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Title of Thesis — Titre de la thèse

EFFECT of THE bithorax MUTATION ON DETERMINATION IN DUPLICATING DROSOPHILA IMAGINAL DISCS

University — Université

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Effect of the *bithorax* Mutation on Determination in Duplicating *Drosophila* Imaginal Discs

by

© Stanley Y.K. Tiong

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Doctor of Philosophy

Department of Genetics

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Effect of the *bithorax* Mutation on Determination in Duplicating *Drosophila* Imaginal Discs submitted by Stanley Y.K. Tiong in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

In the development of *Drosophila melanogaster*, the imaginal discs which give rise to the adult exoskeleton must make developmental commitments in a process known as "determination". It has been suggested that determination is encoded combinatorially by the states of a series of genetic loci called selector genes, of which each is switched on or off in a given cell lineage in a manner dependent upon position in the embryo or disc at a unique stage in development. Such systems are thought to underlie the phenomenon of progressive compartmentalization as detected by clonal analysis. Somatic clones induced as early as the cellular blastoderm stage already define homologous anterior and posterior compartments in the thoracic discs. The phenotypes of *bithorax* and *postbithorax* mutants suggest that these loci are somehow involved in anterior and posterior compartment specific patterns of determination in the metathorax. Hence, according to the selector gene model, the heritability of the determined state in this segment might require the heritable activation of these loci at blastoderm stage. Compartmental commitments are apparently lost when disc fragments duplicate or regenerate. To test for heritability of the metathoracic determined states under these circumstances pattern duplications were induced in *bithorax* or *postbithorax*-transformed third leg discs and in wild-type controls using a temperature-sensitive cell lethal system. In *bithorax* duplicated legs, transformation of the entire duplicate was sometimes observed. Cell lineage analysis of these duplicates demonstrated that anterior *bithorax*-transformed cells could contribute to both mesothoracic and metathoracic posterior duplicate compartments. The results suggest that: (1) *bithorax*$^+$ is required in the establishment of the posterior metathoracic state in the duplicate by activation of *postbithorax*$^+$; (2) cells, regardless of their compartmental origin, make a co-ordinated determinative decision in the duplication blastema which is influenced by the allelic state of *bithorax* locus, in interaction with the positional information system in the blastema; (3) The determinative distinction between mesothorax and metathorax is encoded by the *bithorax* locus itself rather than by a separate locus as would be the case according to a combinatorial binary switch model.

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I. INTRODUCTION

The *Drosophila* adult integument is formed from nests of cells known as the imaginal discs and abdominal histoblasts. Each imaginal disc gives rise at metamorphosis to a particular part of the cuticular surface which contains cuticular elements such as bristles, hairs and sensilla in an highly ordered spatial arrangement. These discs are first histologially evident as invaginations in the epithelium of the late embryo (Lauge, 1967) or early larva (Auerbach, 1936). They each develop as a single layer of cells attached to the larval body wall by a stalk and have no apparent function throughout larval development (Shearn, Rice, Garen and Gehring, 1971).

The number of progenitor cells that form an imaginal disc is small. Using gynandromorph analysis, the number of progenitor cells giving rise to an imaginal disc has been estimated as the reciprocal of the smallest fraction of genetically labelled XO (male) or XX (female) mosaic tissue marking a structure (Patterson and Stone, 1938; Stern, 1968; see Hall, Gelbart and Kankel, 1976, and Janning, 1978 for review). The estimation for various discs ranges from 2-40 cells (Nothiger, 1972; Madhaven and Schneiderman, 1977 for review). Direct cell counts using a histological approach on newly hatched larvae (Madhaven and Schneiderman, 1977) yield approximately the same range of numbers of progenitor cells for different discs.

As estimated from the frequency and size of x-ray induced somatic cross-over clones, the first detectable increase in cell number for different imaginal discs appears to take place towards the end of the first instar. The disc cells then continue to divide exponentially during the remainder of larval life. At pupariation, each imaginal disc evaginates and secretes first pupal and then adult cuticle.

Since each kind of disc has its own characteristic shape, size and morphology and adult fate, each must have acquired a different set of developmental instructions during development. We can conceive of two important steps in the development of an imaginal disc. These are 'determination' and 'differentiation'. The term determination has been defined as "a process which initiates a specific pathway of development by singling it out from among various possibilities for which a cellular system is competent" (Hadorn, 1965). In other words, it is a developmental commitment made by a cellular system to follow a particular developmental pathway. It has been found however that the

commitments made by the disc cells may either be heritable or non-heritable For instance, the decision to be a particular disc is heritable but the decision to form a certain part of a disc such as a particular bristle is non-heritable So that the term determination may be reserved for the heritable commitment, Bryant (1974) coined the term "specification" to refer to the process that gives rise to the spatial pattern within an imaginal disc. Let us first consider the origin and nature of heritable determinative commitments

One way to assess the determinative status of an imaginal disc at a particular time is to look at the structures differentiated by isolated fragments cultured until metamorphosis in an abnormal location in a host. The ideal assay would be to take single genetically marked donor cells (imaginal or embryonic) and transplant them to different locations in a wild-type host where they would be allowed to proliferate until metamorphosis. If the outcome at a different location were always as expected for the normal position, one could conclude that the donor cells were already heritably determined at the time of transplantation. However, if a different result were obtained, one could not then conclude that the cells were not committed at the time of implantation, as a new determined status might have been imposed as a result of the new surroundings. One could only conclude that any earlier commitments were non-heritable under these conditions.

Since it is rather difficult to manipulate and culture single cells in this way, larger fragments have generally been used in experiments described to date to test for determination. Another way of assessing determination which overcomes this technical problem is to label single cells genetically at known stages of development using the somatic recombination technique (Stern, 1936, 1968; Becker, 1957) and to look for clonal restrictions in developmental potential at metamorphosis. The rationale is that if a particular labelled cell were committed to form a particular structure at the time of labelling, all its progeny would be confined to that structure at metamorphosis. If the cell were not so determined, one might be able to find some of its descendants marking other structures also.

