Genes That Control Development

The bithorax complex of the fruit fly is providing a close look at genes that control the development of a higher organism

The Mediterranean fruit fly, which is threatening the multibillion-dollar agricultural industry of California, has dominated the headlines this summer. Meanwhile, the Medfly's benign cousin *Drosophila melanogaster*, long the geneticist's best friend, has been quietly making some news of its own.

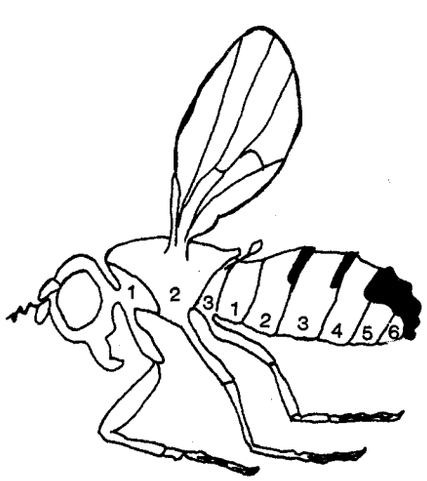
In work that began more than 65 years ago, investigators have identified in *Drosophila* a rare commodity—a set of genes called the bithorax complex—that controls a major part of the development of the segmented body of this insect. Aided by recombinant DNA technology, they are now taking a look at the actual DNA of these genes and beginning to identify the gene products. This represents one of the very few cases in which investigators have been able to directly analyze genes that control the development of a higher organism.

They have already found some surprises in the bithorax complex. Most mutations of the complex genes have turned out to be caused by insertion or deletion of large segments of DNA and not just by changes in one or a few of the nucleotide building blocks of DNA. In addition, the complex contains by far the longest intervening sequence yet encountered. Finally, although more work will be needed to confirm this, some of the bithorax genes do not appear to be copied into RNA transcripts, raising the possibility that they do not act in the ordinary way by directing protein synthesis.

The bithorax complex genes were originally identified, in the manner of classical genetics, by analyzing mutant flies whose body segments had formed abnormally. Calvin Bridges discovered the first such mutant in 1915, but the developmental aspects of the genes received little systematic attention until the mid-1940's, when Edward Lewis of the California Institute of Technology took up the cause. As Lewis explains his lifelong fascination with the bithorax complex, "It is interesting to have a knowledge of genes working very early in development. By the nature of the mutations of these genes, you are sure they are affecting development."

The bithorax mutations disrupt the formation of the individual segments of the fruit fly body, each of which has its own characteristic anatomy and appendages. Usually the mutations cause posterior segments to become more like anterior segments. For example, the first mutation discovered, which was designated *bx* for bithorax, transforms the front portion of the third thoracic segment so that it resembles the front portion of the second thoracic segment. Normally only the second thoracic segment carries a pair of wings; the third has a pair of smaller structures called halteres (from the Latin for swinging weights), which help to stabilize the fly in flight. As a result of the *bx* mutation, the front portion of the halteres develops like the front half of wings.

A second mutation, *pbx* (for posterior bithorax), produces an analogous transformation of the back half of the third thoracic segment. When both mutations are bred into the same fly, an animal with two pairs of wings results, although the second pair is not quite perfect unless a third mutation (*abx* for anterobithorax) is also included.



Fruit fly segmentation

Although the fruit fly is said to have eight abdominal segments, the seventh segment does not develop in the adult male (shown here). The eighth segment forms only the genital structures, which are located on the lower portion of the end of segment six. The small appendage on the upper surface of the third thoracic segment is the haltere.

Flies, which are among the most evolutionarily advanced members of their phylum (Arthropoda), probably evolved from insects that have two pairs of wings instead of one pair of wings and one of halteres. Insects themselves, which characteristically have three pairs of legs, one on each thoracic segment, are generally thought to have evolved from simpler arthropods, like centipedes or millipedes, that have legs on all the segments of the body. In this view, increased specialization of the body segments—replacement of wings by halteres, for example, and loss of legs on abdominal segments—can be considered evolutionary advances.

Lewis's work on the bithorax complex has led him to propose that the second thoracic segment, which bears both wings and legs, is the least advanced segment of the fruit fly body, a kind of evolutionary ground state that may be acted upon by the genes of the bithorax complex to form more advanced posterior segments. A mutation in one of the genes results in the failure of a segment to reach its appropriate developmental level and it becomes more like the second thoracic segment. "The mutants are throwbacks to the ancestral state," Lewis says.

