of a subsequent childhood cancer.

THE QUESTION OF ADAPTIVE EFFECTS

The UNSCEAR 1994 report[17] devotes considerable space to describing experiments that report that some cell types have the ability to adapt to very low doses of radiation. Epidemiological studies lack the power to detect such adaptive effects in the case of cancer induction. Moreover, in reference to the low dose implicated in inducing childhood cancers following exposure of the fetus to maternal diagnostic radiography, the report stresses that: "There is nothing to support the assumption that adaptive processes could be operating after irradiation that could reduce the incidence of radiation-induced cancers."[24]

RADIOACTIVE FALLOUT EPISODES

Widespread releases of radioactive fission products into the environment provide an opportunity for determining whether chronically delivered radiation doses are as effective as acute doses for the same total dose delivered. It is now clear that the radioiodines, especially I-131, are particularly valuable for this purpose.

It has long been known that radioiodine is potentially a special hazard to infants and young children.[25] Cattle and goats feeding on pasture contaminated with radioiodine (primarily the I–131 isotope) concentrate it in their milk, and persons drinking such milk concentrate it in their thyroid glands. Finally, an infant receives a dose to its thyroid gland that is a factor of 10–20 times the dose that an adult thyroid receives for the same amount of I–131 taken up. This factor arises because the beta particles from the decay of I–131 are of such short range that they are effectively all absorbed within the infant thyroid, whose mass is one to two grams, compared to about 20 g for the adult thyroid.

Large-scale releases of radioiodine occurred in the United States during several years of testing of atomic weapons in Nevada. In a 16-month period ending September 1958 such releases were estimated to have resulted in an average annual dose to the thyroid of infants and young children in the United States of 0.1–0.2 cGy, with individual doses expected to vary widely in either direction dependent on many factors.[25]

In some areas of the United States, it would probably have been wise to try to reduce the thyroid dose by limiting the intake of contaminated milk. However, the hazard was not recognized by the US Atomic Energy Commission, which assured the public that fallout doses were far below natural background levels (0.1 rem per year).

The accident at Chernobyl in 1986 is estimated to have released large amounts of radioiodines into the atmosphere with resultant contamination of many parts of Northern Europe, especially Belarus. A recent case-control study[26] has identified a significant increase in thyroid cancer among persons in Belarus who were infants or young children at the time of the accident and who are presumed to have ingested fresh milk contaminated with radioiodines, presumably primarily the I-131 isotope. The observed high risk of thyroid cancer in this young age group is assumed to result not only from the higher dose the gland experiences compared to that of older age groups, but to a greater sensitivity of young age groups to radiation-induced thyroid cancer as observed in a number of other irradiated populations.[27]

It will be important to continue the follow-up of the more heavily exposed populations to fallout from the Chernobyl accident. In this way better estimates can be expected for the carcinogenic risk associated with radiation delivered at very low dose rates.

The Chernobyl accident has again called attention to the special hazard posed by radioiodine to young people. Simple measures to reduce such exposure can be instituted, and were in some localities, although not soon enough, owing to delays in announcing the accident.

In Poland, Prof. Nauman has reported on efforts to reduce the risks to young children, including supplying potassium iodide as a dietary supplement, and has discussed the logistical difficulties in achieving effective prophylaxis.[28] Fortunately, avoidance of contaminated milk and fresh produce are obvious steps that reduce the hazard from ingesting radioiodine. All such efforts, however, are fraught with logistical problems and the danger of causing panic in the population. Hence governments and societies will need to be better prepared to cope with large-scale releases of radioactive elements that may occur in the future.

SUMMARY

One hundred years of observations of the effects of ionizing radiation have led to a number of major discoveries, including (1) ionizing radiation induces gene mutations in animals at a rate linearly proportional to dose, (2) certain cancers are induced by relatively high doses at a rate that is linearly proportional to the dose, (3) exposure of

the fetus to very low doses of X-rays has been associated with an increased risk of a cancer developing during childhood, and (4) many cancers result from somatic mutations in genes that control the normal growth and differentiation of cells. Cancers induced by ionizing radiation are therefore likely to result from somatic mutations, as first predicted by Muller in 1927. Large-scale man-made releases of radioactive fallout have occurred and pose a cancer hazard. A special case is the risk of thyroid cancer to infants who ingest the radioiodines in such fallout. Ongoing epidemiological studies of persons exposed to fallout from the Chernobyl accident in 1986, may eventually help to quantify risks associated with very low radiation doses and low dose rates. Governments and societies will need to be better prepared to cope with any future releases of radioactive fallout.

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SECTION V: HISTORICAL PERSPECTIVES

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HOMEOSIS: THE FIRST 100 YEARS

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In 1894, William Bateson[1] coined the word "homeosis" in his monumental work *Materials for the Study of Variation, Treated with Especial Regard to Discontinuity in the Origin of Species.* He gave a surprisingly broad definition of the term as a type of variation in which "something has been changed into the likeness of something else." Goethe[2] had already described the phenomenon some 104 years previously in his treatise *The Metamorphosis of Plants*, and indeed Masters[3] made more extensive studies in plants and termed it "metamorphy". However, as metamorphy had quite different meanings in other branches of biology, Bateson proposed "homeosis" as a more specific and useful term, and this name has certainly stood the test of time.

Bateson's purpose in writing his text was to provide evidence in support of Darwin's theory: Bateson saw in homeotic variations the kind of dramatic changes that he felt were needed to explain how evolution could occur. Darwin had cited variations in domesticated plants and animals as evidence for evolution and Bateson felt that he could further strengthen Darwin's case by exhaustively compiling the discontinuous variations that occur naturally within a species. The dogma at the time was that the characteristics of the parents blended in the offspring, hence Bateson's emphasis on the discontinuous nature of homeotic variations and meristic variations (changes in the number of body parts, such as vertebrae).

Bateson's book pre-dated by 7 years the rediscovery of Mendel's work and de Vries' mutation theory. In a prophetic passage, Bateson wrote: "So long as systematic experiments in breeding are wanting, and so long as the attention of naturalists is

limited to the study of normal forms, *in this part of biology which is perhaps of greater theoretical and even practical importance than any other*, there can be no progress." [p. 76; italics mine].

When Mendel's work was rediscovered, Bateson immediately recognized its importance; indeed, Haldane[4] has speculated that Bateson may already have himself rediscovered Mendel's laws, but was too modest to have claimed to have done so. Bateson wrote a textbook of heredity[5] and coined other terms that are also still in use, including heterozygote, homozygote, F1, F2, and even the term genetics itself (Fig. 1). After 1901, he was involved in countering an attack on Mendelism by the English school of biometricians, who argued that the gradual variations needed for their theory of evolution could not be explained by the discontinuous types of variations that Mendel used to elucidate the laws of inheritance. Much later, an obstructionist attack on Mendelism came from a school of biologists who argued that the development of an organism was controlled solely by cytoplasmic particles, rather than by nuclear genes. However, Bateson failed to pursue his studies of homeosis; this is perhaps not surprising given that only one case of a homeotic mutant, mentioned below, had actually been discovered before he died in 1926.

Twenty-one years had elapsed from the publication of Bateson's "Materials" before the first established case of a homeotic mutant was described by C. B. Bridges in the fly[6]. He named it *bithorax (bx)*, somewhat of a misnomer as the number of thoracic segments is not affected; rather, there is a conversion, albeit weakly, of the third segment into the second, producing flies that have a small second pair of wings in place of the halteres. Characteristically, Bridges mapped the mutation, established multiple mutant stocks and saw to it that all the available mutations were saved and made available to the community through the Caltech stock center, which he had founded. Data suggesting that *bx* mutations did not involve a simple multiple allelic series came in 1919 from Bridges, who found the *bithoraxoid (bxd)* mutant, which fully complemented *bx* mutants[6]. However, while on a sabbatical at Caltech, W.F. Hollander discovered a dominant loss-of-function mutant, *bithorax-dominant* (bx^D), which Bridges showed failed to complement either *bx* or *bxd*. (The bx^D mutation was renamed *Ultrabithorax* when it was shown to lie between *bx* and *bxd*.) Fuller descriptions of these and other mutants mentioned in this article may be found in the work of Lindsley and Zimm[7]. Both these mutants and others found since 1945 are now known to belong to the bithorax complex (BX-C), a gene cluster that controls not only the identity of the third segment, but also that of the abdominal segments (reviewed in Ref. 8).

I have given a brief history elsewhere[9] of how the discovery of the BX-C did not grow from an attempt to study homeosis, nor even development, but rather to test a hypothesis of Bridges, which he based on cytological evidence[10], that the genome contains naturally occurring gene duplications, often tandemly arrayed. I believe Bridges was aware that the *bx* mutants might indeed illustrate just such a case.

In 1926, Balkaschina[11] discovered the second homeotic mutant, which she named *aristapedia*, and was later[7] renamed *spineless-aristapedia* (ss^a). In describing this mutant,

Figure 1. W. Johannsen (*left*) and W. Bateson in Bateson's garden in Merton, England, circa 1924. In 1909, Johannsen, a professor of plant physiology at the University of Copenhagen, Denmark, coined the word "gene", which came to replace "factor", Bateson's term for Mendel's "Merkmal". Bateson coined the word "genetics", as well as "homeosis", and many other important terms still in use today.

Balkaschina paid tribute to Bateson and even proposed the term 'erbhomeosis', meaning inherited homeosis. She then subdivided erbhomeosis into two categories, Ersatz-heteromorphoses, which involved changes caused by substitution of body parts, and Zusatz-heteromorphoses, which were changes involving reduplication of parts. *aristapedia* belonged to the Ersatz type, as its effect was that a tarsus substituted for an antenna. Too bad that Bateson did not live long enough to use his brilliant command of language to give us equivalent terms in English!

A third homeotic mutant, *proboscipedia* (*pb*), a weak transformation of the proboscis toward a tarsus, was discovered in 1931, again by Bridges[12]. The mutation that underlies this phenotype has turned out to affect a gene within the Antennapedia complex (ANT-C)[13].

Together, the genes of the ANT-C and BX-C control the development of all but the most terminal portions of the fruitfly body. Moreover, they have been found to do so in a colinear manner: the order of the genes along the chromosome tends to correlate with their order of expression along the body axis. It must be pointed out that in the case of the BX-C, these conclusions were all deduced on the basis of genetic data, derived using very simple and rapid techniques for analysing the cuticle of embryos lacking one or more genes of the complex[14].

Molecular analysis of the BX-C had to wait until the development of recombinant DNA techniques, and the pioneering work of Hogness and his colleagues[15]. One surprising development was the high proportion of the ANT-C and BX-C that is involved in *cis*-regulation: in the BX-C, for example, the transcriptional units that encode protein comprise less than 5% of the complex. The remaining regions are thought to include enhancer-like sequences to which regulatory proteins bind, thereby conferring spatial- and temporal-specific production of the proteins encoded by the complexes. Many of the homeotic mutants with the most striking phenotypes involve rearrangements of these *cis*-regulatory regions rather than alterations in the coding regions of the complexes[16]. A quite different class of homeotic mutants was discovered that has disruptions of global *trans*-regulation of ANT-C and BX-C genes. The first such mutant, *Polycomb (Pc)*, found by P. H. Lewis[7], causes derepression of many of the genes of the complex, indicating that the product of the wild-type *Pc* gene is a *trans*-regulatory repressor of genes within these complexes.

Originally, because it was the molecular dogma of the time, we proposed[17] that the genes of the BX-C functioned like the genes of operons by repressing other genes. Now, some thirty years later, it seems likely that while in certain cases they do act as repressors, they can in other instances activate downstream genes[18]. Much current work is aimed at identifying the downstream targets of these gene.

In vertebrates, and in certain invertebrates (but not the *Diptera)*, ANT-C and BX-C form a single tightly linked complex, or homeotic complex[19] (HOM-C). Unfortunately for the developmental geneticist, not one but four copies of the HOM-C are present in vertebrates. In the case of mice and humans, the four partially identical clusters are all on different chromosomes. Nevertheless, despite this redundancy, much progress is being made in understanding how the HOM-C controls specification of the body plan of vertebrates.

McGinnis[20], in a valuable perspective, shows how the pursuit of homeotic mutants also culminated in the discovery[21,22] of the homeobox. This highly conserved DNA sequence is present in each HOM-C gene and encodes a DNA-binding region, or homeodomain. Two very important realizations followed. First, the genes of the ANT-C and BX-C, or at least their homeobox-containing regions, must have arisen from a common origin as the result of tandem gene duplication; this was exactly

what had been surmised on the grounds of purely genetic data. Second, the extreme conservation of the homeobox across many phyla of the animal kingdom shows that both this motif and the gene complexes that contain it pre-date the separation of the invertebrates and vertebrates, estimated to have occurred over 500 million years ago. The homeobox sequence has thus become an invaluable DNA probe for studying evolutionary relationships throughout the animal kingdom.

Sturtevant[23] pointed out how the "development of genetics is one of the striking examples of the interaction between different disciplines. After 1900, the first such interaction was with cytology, which led to a very rapid development of both subjects. Later interactions were with statistics, practical breeding, evolution theory, immunology, and biochemistry. All of these uses have led to the utilization of new ideas and new techniques, and to rapid—sometimes spectacular—advances in genetics and in the other fields concerned" (p. 135).

He goes on to cite the introduction of new types of organisms that proved to be especially favorable for the study of particular problems: "such as *Drosophila, Neurospora, Paramecium*, bacteria or bacteriophages."

To Sturtevant's list of disciplines we can now add the study of development and cite examples of organisms that are proving to be favorable for the molecular and genetic study of homeotic genes, especially those containing DNA-binding motifs: *Arabidopsis* [24],sea urchins[25], nematodes[26], beetles[19,27], locusts[28], frogs[29], mice[30–32] and humans[33].

What, then, has been the legacy of homeosis? Besides giving us the homeobox, it has opened up a completely new approach to the study of development. And over the last 15 years, it has led to the realization that the body plan of most animals, and presumably of plants as well, is controlled by a set of master regulatory genes, first identified by their homeotic mutations.

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REMEMBERING STURTEVANT

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Alfred Henry Sturtevant (1891–1970) was the youngest of six children of Alfred Henry and Harriet (Morse) Sturtevant. His grandfather, Julian Sturtevant, was a Yale graduate, a Congregational minister, and one of the founders and later president of Illinois College in Jacksonville, Illinois. Sturtevant's father taught mathematics for a while at that college, but later took up farming, first in Illinois and later in southern Alabama, where the family moved when Sturtevant was seven years old. Sturtevant went to a one-room country school and later to a public high school in Mobile.

At the age of 17, Sturtevant entered Columbia University, where his brother Edgar, who was 16 years older, was teaching at Barnard College. Edgar and his wife took the young Sturtevant into their family, and Alfred lived with them while attending the University. Edgar was a scholar who later became a professor of linguistics at Yale and an authority on the Hittite language. Sturtevant said that he learned the aims and standards of scholarship and research from Edgar. It was a great pleasure for Sturtevant when he and Edgar were awarded honorary degrees at the same Yale commencement many years later. Also present at the ceremony were Sturtevant's nephew, Julian (Edgar's son), Professor (now Emeritus) of Organic Chemistry at Yale, and Sturtevant's elder son, William, then a graduate student in Yale's department of anthropology and now curator of anthropology at the Smithsonian Institution in Washington.

Sturtevant said that he became interested in genetics as the result of tabulating the pedigrees of his father's horses. He continued this interest at Columbia and also

collected data on his own pedigree. At Edgar's suggestion he went to the library and read some books on heredity, with the result that he read the textbook on Mendelism by Punnett.

Sturtevant saw at once that Mendelism could explain some of the complex patterns of inheritance of coat colors in horses that he and others before him had observed. Edgar encouraged Sturtevant to write an account of his findings and take it to Morgan, who at that time was Professor of Zoology at Columbia, and from whom Sturtevant had taken a course in zoology during his freshman year. Morgan encouraged Sturtevant to publish the paper, and it was submitted to the *Biological Bulletin* in June, 1910, at the end of his sophomore year. The paper appeared that same year (Sturtevant 1910). The connection between the genetics of horses and that of Drosophila will be familiar to readers of this column from the *Perspectives* by Snell and Reed (1993) on the mouse geneticist W. E. Castle.