Using techniques of these kinds, it has been found that the determination processes of imaginal disc cells occur in a stepwise fashion Chan and Gehring (1971)

dissociated anterior or posterior halves of genetically marked embryos at the blastoderm stage into single cells and then mixed them with cells from wild-type whole embryos. After reaggregation, the cell mixtures were cultured in fertilized adult female abdomens where the cells proliferate. They then recovered the implants and transplanted them into late third instar larvae where they metamorphosed with the hosts. It was observed that the anterior and posterior cellular blastoderm cells were already committed to differentiate either anterior or posterior structures. Their results do not tell us however that the imaginal discs are already separately determined at this stage. Illmensee (1976) transplanted single genetically marked cells from blastoderm stage into hosts of the same age. He found that at metamorphosis, the kind of imaginal disc structures formed were dependent upon the location of origin of the donor cells regardless of the location in the hosts where the cells were transplanted. The results are therefore in agreement with the idea that cellular blastoderm cells are already determined to form specific discs.

With the somatic recombination technique, it is possible to produce a genetically marked homozygous clone of cells in an otherwise heterozygous individual at any stage of development. Using this technique, a number of people have shown that clones induced as early as the cellular blastoderm stage are already confined to a single thoracic segment. However, these clones can still extend from the dorsal disc into the ventral disc in the same segment. Also left-right overlaps of clones within a segment can be found at this stage (Steiner, 1976; Wieschaus and Gehring, 1976). These results suggest that at cellular blastoderm stage, the cells are already determined to form different segments but not individual discs. The dorsal-ventral segregation occurs only one or two cell divisions later.

Using mechanical extirpation techniques such as micro-beam irradiation (Lohs-Schardin, Sander, Cremer, Cremer and Zorn, 1979; Sander, Lohs-Schardin, Nusslein-Volhard and Cremer, 1980) or pricking (Bownes and Sang, 1974; Mertens, 1977), it was shown that at cellular blastoderm stage the defects observed in the adult could be correlated with the segmental location of the injury induced on the embryo but not with dorso-ventral location within a segment until after gastrulation. These results are also consistent with the previous conclusion that cells are already restricted or determined to form a certain part of the adult structures at cellular blastoderm stage.

They also suggest that cells acquire their final states of determination progressively in a stepwise manner.

Although apparently heritable via mitosis, these progressive restrictions in developmental capacity probably do not represent a loss or permanent inactivation of different genetic elements in the determined cell. Nuclear transplantation experiments have been used to show that some nuclei obtained from nuclear multiplication, syncytial blastoderm or even gastrulation stages of embryonic development, when implanted into a fertilized or unfertilized egg are able to support the entire development of the egg and to contribute to the development of any adult body part (Illmensee, 1968, 1970, 1972, 1973, Okada, Kleinman and Schneiderman, 1974). These results not only demonstrate the reversibility of the restrictions imposed on the nuclei during different stages of development, they also demonstrate that the kind of determination acquired is dependent on the position in the egg at which the nuclei were implanted.

The idea of progressive stepwise determination is also encountered in the phenomenon known as compartmentalization. Using the somatic recombination technique, it has been shown that the number of structures a clone can form in a disc becomes more and more restricted as development proceeds (Becker, 1957; Bryant, 1970; Garcia-Bellido and Merriam, 1971; Garcia-Bellido, Ripoll and Morata, 1973). However, this restriction may be attributed to the smaller size of clones induced later in development. Such problems have been overcome by using the "*Minute*-technique" (Kaplan, 1953; Morata and Ripoll, 1975). *Minutes* are a class of dominant mutants causing small bristles, which are also recessive lethals (Lindsley and Grell, 1968). One can use mitotic recombination to induce genetically labelled *Minute*$^+$ homozygous clones in a *Minute* heterozygous background at different stages of development. As cells heterozygous for *Minute* have slower division rates, these marked *Minute*$^+$ clones tend to label much larger areas on the adult cuticle than clones produced at the same larval stage in non-*Minute* individuals. It was observed in the wing disc that clones induced as early as cellular blastoderm stage, would already define an anterior-posterior boundary. They would run along the restriction line for hundreds of cells without crossing it (Garcia-Bellido, Ripoll and Morata, 1973, 1976). Garcia-Bellido coined the term 'compartment' to define such a region of apparent developmental restriction on the adult

exoskeleton. At a later time, as the wing disc grew, a wing-notum restriction was established subdividing the existing anterior and posterior compartments to form four compartments in which no clone could transgress either anterior-posterior or wing-notum restriction lines. At about the same time as wing-notum compartments were formed, a dorsal-ventral restriction also appeared giving a total of eight compartments. Other compartments in the wing were also observed at later times in development. Compartmentalization processes seem to be a generalized phenomenon in *Drosophila* imaginal disc development. Apart from the wing disc, compartments have also been found in all three thoracic leg discs (Steiner, 1976), eye-antennal disc (Baker, 1978) and labial disc (Struhl, 1977). It has been suggested that compartmentalization events may represent progressive heritable determinative restrictions allowing sub-division of larger developmental units into smaller ones perhaps to mediate control and regulation of gene activity in pattern formation (Crick and Lawrence, 1975).

A compartment is derived from a group of founder cells which are not necessarily clonally related, because clones induced prior to the establishment of compartments never fill the entire compartment without also labelling other compartments. Therefore, each compartment is referred to as being 'polyclonal' in origin (Crick and Lawrence, 1975). One interesting aspect of compartment formation is that each compartment is derived from one existing earlier through a subdivision into two new polyclones (Garcia-Bellido, Ripoll and Morata, 1973, 1976). For instance, the anterior wing polyclone is subsequently split into two clonally distinct groups of cells, one dorsal and the other ventral. Garcia-Bellido proposed that whenever a group of cells is subdivided into two distinct compartments, a regulatory gene known as the 'selector gene' is heritably activated in one compartment, but remains switched off in the other, and controls the developmental distinction between them (Garcia-Bellido, 1975a). Depending on the number of compartmentalization events for the disc concerned, the final result would be that each compartment is under the control of a combination of selector genes active within it (see Figure 1). A combinatorial scheme of this kind would be a very economical way to build a genetic program controlling development, as only n selector genes would be required for 2exp(n) distinct compartments.