Additional examples of this include the *bxd* (bithoraxoid) mutation, which makes the first abdominal segment resemble the third thoracic. These mutants may have a fourth pair of legs on the transformed segment. The most extreme bithorax mutants, those in which the entire complex is missing, do not develop beyond the larval stage. Nevertheless, all the larval segments behind the second thoracic have the characteristic features of the second thoracic segment.

According to Lewis's model, the bithorax complex ought to contain at least one gene for each body segment below the second thoracic, or a minimum of nine genes (for one thoracic and eight abdominal segments). Development of the third thoracic segment requires the activation of the first gene of the complex in the cells that produce that segment. To achieve normal development one more gene must be turned on in each

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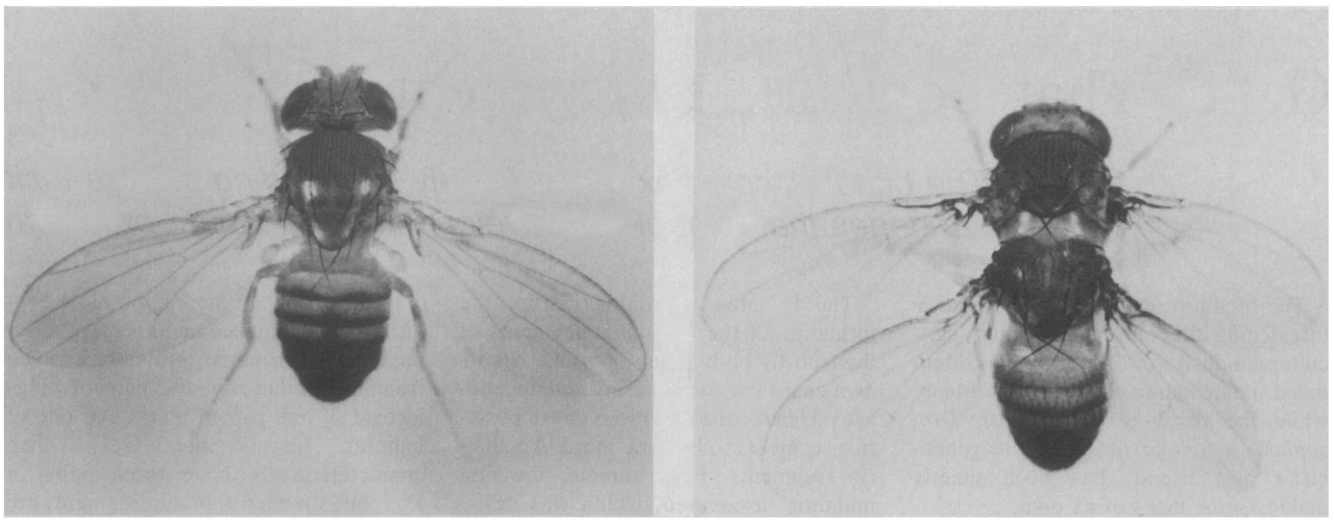


Wild-type fruit fly and bithorax mutant

Three separate bithorax mutations can be combined in one fruit fly to produce an animal with a second, almost perfect, pair of wings. [Source: Edward Lewis, California Institute of Technology]

additional segment until they are all activated in the last segment.

So far, direct evidence in the form of mutants has been obtained for only eight genes but there is plenty of room for more in the complex. David Hogness of Stanford University and Welcome Bender, who recently moved to Harvard Medical School, found that the complex DNA is more than 200, and perhaps as many as 300, kilobases (kb) in length. If a gene contains no intervening sequences (introns) of untranslated DNA, 1 kb is large enough to code for an average-sized protein containing about 300 amino acids. Lewis says, "The gene cluster is immense in terms of what we might have once thought."

By standard genetic breeding experiments Lewis deduced the relative positions of the known genes of the complex. "The remarkable thing," he explains, "is that they are lined up on the chromosome in almost the same order as the segments of the body that they transform."

To explain the orderly activation of progressively more genes in each additional posterior segment, Lewis has proposed the existence of a repressor substance that keeps genes in an inactive state. He postulates that this repressor is more concentrated in the anterior segments of the insect, where fewer genes are turned on, than in the posterior segments.

The repressor might be a product of the polycomb (*Pc*) gene, which is located outside the bithorax complex but which helps to control the activity of the bithorax genes. All the body segments of mutants in which this locus has been deleted resemble the last abdominal segment, a finding which suggests that all of the genes are turned on in all segments.