The other result of Sturtevant's interest in the pedigrees of horses was that he was given a desk in the famous fly room at Columbia University where, only 3 months before, Morgan had found the first white-eyed fly. These stories and more about the early days at Columbia, when modern genetics was in a very real sense born, are a matter of record, especially in the writings of Sturtevant himself (1965a,b).

Sturtevant once wrote that he knew of no one else at the time who was so thoroughly committed to the experimental approach to biological problems as was Morgan. It was Morgan's aim to produce a mechanistic, as opposed to a purposive, interpretation of biological phenomena. A great deal of this approach clearly rubbed off on Sturtevant.

Sturtevant had a remarkable memory. It was as if his memory were composed of a plethora of matrices waiting to be filled with any data that lent themselves to classification into discrete categories. The data might be in the form of numbers and kinds of bristles missing in a mutant fly; numbers of snails with a right-handed coil vs. a left-handed coil, the genetics of which Sturtevant was the first to explain; the relation between inversion sequences in different species; or the host of other characteristics he investigated not only in Drosophila, but in irises, evening primroses, snails, moths, and many other creatures, including human beings. Whatever form the data took, the observations fell into the appropriate matrix in his memory, from which they were readily retrievable to a degree that was truly phenomenal. Sturtevant liked to refer to this as the "blockhead" approach.

The Caltech period was a time of collaboration, especially with Sterling Emerson, Theodosius Dobzhansky, George Beadle, and Jack Schultz. It was Sturtevant's style, at least after he came to Caltech in 1928 with Morgan and Bridges, to spend his mornings doing experiments. Afternoons were spent in the biology library checking on any incoming journals, few of which in any phase of biology he did not at least dip into. The pace of science was not so frenetic as it is nowadays, so there was time for extended afternoon tea sessions at which Sturtevant might bring up a paper he had read that afternoon and that had attracted his attention. These sessions were very stimulating for the graduate students in genetics and embryology who usually

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attended them; among the faculty in genetics, Schultz, Emerson and Dobzhansky were likely to be present in addition to Sturtevant, and in embryology, Albert Tyler, who was working on the biochemistry of fertilization. Although a rift had developed between Sturtevant and Dobzhansky, there was no sign of it in front of the graduate students.

Sturtevant taught the undergraduate course in genetics at Caltech for many years. From time to time he also gave a course for undergraduates in entomology, complete with a field laboratory session. His lectures on topics in advanced genetics were scholarly reviews of special areas of genetics, often dealing with organisms with bizarre genetics, such as the protozoa. His lectures were especially valuable because he covered areas of research not ongoing at Caltech. The elementary course in genetics that Sturtevant taught was based on a textbook that he and George Beadle wrote (1939). It was not so widely used as perhaps it should have been, probably because it was considered too difficult for the average student. It was tailored for Caltech students, and the problems especially were a challenge, even for Caltech undergraduates.

Sturtevant and Beadle planned to revise the textbook, but the pressure of other work and the rapidity of developments that followed the discovery of the role of DNA prevented the revision. Sturtevant also liked to point out that both he and Beadle found after writing the book that each had used the term "gene" differently. For example, the *white* gene to Sturtevant was the specific *white* mutant, but to Beadle it

represented the constellation of *white* alleles including the wild-type allele. Sturtevant facetiously blamed their inability to get out a second edition on this difference in thinking about the gene. Characteristically, he would ask each geneticist whom he met how he or she used the term, and he then promptly catalogued such persons according to whether they thought of the gene the way he did or the way Beadle did. The person asked did not, of course, need to worry about his answer being in good company in either case.

Sturtevant read widely and kept abreast of many topics of current interest, especially politics. He would, for example, read the Sunday *New York Times* and the *Manchester Guardian Weekly* virtually from cover to cover. He was especially happy if he could do the crossword puzzle in the *Guardian* at one sitting. Those who know those puzzles will understand that only a very special breed of person attempts them, let alone solves them in one sitting. In the evening he would browse through the *Encyclopedia Britannica*, which was shelved next to his easy chair. He complained one time, and he was not bragging, that he had difficulty in finding an article which he had not already read.

Sturtevant was fascinated with puzzles of all kinds, especially puzzles involving three-dimensional objects. When Anne Roe (1953) made a study of what makes scientists tick, she chose Sturtevant as one of her subjects. He was not only flattered, but overjoyed at the opportunity to take the tests, which he viewed simply as a new set of puzzles to work out.

Sturtevant would develop a topic logically and succinctly, whether he was publishing a paper or giving a formal lecture. In private conversation, however, he always seemed to assume that the listener was at least as well versed in the subject as he was, so he would leave out the preliminaries and get right to the point. This could be mystifying to some. For others it was a challenge to try to become sufficiently versed to profit by listening to his ideas or tapping the tremendous store of information at his fingertips on almost any topic of substance. His papers were so well written that one would assume that he had labored over each word. His penciled manuscripts rarely contained more than a few minor changes inserted into the original draft, which was done in longhand on foolscap. When asked how he did this, he told me that he usually spent many days mulling the paper over in his mind until all the words fell into place, and then all he had to do was write it down from memory.

Sturtevant developed a keen interest in the history of science; his book, *A History of Genetics* (1965a), is a classic. His main purpose in writing it, I believe, was to give credit where he thought it was due, always a difficult task, and at the same time to trace the history of the ideas underlying scientific discoveries. I believe he would have decried a tendency in some quarters to relate scientific discoveries to the sociopolitical views of the discoverers themselves. His fascination with pedigrees, including his own, led him to compile an appendix that contained a series of "intellectual" pedigrees. Sturtevant, of course, was a direct descendant of Morgan and Wilson, another eminent biologist who was a contemporary and friend of Morgan's at Columbia. Morgan and Wilson were, in turn, direct descendants of Martin and Brooks, two men

who were at Johns Hopkins University where Morgan had obtained his doctorate; Martin was descended from T. H. Huxley and Brooks from Louis Agassiz; and so it went.

Sturtevant had a fund of aphorisms and anecdotes that he liked to spring whenever an occasion arose. Three of his favorites were from Morgan: "Establish a point and publish it;" or, when trying to overcome the difficulty in starting to write a paper, "Compose a flowery introduction, then throw it away and write the paper;" or, when a Drosophila experiment gave a totally unexpected result, "They will fool you every time." Sturtevant had one that pertained to his own marriage to Phoebe Reed Sturtevant and to that of a number of their friends, namely, "Marriages are made in heaven but there is a branch office in Woods Hole." A few were deliberately outrageous in order to make a subtle point: "Too bad graduate students are people;" or "Vertebrates are a mistake and should never have been invented." He liked to deflate pomposity whenever he ran across it and referred to pompous persons as "stuffed shirts." Echoing his contempt for profundity, he would say, "Something is profound if it reaches conclusions which I like by methods I don't understand."

Sturtevant's love for all living things, including people, was expressed in many ways. For example, in 1954 he gave the presidential address before the Pacific Division of the American Association for the Advancement of Science, where he warned of the potential hazards to human beings of the fallout from the atmospheric testing of atomic bombs. What had provoked Sturtevant was a strong statement issued by the executive branch of the government that the fallout levels from testing were far below any that could cause damage to human beings. This assumption, that there is a threshold for damage from ionizing radiation, had no evidence to support it and clearly was being used to justify testing of nuclear weapons.

Although I know that some assumed that the only purpose of Sturtevant's remarks was a desire to see a halt to bomb testing, this was not the case. He took a neutral stance and, although he felt there might be a need for testing, the public should be given the best estimate that scientists could make about the nature of the danger to the unborn from fallout levels of radiation. In "Quarreling Geneticists and a Diplomat," Crow (1995) has described in more detail the ways in which Sturtevant and other geneticists interacted in assessing radiation risks to the germ plasm.

I am indebted to Sturtevant's son, William, for pointing out in a personal communication that his father "had deep disdain for eugenics and a strong contempt for all forms of social discrimination," sentiments that perfectly sum up Sturtevant's position on these matters. Indeed, most of the chapter on the "Genetics of Man" in Sturtevant's *History of Genetics* (1965a) is devoted to a balanced treatment of the nature–nurture question.

Sturtevant's scientific accomplishments have been reviewed elsewhere, by himself (1965a); by Sterling Emerson (1971), who first became acquainted with him in 1922; by Beadle (1970), who first came to Caltech in 1931 as a National Research Council Fellow; and by me (1976). Some of his most important papers were reprinted in a book, *Genetics and Evolution* (1961), on the occasion of his 70th birthday. Sturtevant

was invited to make addenda to those papers as he saw fit; characteristically, he made only the briefest possible ones.

In a *Perspectives*, Crow (1988) stressed Sturtevant's remarkable contributions to virtually every branch of genetics. One of Sturtevant's most enduring scientific interests was that of evolutionary theory and how to approach it experimentally. One of his first contributions relevant thereto was his discovery and analysis of hybrids between *Drosophila melanogaster* and *D. simulans*, for which there is a valuable *Perspectives* by Provine (1991).

Sturtevant's research style was to let the experiments lead the way. In this respect he was not restrained by having to write grant proposals, and a decline in his rate of publishing after 1945 might have resulted in a low score anyway. Bateson is often cited for having said, "Treasure your exceptions." I believe Sturtevant's admonition would be, "Analyze your exceptions," for it is his remarkable analytical ability that shines through all his work.

For Sturtevant, science must have been an exciting and rewarding journey into the unknown. It was fortunately a long journey, with detours to many realms, and I am sure he savored every minute of it.

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C. B. BRIDGES' REPEAT HYPOTHESIS AND THE NATURE OF THE GENE

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In producing the definitive maps of the giant salivary gland chromosomes of *Drosophila melanogaster*, C. B. Bridges (1935) interpreted certain structures as tandem gene duplications that had become established in the species. He wrote:

> In my first report on duplications at the 1918 meeting of the A.A.A.S., I emphasized the point that the main interest in duplications lay in their offering a method for evolutionary increase in lengths of chromosomes with identical genes which could subsequently mutate separately and diversify their effects. The present demonstration that certain sections of normal chromosomes have actually been built up in blocks through such "repeats" goes far toward explaining species initiation (p. 64).

I will call this Bridges' repeat hypothesis, and in this article I show how Bridges had hoped to test it. Although he was unsuccessful, it led others to discover (1) recombination within the gene and (2) the existence of gene complexes, or clusters of closely linked and functionally related genes.

Bridges had, in fact, provided a cytological basis for challenging the concept of multiple allelism that had dominated research on the nature of the gene for nearly 40 years. Sturtevant (1965), who defined the concept, succinctly describes the difficulties it began to present:

> Multiple alleles had been supposed to represent changes in a single original gene, and there were two criteria for their recognition: they occupy the same locus in the chromosome and

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were not separable by crossing over; and their heterozygote (trans type) was mutant with respect to their common recessive phenotype, since neither carried the wild-type allele of the other (p. 89).

Sturtevant points out that these two criteria failed to agree when, in the 1940s, crossing over was shown to occur within what had been considered single genes, namely *Star* (*S*) (Lewis, 1942, 1945); and *lozenge* (*lz*) (Oliver, 1940; Green and Green, 1949). Both cases cited Bridges' repeat hypothesis.

In preparing a talk on the immense indebtedness of the genetics community to C. B. Bridges, I had occasion to examine the vast collection of cards[1] on which he recorded the data from his experiments. They show that, as early as 1938, he had begun a deliberate attempt to subject another multiple allelic series to a recombination analysis, namely that of *bithorax* (*bx*), whose prototypic allele, *bx*, he had discovered in 1915. That was also the year in which he was awarded the Ph.D. degree at Columbia University. His doctoral thesis, "Non-disjunction as proof of the chromosome theory of heredity," became a classic and appeared the next year as the first article in the first volume of Genetics (Bridges, 1916).

In 1919, Bridges discovered another mutation, *bithoraxoid* (*bxd*), which fully complemented *bx*.[2] These mutations cause partial transformation of the third thoracic segment of the fly into the second. As such, they are examples of homeosis, a phenomenon that Bateson had described in 1894 (see reviews in Lewis, 1994; McGinnis, 1994). Bridges' *bx* and *bxd* mutants were the first examples, at least in animals, of homeosis that were the results of gene mutation, as opposed to being accidents of development.

Bridges' renewed interest in *bx* mutations was prompted by W. F. Hollander's discovery of two new *bx* mutations in the summer of 1934. At the time, Hollander was a graduate student of L. J. Cole at the University of Wisconsin, and, as an assistant in the laboratory course in genetics, Hollander maintained stocks of Drosophila.

Dr. Hollander informed me that he had found the mutations in a single fly and was able to set up stocks of them that he then sent to the stock center at the Cold Spring Harbor Laboratory, probably in 1935. Bridges was at the time a research associate of the Carnegie Institution of Washington at Caltech, but he spent summers at Cold Spring Harbor (CSH), where, along with Demerec, he helped

[1] Bridges' records are held at Caltech in their original filing cabinet and consist of thousands of 3 × 5 in. cards that are numbered consecutively, commencing with number 7662, *ca.* 1917 (since cards starting with 7700 are dated October 1917), and terminating with card number 22,002, dated June 1938. The fate of cards 1–7661 from the Columbia University period is not known. Cards 7662–22,002 contain mainly the raw data on which the linkage maps of *D. melanogaster* were based. Not every number is represented. Other cards, often 3 × 5 in. pieces of paper, provide summaries and raw data on which Bridges based the map locations for almost all mutants known at the time of his death.

[2] Unfortunately, the figure of the mutant in Bridges and Brehme (1944) and in later compendia is that of an early bx^b mutant allele, now lost, which apparently became confused with *bxd*. A penciled drawing, labeled *bxd* by Lillian V. Morgan's artist, E. M. Wallace, was never finished. It showed a cup-shaped halter wing and a missing first abdominal segment. The latter was overlooked in the description of the mutant. Figures of the halteres of the *bx* and *bxd* mutants (Lewis, 1951) show that *bx* and *bxd* have mutually exclusive phenotypes. Thus, *bx* mutants have only the anterior half of the halter transformed toward an anterior-like wing, while *bxd* mutants have only the posterior half transformed to a posterior wing.

maintain the CSH stock center and thus would have become aware of Hollander's stocks.

Hollander has kindly shared with me handwritten letters that Bridges sent him, concerning these mutations. In the first letter, dated March 8, 1935, Bridges indicates that he is sending Hollander stocks of *bx*, bx^{34e}, an allele found by J. Schultz (Bridges and Brehme, 1944), and *bxd*. Hollander followed Bridges' advice and tested all possible combinations of these mutations with his two new mutations. Hollander named one of the mutations *bithorax-Wisconsin* (bx^{w}). In a letter dated August 10, 1937, Bridges suggested that the other mutation be named *bithorax-Dominant* (bx^{D}), since $bx^{D}/+$ flies have slightly larger halteres than wild-type flies.

Clearly Bridges was intrigued with bx^{D}, since it behaved as allelic to both *bx* and *bxd*, even though all previously known *bx* mutations fully complemented *bxd*. In the same letter (in which "allel" was the old abbreviation for allelomorph), Bridges wrote: "Whether it really is an allel nevertheless is one of those unsettled questions; I should say not an allel by preference until proved otherwise."

At Bridges' suggestion, Hollander (1937) described the phenotypes of all the possible *trans*-heterozygotes involving his mutations and those that Bridges had sent him. Bridges also advised Hollander to examine the salivary gland chromosomes of bx^{D}; however, Hollander was awarded the Ph.D. degree in 1937 and had to give up work with Drosophila. He has pursued a long career studying the genetics of pigeons, a field in which he was one of the pioneers and in which he is still actively engaged. For a history of pigeon genetics at Wisconsin, in which Hollander participated, see Owen (1989).

In early 1938, Bridges began a genetic and cytological analysis of Hollander's mutants. I believe he saw it as a way to test his repeat hypothesis. One of the data cards (undated) has a diagram that shows that Bridges had repeated, and essentially confirmed, all of the interactions that Hollander (1937) had described, namely, that *trans*-heterozygotes involving bx^{D} and either *bx* or *bxd* have, in every case, a more extreme mutant phenotype than that of $bx^{D}/+$, whereas *trans*-heterozygotes involving a given *bx* mutation and *bxd* are in every case wild type.