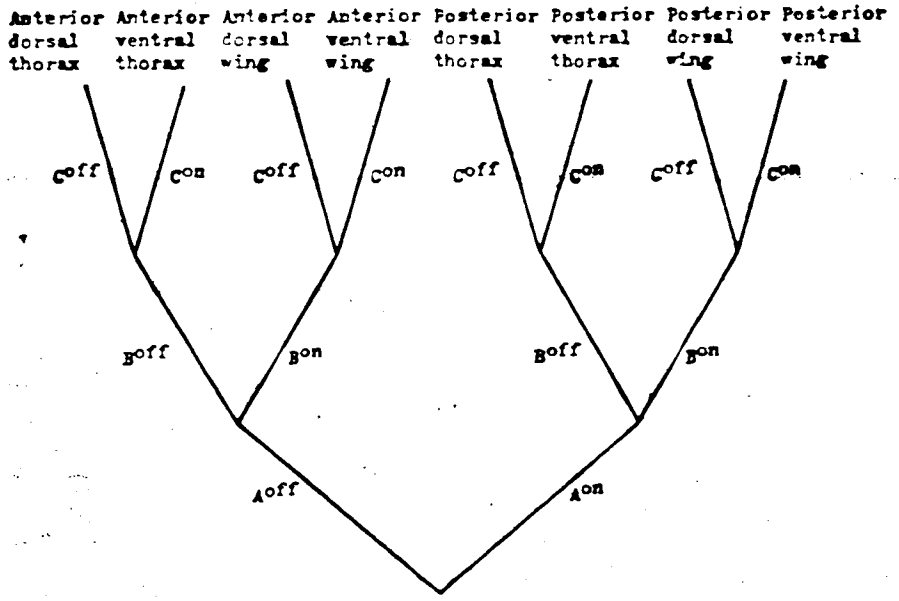


Figure 1. A hypothetical scheme involving the "selector gene model" for compartmentalization of the wing disc (After Garcia-Bellido, 1975). A, B and C are hypothetical selector genes.

On the basis of the properties of late larval lethal mutations affecting imaginal disc development and 'transdetermination' patterns, Kauffman (1973 1975) has proposed a "binary switch epigenetic code" similar to the selector gene model to account for the determinative state of each imaginal disc. Shearn *et al* (Shearn, Rice, Garen and Gehring, 1971) had isolated 34 late larval lethals that affect disc development. These lethals can be organized into 13 classes on the basis of the subset of discs affected. In 8 of the 13 classes, complementary pairs of discs are affected. Kauffman interpreted these observations as an indication that the determinative distinctions between discs are based on a combination of alternative states of a series of bistable genetic circuits perhaps similar to the system controlling maintenance of the immune or lytic states in bacteriophage *lambda*. Interestingly, when the boundary lines between the subsets of discs affected in the four complementary classes of mutants were transposed onto the embryonic fate map (Garcia-Bellido and Merriam, 1969), the geometry of the lines formed a surprisingly simple pattern (see Figure 2). This provided some support for the model as it suggested that activation of the different controlling genes might be based on position in the embryonic system.

A further indication that determinative decisions are made between two alternative states came from analysis of the phenomenon known as transdetermination (Hadorn, 1965). Imaginal discs can be propagated for many years by serial transplantation and culture in the abdomens of a series of fertilized adult female hosts where they proliferate without differentiation. These implants can be recovered for assessment of their determined states by transplanting them into late third instar larvae where they metamorphose with the hosts. Hadorn and co-workers observed that the determined state of a disc is very stable even after many generations of fragmentation and culturing. However, occasionally the determined state of some cells may be changed to a different state after culturing. The new determined state is also normally very stable and heritable but may also change to another different state after subsequent culturing. Such a change, for instance from 'legness' to 'wingness', is known as transdetermination and apparently occurs only if cell proliferation is permitted (Schubiger, 1973). Cell lineage analysis using the somatic recombination technique has demonstrated that transdetermination is not due to a somatic mutational event as groups of cells which do not belong to a single clone

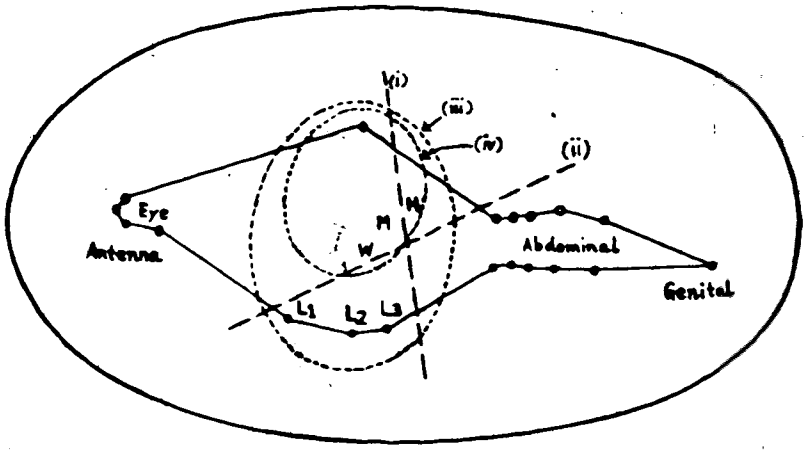


Figure 2. The four boundaries dividing sets of discs affected by disc-defective lethal mutations transposed onto the fate map of the egg showing the relative geometric positions of various disc primordia. W = wing, M = mesothorax, H = haltere, L1 = first leg, L2 = second leg, L3 = third leg (From Kauffman, 1973, 1975).

may transdetermine together (For a review of transdetermination see Hadorn, 1978)

Transdetermination patterns exhibit three special properties. Firstly, transdetermination occurs one step at a time and often in specific sequences for a particular disc. For instance, genital disc transdetermines first to leg which may then transdetermine to wing which may transdetermine further to mesothorax. Secondly, the direction of transdetermination in all such sequences is oriented towards mesothorax. Thirdly, most steps are reversible. Therefore, the number of steps required to eventually transdetermine to mesothorax is state characteristic. For example, it takes three and two steps respectively for genital and prothoracic leg discs to reach mesothorax. The forward transdetermination step towards mesothorax is more probable than the reverse. These results also suggested to Kauffman that the determined state of an imaginal disc is encoded combinatorially by the states of a number of "bistable memory circuits" in which each circuit is set at either a less stable (0) or more stable stationary state (1). Assuming a minimum of four circuits (capable of specifying $2^4 = 16$ different determined states) Kauffman was able to assign code words for the various discs, which would account not only for Shearn's lethals, but also imaginal disc transdetermination sequences and frequencies.