Finally, Lewis's model requires that the bithorax complex contain regulatory sites that interact with the repressor to keep the bithorax genes turned off. Lewis postulates that the regulatory regions of the most distal genes, those that are presumed to be active in the most posterior segments, have a higher affinity for the repressor, with the result that the regulated genes can be turned on only in the posterior segments, where the repressor concentration is lowest.

The classical geneticist such as Lewis attempts to explain gene function and arrangement by looking at mutants that have altered morphologies and working back to the genome. The perspective can be considered to be looking in from without. This approach is now complemented by developments in molecular biology that permit investigators to proceed directly to the heart of the matter, to analyze the DNA itself. Aided by the well-characterized mutants and genetic map for the bithorax complex, the Hogness and Bender groups are now analyzing the genes of the complex with results that have often been surprising. The workings of the bithorax complex may not be as straightforward as the genetic studies have indicated.

The identification and isolation of bithorax complex DNA were feats in themselves. When Hogness and Bender started the work about 3 years ago, they did not have a probe for detecting the desired segment of DNA.

They did not start with the bithorax complex itself, however. Bender and Pierre Spierer of Stanford were studying a section of fruit fly chromosome 3, which is about 4000 kb away from where Lewis had mapped the complex. They started there, Bender says, "because John Lis (another member of the Hogness group) just happened to have a cloned DNA that hybridized with the region" and it was close to two other gene loci in which they were interested.

Bender and Spierer refer to the procedure with which they analyze the DNA as "walking along the chromosome." After digesting the cellular DNA to produce a large number of fragments, they used a probe, in this case the piece of DNA cloned by Lis, to pick out pieces from the region of interest. They then used the piece that extends farthest beyond the ends of the original probe to pick out additional DNA fragments. By repeating these steps, each time using the longest fragment as a new probe, Bender and Spierer inch their way along the chromosome and collect overlapping fragments of DNA.

The appeal of continuing their chromosome walk into the bithorax complex was obvious. But a stretch of 4000 kb is longer than it might seem. Since the walk can cover about 50 kb per month at best, 4000 kb represents many years of work.

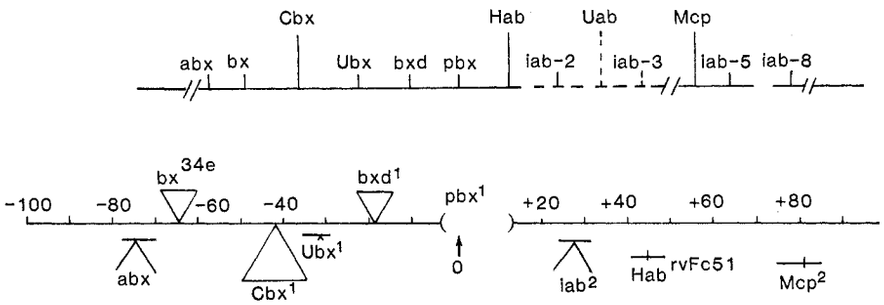
However, Lewis suggested that it might be possible to "jump" into the bithorax complex with the aid of a mutation of the inversion type. In an inversion a piece of DNA is snipped out of the chromosome, flipped over, and put back in with its original polarity reversed. Using two mutants with bithorax inversions that were supplied by Lewis and

one provided by Thomas Kaufman of Indiana University, the Hogness group identified a mutant in which bithorax complex DNA had been brought into close proximity to the region they were mapping. A probe they already had enabled them to detect a DNA fragment that was a hybrid of DNA from the mapped region and bithorax DNA. This, in turn, served as a probe for entry into the complex. "At the time," Hogness said, "we had no knowledge of the DNA of the complex. We had no other way of getting in."

Currently, more than 200 kb of the complex have been mapped and the locations of individual genes determined by locating the sites of DNA changes in various mutant flies. The order of the genes presented no surprises, as it confirmed the order already established by Lewis. What was unexpected, in addition to the large size of the complex, was the nature of most of the mutations. Many of them had been thought to be point mutations, caused by changes in one or a few nucleotides. But, Hogness says, "Most turned out not to be true point mutations. They were insertions of a few kilobases of DNA."

In several mutants, the insertions had the characteristics of the movable elements that are being identified in *Drosophila*, yeast, and other higher organisms (*Science*, 9 January, p. 153). Many investigators think that movable elements are important mediators of evolutionary change. In the bithorax complex of *Drosophila*, at least, they appear to be important mediators of mutation.