Bridges recorded, on a card dated December 25, 1937, "no aberration" associated with bx^{D} in the salivary gland chromosomes. Thus, he ruled out at least a gross deficiency or chromosomal rearrangement as an explanation for the failure of bx^{D} to complement *bx* or *bxd* in *trans*. A card dated March 9, 1938 (Fig. 1) shows that he had already completed the scoring of progeny from matings of *Sb* $bx^{D}/\ bx^{w}$ females to a homozygous bx^{w} male. *Stubble* (*Sb*), located at 58.3, provided a closely linked flanking marker to the left of the locus of bx^{D} at 58.8. Among 1169 progeny, Bridges reports an *Sb* fly that was wild type for *bx*. He bred the fly, confirmed its genotype, and on another card (not shown) labeled it a "revertant." Bridges' diagram of the maternal genotype (Fig. 1) shows that he also considered it to be a possible crossover between bx^{w} and bx^{D}, provided that bx^{w} lies to the left of bx^{D}. It is interesting that the diagram allows for the possibility that bx^{w} lies to the right of bx^{D}. The reason must have been that Bridges recorded on a raw data card (Fig. 2) a

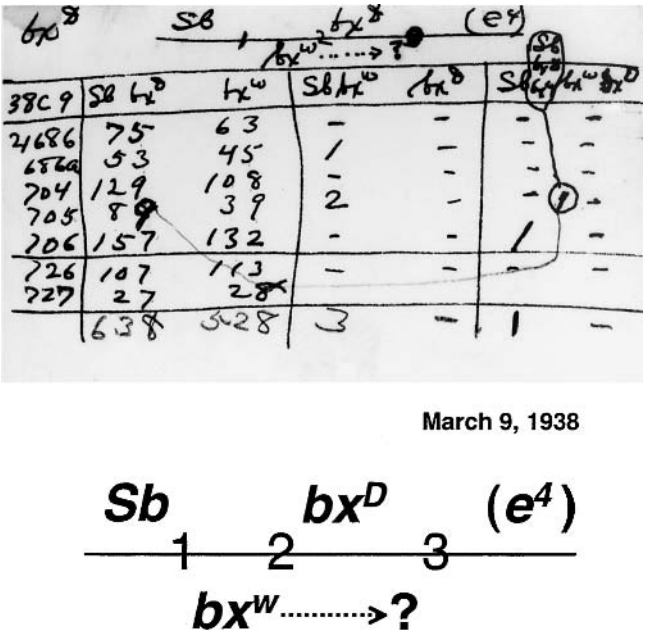

Figure 1. (*Top*) Bridges' 3 × 5 in. data card showing progeny of a mating of an *Sb* bx^D (e^4)/bx^w female(s) to bx^w males that gave a single Sb fly (column 6). Column 1 is dated March 9, 1938 (38c9) and shows culture numbers that contain the raw data on which the summary is based. Columns 2–7, inclusive, show the number of progeny having the phenotype at the head of the column (with the paternally derived bx^w omitted). Inserted between column 6 and 7 in the header is the genotype of a possible double mutant from culture 21705 (see Fig. 2). Flies were not scored for *ebony-4* (e^4) since not all parental females carried it. (*Bottom*) Interpretation of the maternal genotype and frequency of the wild-type recombinant (1 in 1169 offspring). See text for description of other mutant symbols.

possible double-mutant fly that he designates *Sb* bx^D bx^w/bx^w. On the reverse side of the card, he sketched the fly (Fig. 3). Since it expressed the *Sb* marker, to be a double-mutant crossover, bx^w would have had to lie to the right of bx^D. Although bx^w is now lost, all other existing *bx* mutations lie to the left of bx^D. It seems more likely, therefore, that the fly was the result of a new mutation, either *bx* or an enhancer.

A frequency of one revertant in only 1169 offspring must have encouraged Bridges to repeat the experiment. He did so rather quickly and added *stripe* (*sr*), at 62.0, as a flanking marker to the right of bx^D. A summary card dated June 14, 1938 (Fig. 4) records his failure to obtain any wild-type or double-mutant crossovers with respect to *bx* from a mating of *Sb* bx^D/bx^w *sr* females to homozygous bx^w *sr* males. There is no record that Bridges recorded the total progeny of this mating. I have therefore calculated from the total progeny listed on each of the 37 summary cards a grand total

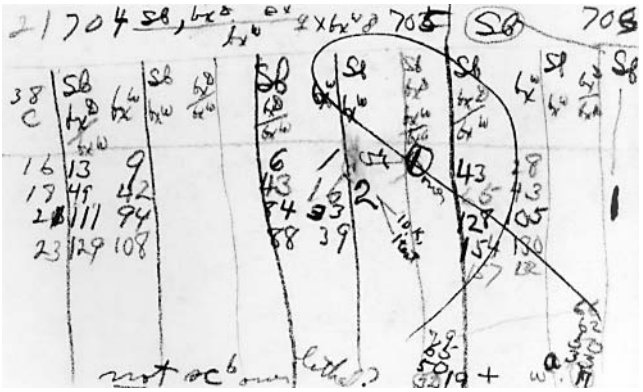

Figure 2. Bridges' card showing progeny scored in March 1938 (38c) from three cultures: 21704; 21705, which produced the possible double-mutant fly of genotype *Sb* bx^D bx^w/bx^w; and 21706, which produced an *Sb* fly, a possible revertant or wild-type crossover between *re* and *y*. A large delete sign is Bridges' indication that the data were copied to a summary card.

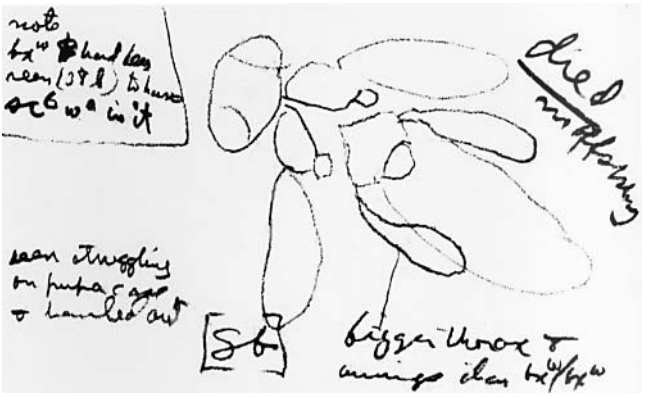

Figure 3. The reverse side of the card in Fig. 2 with a sketch of the possible double mutant. Bridges' comments are "died no offspring"; "bigger thorax & wings than bx^w/bx^w"; "seen struggling on pupa case & hauled out"; "bx^w stock had been seen (38b) to have sc^6 w^a in it." The last comment suggests that the stock had been contaminated at some point.

of 6753 progeny. The result must have been very discouraging.[3] Bridges became ill at about that time and on December 27, 1938, he died of a heart infection at the age of 49.

[3]Bridges' failure to obtain any more recombinants in 6753 offspring is not unexpected, since the frequency of crossing over between *bx* and *Ultrabithorax* (*Ubx*) is estimated to be 0.01% on the basis of experiments (Lewis, 1954) in which the use of the method of Steinberg (1936) resulted in an approximately threefold increase in crossing over in the *bx*-*Ubx* region.

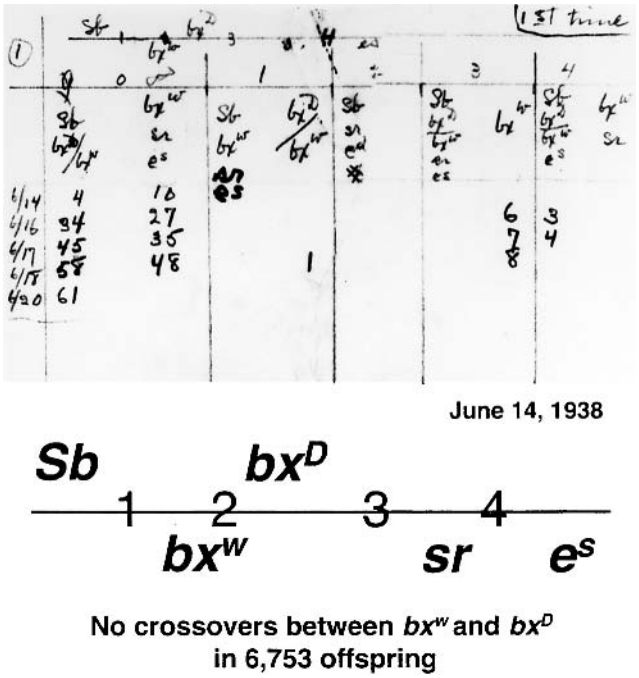

Figure 4. (*Top*) The first of 37 summary cards showing F_1 progeny (cumulatively totaled) from a mating of *trans*-heterozygous females backcrossed to bx^w *sr* e^s males. (*Bottom*) Interpretation of the parental genotype. Numbers inserted on the line are the C-o intervals. No crossovers were recorded in interval 2. e^s, *ebony-sooty*.

In spite of Bridges' failure to repeat his success in obtaining a possible recombinant between two *bx* mutations, his repeat hypothesis became the catalyst that motivated subsequent successful attempts to use crossing over as a tool to dissect "multiple allelic" series. Those attempts have been reviewed in three *Perspectives*: by Duncan and Montgomery (2002a, b) for the *Star* and *bithorax* series and by Green (1990) for the *lozenge* series. Not only were wild-type crossovers between "alleles" obtained in those early studies, but the reciprocal double-mutant crossovers were derived as well. Without the latter, the possibility remained that the wild-type products were instead reverse mutations associated with crossing over (Oliver, 1940).

Recovery of the double mutant has usually proved difficult because of a striking position effect, or "*cis–trans* effect," in which the *cis*-heterozygote for recessive mutations is wild type, and the *trans*-heterozygote is mutant in phenotype. In the Star-asteroid case, the *trans*-heterozygote, $S + / + ast$, is nearly eyeless, while the *cis*-heterozygote, $S\ ast\ / + +$, has a nearly normal eye like that of S/+ (figured in Lewis, 1951). Similar strong *cis–trans* effects were found for heterozygotes involving bx^D and for each of the various *bx* mutations (*bx* and *bxd*). However, in this case,

cis-and *trans*-heterozygotes for *bx* and *bxd* are both wild type (Lewis, 1951).[4] Two other series of mutations were found to be quite similar in complementation and recombination properties to those of the *bx* series, namely the *Notch* series (Welshons, 1958; Welshons and Von Halle, 1962) and the *dumpy* series (Carlson, 1959); however, critical tests for *cis–trans* effects were not carried out, because of failure to obtain the double-mutant recombinant class.

Subsequently, many multiple allelic series proved divisible by recombination not only in Drosophila, but also in microorganisms, phage, and even in maize (see review in Lewis, 1967). In an ingenious experiment, Nelson (1962) used iodine staining of maize pollen from a *trans*-heterozygote for two waxy (*wx*) mutations to obtain high yields of the non-*wx* (wild-type recombinant) pollen. Similar high yields of wild-type recombinants between alleles were readily obtained in many examples of multiple allelic series involving microorganisms and phage. However, recovery of the reciprocal double-mutant class was rarely achieved or even attempted.[5] Instead, it came to be taken for granted that the *cis*-heterozygote would be wild type for two recessive mutations.

In Drosophila, several cases of recovery of the *cis*-heterozygote were made possible by the technique of half-tetrad analysis.[6] Thus, from *trans*-heterozygous attached-X females that are mutant with respect to two sex-linked mutations, rare wild-type females can be recovered that carry the wild-type recombinant in one arm of that chromosome and the reciprocal, or double-mutant, recombinant in the other arm. In this way, the double mutants for two different *white* (*w*) mutations were obtained (Lewis, 1952),[7] as were two different *garnet* (g) mutations (Hexter, 1958; Chovnick, 1961). In both cases, there was the typical strong *cis–trans* effect; namely the *trans*-heterozygote was mutant and the *cis*-heterozygote was wild type, being in fact the basis on which it was selected. Many multiple-mutant combinations of *bx* mutations, with resultant *cis–trans* effects, were derived using attached right arms of the third chromosome (Lewis, 1967).

Working with the ascomycete, *Aspergillus nidulans*, Pritchard (1955) used a different type of half-tetrad analysis that involved mitotic recombination to derive the double mutant for two *adenine* (*ad*)-requiring recessive mutations. Pritchard was able to obtain the *cis*-heterozygote for two of the *ad* mutations and established a *cis–trans* effect.

[4] bx^D was renamed *Ultrabithorax* (*Ubx*) when it was found to lie between *bx* and *bxd* (Lewis, 1954a, b). The mutation is now known to be the insertion of a transposable element into the 5′ exon of the *Ubx* transcription unit (Bender et al., 1983). Bridges' *bx* and *bxd* mutations are now known to be insertions of such elements in large *cis*-regulatory regions that control where and when the protein coded by the *Ubx* gene is expressed during development. Intriguingly, though, as shown by Lipshitz et al. (1987), the wild-type *bxd* region codes for a 26.5 kb transcript that results in a family of non-protein-coding RNAs of unknown function.

[5] Another difficulty encountered with microorganisms was the phenomenon of gene conversion, which made it difficult to order the alleles even when there were flanking markers. Gene conversion, however, did not complicate the analysis of *S*, *lz*, and *bx*. The mutations, at least in the case of *bx*, are either deletions of many kilobases of DNA or insertions of transposable elements (Bender et al., 1983). Apparently such large lesions cannot be readily corrected by gene conversion.

[6] The technique was pioneered by Lillian V. Morgan (1931), who used it to obtain, simultaneously, the reciprocal products of unequal crossing over at the *Bar* locus.

[7] Wild-type recombinants between two *w* mutations were reported by MacKendrick and Pontecorvo (1952).

Thus, the *trans*-heterozygote required the addition of adenine to grow, whereas the *cis*-heterozygote was wild type and grew without addition of adenine.

Historically, Bridges' repeat hypothesis stimulated research that led to the discovery of recombination within a multiple allelic series at first in Drosophila and later in many other organisms. Similarly, it stimulated work that eventually led to the discovery of the tightly linked clusters of related genes known as the bithorax complex (BX-C) (Lewis, 1978) and the Antennapedia complex (ANT-C) (Kaufman et al., 1980). Finally, McGinnis et al. (1984) and, independently, Scott and Weiner (1984) discovered the homeobox (HOX), a DNA motif in the protein-coding region of the genes of these two complexes. In most animals the BX-C and ANT-C form one continuous complex known as the homeobox complex (HOX-C). The incredibly high degree of conservation of the HOX in all higher animals, including humans, points to the evolution of the HOX-C by a process of repeated gene duplication and diversification.

I believe Bridges would have liked that!

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DID DEMEREC DISCOVER INTRAGENIC RECOMBINATION IN 1928?

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Sturtevant's multiple allelism hypothesis (Sturtevant 1913) led to the assumption by geneticists during much of the first half of the twentieth century, that genes were units indivisible by recombination (reviewed in Lipshitz 2004, pp. 13–18). Disproof of this hypothesis required the discovery of the *cis–trans* position effect in Drosophila and the recovery of both wild-type and double-mutant recombinants from flies heterozygous for the *Star* and *asteroid* rough-eye mutations (Lewis 1942, 1945). A decade later, fine-structure mapping of the *r* II locus in phage provided definitive proof of intragenic recombination (Benzer 1955). In fact, Oliver (1940) had reported the occurrence of wild-type flies from females heterozygous for the mutations of the lozenge rough eye series. He interpreted these as revertants, as the title of his paper indicates, but he does discuss the possibility that they were the result of recombination. Green and Green (1949) later obtained double- and even triple- *lozenge* mutant combinations, thus establishing recombination as the source of the wild-type flies Oliver had discovered.

There are now many examples in which a gene is made up not only of a protein-coding region, but enhancer regions that, remarkably, recombine as if they are separate genetic loci. Not only do such regions recombine to produce wild-type crossovers but there are examples in which double- and even triple-mutant enhancer regions were derived by crossing over, such as in the case of the *bithorax* gene of the *bithorax* gene complex (Lewis 1955, 1967; reviewed in Lipshitz 2004, pp. 23–29).

[1]Deceased July 21, 2004.

Demerec's name may be familiar to may modern Drosophilists because of the 1994 reprinting of the book, *Biology of Drosophila*, which Demerec edited (Demerec 1950). Others may know of him through this tenure as director of the Cold Spring Harbor Laboratories from 1941 to his forced retirement in 1960. However, many do not know that he was a highly accomplished Drosophila geneticist in his own right.