Both the selector gene and the binary switch epigenetic code models therefore suggest that imaginal disc determination occurs by a series of bifurcations of developmental pathways with the activation of a controlling gene to distinguish the two states generated at each step. Consistent with these ideas, there exist a number of gene loci known as the homeotics which, when mutant, cause the replacement of one body structure by a different one which is normally found somewhere else (Ouweneel, 1976, for review). Some such loci may be selector genes. The transformations caused by homeotic mutants may be interdisc or intra-disc and may involve a whole disc, more than one disc, or only a part of it, such as a compartment. For instance, some *Antennapedia (Antp)* mutants can transform the entire antenna into a second leg. *engrailed (en)* transforms posterior wing into a mirror image copy of the anterior wing while *bithorax (bx)* and *postbithorax (pbx)* cause replacement of anterior and posterior haltere by anterior and posterior wing respectively (see Lindsley and Grell, 1968, for more details of mutants). Interestingly, the transformations caused by *bx*, *pbx* and *en* correspond well

to the compartments as defined by clonal analyses.

Further evidence which suggests that these three homeotics may be important controlling genetic factors for compartments also comes from clonal analyses. Homozygous *bx* and *pbx* clones induced at any time from the blastoderm stage until late third larval instar differentiate autonomously only in the anterior and the posterior compartments of the haltere respectively (Morata and Garcia-Bellido, 1976). In addition, clonal analysis of the *bx pbx* double mutants indicates that the number of progenitor cells as well as the clonal parameters of development in the transformed haltere disc are similar to those of the wild-type wing disc. Hence bx^+ and pbx^+ must function from the time these compartments are set aside, until the time they start to differentiate, to prevent their transformation into the corresponding mesothoracic compartments. Thus bx^+ and pbx^+ appear to be necessary for the heritable maintenance or expression of these determined states. These results satisfy some of the criteria used to define a selector gene, that is, compartment specificity and continuous activity in the compartment from the time it is formed until the time it differentiates.

In the case of *en*, homozygous *en* clones induced after the anterior-posterior restriction has been set up in the wild-type wing, not only differentiate autonomously in the posterior compartment only, they also fail to respect the anterior-posterior restriction line if they originate in the posterior compartment. These results suggest that the only difference between anterior and posterior wing compartments lies in en^+ being switched on in the posterior compartment (Morata and Lawrence, 1975; Lawrence and Morata, 1976). Therefore, the *en* locus might well be a selector gene for posterior wing.

Clonal analysis is not however a sufficient basis for us to draw definitive conclusions about the normal function(s) of a wild-type gene. One also has to know the nature of the mutation one is dealing with. Gene dosage analysis (Muller, 1932) applied to a number of homeotic mutants has revealed that transformations may be the result of either a loss of a gene function, or of a gain of a function not normally expressed in the particular compartment affected (Russell and Hayes, 1980, for review). The loss of a gene function due to a mutation may be partial (a hypomorph) or complete (an amorph). Therefore, the completeness of transformation may depend on whether the mutant is a hypomorph or an amorph. In the case of gain of a gene function, the mutant is termed a

neomorph. The effects of a neomorph can be reverted by deleting the mutant gene locus (Denell, 1972). Since transformations caused by homeotics may be due to various genetic states of a gene, it is very important to know the type of mutation we are dealing with before a conclusion is drawn about the normal function of the gene.

Now let us consider non-heritable commitments of cells involved in pattern specification. The kind of structure formed by a cell in a disc is dependent on its location within the disc as well as its previous developmental history. The position specificity of structures differentiated by cells within discs allowed the construction of fate maps by observing the patterns differentiated by particular mature disc fragments implanted into late third instar larvae for immediate metamorphosis (Schubiger, 1968; Ouweneel and van der Meer, 1973; Bryant, 1975; Gehring and Nothiger, 1973, for review). At this stage, the specification is so detailed that even a single bristle such as the 'edge bristle' or the 'hairy island bristle' in the leg can be localized to a single small region of the disc. However, the behavior of disc fragments can be altered if they are cultured in fertilized adult females for a period of time allowing cell-proliferation to occur, before inducing metamorphosis in late third instar larval hosts. Typically, when a disc is bisected into two fragments and cultured in this way, one fragment regenerates the missing part of the pattern which that fragment does not normally form, and the other fragment duplicates the fate map elements it contains in mirror image symmetry. The ability of disc fragments to regenerate, suggests that each imaginal disc constitutes a single developmental "field" within which regulation can occur to alter the fates of different constituent parts after surgical intervention (Weiss, 1939).

In order for a group of genetically identical cells with the same determinative state to differentiate differently, forming highly ordered spatial patterns, it is thought that they must be able to assess and measure their positions with respect to each other and respond in a certain way at differentiation. This idea led to the theory of 'positional information' which has been formulated most rigorously by Wolpert (1969, 1971). He argued that cells may have their positions specified by the acquisition of positional information defining a co-ordinate system constituting a developmental field.

In the light of extensive experimental results, French, Bryant and Bryant proposed an explicit positional information model known as the "Polar co-ordinate model" to

account for pattern regeneration and duplication in imaginal discs, cockroach legs and amphibian limbs (French, Bryant and Bryant, 1976; Bryant, French and Bryant, 1981). They suggested that cells in the two dimensional imaginal disc epithelium must be specified by two positional values -- a circumferential value and a radial value (see Figure 3). This model has gained a lot of attention as it can explain a great variety of otherwise puzzling results from grafting and regeneration experiments with imaginal discs and cockroach and amphibian legs on the basis of just two formal rules (see reference for further details).

On the basis of experimental evidence, Weiss (1939) recognized that there may be at least two kinds of fields: primary fields such as the preblastoderm insect embryo, and secondary fields such as the various imaginal discs, which become independent of the embryonic field later in development. The experimental evidence which suggests that the *Drosophila* preblastoderm acts as a single primary field comes from ligation experiments (Schubiger, 1976; Vogel, 1977; Newman and Schubiger, 1980). When embryos are ligated before the blastoderm stage, larvae with several missing segments result. This is otherwise known as the "gap phenomenon". The blastoderm cells that normally form the missing segments instead form segments ordinarily located far from the site of ligation. This indicates that the pattern of segmentation of the whole embryo is specified as a single unit, suggesting that the embryo as a whole is a single field. The size of the gap decreases if the ligation is done later and later until at cellular blastoderm stage, the defects are limited to the site of ligation. Normal segmentation is restored if the barrier formed after ligation is destroyed by poking a hole in it using a glass needle (Schubiger, Moseley and Wood, 1977). These results have been interpreted as meaning that establishment of a normal segmentation pattern requires the interaction of two determinants, perhaps in a gradient, arising from the two ends of the embryo. Interaction between the parts is also a characteristic expected of a developmental field. Two mutants that may be involved in establishing or interpreting the anterior-posterior positional information in the embryo are *bicaudal* (Bull, 1966; Nusslein-Volhard, 1977 and 1979 for review) and *dicephalic* (Lohs-Schardin and Sander, 1976). These cause mirror image transformation of anterior to posterior and posterior to anterior patterns respectively in larvae.