Not all of the bithorax mutations were insertions, however. Some were deletions, often of sizable pieces of DNA. The *pbx*^{1} mutation, for example, was caused by deletion of a 17-kb fragment. Interestingly, another type of mutation, *Cbx*^{1} (for first contrabithorax), arose at the same time as the first *pbx* mutation. *Cbx*^{1} was caused by the insertion of the 17-kb fragment back into the complex about 40 kb from its original position. Whereas the *pbx* deletion causes the posterior portion of the third thoracic segment to resemble the posterior portion of the middle segment (rear haltere into rear wing), the *Cbx* insertion has exactly the opposite effect, making the back half of the middle segment look like the back half of the third. "The now obvious model," Bender concludes, "is that the 17-kb piece of DNA encodes the directions to develop posterior haltere." And Hogness says about the double mutation, "It is interesting . . . by changing the position of the 17-kb segment you change its regulation so that it is now expressed in the wrong place."



The bithorax complex

(Top) Genetic map of the bithorax complex. Symbols in the lower row represent loci of mutations that cause posterior segments to become more like anterior segments. Those in the upper row represent mutations that transform anterior segments into posterior ones. The order of mutations in the region of the broken line has not yet been established. (Bottom) Locations of various bithorax mutations as mapped by the "chromosome walk" (see text). The arrow at 0 indicates the point of entry for the walk. The relative order of mutations is the same in both maps, although direct examination of the chromosomal DNA has shown that mutations giving rise to certain anatomical changes may be spread over large stretches of DNA. In the most extreme case known, Ubx^1, *at about −30 kb, represents the right end of a region at least 70 kb long containing several* Ubx *mutations. (Triangles and inverted V's represent insertions, lines with crossbars are inversions, and* pbx^1 *is a deletion.)*

Also surprising is the occurrence of mutations that give rise to a particular anatomical change at any of several sites over a large stretch of bithorax DNA. The ultimate in this regard are the *Ubx* (ultrabithorax) mutations, which are generally lethal in the larval or pupal stages if they are carried on both members of the chromosome pair. Examination of the immature flies suggests that *Ubx* mutations transform both the third thoracic and first abdominal segments to the second thoracic state.

Ubx mutations, many of which are caused by breaks in the DNA, have been mapped throughout a 70-kb section of the bithorax complex. Hogness says, "We have 12 of the mutants [the breaks] and they are just scattered randomly. *Ubx* is not a small gene but it appears to be a unit of function." He suggests that the entire 70-kb segment is transcribed as a unit. "It requires the ends," he explains. "If you break and separate the ends, you destroy the function."

Searching for RNA copies of the bithorax DNA is difficult because they are present in very low concentrations. Nevertheless, Robert Saint, another member of Hogness's group, began looking for transcripts of the 70-kb region containing the *Ubx* mutations. He eventually found three small messenger RNA's, 3.7, 4.3, and 4.7 kb long, which were detected by a 1.7-kb probe made from the extreme right side of the *Ubx* region. The same three messengers were detected by a probe from the extreme left end of the *Ubx* region. No messengers were detected from any DNA in between. "One interpretation of this," Hogness says, "is that the primary transcript is 70 kb long, which is very large, and that all but the ends are spliced out."

In the past few years, molecular biologists have found that the genes of higher organisms contain intervening sequences that do not code for the amino acid sequences of proteins. The introns are transcribed into messenger RNA's, but their RNA transcripts must be spliced out before the messenger directs protein synthesis. Most introns are just a few kilobases long. The largest found before now, Hogness notes, was 26 kb. So a 70-kb intron is unusually large.

There is also the possibility that the DNA itself is rearranged during development, as it is during the development of antibody-producing cells. However, no such rearrangements have been found so far in bithorax DNA.

"The curious thing," Hogness continues, "is that the *abx* and *bx* gene loci are in the region that gets spliced out. We find no messenger RNA's for them. Are they genes in the sense that they each have a product that is expressed in the appropriate segment?"

There is currently no answer to this question. Mutations in the *abx* and *bx* loci are known to affect fruit fly development, so the regions must play some role in the appropriate segments. Nevertheless, additional evidence supporting the idea that the *bx* locus does not code for a protein product comes from the laboratory of Bender. Some bithorax mutants may spontaneously revert to the wild type. According to Bender, one revertant of a *bx* mutation still has two pieces of inserted DNA, one of them 4 kb long,

but the flies appear completely normal. "If there is such a thing as a *bx* protein," Bender says, "it is not coded at either site [of the insertions]."

Hogness speculates that the large *Ubx* intron might be spliced out by different pathways in the embryonic cells that give rise to various segments, with the nontranscribed genes somehow specifying the pathway to be activated. If a mutation in one of these genes blocked or altered the pathway, then a developmental abnormality might result.