Commencing in 1926, Demerec reported the existence of three mutable genes in *Drosophila virilis*: *reddish-α* (Demerec 1926a), *miniature α* (Demerec 1926b) and *magenta-α* (Demerec 1927). In 1928 he provided an extensive analysis of two strains carrying the *reddish-α* body color mutation, abbreviated here as *re*. Demerec had introduced into both strains the closely linked flanking markers *sepia* eye color (*se*) located at 0.2 and *scute* (*sc*) at 3.0 in the X chromosome. In one strain *re* behaved as an allele of the yellow body color mutation, *y*, at 2.4 in the X chromosome. Among 2,252 male progeny of *re/se y sc* females there were no wild-type recombinants between *re* and *y*. In the second strain, however, Demerec observed a remarkably high frequency of reversions of *re*: namely 171 reversions (17.1%) among a total of 9,988 male progeny of *re/se y sc* females. Among the 171 revertants of *re*, 148 were wild type with respect to *se* and *sc*, and hence were true revertants of *re*, 41 of which Demerec confirmed by actual progeny testing. The remaining 23 revertants of the original 171 carried the flanking marker *sc* and constituted 0.23% of the total offspring. Revertants occurred during the meiotic divisions in the female, and were reported not to occur in the male germ line or in somatic cells of either sex.

Demerec found that, unless he selected for instability of *re*, the frequency of revertants fell to nearly zero after about seven generations. The results involving two such lines that had stabilized have direct relevance to this Perspective.

1. From one such stabilized line among 24,823 male progeny of *re/se y sc* females, Demerec (1928, p. 376) states: "there were only three reversions. It might be questioned, however, whether these three reversions did not originate in a different way to the others, since all three were crossovers in the yellow-scute region."
2. In a second stabilized line Demerec sought to determine if there were modifier genes that induced the high frequency of reversions. Among a total of 9,101 male offspring of *re/se y sc* females, he observed two reversions.

Thus from the two stabilized lines Demerec actually had found a total of 5 revertants among 33,924 offspring, or 0.015% that were wild-type revertants of *re*. They must have carried the *sc* marker, since he states that all of them were associated with crossing over in the *y-sc* region. There is therefore a strong hint that "in a different way" was by crossing over between *y* and *re*.

Even though the salivary gland chromosomes are the same length as the corresponding arms of *D. melanogaster*, in *D. virilis* the genetic length is much greater. In her review of *D. virilis* genetics, Mary Alexander mentions that a factor of 2.8 more recombination occurs than in *D. melanogaster* (Alexander 1976). That factor can vary

widely; for example although there is 0.6 MU between *sc* and *y* in *D. virilis*, the map distance in *D. melanogaster* is effectively zero. For example, the double mutation y^2 *sc* was first obtained as a rare male crossover from large scale mating of y^2 *sc* females in which all of the other major chromosome were structurally heterozygous for chromosomal rearrangements (E. B. Lewis, unpublished results).

The high frequency with which *re* reverted may have been the result of jumping of a transposable element near if not at the locus of *re*. Any explanation for the high reversion rate must also account for the higher frequency of the *sc*-bearing revertants in the unstable lines (156/14,599), based on a summary of two experiments presented in Tables 2 and 3 of Demerec (1928), relative to the frequency observed in the stable line (7/2252). A possible explanation of the high reversion frequency is the presence of a mobile element near the unstable *re*. It is known that in *D. melanogaster* the P transposable element not only jumps at high frequency in dysgenic crosses, but also stimulates recombination in the vicinity of its insertion site (Sved et al. 1991). An element in *D. virilis* that may possess similar properties to the P element is *Penelope* (Evgenev et al. 1997). Together with the higher overall recombination frequency in *D. virilis*, local stimulation of recombination by *Penelope* may underlie any explanation of Demerec's results.

Demerec did not pursue his findings on *re*, perhaps because he was preoccupied with determining the cause of the instability of *reddish-α*. He also had to maintain many lines of *re* to be sure that some were still unstable.

I knew about Demerec's work on mutable genes from Oliver, not having read any of Demerec's papers. I was fortunate to have spent the summer of 1939 at Cold Spring Harbor. At the time, I brashly thought that, if I had the raw data for the experiments Demerec had carried out with *re*, I would be able to figure out what had caused the high reversion frequency. Demerec generously provided me with the data, which formed a considerable pile, as I remember. I spent many days poring over them but eventually gave up. I mention this episode as an example of Demerec's legendary generosity and strong interest and support of young scientists. He certainly helped one beginner on his way!

In conclusion, Demerec in 1928, a dozen years before Oliver, probably had the first documented case of intragenic recombination.

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EDWARD B. LEWIS: AUTOBIOGRAPHY

I was born May 20, 1918 in Wilkes Barre, Pennsylvania. My father was a watchmaker and jeweler. After completing grammar school he had to drop out to learn the trade of watchmaking in order to do his share of supplementing his parents' income. My mother finished high school and married my father in 1910. He worked in a jewelry store in Wilkes-Barre until it was forced to close during the Great Depression. It was a tragic period for my parents, who then had to struggle to make ends meet. They never wavered in their devotion to my brother and me.

My brother, who was $5\frac{1}{2}$ years older, earned a Masters degree in international law from George Washington University and had a long career in the Foreign Service, as an economic counselor in our embassies, first in London during the days of the Blitz, and after World War II in Copenhagen. He was a delegate to the Paris Peace Conference held in 1946. Later, he was Minister-Counselor for Economic Affairs in Geneva and the Deputy Director-General of the GATT (General Agreements on Tariffs and Trade) with the rank of Ambassador. His final assignment was as our Deputy Ambassador in Helsinki. It was a financial struggle for him to complete his college education during the height of the depression. He was a great inspiration in my life. Unlike me, he read rapidly and widely. I often sought his advice and recall his once explaining to me what was meant by natural selection.

From an early age, I remember having a keen interest in animals, especially toads and garter snakes, which could easily be found in vacant lots in Wilkes-Barre. During my high school days, I kept large snakes in my bedroom in terraria which I built for

them. I mention this to illustrate that my parents never discouraged my interest in animals.

Particularly important for me during my junior and senior high school years was the Osterhout Library, a first class library for a relatively small city. There I found books on how to make aquaria, which I then built and stocked with tropical fish. I soon discovered books by Bertrand Russell and avidly read many of them. I was fascinated by his iconoclastic views of society, religion, economics, law and human affairs in general. One book of his, The Scientific Outlook, had a strong and lasting influence. It lucidly explained the nature and philosophy of the scientific method, emphasizing its inductive rather than deductive nature, and the logical difficulties that are involved. Of more immediate influence at the time was a book by H. S. Jennings, The Biological Basis of Human Nature. It used simple diagrams to illustrate the sex-linked inheritance of white eyes in Drosophila. The book described how T. H. Morgan and his students, especially Bridges, had established the chromosomal basis for such inheritance. I then was fortunate to discover from an advertisement in *Science* magazine, which the Osterhout Library subscribed to, that cultures of Drosophila could be obtained from a Professor Rifenburgh at Purdue University for $1.00 each. My friend, Edward Novitski, and I ordered cultures and began growing Drosophila in the biology laboratory of our high school (E. L. Meyers) in Wilkes-Barre. Our biology teacher was the athletics coach. By allowing Novitski and me freedom to use the biology laboratory and its supplies after school hours to carry out experiments with Drosophila, he could not have been more helpful in furthering our careers. There was none of the present attitude that one cannot become a scientist without having had the benefit of teachers skilled in the art of keeping their students constantly motivated. For as long as I can remember I was "turned on," to use the language of today.

When I was 10 years old, I was given a wooden flute by my great-uncle Thomas D. Wyllie, who was president of the Pittston Stove Co. A few years later, my father purchased a silver flute for me at, I am sure, a considerable sacrifice. I had a few lessons while in grammar school. Playing the flute became a hobby along with keeping animals. I played in the high school orchestra and the Wilkes-Barre Symphony. I then went for one year to Bucknell College on a music scholarship before transferring to the University of Minnesota in 1937, where I continued my interest in music by playing second flute in the University Symphony. In recent years, I have kept up this interest by playing in small chamber music groups and in Caltech musicals, but only after having taken some much needed lessons from professional flute players.

I chose the University of Minnesota because it had one of the lowest out-of-state tuition fees of any major state university in the country and it also did not have a compulsory ROTC program. It proved to be an excellent choice in many ways. Especially important was the kindness extended to me by Dr C. P. Oliver, who was Professor of Genetics. He was one of H. J. Muller's outstanding students. He gave me a desk in his laboratory and allowed me complete freedom to resume work on Drosophila. In my Nobel lecture I have given a history of how I came to start work

in his laboratory and how it eventually led to the work for which I was awarded the Prize.

While at the University of Minnesota, my brother helped me financially or I could not have completed my education there. Also helpful was the newly created National Youth Administration, which provided funds to support college students. I obtained part-time employment on a NYA-supported project in the biostatistics department, where I worked on an interesting biomedical problem related to women's health.

By passing examinations in a number of courses for credit without actually taking the courses, I was able to complete the requirements for a B.Sc. degree in biostatistics after two years at the University of Minnesota. With Professor Oliver's help, I was offered a teaching fellowship at Caltech and went there in August of 1939. I was one of A. H. Sturtevant's graduate students and in the Morgan tradition he allowed me complete freedom to carry on work that I had begun in Oliver's laboratory. In many ways, graduate life at Caltech could not have been more ideal. One met with faculty and other graduate students daily at an afternoon tea session. In attendance were usually Sturtevant, the embryologist, Albert Tyler, and other geneticists, Jack Schultz, Sterling Emerson and, before he left for Columbia University, Theodosius Dobzhansky. It was a remarkably friendly and intellectually stimulating atmosphere. I also benefited greatly from an opportunity to attend the 1941 Cold Spring Harbor Symposium and to stay on that summer, learning salivary gland chromosome analysis of complex rearrangements from Dr. Berwind Kaufmann.

My doctoral thesis dealt with a new kind of position effect that came to be known as the *cis–trans* effect. It was for me a very exciting discovery, partly because Sturtevant had discovered the position phenomenon in 1925, and the *cis–trans* effect came to be a new and especially dramatic example of it. My minor was in bioorganic chemistry under Professor Haagan-Smit, who later determined the chemical basis of smog.

Although the United States entered World War II in December, 1941, I was able to obtain a deferment to complete graduate work, receiving the Ph.D. degree in June, 1942. There were very few opportunities for a biologist to serve in the armed forces, so I chose to enroll as a cadet in the United States Army Air Corps training program in meteorology at Caltech. I completed the program and was awarded the M.Sc. degree in meteorology in 1943. I then was sent to the University of California at Los Angeles for a short course in oceanography. It was taught by the distinguished Norwegian oceanographer, Harald Sverdrup. I was stationed for a time at bases in Hawaii, first at Hickam Field where I prepared weather forecasts for planes flying on the route between San Francisco and Canton Island. I was then assigned as a weather officer to the G2 section of the Tenth Army, with headquarters at Scofield Barracks. The Tenth Army was at that time preparing for the invasion of Okinawa. I arrived Okinawa shortly after D-Day, April 1, 1945, and was stationed on one of the command ships anchored in the harbor. These ships had the communication facilities to receive weather data, meager as they were, for that area of the world. I arose every morning at 4.00 a.m. and prepared a weather forecast that was then relayed to reconnaissance planes that daily flew over the battle zones on Okinawa.

After the war ended, I returned to the United States and in January, 1946, took up the position of instructor at Caltech, having been promised the job before leaving Caltech in 1943 by its president, R. A. Millikan. He had called me into his office and simply said "this war will not last forever, when it is over we would like you to come back as an instructor." Obviously, Sturtevant had had a hand in this style of making appointments that was typical of the times. I helped teach the introductory genetics laboratory before taking over the teaching of the entire course some years later.

The year 1946 was most notable as the year I met and married Pamela Harrah, who had been invited to come to Caltech by the new Biology Division chairman, George Beadle. She was a Stanford graduate and had studied genetics. For a while, she was curator of the Drosophila stock collection, before becoming a research assistant to Sturtevant. She has been a constant inspiration and source of strength. We have two sons living, Hugh and Keith. A third, Glenn, died in a mountaineering accident in 1965.

In 1948, I was awarded a Rockefeller Foundation Fellowship and chose to spend it at Cambridge University in Professor David Catcheside's laboratory of genetics in the Botany School. In September of that year, my wife and I embarked for England on the Queen Mary along with Hugh, who was then only 3 months old. I continued work on the bithorax mutants and presented a paper at the annual meeting of the Genetical Society of Great Britain., where I met for the first time, G. Pontecorvo and J. B. S. Haldane. My main accomplishment on the Fellowship was to write a review of the position effect phenomenon that later appeared in *Advances in Genetics.* In doing so I made extensive use of the splendid libraries at Cambridge as well as at the British Museum in London.

When the United States began testing atomic weapons in Nevada, I became intrigued with the possibility that the induction of cancers by ionizing radiation might be linearly related to the dose, just as Muller had shown for mutations in the germ line of Drosophila. I was surprised to find that it was generally assumed that there would be a threshold dose below which there would be no induction of cancer. Already there were studies linking leukemia to radiation exposure, so I summarized much of the available data and published it in an article in *Science* in 1957. Four population groups were examined: survivors of the atomic bombing of Hiroshima and Nagasaki; patients treated with X-rays for ankylosing spondylitis; children X-irradiated for enlarged thymus glands; and radiologists. The results were consistent with a linear dose response from which a risk estimate could be derived of one to two cases of leukemia per million persons per rad per year.

In radioactive fallout, the bone-seeking Strontium 89 and 90 isotopes are of particular concern. The literature contained the extraordinary statement that one would not expect to see any damage from these isotopes at doses to the bone below 1,000 rads. In addition it was not realized that not only bone cancer but leukemia could result from the radiation from these isotopes. I was indebted to two professors of physics at Caltech, William Fowler and Thomas Lauritsen, who showed me how to calculate the absorbed doses from these Strontium isotopes (and their Yttrium decay

products). I circulated a draft of the paper to a number of Caltech colleagues, including Linus Pauling and Harrison Brown. Pauling used the risk estimates for leukemia to calculate the number of leukemia deaths worldwide from fallout. Brown informed Albert Schweizer of my findings. Pauling and Schweitzer were, of course, leaders in attempting to stop the testing of atomic weapons. However, it was many years later before above ground testing was stopped by President Kennedy.

I testified on my findings before a Congressional joint committee on Atomic Energy in June, 1957. It was the beginning of a period of service on a National Advisory Committee on Radiation of the U.S. Public Health Service and committees of the National Academy of Science concerned with estimating genetic and somatic risks of ionizing radiation.

In 1959, I reported in the *Proceedings of the National Academy of Sciences* on the special hazard to infants and children exposed to another prevalent isotope in fallout, radioiodine, I-131. Based on data from a 16-month period ending in September 1958, I estimated that, over a period of several years commencing in 1954, the average annual dose to infants and children in the United States from fallout from atom-bomb tests in Nevada was equal to 0.1–0.2 rad per year. This rate amounts to one to two times the natural background dose to the thyroid. The public had been assured that fallout dose rates from the A-bomb tests would be well below natural background rates. Several factors contributed to the higher rates to thyroid glands of young age groups. Cows feeding on grass contaminated with I-131 concentrate it in their milk. Persons consuming such milk not only concentrate the isotope in their thyroid glands but, owing to the short range of the beta particles, the smaller the gland the higher the absorbed dose. Thus, for the same amount of I-131 taken up, an infant thyroid of 2 g receives 10 times the dose that the average adult thyroid of 20 g receives.

In 1959, as a member of an ad hoc advisory committee of the International Commission of Radiation Protection, I attended a meeting in Munich to discuss the current status of genetic and somatic effects of ionizing radiation. Among those discussing genetic effects were the mouse geneticists, W. L. Russell and K. Luning. Also present was J. Lejeune who had discovered the presence of an extra chromosome in Down's disease. He had shown me a slide of it while on a sabbatical in my lab in 1958. Among those discussing somatic effects were W. M. Court-Brown and Richard Doll, who had shown the elevated risk of leukemia and aplastic anemia in patients undergoing spinal irradiation for ankylosing spondylitis, and Alice Stewart, who had shown that the very low doses associated with pelvic X-ray examinations of pregnant women were associated with a significantly elevated risk of leukemia and certain other childhood cancers in children who had been irradiated in utero during such examinations.