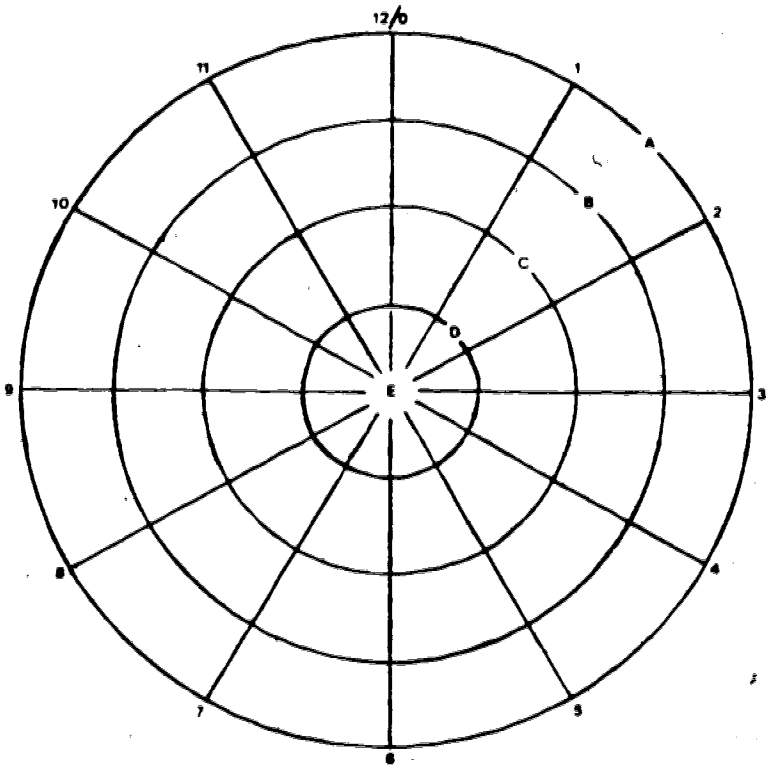


Figure 3. The polar co-ordinate system for specification of positional information. Position of each cell is specified by a radial value (A-E) and a continuous circumferential value (12 through 0) where positions 12 and 0 are the same (From French, Bryant and Bryant, 1976).

The primary embryonic field may subsequently be subdivided into smaller secondary fields. Nusslein-Volhard and Wieschaus analyzed fifteen different embryonic lethals which give abnormal segmentation patterns in larvae (Nusslein-Volhard and Wieschaus, 1980). These lethals fall into three general classes. The first class of mutants have the normal number of segments but each segment is partially deleted with the remainder of the segment duplicated in mirror image symmetry. The second class causes deletions in alternating segments. The deletion patterns can be in odd or even segments or in parts of pairs of odd and even segments causing segment-fusion patterns. The third class of mutants causes gaps as large as eight adjacent segments in the larvae and these phenotypes are very similar to the effect caused by ligating preblastoderm embryos. The phenotypes of these three classes of mutants were taken to mean that at least three levels of spatial organization are responsible for the segmentation process. These are: the whole egg as a single developmental unit; a repeating unit of two segments; and finally the individual segment. These developmental units may represent a hierarchy of developmental fields but evidence in addition to mutant phenotypes would be necessary to confirm this. As discussed earlier, experimental results from clonal restriction analysis of blastoderm cells (page 3) and from UV-laser microbeam extirpation experiments (page 3) both suggest that the imaginal discs are only formed at the partitioning of the individual segmental units. From this time until the end of larval development each disc behaves as an independent developmental field. The successive subdivision of larger embryonic fields into smaller ones may perhaps be related to the progressive stepwise acquisition of determined states and the combinatorial epigenetic code models mentioned earlier (pages 3-9).

There are two lines of evidence suggesting that the positional systems in different imaginal discs may be homologous. Firstly, comparison of normal appendages and structures partially transformed by homeotic mutants demonstrated that a one to one correlation can be made between the transformed structures and location in the disc. For instance, in the *Antennapedia* mutants, the proximal part of antenna is always transformed into proximal second leg while the distal antenna (the arista), is always transformed into distal leg (the tarsus)(Postlethwait and Schneiderman, 1971; Struhl, 1981). Also *bx* and *pbx* clones induced in a wild-type background differentiate only

proximal wing in the proximal haltere and distal wing in the distal haltere (Morata and Garcia-Bellido, 1976) Therefore, there may be a one to one correspondence of positional information in homologous regions of different discs

A second line of evidence for homology in positional information in different discs came from grafting experiments As discussed earlier, when an imaginal disc is bisected and cultured, one fragment will duplicate and the other will regenerate The behavior of the duplicating fragment can be modified by a process known as intercalary regeneration using a grafting technique (Haynie and Bryant, 1976) A number of groups have observed that appropriate fragments of all discs tested can provide a stimulus which causes a normally duplicating fragment to regenerate instead (Wilcox and Smith, 1977, Bryant *et al*, 1978) These results are also consistent with the idea that different discs make use of homologous positional information systems This approach should allow one to define and map the homology between discs fragments with respect to their regulative interactions However, at this stage, it is not entirely clear if the patterns of regional homology as deduced from homeotic transformations and from pattern regulation experiments, are always consistent (Karlsson, 1979)

Imaginal discs are subdivided into compartments The question arises as to whether each compartment of a disc may also represent a separate field The observation that a disc fragment which comes entirely from within a given compartment can regenerate across the compartment boundary argues against the view that compartments are sub-fields of a disc (Schubiger, 1971, Bryant, 1975) However, experiments designed more specifically to test this point have not so far been reported The observation that cells can regenerate or duplicate across the compartment boundary suggested compartmental distinctions are non-heritable and therefore might not be important in determinative decisions However, the re-establishment of compartment boundaries during pattern regeneration and duplication (Szabad, Simpson and Nothiger, 1979, Girton and Russell, 1981, Abbott, Karpen and Schubiger, 1981) argues against this conclusion Compartmentalization seems to be a necessary event in the initiation of a pathway of development, but the decisions are evidently only heritable under normal conditions and can be reassessed after surgical intervention

Current models suggest that selector genes (possibly represented by some homeotics) play an important role in determination. The probable function of selector genes in the light of positional information theory, would be to maintain a heritable memory of position in a field at some earlier stage in development, so that the cells could appropriately interpret their positional values in one of a set of homologous fields at a later stage.