Clearly, much more work will be needed before this and other aspects of the operation of the bithorax complex are understood at the molecular level. For example, the Hogness group is identifying the RNA transcripts of the complex, but the functions of the ultimate gene products are unknown. Since the transcripts have all the earmarks of messenger RNA's, they probably direct protein synthesis. Identification of the protein products and determination of their functions may prove difficult, however.

The messengers, incidentally, appear about 2 to 4 hours after the egg starts to divide and remain present throughout embryogenesis. This means that they first appear about the time when individual cells are becoming committed to developing into particular segments. The messengers are also present in elevated concentrations in the imaginal disks, embryonic structures that give rise to specific adult body parts.

Even though the bithorax complex may not give up its secrets easily, the researchers are not dismayed. As Hogness puts it, "It is rare that you can find mutants that control events very early in development. It is kind of fun to study a locus that has such fundamental effects."—JEAN L. MARX

Globin Gene Transferred

Investigators at Ohio University and the Jackson Laboratory have successfully transferred into mice a functional rabbit gene for the protein β-globin. Not only did the foreign gene work in the mice, where it directed the synthesis of the rabbit protein, but it was also transmitted through the germ line to a second generation of animals.

"The current work was perceived as part of a long, quiet series of experiments on how the sperm transfers its genetic information to the egg. Ideally we would like to introduce new DNA directly into sperm, but this is impossible," explains Thomas Wagner of Ohio University. When a sperm fertilizes an egg the nuclei of the two germ cells do not fuse for at least 8 hours, however. During this time the male pronucleus, as the sperm nucleus is now called, can be identified and injected with DNA.

Wagner, with collaborator Peter Hoppe of the Jackson Laboratory, Bar Harbor, Maine, injected the male pronuclei of recently fertilized mouse eggs with foreign DNA containing the gene for rabbit β-globin. The source of the DNA was a hybrid plasmid, consisting of bacterial DNA plus the rabbit gene, which was supplied by Richard Flavell of the National Institute of Medical Research in London and Charles Weissmann of the University of Zurich.

The eggs were injected either with the intact plasmid or with a fragment containing the β-globin gene plus some of its leader sequence, which is thought to carry control signals needed for gene expression. Each egg received about 20,000 copies of the gene.

All together, 312 eggs were injected and 211 of the resulting embryos were transplanted into a total of 21 foster mothers. Eleven of the foster mothers became pregnant and eventually delivered 46 pups.

The investigators took blood samples from the animals as soon as they were old enough to provide sufficient material for analysis. The presence of rabbit β-globin in the blood was detected by a standard immunological test, using antibodies directed only against the protein. "We think 15 to 20 percent of the mice were producing some level of the protein," Wagner says, "but we are only reporting* on five that gave clear and definite evidence of expression."

*To be published in the October issue of the *Proceedings of the National Academy of Sciences*.

Wagner hypothesizes that injecting the DNA into the male pronucleus may have increased the chance of achieving successful gene transfer. Work by other investigators with frog and sea urchin eggs suggests that the DNA of the male pronucleus is extensively processed by egg enzymes before fusion occurs. The processing might facilitate incorporation of the foreign DNA into a male chromosome and this might be "a way of fooling the embryo into taking the genes."

Wagner notes that the five animals that were making large quantities of rabbit β-globin were somewhat anemic, which suggests that control of hemoglobin synthesis might have been adversely affected by the presence of the foreign gene. On the plus side, preliminary evidence indicates that the rabbit protein "was not made indiscriminately throughout the mouse," as Wagner puts it, but only in the precursors of the red blood cells.

Despite their mild anemia, two of the animals were mated with each other and produced eight offspring. Five of the pups also made the rabbit protein, although the concentrations were somewhat lower than that in the mother.

The first group of animals were born in November and December 1980 and the second generation in January and February of this year. The investigators submitted their report to the *Proceedings of the National Academy Sciences* in February, but it will not be published until this October because they had to perform additional experiments that were requested by the journal's referees.

The decision to release the results before publication was made by administrators at Ohio University, which provided the support for much of the research. Wagner agreed to the release but says that Hoppe did not.

Progress in attempts to introduce working foreign genes into living animals has been very rapid over the last year or so. The latest work represents another significant advance in that introduction of a new gene into a recipient of a different species and germ line transmission were achieved in the same experiment. Both of these milestones had been accomplished separately (*Science*, 28 August, p. 996) but, at least as far as published reports go, not together.

—JEAN L. MARX

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