On the way home I stopped in Copenhagen and visited my brother and mother who lived in one the Embassy residences. I used the occasion to visit the new Institute of Genetics of the University of Copenhagen and met with Professor Mogens Westergaard, a plant geneticist, who headed the Institute and was later to visit Caltech

on a sabbatical. I was to return there in 1975–1976 as a visiting Professor, as described below.

In 1963, I published in *Science* a further study of the mortality experience of American Board-certified radiologists during the period, 1948–1961. I obtained a small grant from the American Cancer Society and used it to obtain 425 death certificates of radiologists who had died in that period. I compared their mortality experience with that of U.S. white males and found that not only were deaths from leukemia significantly elevated in the radiologist group but so were deaths from multiple myeloma, and aplastic anemia. The results were especially significant since radiologists and other medical specialists have lower mortality rates from all causes combined than are experienced by U.S. white males. The onset of multiple myeloma does not occur until relatively late in life and it evidently was for this reason that my study of a population of radiologists, fifteen percent of whom were in age groups above 60 years, was the first to establish a significantly increased risk of this disease following exposure to radiation. Subsequently, as certain other irradiated populations, such as the ankylosing spondylitis patients, have aged the risk of multiple myeloma has also been shown to be significantly elevated.

These radiation-related studies and service on national committees were carried out as extracurricular activities. I continued to carry on a teaching and research program at Caltech, as well as overseeing the Drosophila stock collection.

The academic year, 1975–1976, was spent in Denmark as a visiting professor at the Genetics Institute of the University of Copenhagen, headed by Dieter von Wettstein. I had the pleasure of interacting with the Drosophila group there, including Erik Bahn, who had earlier spent a year in my group at Caltech, Leif Søndergaard, Søren Nørby, Knud Sick, and Penny von Wettstein, as well as other members of the Institute. The group met for morning and afternoon tea and lunched in the canteen that adjoined the laboratories. It reminded me of my halcyon days as a graduate student. My wife and I often went to the opera and ballet, living only a short block away on Nyhavn in a penthouse provided free to visiting professors by the Danish National Bank. We also frequently took the ferry to Malmo, Sweden, to attend the opera there. The year was a welcome and intellectually stimulating one.

In 1968, David Hogness of Stanford University spent 3 months of a sabbatical year in my laboratory. He had decided to switch his research interests to molecular studies of Drosophila. In 1972, he proposed recombinant DNA methods that included construction of genomic DNA libraries and chromosomal "walking" and "jumping", which later came to be known as positional cloning. To accomplish the first positional cloning Hogness and his postdoctoral fellow, Welcome Bender, selected the bithorax complex because of its developmental phenotypes and the availability of a large collection of chromosomal rearrangements. Thus, in 1978 Bender, Hogness, and colleagues initiated the chain of events, described in my Nobel lecture, that finally showed that the homeotic gene complexes are highly conserved throughout the animal kingdom and that they act as master regulators of the development of the

head, thorax, and abdomen of all animals from primitive worms to human beings. Particularly exciting for me was the completion of the sequencing of the bithorax complex and a search for regulatory sequences in it as described in my Nobel lecture.

The excitement of doing science has never diminished for me. The genetic studies and genome analyses which I am currently engaged in will, I hope, uncover new phenomena that will require investigation at the molecular level.

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THE BITHORAX COMPLEX: THE FIRST FIFTY YEARS

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Nobel Lecture, December 8, 1995

> "The power of using abstractions is the essence of intellect, and with every increase in abstraction the intellectual triumphs of science are enhanced."
>
> Bertrand Russell

INTRODUCTION

Genetics is a discipline that has successfully used abstractions to attack many of the most important problems of biology, including the study of evolution and how animals and plants develop. The power of genetics to benefit mankind was first recognized by the award of the Nobel Prize in physiology or medicine in 1933 to T. H. Morgan. In the 23 years that had intervened between the time Morgan introduced *Drosophila* as a new organism for the study of genetics and the award of the Prize, he and his students, especially, A. H. Sturtevant, C. B. Bridges, and H. J. Muller, had vastly extended the laws of Mendel as the result of a host of discoveries, to mention only a few: that the genes (Mendel's factors) are arranged in a linear order and can be placed on genetic maps, that they mutate in forward and reverse directions, that they can exist in many forms, or alleles, and that their functioning can depend upon their position. Purely on the basis of breeding experiments, these early workers were able to deduce the existence of inversions and duplications, for example, before it became possible to

demonstrate them cytologically. The list of their achievements is a long one and one that has been put into historical perspective by Sturtevant in A History of Genetics [1].

All of these discoveries were made with *Drosophila* by taking advantage of its small size, ease of culturing, high fecundity, short life cycle, small chromosome number, wealth of spontaneous and induced mutations, and, after their discovery in 1935, its giant salivary gland chromosomes. Of immense importance also was the existence of standard or "wild-type" strains.

That Morgan's contributions satisfied the criterion of being of benefit to mankind was evident by the remarkable extent to which the new discoveries with *Drosophila* had direct application to the understanding of the inheritance of many human traits. For example, the inheritance of colorblindness and hemophilia in human beings could be understood for the first time.

The second Nobel Prize for work in the genetics of *Drosophila* was awarded in 1946 to H. J. Muller for his discovery in 1927 that X-rays produce gene mutations and do so in direct proportion to the dose [2]. Muller called attention to the genetic risks to the human race posed by indiscriminate use of ionizing radiations, and, prophetically, he argued that such uses would also increase the risk of cancers, if cancer is the result of somatic mutations. The implications of Muller's work were not overlooked with the advent of the atomic age. As a result, extensive genetic studies were carried out in *Drosophila* and mice to assess the relative rates of mutation in these organisms as a means of assessing the genetic risks to human beings from the use of atomic energy.

The award of the Prize in 1995 for work with *Drosophila* recognizes the growing importance of a field that has come to be called developmental genetics. The work of my cowinners, Eric Wieschaus and Christiane Nusslein-Volhard, has identified crucial steps in the early development of the organism. Specifically, they have identified major genes involved in setting up the initial axes of the embryo and its germ layers [3] thereby setting the stage for groups of master control genes that then program the final body plan of the organism. It is this latter group of genes with which we will be concerned here: what they do and how they came to be discovered. My part in this story began in the late 1930s and it will be first examined in relation to the concept of the gene at that time.

THE GENE CONCEPT

Johannsen coined the term, "gene," in 1909 and it quickly replaced Mendel's "factor" [4]. The concept of the gene is one of the most powerful abstractions in biology and one of great utility. For many years the gene could be satisfactorily defined as a unit within which genetic recombination, or crossing over, does not occur. The unit defined in this way tended to correspond to a unit of function, as defined by the standard phenotypic test for allelism, or the "complementation" test, to be discussed below.

In 1925, Sturtevant made two important discoveries that were eventually to lead to a reexamination of the gene concept in terms of the gene's function [5]. In

analyzing the progeny of females homozygous for the unstable eye mutation, *Bar* (*B*), he predicted that a rare mutation, *double-Bar* (*BB*), was a tandem duplication that arose in the progeny of homozygous *B* females as the result of "unequal crossing over." He then showed that the eyes of *BB*/+ females are slightly smaller than those of *B*/*B* and deduced that the function of a gene can depend upon its position with respect to is neighbors, the first example of the "position effect," as he named it.

Eleven years later, using the giant salivary gland chromosomes of the *Drosophila* larva, Bridges [6] and Muller and Prokofyeva [7] reported that the *B* mutant was actually a tandem duplication of seven bands in the X chromosome and that *BB* was a triplication for that region. Hence *BB* was arising from unequally paired duplicated regions accompanied by normal rather than "unequal" crossing over. Interestingly, Wright had predicted that *B* itself would be a duplication before it was demonstrated cytologically [8].

Bridges had earlier called attention to duplication-like structures in the salivary gland chromosomes of wild-type larvae [9]. In particular, he interpreted numerous double banded structures, or "doublets," as two duplicated bands fused along their edges. Their structure suggests that they are reverse (ABBA), rather than direct (ABAB), repeats of single bands (Fig. 1). Bridges' cytological evidence for such repeats combined with Sturtevant's demonstration of position effect suggested that multiple alleles of a given gene might in some cases be resolvable into two or more repeated genes that acted like one because of a position effect. Evidence that multiple alleles might be resolvable into separable loci began to be obtained in the late 1930s by C. P. Oliver at the University of Minnesota. He found a low frequency

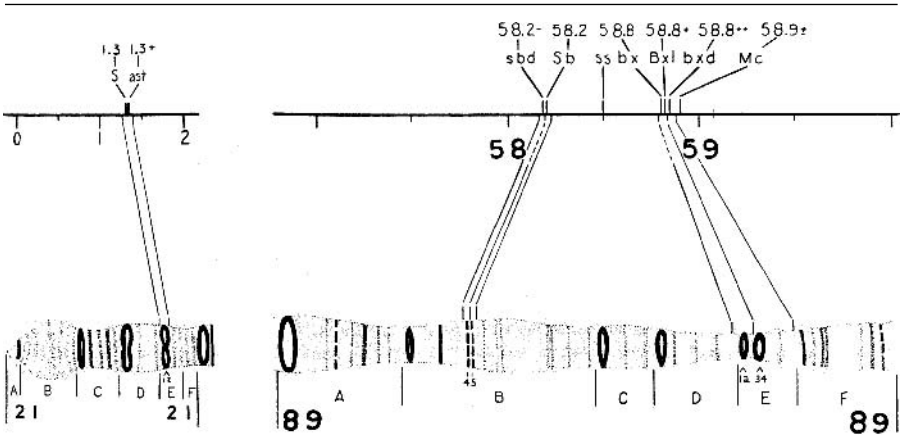

Figure 1. A correlation of the genetic and salivary gland chromosome locations of the three sets of pseudoallelic genes studied for *cis–trans* effects. At the left are the correspondences found near the extreme left end of the second chromosome; at the right is shown a section from the middle of the right arm of the third chromosome. The symbols *ss* and *Mc* refer to the loci *spineless* and *Microcephalus*, respectively; other symbols are described in the text. (Reprinted from [11].)

of revertants to wild type in the offspring of females heterozygous for two recessive *lozenge* (*lz*) eye mutations. Although the revertants were invariably associated with crossing over in the region, he was unable to detect a reciprocal crossover having both mutants in the same chromosome. He therefore could only suggest that the revertants could be explained as the result of "unequal crossing over or crossing over between 'repeats.' "[10].

STAR AND ASTEROID

I was an undergraduate at that time and Oliver generously gave me a desk in his laboratory and allowed me to work on a new rough-eyed mutant that had been given to me by E. Novitski, who was then at Purdue University. [Novitski and I had begun our work with *Drosophila* in high school around 1935]. Bridges had suggested that it be called *Star-recessive* (S^r), since it acted as an allele of a weakly dominant rough eye mutant, *Star* (*S*). Thus, $S/+$ flies have slightly smaller eyes that are slightly roughened; S^r/S^r flies have eyes reduced to about half their normal size and with a very roughened surface; while S/S^r flies are nearly eyeless (figured in [11]). Although in a preliminary test, I had found a revertant of S^r or of *S* in 3,235 offspring of S/S^r females, when flanking markers were introduced I obtained no more wild-type products among 9,294 offspring [12].

In spite of these inconclusive results, I continued the study of *S* and S^r as one of Sturtevant's graduate students at Caltech, commencing in 1939. In the tradition of Morgan, Sturtevant allowed his students considerable freedom to choose their thesis research projects. Quite a risk was involved in choosing to work on *S* and its "alleles." Crossovers between them would be rare if they were to occur at all. Even if the wild-type crossover could be recovered, it was expected that it would be very difficult to detect the reciprocal, or double-mutant crossover.

To increase the resolving power of the analysis, I made use of the interchromosomal effect of rearrangements on crossing over. Introduction of heterozygosity for inversions in chromosome arms other than the left arm of the second chromosome, in which *S* is located, resulted in an approximately fourfold increase in the frequency of crossing over in the vicinity of *S*. As in Oliver's work on *lz*, the revertants were invariably associated with crossing over between *S* and S^r. I renamed the latter "allele," *asteroid* (*ast*).

A tandem duplication for the *S* region which I had found as an X-ray-induced revertant of *ast* [13] lent itself to the recovery of the *S ast* double mutant chromosome [14]. A striking position effect was in evidence: whereas, $S +/+ ast$ is nearly eyeless, the complementary genotype, *S ast* / + +, is nearly wild type, except for a slightly smaller and slightly roughened eye indistinguishable from that of $S/+$ [14], figured in [11].

S and *ast* proved to be localized to the 21 E 1–2 doublet of the salivary gland chromosomes (Fig. 1), the doublet which Bridges had singled out as being a representative

example [9]. These cytogenetic studies of *S* and *ast* formed my doctor's thesis [15] published in part in 1945 [16].

Comparison of the difference in phenotype between *cis* vs *trans* genotypes is usually referred to as the *cis–trans* test, and the position effect, if present, as the *cis–trans* effect. For a history of this terminology see Hayes [17].

EARLY STUDIES OF THE BITHORAX MUTANTS

In 1945, the time seemed ripe to look for more examples of the *Star-asteroid* type in the genome. An intriguing region of the third chromosome included three loci within less than one centiMorgan; namely, the bristle mutations, *Stubble* (*Sb*) and *spineless* (*ss*), and a homeotic mutation, *bithorax (bx)* (Fig. 1). Certain useful combinations of these mutants had already been synthesized by Bridges and maintained in the Caltech stock collection. The recessive alleles of *Sb* proved to be at a separate locus, that I named *stubbloid (sbd)*, less than 0.1 cM to the left of the *Sb* locus. An especially striking position effect occurs: sbd^2 +/+ *Sb* flies have extremely short blunt bristles, while sbd^2 *Sb*/++ flies are wild type with no trace of the dominant short-bristle phenotype of *Sb*/+ flies.

It soon became evident that the diverse array of existing mutations of the bithorax type held considerable promise of being a cluster of genes rather than a multiple allelic series. It was for this reason that they were chosen for study rather than with any belief that they would tell us something about how genes control development.

The original *bx* mutant had been found by Bridges in 1915 as a transformation of the third thoracic segment (T3) toward the second (T2), notably causing the halteres to become partially wing-like. Body segments and structures of the wild-type adult are correlated with those of the late embryo in Fig. 2. *bx* was the first example of a mutant that exhibited homeosis, a term Bateson had first coined for conversion of one structure into an homologous one (discussed in [18]). In 1919, Bridges found a somewhat similar mutant that fully complemented *bx*, so he named it *bithoraxoid (bxd)*; i.e., *bx/bxd* is wild type in phenotype. However, he later showed that bx^D, which W. F. Hollander had found, failed to complement either *bx* or *bxd* [19].

Although the original *bx* mutant has 100% penetrance, it is a highly variable transformation of, as it turns out, only the anterior portion of T3 toward anterior T2. Fortunately, two other *bx*-like mutants, bx^{34e} (J. Schultz) and bx^3 (C. Stern) had also been saved by Bridges [19]. These have 100% penetrance and nonvariable weak and strong transformations, respectively, of anterior T3 toward anterior T2. The wing-like halter of the bx^3 homozygote is shown in Fig. 3.

Flies homozygous for *bxd* show 100% penetrance for a partial transformation of only the posterior portion of T3 toward posterior T2. The wing-like halter of a homozygote for an extreme *bxd* mutation, bxd^{100}, is shown in Fig. 3. In addition, *bxd* flies also have the first abdominal (A1) segment transformed toward T3, occasionally producing tiny rudimentary T3-like legs. A *bxd* hemizygote has a well developed T3-like leg on the transformed A1 (Fig. 4D).