A test of this hypothesis would be to look at the heritability of the determined state in cells transformed by selector genes. Some mutants of the *bithorax* complex provide a good system for this approach for the following reasons. Firstly, the genetics of *bithorax* complex has been elegantly analysed by Lewis (1963, 1964, 1967, 1968, 1978). The complex contains at least eight tightly linked loci which all map to the right arm of chromosome 3 at 58.8 (see Figure 4). These loci seem to be involved in the compartmentalization and segmentation patterns of thoracic as well as the abdominal segments. Two mutants, *bithorax* (*bx*) and *postbithorax* (*pbx*), are of particular interest in the present context as they transform respectively the anterior and posterior metathorax into mesothorax. Gene dosage analysis has shown that these mutants are hypomorphs(Lewis, 1963, 1978). Two other dominant mutants, *Ultrabithorax (Ubx)* and *Contrabithorax (Cbx)* behave as an amorph and a neomorph respectively. *Ubx* mutants transform the whole metathorax to mesothorax (Lewis, 1963) and *Cbx* can transform distal parts of the wing into haltere (Lewis, 1963, 1978; Morata, 1975). Hence, these two mutants may represent regulatory site(s) for *bx* and *pbx* loci. Gene dosage studies with these two mutants are also consistent with these views.

Since the action of *bx* and *pbx* are anterior and posterior compartment specific respectively, one can look at the heritability of the determined state of cells in the compartments transformed by these mutants when they regenerate or duplicate across the compartment boundary. These mutants might affect either the activation of a selector gene, maintenance of a determined state, interpretation of position at terminal differentiation, or a combination of all of these. Some of these possibilities would be distinguishable in pattern duplication. The experiments reported below were attempted with this idea in mind.

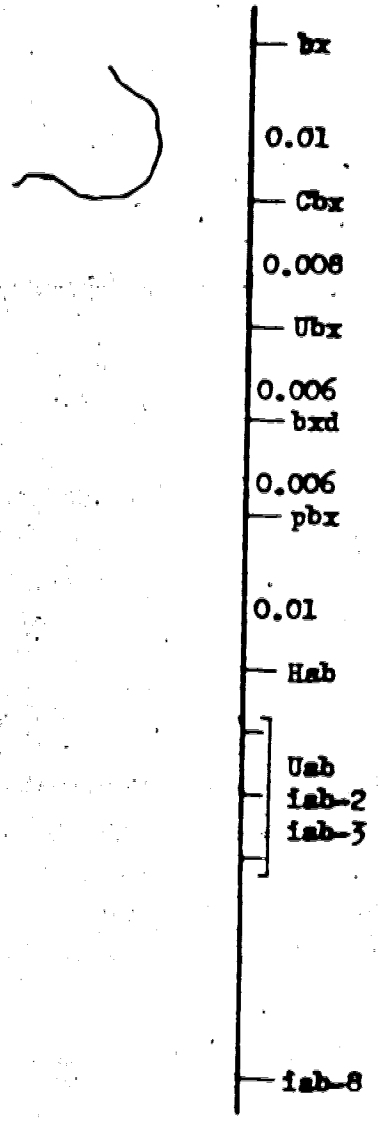


Figure 4. A genetic map of the *bithorax* complex. *Ultra-abdominal* (*Uab*), *Hyper-abdominal* (*Hab*, formerly *Contrabithoraxoid*, *Cbxd*), *bithoraxoid* (*bxd*), *infra-abdominal* genes (*iab-2*, *iab-3*, *iab-8*) appear to control abdominal segment patterns. See text for description of other loci shown. Map distances given where known (Redrawn from Lewis, 1978).

II. MATERIALS AND METHODS

Drosophila strains

The *Drosophila melanogaster* strains used in these studies were synthesized from mutants obtained from the California Institute of Technology, and Bowling Green *Drosophila* stock centers and from strains that were kept in Dr. M.A. Russell's laboratory. A list of the *Drosophila* strains used is given in Table 1.

Mutations and chromosomes

A detailed description of the majority of the mutations and special chromosomes can be found in Lindsley and Grell (1968). *Dp(3;1)mwh*$^{+}$ was kindly supplied by Dr. John Merriam (Merriam, 1969). *l(1)ts726* and *DTS-4* were isolated by Dr. M.A. Russell (Russell, 1974) and Drs. J.J. Holden and D.T. Suzuki (Holden and Suzuki, 1973), respectively. A brief description of the mutations and chromosomes used are listed in Tables 2 and 3.

Culture conditions

All *Drosophila* strains were raised on a standard yeast-agar-sucrose culture medium, in either half-pint glass bottles containing about 50 ml, or glass vials with about 8 ml of medium. The medium contained 1.5 grams of agar, 10 grams of sucrose, 10 grams of brewers yeast, 1 ml of propionic acid, and 10 micrograms of chloramphenicol per 100 ml of distilled water (Nash and Bell, 1968). Propionic acid and chloramphenicol were added when the mixture had cooled to a temperature of about 60 degrees before pouring.

The stocks were kept at room temperature (20-22degrees C) and all the experiments were performed in incubators at 22±1degrees C and 29±1degrees C.

Mating and egg collection

Matings were done either in the glass vials or half-pint bottles at 22±1degrees C. After two days, or when mated females began to lay a reasonable number of eggs, the flies were transferred to fresh bottles and eggs were collected at intervals of 12, 24, or 120 hours depending on the nature of the experiments as described in the results.