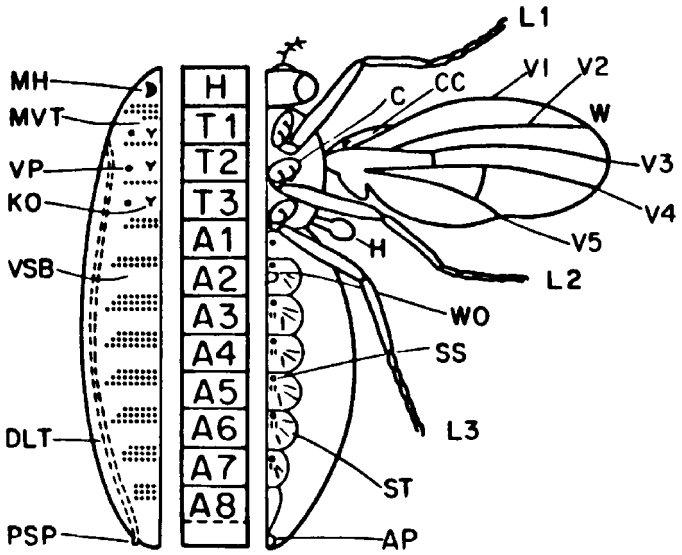

Figure 2. Comparison of the ventral cuticular pattern of the late embryonic stage with that of the adult stage. MH = mandibular hooks; MVT = midventral tuft; VP = ventral pits; KO = Keilin's organ; VSB = ventral setal belts; DLT = dorsal longitudinal (tracheal) trunk; PSP = posterior spiracle; H = head; T = thoracic; A = abdominal; L = leg; W = wing; H = halter; C = coxa; CC = costal cell (of wing); V = vein; WO = Wheeler's organ; SS = sensillum (on segments A1 to A7, inclusive); ST = sternite; AP = anal plate. (Modified from [103].)

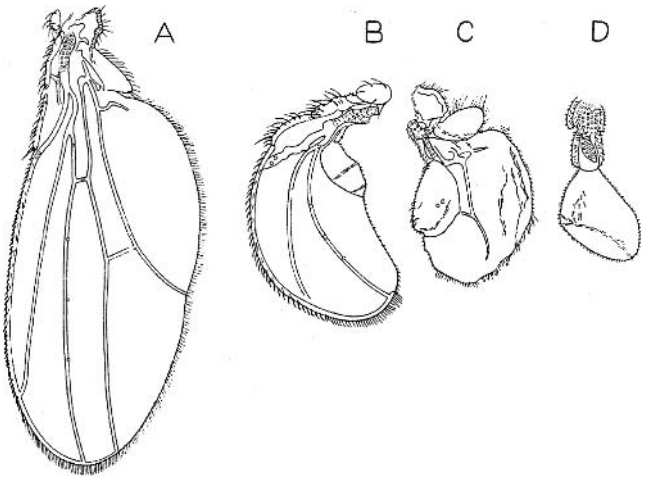

Figure 3. Camera lucida drawings of: (A) the wild type (T2) wing; (B) the corresponding appendage on T3 of a bx^3 homozygote; (C) the corresponding appendage, on T3 of a bxd^{100} homozygote; (D) the wild-type T3 halter. Only (B) and (C) are drawn to the same scale. (Reprinted from [11].)

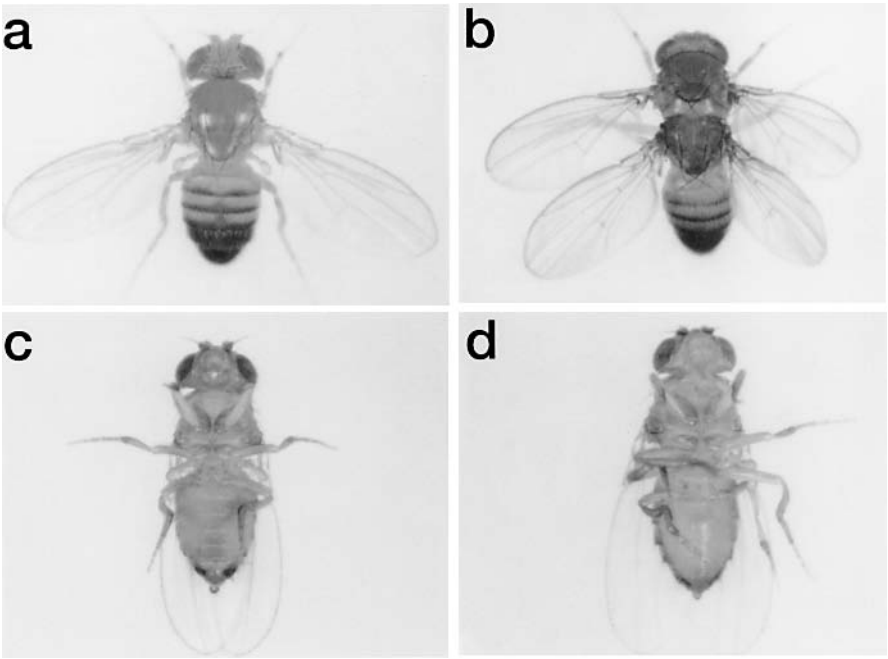

Figure 4. Extreme segmental transformations. (a) Wild-type male. (b) *abx bx*^{3} *pbx* homozygote, in which T3 is transformed toward T2. (c) Wild-type female, ventral view. (d) *bxd/Df-P2* female, ventral view having an extra pair of T3-like legs on A1 (unpublished).

A crossing-over analysis showed that *bx*^{D} occupies a separate locus between the *bx* and *bxd* loci, and therefore it was first renamed *Bithorax-like (Bxl)* (Fig. 1), and later, *Ultrabithorax (Ubx)* [11]. This analysis provided a number of *cis* and *trans* genotypes that exhibited position effects. Examples are shown in Fig. 5.

GENE EVOLUTION BY TANDEM DUPLICATION

These early studies were viewed as supporting a simple hypothesis about how new genes arise from preexisting genes. Based on the work of Sturtevant and Bridges, already cited above, the hypothesis proposed that new genes evolve from old genes by a two-step process: tandem gene duplication followed by one of the resulting duplicates mutating to a new function [11]. This "new" gene would generally not be easily established in the population unless the other, or "old" gene, was retained to carry out the old function. As a result the genome would be expected to contain clusters of closely linked and functionally related genes that superficially act like a single gene. At the Cold Spring Harbor in 1950, I reported [11] on the evidence in support of this hypothesis from three studies: of other organisms; of the above mentioned *S*, *Sb* and *bx* regions; and of *lz* mutants by Green and Green [20].

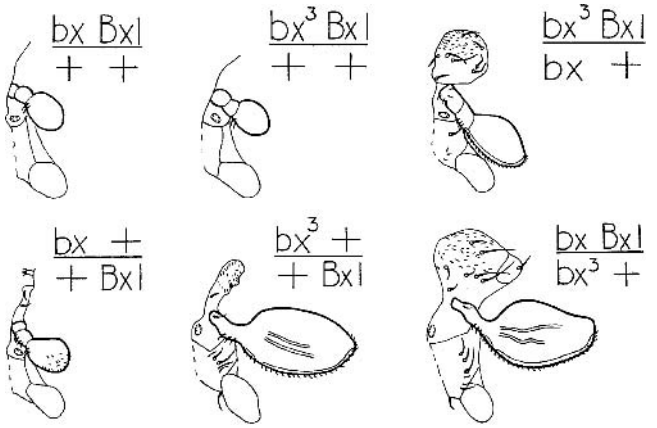

Figure 5. *Cis–trans* effects involving the *bithorax (bx)* and *Ultrabithorax* (here designated *Bxl*) mutants, illustrated by camera lucida drawings of the dorsal and lateral region of T3 of the adult fly. The pair of genotypes in each vertical column are identical except for the way in which the alleles are distributed between homologous chromosomes. (Reprinted from [11].)

AN EARLY MODEL OF THE *CIS–TRANS* EFFECT

A model (Fig. 6) was also presented at that Symposium to account for the *cis–trans* effect [11]. It was based on the then generally accepted biochemical dogma that genes were proteins, and that they could catalyze enzymatic reactions. The wild-type alleles of *a* and *b* were assumed to control sequential steps in a biochemical pathway in which a substrate, S, is converted into two products, A and B, that are produced at the site of the genes in the chromosome. The *a* and b mutants are assumed to lower production of A and B, symbolized as <A and <B, respectively (Fig. 6b). As a result, *a b*/+ + (Fig. 6a) is expected to produce enough B to be wild type, or nearly so. By contrast, *a* +/+ *b* (Fig. 6b) would produce insufficient B, and therefore be mutant in phenotype [11].

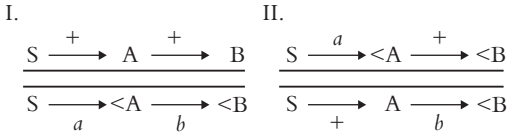

Figure 6. An early model to explain *cis–trans* effects. Paired homologous chromosomes are diagrammed by the long horizontal lines. Two adjacent loci are shown with either wild-type (+) or mutant (*a* or *b*) alleles. The genes at these loci are assumed to catalyze the reaction of the substrate S into product A, and product A to product B. The A product is assumed to remain in the vicinity of the locus where it is produced. The *cis* configuration (a) produces sufficient B to give a nearly wild-type phenotype. The *trans* configuration (b) produces insufficient B resulting in a mutant phenotype. (Reprinted from [11].)

The model could therefore also account for polarized *cis–trans* effects. For example, when bx^3 is opposite an extreme X-ray-induced *bxd* allele, such as bxd^{100}, bx^3+/+ bxd^{100} flies have a very slight wing-like transformation of the posterior portion of the halteres. On the other hand, they have no trace of the *bx* phenotype, even though the latter phenotype is a more sensitive one for the detection of slight effects than is the *bxd* phenotype. Hence bx^3 appears to weakly inactivate bxd^+, but even extreme *bxd* mutants do not inactivate bx^+.

In retrospect the model is no longer compatible with our present knowledge of the structure and function of the gene. However, since no assumptions were made about the nature of the products S, A and B, the model might still be tenable if S, A and B correspond to noncoding RNA transcripts. The real value of this hypothesis was that it led to an experiment that revealed a new phenomenon of "transvection," to be discussed below.

CONTRABITHORAX—A GAIN OF FUNCTION MUTATION

In 1954 [21], an X-ray-induced mutation was found that had T2 transformed toward T3. This "gain-of-function" [22] phenotype was therefore the inverse of the T3 to T2 transformation characteristic of the *bx* and *bxd* mutations. Surprisingly, mapping showed it to be a double mutation made up of a gain-of-function mutation, *Contrabithorax (Cbx)*, the locus of which lies between the *bx* and *Ubx* loci, and a recessive loss-of-function mutation, *postbithorax (pbx)*, that occupies a new locus distal to that of the original *bxd* mutation. Thus the map expanded to five loci, at which there were mutations with effects on one or more of the segments, T2, T3, and A1 [23].

This cluster of mutant loci came to be called the *Ubx* domain of a much larger cluster, the bithorax complex (BX-C) (Fig. 7). The latter name is derived from "gene complex," a term invented by Brink for a closely linked cluster of genes that he predicted would be closely related in function [24]. Kaufman and his coworkers defined the Antennapedia-complex (ANT-C) that controls the identity of segments anterior to those controlled by the BX-C [25].

Unlike the *bxd* mutant, *pbx* has only a transformation of the posterior portion of T3 toward posterior T2. The *trans* heterozygote, *bxd* +/+ *pbx* shows a *pbx* phenotype but no trace of the transformation of A1 toward T3 that is typical of the *bxd* homozygote. Furthermore, bx^3+/+ *pbx* also shows, albeit weakly, a *pbx* phenotype, but no trace of a *bx* phenotype. In both of these examples the *cis*-heterozygotes are wild type. Thus, polarized inactivation of pbx^+ function can be effected in *cis* by either *bxd* or bx^3.

THE TRANSVECTION PHENOMENON

One of the predictions of the early model of the *cis–trans* effect (Fig. 6) was that disruption of somatic pairing might intensify the difference between *cis* and *trans* types. Specifically, heterozygosity for a chromosomal rearrangement that would disrupt

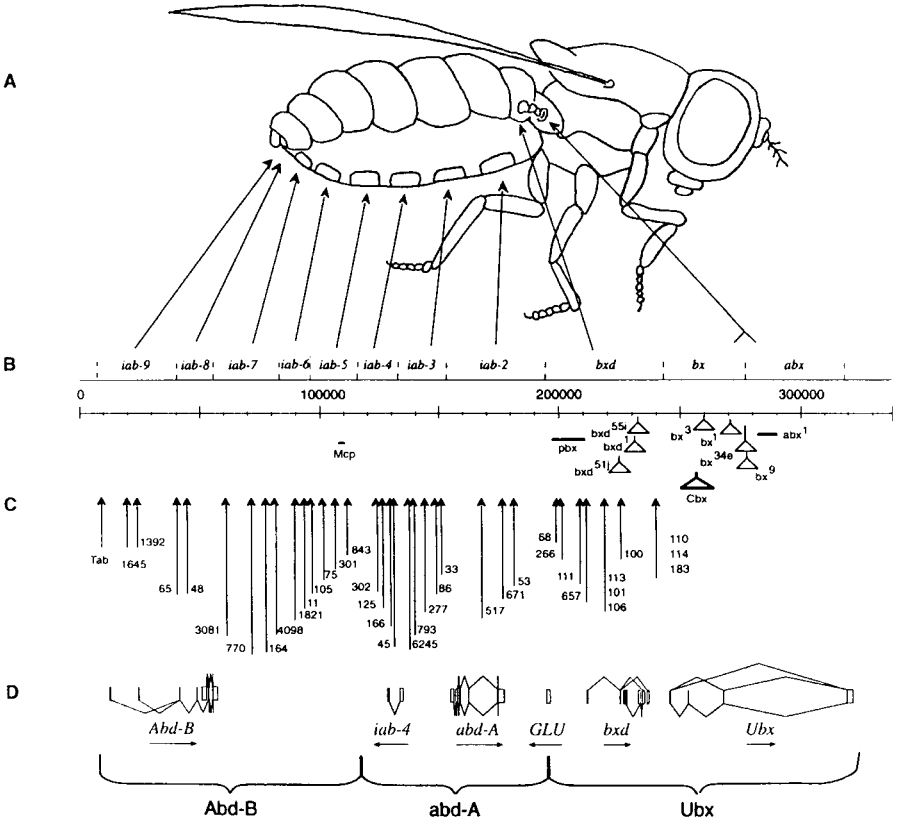

Figure 7. Genetic and Molecular maps of the BX-C. (A) Adult female, showing the segments affected by BX-C mutations. (B) Regulatory regions aligned to the DNA map which covers 338 kb [101]. (C) Mutant lesions. Insertions are indicated by triangles, deletions by horizontal bars, and rearrangement breakpoints by vertical arrows. (D) Transcription units within the three domains, *AbdB*, *abdA*, and *Ubx*. Alternate promoters and alternate splicing patterns are indicated. GLU marks a sequence predicted to encode a homolog of a mammalian glucose transporter protein; the fly sequence has no apparent function in segmental specification [101]. The *iab-4* and *bxd* transcription units do not encode proteins (see text). The *iab-9* through *iab-5* regulatory regions control expression patterns of *Abd-B*; *iab-4*, *iab-3*, and *iab-2* regions control *abd-A*; and the *bxd*, *bx*, and *abx* regions control *Ubx*.

pairing in an *a* +/+ *b* individual would be expected to cause a more extreme *b* phenotype. The prediction was borne out, and a powerful new method was discovered for detecting chromosomal rearrangements in the first generation after their induction. The method was first used to measure the frequency of induction of such rearrangements in the progeny of males exposed to neutrons from an atomic bomb test [26].

The method detects only the majority of rearrangements having one breakage point in a "critical" region of some 500 bands of the salivary gland chromosomes;

namely, the region between the centromere of the third chromosome and the locus of the BX-C. Similar findings were later obtained for the *decapentaplegic (dpp)* region in 2L [27] and for the *eyes-absent (eya)* region in that arm [28].

Although at first only *trans* genotypes showed the phenomenon, it was soon found that *Cbx Ubx*/+ + was also subject to transvection [23]. *Cbx* in this genotype was found to exert a slight gain of function of Ubx^{+}, chiefly expressed by spread wings and a reduced alula, when the chromosomes are paired. That effect is abolished (wings normal) when pairing is disrupted by transvection-suppressing rearrangements. As a result, it became possible to mutagenize wild type and to select rearrangements that abolished the weak *Cbx* effect of the *Cbx Ubx*/+ + genotype. Among the resultant rearrangements, some, as expected, had breaks within the BX-C. These breaks were unselected for any effect on function in the BX-C other than suppression of transvection. Such rearrangements, when subsequently tested over deletions of the BX-C, provided the basis for discovering additional *infra-abdominal (iab)* regions and ordering all of the known regions from *iab-2* to *iab-8*, inclusive. The *iab-9* region has been identified by means of breakpoints associated with gain-of-function mutants in that region, namely *Uab* [29] and *Tab* [30] (Fig. 7).

In the process of isolating transvection-suppressing rearrangements, a sex-linked mutant was recovered in two independent cases, whose effect was to enhance the *bithorax* phenotype of bx^{34e}/*Ubx*. This mutant, originally named, *enhancer-bithorax (e-bx)*, proved to be an allele of the *zeste* (*z*) gene [31] and to be like the z^{a}, or null, alleles of Gans [32]. It was soon found that z^{ae-bx}, as it is now symbolized, suppresses transvection not only in the case of the BX-C but also *dpp*. The *z* protein has been shown to be a DNA-binding protein that binds *in vitro* to the *Ubx* gene as well as to other genes [33]. Benson and Pirotta suggest that "transvection effects are a by-product of normal intragenic *z* action" [34].

Remarkably, tandem duplications for the BX-C region act as powerful suppressors of transvection, when placed opposite the *Cbx Ubx* chromosome (Lewis, unpublished). Evidently, the duplicate regions pair intrachromosomally with one another and prevent the *Cbx* mutant from gaining access to the Ubx^{+} regions. In organisms which lack somatic pairing between homologous chromosomes, such as the vertebrates, intrachromosomal pairing of tandem repeats may still occur. In that event, transvection may prove to be a general phenomenon applicable to tandemly repeated regions in all organisms.

MOBILE ELEMENTS IN THE BITHORAX COMPLEX

In 1932 Bridges [35] reported the discovery of one of the first suppressor mutants in *Drosophila*. He named it *suppressor-of-Hairy wing* (now symbolized *su(Hw)*) and found that it acted as a recessive suppressor of certain alleles of a number of other genes. Although the bx^{3} mutation had been saved as a balanced stock, when I used it in 1946 the homozygote appeared wild type in phenotype, as if the mutant had reverted. In fact, the stock had acquired a suppressor that mapped to the same locus as that of

Bridges' *su(Hw)*. His mutant had been lost, but the new occurrence, named *su^2-Hw*, suppressed the same group of specific alleles as was reported for *su(Hw)*. In addition, we found that it not only suppressed *bx^3*, *bx^{34e}* and *bxd*, but also specific alleles of many other genes [36].

The mechanism by which *su^2-Hw* suppresses specific alleles proved elusive until many years later, when it was shown that such alleles are the result of an insertion of the mobile element, *gypsy*, almost invariably in the noncoding portion of the gene [37]. The wild-type *su^2-Hw* gene codes for a DNA-binding protein [38] that is assumed to bind to specific sequences in the *gypsy* element, thereby lowering the rate of transcription of the gene containing that element [39]. Hence, in the *su^2-Hw* homozygote, failure of the mutant protein to block transcription of that gene would restore the wild-type phenotype.

In retrospect, it now seems extremely fortunate that the early mapping of mutants in the *Ubx* domain was carried out using mutations that were insertions or deletions. Thus, *bx^3* and *bxd* are *gypsy* insertions (7 kb in length), *Ubx* is a "*Doc*" mobile element [40], and *pbx* and *Cbx* are a deletion and insertion, respectively, of a 17 kb segment of DNA [40]. Had they been true point mutations, they might then have been subject to gene conversion, a phenomenon first discovered in fungi and characterized by high negative interference over short map regions and aberrant segregation of alleles in a meiotic tetrad (reviewed by Holliday [41]). As a result, unambiguous ordering of mutants in the *Ubx* domain would probably not have been possible.

HALF-TETRAD MAPPING OF THE ULTRABITHORAX DOMAIN

The great diversity of phenotypes represented by mutants at the five known loci of the *Ubx* domain made it relatively easy to derive double mutants and, in turn, higher multiples, including the quintuple mutant, *bx^3 Cbx Ubx bxd pbx*. Although flanking marker recombination provided unambiguous ordering of these loci, the possibility of gene conversion was of sufficient concern that I undertook a half-tetrad analysis of that domain.

Attached autosomal arms had been synthesized, partly to be able to perform such an analysis, by I. Rasmussen and E. Orias, working in my laboratory [42]. Females were constructed with the quintuple mutant combination in one of the attached arms and the corresponding five wild-type alleles in the other arm, along with appropriate flanking markers (Fig. 8); their phenotype was indistinguishable from that of *Ubx*/+ [43]. Among approximately 221,000 female offspring, 19 were the result of exchanges in the regions between the loci of *bx^3* and *pbx*. Reciprocal crossovers were recovered simultaneously from four out of five of the regions and were easily detected by their having strong *cis–trans* effects when compared with the maternal *Ubx*/+ phenotype. None of the half-tetrads showed evidence of gene conversion. As one possible explanation it was suggested that "one or more of the mutants are associated with minute rearrangements which have precluded the occurrence of intragenic recombination" [43].

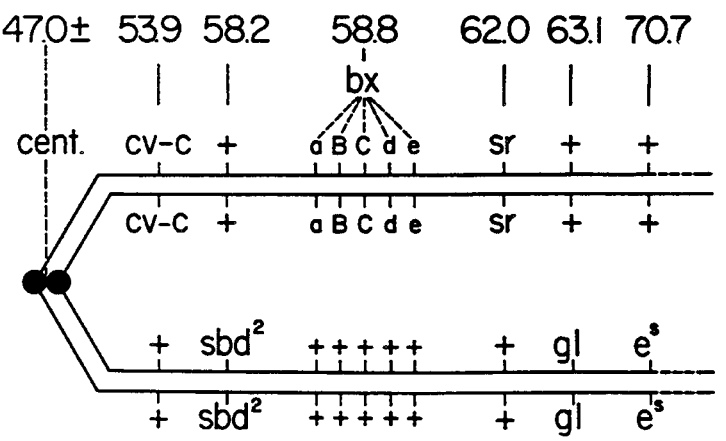

Figure 8. Diagram of the genetic constitution of attached 3R chromosomes heterozygous for a quintuple bithorax mutant combination and for closely linked marker genes. The symbols are: cent., centromere; cv-c, *cross-veinless-c*; +, wild type allele; bx, the BX-C; a, *bithorax-3*; B, *Contrabithorax*; C, *Ultrabithorax*; d, *bithoraxoid*; e, *postbithorax*; sr, *stripe*; sbd^2, *stubbloid-2*; gl, *glass*; e^s, *ebony-sooty*. The standard map locations are shown above the mutant symbols, in centiMorgan units. (Reprinted from [43].)

I had earlier used attached-X females to perform half-tetrad analyses of the *white* (*w*) eye mutant and its "allele," *white-apricot* (w^a) [44]. Exchanges between *w* and *apricot* (*apr*), as I renamed w^a, were detected in the progeny of *w* +/+ *apr* attached-X females carrying closely linked flanking markers. Reciprocal crossover products of such exchanges were recovered simultaneously in several daughters. Whereas, *w* +/+ *apr* females flies have a pale pink eye color, *w apr*/+ + females have the red eye color of wild type. Flanking markers indicated that *apr* lies to the right of *w*, the map distance being about 0.01 cM. No evidence of gene conversion was detected.

THE BITHORAX COMPLEX AND ITS ORGANIZATION

Duncan has provided a comprehensive and thorough review of the complex [45]. I have recently given a brief historical review of work on the homeotic clusters in a number of organisms [46]. The following sections will be concerned chiefly with the organization and function of the BX-C.

By generating somatic mosaics for the *bx* phenotype. I was able to show that the effects of the *bx* mutants are highly autonomous [47]. Thus, when cells mutant for the bx^3 function arise from induced somatic crossing over in bx^3/+ animals, the cells express the expected mutant phenotype, namely T2-type bristles on T3, which normally lacks any bristles. Morata and Garcia-Bellido provided additional examples and showed that the mutant tissue could arise from exchange events induced as late as the last larval instar [48]. Thus, the wild-type products of at least the *Ubx* domain are not diffusible to any appreciable extent, and such products continually regulate the development of cuticular structures of T3 into late larval life.

In 1964, borrowing from the then-prevailing biochemical dogma based on the operon model, I interpreted the function of the genes of the BX-C to be to "repress certain systems of cellular differentiation and thereby allow other systems to come into play" [49]. Clearly, that function could also be to activate other systems, as Garcia-Bellido later pointed out [50].

Early studies of the BX-C had reached an impasse until homozygotes for deletions of parts, or of all, of the complex were found to have striking effects on cuticular structures of the late embryo. Simple preparations of late embryos cleared in a drop of lactic acid permitted the study of many embryonic lethal phenotypes.

It became evident that the BX-C included genetic material that programmed the development of not only T3 and A1, but also all of the remaining abdominal segments from A2 through A9, inclusive [29]. Thus, animals lacking the entire BX-C, as the result of being homozygous for deletions that removed all of the 89E1-4 bands, were found to die at the end of embryonic development and to have a striking transformation of the first seven abdominal segments toward the T2 segment. The cuticular structures involved include anterior spiracles, ventral pits, Keilin organs and other sense organs. The A8 and A9 segments transform even more anteriorly toward a head segment, based on their developing tiny rudiments of the mandibular hooks (Fig. 2).

It is always dangerous to deduce the wild-type function of a gene from a loss-of-function mutations, especially for genes which affect morphology. The wild-type function of major regions of the BX-C could be inferred by adding them to a homozygous deletion of the BX-C (*Df-P9*) [29]. For example, a duplication, $Dp(3)bxd^{100}$ that includes a wild-type copy of the *Ubx* domain proximal to the *bxd* region, restores the longitudinal tracheal trucks in all segments from T2 to A8, inclusive. The genes of the BX-C control the development of specific structures and organs of the segments rather than segmentation *per se*. The particular segments in which a given BX-C gene is expressed is determined by the combined action of *trans*-regulatory genes.

The analysis of the functions of *cis*-regulatory regions located distal to the *Ubx* domain, made use of chromosomal rearrangements having breakpoints in those regions. Such rearrangements have a recessive loss-of-function expressed as a transformation of a posterior segment toward a more anterior one; thus rearrangement breakpoints in the *iab-2 cis*-regulatory region cause A2 to transform toward A1. By 1978, three *iab* regions had been identified, *iab-2*, *iab-3*, and *iab-8*, and a fourth, *iab-5*, was inferred from an analysis of revertants of a dominant gain-of-function mutation, *Miscadestral pigmentation (Mcp)* by M. Crosby [29]. Subsequently, the regions of the BX-C controlling abdominal development were divided into two domains, *abdominal-A (abd-A)* and *Abdominal-B (Abd-B)*, based on lethal complementation studies [51, 52].

RULES GOVERNING *CIS*-REGULATION OF THE BX-C

The BX-C is regulated in *cis* and in *trans*. Rules governing its *cis*-regulation are considered first and were deduced from genetic analysis. Many of the rules are highly

unusual and possibly unique. It seems likely that their molecular analysis will reveal hitherto unsuspected regulatory mechanisms.

Colinearity

The rule of colinearity (COL) states that the order of the BX-C loci in the chromosome parallels the order in which the units at those loci are expressed along the antero-posterior axis of the body. Two types of gradients had been invoked to explain this rule: "an antero-posterior gradient in repressor concentration along the embryo and a proximo-distal gradient along the chromosome in the affinities for repressor of each gene's *cis*-regulatory element" [29].

Molecular studies confirmed the rule and extended it to all of the abdominal *cis*-regulatory regions from *iab-2* to *iab-8*, inclusive [53]. Associated with the COL rule is the strong tendency for the proteins of the BX-C, once expressed to continue to be expressed more posteriorly in the body except for the terminalia. This is elegantly shown in Fig. 9, for the *Ubx, abd-A* and *Abd-B* proteins visualized by the use of immunostaining with antibodies specific to each.

Cis-inactivation

The second rule of *cis*-inactivation (CIN) states that loss-of-function mutations in a given *cis*-regulatory region tend to inactivate the next more distal region of the complex. Examples have already been cited for the polar inactivation of the pbx^{+} function by bx^{3} and by *bxd*. Other examples were later found in analyzing rearrangement breakpoints in the *iab* regions of the BX-C [54]. It has not been

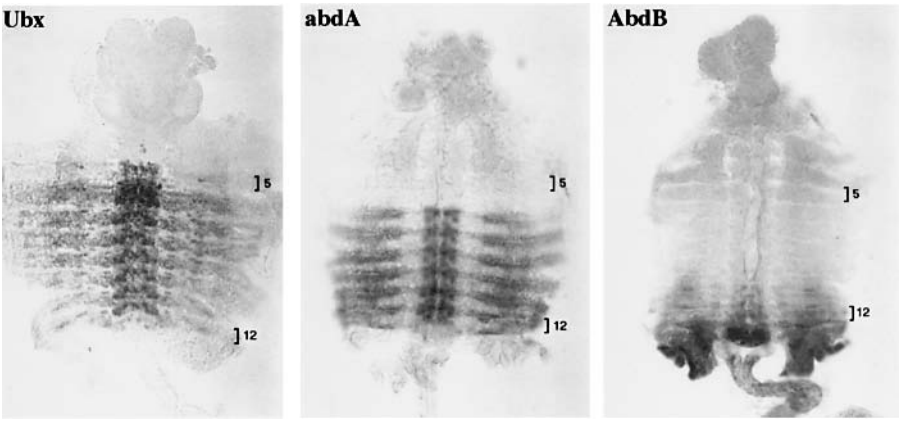

Figure 9. Embryos stained with monoclonal antibodies to the protein products of the BX-C. Preparations are of 10–12 h embryos, split along their dorsal midlines and flattened. Brackets indicate parasegments 5 and 12, which correspond approximately with the third thoracic and seventh abdominal segments, respectively. *Ubx* protein appears in parasegments 5–13, *abd-A* protein in 7–13, and *Abd-B* protein in 10–13. (From W. Bender, unpublished.)

possible to establish whether there are CIN effects between major domains of the BX-C.

Cis-overexpression

The third rule of *cis*-overexpression [COE] is a quite surprising one. The rule states that the loss of function associated with a given *cis*-regulatory region tends to be accompanied by an overexpression of the function associated with the *cis*-regulatory region that lies immediately proximal to it. In the abdominal domains, rearrangements with breaks in the *iab-3* region, for example, not only have a loss-of-function *iab-3* phenotype (A3 transformed toward A2) but a gain-of-function of the *iab-2*$^{+}$ region that is manifested as a transformation of the A1 segment toward A2. Other examples have been described [54].

COE effects are known not only for breakpoints of chromosomal rearrangements but for gypsy insertions. An important one is a COE effect of *bx*^{3}. Flies homozygous for *bx*^{3} have a reduction in the extreme anterior region of T2. This effect is dominant since it is not suppressed by duplications that totally suppress the recessive *bx*^{3} transformation of T3 toward T2. An X-ray-induced mutant, *anterobithorax (abx)*, was discovered that had a weak bithorax-like phenotype. It is located just proximal to *bx*, and *abx bx*^{3} double mutants lack the COE effect on T2 seen in the *bx*^{3} single mutant genotype.

Until *abx* had been found, it was not possible to achieve a full transformation of T3 toward T2; i.e., the *bx*^{3} *pbx* double mutant homozygote fails to transform the most anterior portion of T3. Flies homozygous for the triple mutant, *abx bx*^{3} *pbx*, were constructed and proved to have virtual complete transformation of the wing and cuticular structures of T3 transformed toward those of T2, resulting in a four-winged fly (Fig. 4).

NEGATIVE *TRANS*-REGULATION OF THE BITHORAX COMPLEX

In 1947, a remarkable X-ray-induced dominant mutant, *Polycomb (Pc)*, was found by P. H. Lewis [55]. It had sex combs on not only on the first, but the second, and third pair of legs, and rudimentary antennal to leg transformations resembling those of *Antennapedia (Antp)* mutants. It also had effects that were only later realized to be gain of function of genes in the *Ubx* domain; namely, reduction in the extreme anterior region of T2 and reduction in the wing similar to that of weak *Cbx* phenotypes, such as in *Cbx Ubx*/+. It was nearly 30 years before it was realized that *Pc* is a mutation in a gene that acts as a negative regulator of the BX-C, and of the ANT-C complex as well. Thus, the homozygous *Pc* embryo has the three thoracic and the first seven abdominal segments all transformed toward A8, presumably as the result of derepression of the *Abd-B* domain [29] (figured in Duncan [56]).