Table 1. *Drosophila melanogaster* **strains**

*bx^{34}

FM7/Y; Sb/TM2 x C(1)RM/Y; Sb/TM2

*Dp(3;1)mwh^{+}; y v f^{36} 1(1)ts726/FM7; mwh/mwh

Dp(3;1)mwh^{+}; y v f^{36} 1(1)ts726/FM7; mwh bx^{34}/mwh bx^{34}

pbx/T(2;3)ap^{xa}

sbd^{2} bx^{3}

w sn^{3} 1(1)ts726/FM7; mwh/mwh

w sn^{3} 1(1)ts726/FM7; mwh bx^{34}/mwh bx^{34}

y v f 1(1)ts726/B^{s} Y x c(1)DX/B^{s} Y

y v f 1(1)ts726/Y; DTS-4/TM3 x C(1)RM/Y; DTS-4/TM3

y v f 1(1)ts726/FM7; bx^{34}/TM2

y v f 1(1)ts726/FM7; pbx/TM2

y v f 1(1)ts726/FM7; sbd^{2}bx^{3}/TM2

* Superscripts 34 and 36 are used throughout this thesis instead of 34e and 36a, respectively.

Table 2. Mutations

Mutation	Symbol & Allele	Map Location	Description	Reference
bithorax	*bx^{1s}*	3-58.8	A weaker pseudoallele of the bithorax complex which transforms anterior metathorax into anterior mesothorax.	Lindsley and Grell (1968)
bithorax	*bx^{1}*	3-58.8	A strong allele of *bx* with high expressivity.	Lindsley and Grell (1968)
Dominant Temperature Sensitive lethal-4	*DTS-4*	3-24.5	A dominant temperature sensitive mutation; homozygotes are lethal.	J.J. Holden and D.T Suzuki (1973)
forked	*f*	1-56.7	Short and forked bristles and trichomes.	Lindsley and Grell (1968)
forked	*f^{36}*	1-56.7	The most extreme allele of forked.	Lindsley and Grell (1968)
lethal(1) temperature sensitive 726	*l(1)ts726 or 726*	1-65.9	A cell autonomous, temperature sensitive lethal at the Suppressor of forked locus.	M.A. Russell (1974)
multiple wing hair	*mwh*	3-0	Each trichome cell secretes three to six hairs instead of one	Lindsley and Grell (1968)
postbithorax	*pbx*	3-58.8	A pseudoallele of the bithorax complex which transforms quite completely the posterior metathorax to posterior mesothorax.	Lindsley and Grell (1968)

Serrate	*Ser*	3-92.5	Nicking and truncation of wing margin; homozygous inviable.	Lindsley and Grell (1968)
stubbloid	*sbd*^{1}	3-58.2	Short bristles	Lindsley and Grell (1968)
Stubble	*Sb*	3-58.2	Short, thick and broken bristles; a fertile allele.	Lindsley and Grell (1968)
singed	*sn*^{1}	1-21:0	Short, twisted and kinked bristles; a fertile allele.	Lindsley and Grell (1968)
Ultrabithorax	*Ubx*^{1}	3-58.8	A pseudoallele of the bithorax complex. Enlargement of haltere with very slight transformation of metathorax into mesothorax; homozygous lethal	Lindsley and Grell (1968)
vermilion	*v*	1-33.0	Eyes bright red.	Lindsley and Grell (1968)
white	*w*	1-1.5	White eyes.	Lindsley and Grell (1968)
yellow	*y*	1-0	Bristles, trichomes and body colour are yellow.	Lindsley and Grell (1968)

Table 3. Special Chromosomes

Special Chromosome	Description	Reference
B^Y	A Y-chromosome that carries the *Bar of Stone* mutation and which also contains a duplication of *Su(f)*$^+$ on YL	Lindsley and Grell (1968)
C(1)Dx	Compound (1) Double X	Lindsley and Grell (1968)
C(1)RM	Compound (1) Reversed metacentric	Lindsley and Grell (1968)
Dp(3;1)mwh$^+$	A translocation of the tip of 3L carrying *mwh*$^+$ to left distal tip of X-chromosome	John Merriam (1969)
FM7a	First multiple-7; multiple inversions act as balancer of X-chromosome	John Merriam (1969)
T(2;3)ap^{Xa}	Reciprocal translocation of 2R and 3R with dominant Xasta wing phenotype	Lindsley and Grell (1968)
TM3	Third multiple-3; a multiply inverted chromosome III used as balancer	Lindsley and Grell (1968)
TM2	Third multiple-2; a multiply inverted chromosome which effectively balances chromosome III. It contains *Ubx*$^{1\,0}$	Lindsley and Grell (1968)

Eggs were then left in the same bottle for further development. For short interval egg collection periods, the same parents were sometimes transferred to new medium several times.

Induction of pattern duplications

In order to generate pattern duplications in the metathoracic legs of the adult flies of different genotypes, eggs carrying *l(1)ts726* in combination with other mutations as specified in the results section were collected in a 22±1degrees C incubator and the culture vessels with larvae of appropriate age were then transfered to 29degrees C for 48 hours and finally returned to 22degrees C to complete development.

Screening and preparation of pattern duplications

Upon eclosion adult flies were screened under a dissecting microscope for duplicated metathoracic legs. Uneclosed pharate adults were washed off the sides of the culture bottles with warm water, collected using a strainer, and preserved in 70% ethanol. Pharate adults were then dissected out of the pupal cases and screened for duplicated metathoracic legs. The flies can be kept in alcohol for an indefinite period of time until scoring.

In order to remove the internal soft tissues, the flies were cooked in 1N NaOH at about 80degrees C for about ten minutes or until clear. The legs were then dissected free from the rest of the fly using a pair of fine irridectomy microdissecting scissors and mounted between coverslips in Gurr's water mounting medium. The coverslips were then pressed with weights and dried on a hot plate at 45degrees C for at least twenty-four hours before scoring. The legs were then scored for morphological cuticular markers under a compound light microscope at a magnification of 400X.