Duncan found a second mutant of the *Polycomb* type, *Polycomb-like (Pc-l)* [56]. *Pc* and *Pcl* have proved to be but two of a family of genes that act as negative regulators [57]. That the *Pc* protein is involved in binding to the BX-C and the ANT-C regions

(as well as to other regions) has been elegantly shown by immunostaining of salivary gland chromosomes with an antibody to that protein [58]. Since the *Pc* protein is a non-histone chromosomal protein, rather than a DNA-binding protein [59] its binding specificity may reside in its complexing with proteins of other genes of the *Pc* family, some of which first bind specifically to BX-C and ANT-C.

POSITIVE *TRANS*-REGULATION OF THE BITHORAX COMPLEX

Positive *trans*-regulators were also found, such as *Regulator of bithorax (Rg-bx)*. An analysis of this mutant, and of deficiencies which include the locus, indicate that the wild-type gene is a positive regulator of the BX-C [60]. A partial loss-of-function allele, *trithorax (trx)*, was then found by Ingham [61]. The *trx* gene has been cloned and is a DNA-binding protein of the zinc finger category [62, 63]. More recently, Kennison and Tamkum have identified a family of genes like *trx* that act when mutated as enhancers of *bx* phenotypes [64].

Additional classes of *trans*-regulators of the BX-C have come from the studies of Nusslein-Volhard and Wieschaus [3]. For example, the gap gene, *hunchback (hb)*, is involved in establishing major subdivisions of the body regions. It encodes a zinc finger protein and acts as a negative regulator of the BX-C, keeping the complex turned off in the anterior regions of the body, presumably by the binding of the *hb* protein to at least one specific motif in the *Ubx* gene [65]. A dominant mutant, *Regulator of postbithorax (Rg-pbx)*, is now known to be a gain-of-function mutation in the *hb* gene [66]. It produces variable *pbx*-like transformations of the halter [67].

Another example is the *Krupple (Kr)* gene of Gloor [68]. It is also a gap gene and encodes a DNA-binding protein [69, 70]. One motif to which it binds is in the *iab-2* region and, on two independent occasions, a mutation in a single specific base pair of that motif has resulted in a dominant *Hyperabdominal (Hab)* phenotype [71]. These gain-of-function mutants have poor penetrance, but in some crosses *Hab*/+ flies occasionally have only four legs and no halteres owing to T3 being transformed toward A2 [29].

MOLECULAR ANALYSIS OF THE BITHORAX COMPLEX

Molecular analysis of the *Ubx* domain of the BX-C was initiated by D. Hogness and coworkers in 1978 and they soon identified the major features of that region. The *bx* mutants, *Ubx*, and several *bxd* mutants all proved to be insertions of transposable elements [40]. Molecular studies revealed a single transcription unit coding for proteins in the *Ubx* domain [72, 73]. The embryonic distribution of the *Ubx* protein products was determined by White and Wilcox [74] and Beachy et al., [75], see also Fig. 8. The transcription unit and protein product of the second domain, *abdominal*-A, were characterized by Karch et al., [76]. The third domain, *Abdominal-B*, produces at least four transcripts [77–81] and two *Abd-B* proteins [80, 82, 83].

Surprisingly, the *cis*-regulatory regions are transcriptionally active, as first shown for the *bxd* region of the BX-C [84]. This region produces a large (26.5 kb) primary

transcript, that is then spliced to yield a family of nonprotein-coding RNAs (i.e., containing multiple stop codons). Similar noncoding transcription units are known for the *iab*-4 region [85].

THE *TRANS*-ABDOMINAL MUTATION

King and Wilson [86] called attention to the possible importance in evolution of creating novel phenotypes solely by rearrangements involving *cis*-regulatory sequences. A striking example was our discovery of an X-ray-induced dominant mutation, *Transabdominal (Tab)*. *Tab*/+ flies have a sexually dimorphic pattern of pigmented bands in the dorsal thorax of T2 (Fig. 10). Unlike the great majority of dominant gain-of-function phenotypes, *Tab*/+ has 100% penetrance and complete expressivity. Molecular and morphological studies [30] indicate that the pigmentation pattern of the bands resembles that normally found in the tergites of segments A5 and A6. Thus, the pigmented bands in the *Tab*/+ male dorsal thorax are broad as in the A5 and A6 male tergites; whereas, in the *Tab*/+ female they are narrow as in the corresponding female tergites. The *Tab* mutant is associated with an inversion having one breakpoint in the *iab*-9 *cis*-regulatory region (Fig. 7) and the other near the *stripe (sr)* locus in 90D which codes for an early growth-response transcription factor [87]. *In situ* studies [88] of the dorsal thoracic disc of T2, which gives rise to the dorsal thorax, show cells in *Tab*/+ animals that express the Abd-BII protein and its RNA. These cells correspond to the sites of the bands in the *Tab*/+ adult thorax and appear to be the sites of attachment for certain thoracic muscles. Our studies of the RNA and protein distributions in embryos and imaginal discs indicate that the *Tab* mutation represents

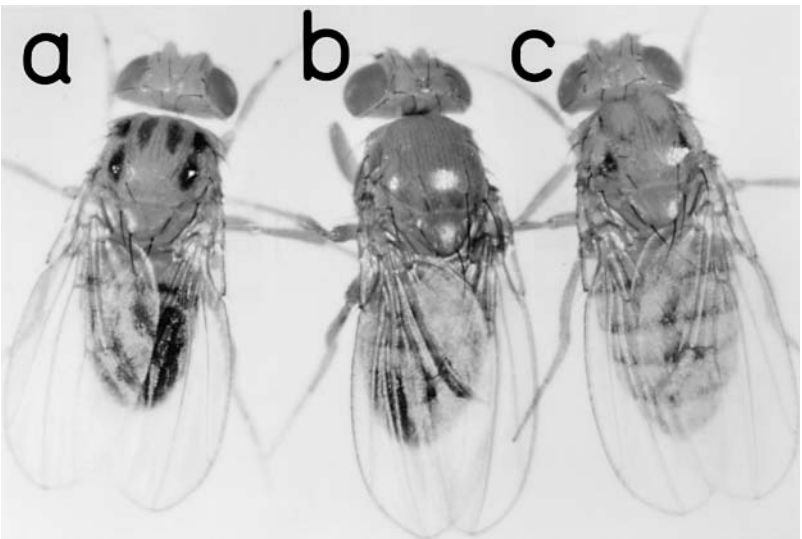

Figure 10. *Transabdominal*, a sexually dimorphic mutant of the *Abdominal-B* domain. (a) Wild-type male. (b) *Tab*/+ male. (c) *Tab* /+ female. Thoracic transformations are described in the text (unpublished).

a case in which *cis*-regulatory regions of a gene involved in defining the development of muscle attachment sites is now driving *Abd-B* protein expression [87]. Other minor disturbances in the abdominal tergites of *Tab*/+ flies are believed to involve ectopic expression of the *Abd-B* protein in such attachment sites for abdominal tergite muscles.

CONTROL OF SOMATIC GONAD DEVELOPMENT IN DROSOPHILA AND BOMBYX

As early as 1943, Itikawa reported [89] on a mutant designated E^N whose phenotype when homozygous parallels closely that of the homozygous deficiency for the BX-C in Drosophila (*Df-P9*). Itikawa's discovery that certain mutants of the "E" series lacked gonads led me to examine a dominant mutant, *Ultra-abdominal*^{4} (*Uab*^{4}), which is associated with a recessive *iab*-3 phenotype. Internally, the *Uab*^{4} hemizygote was found to lack gonads [29]. Subsequently, I found that rearrangements with breakpoints in the *iab*-4 region of the BX-C, when viable as homozygotes appear virtually wild type, but internally they lack gonads [54]. Loss of gonads in *iab*-2 and *iab*-3 mutant animals results from *cis*-inactivation of the *iab*-4 region (Lewis, unpublished). Since the gonad is of mesodermal origin, its loss was one of the first indications that the BX-C phenotypes were not limited to ectodermal tissues.

A comparative molecular analysis of the *iab*-4 *cis*-regulatory with regions controlling gonad formation in *Bombyx* and other animals may show how the homeotic genes control the development of a specific structure. Thus, since some of the more primitive nonsegmented animals, such as the nematode, have somatic gonads, it is likely that control of the initiation of their development will have common features. Of great interest will be the target genes in *Drosophila* that accomplish such initiation. A promising approach to understanding the process in human beings can be expected to come from analyzing molecularly the basis of inherited defects in the human gonad.

THE HOMEOBOX AND TANDEM GENE DUPLICATION

Molecular support for the assumption that tandem gene duplication was responsible for at least the coding portions of the BX-C and the ANT-C complex finally came with the discovery of the homeobox in 1984, by McGinnis et al. [90] and Scott and Weiner [91] who independently showed that the proteins encoded by the *Ubx* and *Antp* genes contain a remarkably conserved group of amino acids, known as the homeodomain. The DNA sequence encoding the homeodomain was named the homeobox [90]. The homeobox sequence is conserved to a remarkably high degree throughout the animal kingdom and it was used to probe for homologs of the BX-C and ANT-C in many other organisms, including vertebrates as well as invertebrates [92–94]. Most of these organisms have the homologs of both the BX-C and the ANT-C in a single complex known as the homeotic complex (HOM-C).

In unsegmented organisms like *Caenorhabditis* [94] there are apparently only a few HOM-C genes. Insects such as the silkworm, *Bombyx* [95] and the flour beetle, *Tribolium* [96] have larger clusters as in *Drosophila*. The most primitive vertebrates

represented by the lancelet, *Amphioxus* [97, 98] also have a single large HOM-C. However, higher vertebrates have four semiredundant copies of the HOM-C. In the mouse and human, each copy is on a different chromosome. This redundancy makes it difficult to dissect the function of a given gene in any one of the sets. Remarkable progress is being made by using gene knock-out techniques in mice, to study the role of the HOM-C genes in development. HOM-C gene expression in the mouse, as in *Drosophila*, obeys the rule of colinearity (reviewed by Lewis [46]). Their segmental expression limits are also regulated in *trans* by genes that are remarkably parallel to those of the *Pc* Group and *trx* Group (reviewed by Simon [99]).

HOM-C genes are now regarded as master control genes whose proteins bind to the *cis*-regulatory regions of target genes. The latter then activate or repress systems of cellular processes that accomplish the final development of the organism. Even minor mutant lesions in HOM-C genes may be expected to have global effects on such systems. An example is a targeted gene disruption of the mouse HOX A3 gene (formerly HOX 1.5) that leads to defects in the thyroid glands and surrounding tissues [100]. The resultant group of defects resembles those seen in the congenital DiGeorge syndrome of human beings.

COMPLETE SEQUENCE OF THE BITHORAX COMPLEX

The DNA sequence of the BX-C has now been completely determined [101] and a preliminary analysis made of it [102]. The protein-coding regions comprise only 2% of the entire sequence. The other 98% is expected to contain a diverse group of motifs to which *trans*-regulatory proteins bind, thereby conferring the specific spatial and temporal expression of the protein products of each domain. There may also be a regulatory role for noncoding RNA's of the type identified in the *bxd* and *iab-4* regions.

THE NEXT 50 YEARS

Only three of the many future challenges will be outlined: (1) molecular and genetic approaches are needed to determine the immediate target genes that are turned on or off by the genes of the HOM-C; (2) since the genes of the HOM-C have tended to remain tightly linked and colinear with their expression patterns along the body axis, it will be exciting to discover the underlying mechanisms that have kept them together, and (3) comparative DNA sequence analysis of the HOM-C among many different organisms may provide evidence that the *cis*-regulatory regions have evolved by tandem duplication. Ultimately, comparisons of the HOM-C throughout the animal kingdom should provide a picture of how the organisms, as well as the genes of the HOM-C, have evolved.

CONCLUSIONS

Basic research concerned with testing a simple hypothesis about how new genes arise from old genes led after many circuitous routes to the discovery of the homeotic

complex (HOM-C). This cluster of master control genes programs much of the development of all higher animal organisms. Each of the genes contain a homeobox, a remarkably conserved DNA sequence that provides molecular support for the hypothesis that the complex itself arose by a process of tandem gene duplication. The high degree of conservation of the HOM-C, itself, between vertebrates and invertebrates indicates that it arose from an ancestral complex over 500 million years ago, the estimated time of separation of these two great groups of organisms.

It is likely that mutations within the HOM-C's of human beings are the cause of certain genetically based abnormalities that arise at various stages of human development. Somatic mutations in genes of the HOM-C may conceivably be involved in the generation of tumors. Meanwhile, future genetic and molecular studies of the HOM-C in lower creatures that have but one set of the complex promise to advance our understanding of its role as a master regulator of development.

Much has been learned about the role of the HOM-C in development, and about its molecular products. Nevertheless, we are still unable to make sense of much of the DNA sequence of the BX-C or to explain how the complex is itself regulated. Progress will still need to be driven by the logic of genetics and by further increases in abstraction.

ACKNOWLEDGMENTS

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APPENDIX

CURRICULUM VITAE OF E. B. LEWIS

Name: Edward B. Lewis
Date of Birth: May 20, 1918
Place of Birth: Wilkes-Barre, Pennsylvania
Date of Death: July 21, 2004
Place of Death: Pasadena, California

Education:

University of Minnesota, Minneapolis, B.A. (Biostatistics)	1939
California Institute of Technology, Pasadena, Ph.D. (Genetics)	1942
California Institute of Technology, Pasadena, M.S. (Meteorology)	1943

Memberships:

Genetics Society of America	
Secretary	1962–1964
Vice President	1966
President	1967
National Academy of Sciences	1968
American Academy of Arts and Sciences	1971
Royal Society (London), Foreign Member	1989
American Philosophical Society	1990
The Genetical Society (Great Britain), Honorary Member	1990
The Genetics Society of Japan, Honorary Member	2001

Professional Experience:

Thomas Hunt Morgan Professor of Biology, Emeritus, California Institute of Technology	1988–2004

Thomas Hunt Morgan Professor of Biology, California Institute of Technology	1966–1988
Guest Professor, Institute of Genetics, University of Copenhagen, Denmark	1975–1976
Professor of Biology, California Institute of Technology	1956–1966
Associate Professor of Biology, California Institute of Technology	1949–1956
Assistant Professor of Biology, California Institute of Technology	1948–1949
Rockefeller Foundation Fellow, Cambridge University, Cambridge, England	1947–1948
Instructor, California Institute of Technology	1946–1948
Served to Captain, U.S. Army Air Corps	1942–1946

Honors:

Honorary Doctor of Philosophy, University of Umeå, Umeå, Sweden	1981
Thomas Hunt Morgan Medal of the Genetics Society of America	1983
Gairdner Foundation International Award (Canada)	1987
Wolf Prize in Medicine (Israel)	1989
Lewis S. Rosenstiel Award in Basic Medical Research	1990
National Medal of Science (USA)	1990
Albert Lasker Basic Medical Research Award	1991
The Louisa Gross Horwitz Prize	1992
Honorary Doctor of Science, University of Minnesota	1993
Nobel Prize in Physiology or Medicine	1995

Publications:

1939 Lewis, E. B. Star-recessive, a spontaneous mutation in *Drosophila melanogaster.* Proc. Minn. Acad. Sci., 7:23–26.

1941 Lewis, E. B. Another case of unequal crossing over in *Drosophila melanogaster.* Proc. Natl. Acad. Sci. USA, 27:31–34.

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1971 Abrahamson, S. and Lewis, E. B. The detection of mutations in *Drosophila melanogaster*. In: *Chemical Mutagens: Principles and Methods for Their Detection,* Vol. II, Edited by Hollaender. Plenum Press: New York, pp. 461–487.

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1975 Lewis, E. B. Possible genetic consequences of successful treatment of childhood tumors with ionizing radiation. Radiology, 114:147–153.

1976 Garcia-Bellido, A. and Lewis, E. B. Autonomous cellular differentiation of homoeotic bithorax mutants of *Drosophila melanogaster*. Dev. Biol., 48:400–410.

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