The morphological markers

The prothoracic, mesothoracic and metathoracic legs each possess a number of characteristic cuticular landmarks and morphological features that are useful in distinguishing the three kinds of leg. Table 4 shows the location of mesothoracic and metathoracic markers used in this study. The nomenclature is essentially that of

Table 4. Morphological markers that are similar or different between mesothoracic (L2) and metathoracic (L3) legs classified by leg segment and compartment

Leg Segment	Landmark	Description	Thoracic Segment	Compartment (A/P)
Thorax	SP	sternal pleural macrochaetes	L2	A
	ThB	Thoracic microchaetes	L2	A
	ST	single sternal bristle	L2	A
Coxa	St4	group of 4 sensilla trichodea	L2 and L3	P
	St8	group of 8 sensilla trichodea	L2 and L3	A
	CoB	coxal bristles	L2 and L3	A
	CoP	v-shaped coxal process	L2	P
	Y-apo	Y-shaped apodeme	L3	P
	BH	single hairy island bristle	L2	P
Trochanter	St1	1 sensillum trichodea	L2 and L3	A
	St5	group of 5 sensilla trichodea	L2 and L3	P
	St6	group of 6 sensilla trichodea	L2 and L3	A
	St7	group of 7 sensilla trichodea	L2 and L3	A
	Sc3	group of 3 sensilla campaniformia	L2 and L3	P
	Sc5	group of 5 sensilla campaniformia	L2 and L3	A
	Sc8	group of 8 sensilla campaniformia	L2 and L3	P
	PrBR2	2 or 3 tiny bristles proximal to Sc3	L2	P

	DBR2	1 tiny bristle distal to Sc3 if present	L3	P
	EB	edge bristle	L2	A
Femur	Sc1	1 sensillum campaniformia	L2 and L3	A
	Sc11	11 sensilla campaniformia	L2 and L3	P
	H'	A trichome filled region between R2 and R3	L2	P
	H	Hairless region between R2 and R3	L3	P
	H B	4 to 5 tiny bristles in the H	L3	P
	R3SB	Row 3 microchaetes	L2	P
	R3TB	Row 3 tiny bristles	L3	P
Tibia	TSt3	3 sensilla trichodea arranged in a triangle	L2 and L3	A
	TSc2	2 large oval sensilla campaniformia	L2 and L3	A
	AB	Apical bristle	L2	A
	PAB	preapical bristle	L2 and L3	A
	Tsp	Tibia spurs or thorn bristles	L2	AP
	TR	A row of transverse bristles	L3	P
Basitarsus	BTR2	A single bristles row	L2	P
	BR	10-12 transverse bristles rows	L3	P
Second Tarsus	STR2	A single bristle row	L2	P
	STR	5-6 transverse bristles rows	L3	P

Hannah-Alava (1958), Schubiger (1971) and Russell, Girton and Morgan (1977). Cuticular markers that have not previously been described are named and added to the list.

The landmarks are further classified as either anterior compartment (A) or posterior compartment (P) markers. Steiner (1976) has shown clearly using clonal analysis the location of the anterior-posterior compartment boundary line in the three thoracic legs. In the present studies the mesothoracic and metathoracic legs landmarks are classified as anterior or posterior according to Steiner's maps. These assignments are shown in Table 4 and Figure 5.

Cell lineage analysis

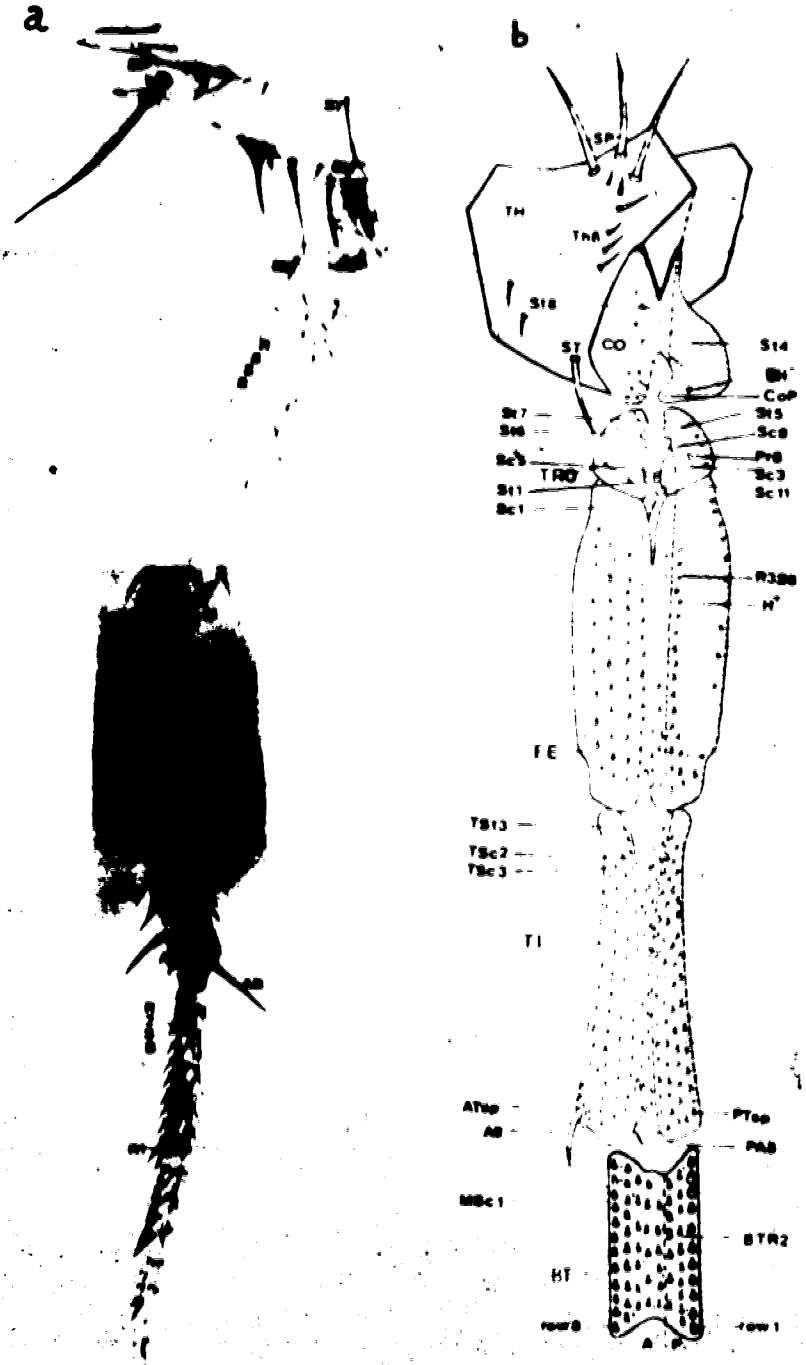
Mitotic recombination normally occurs at very low frequency in the somatic cells of *Drosophila*. However, the frequency can be greatly enhanced by radiation. In the cell lineage analysis of pattern duplications *Dp(3;1)mwh⁺, y v f³⁶ /(1)ts726/FM7; mwh bx³⁴/mwh bx³⁴* virgin females were crossed to *w sn³ /(1)ts726/Y; mwh bx³⁴/mwh bx³⁴* males.

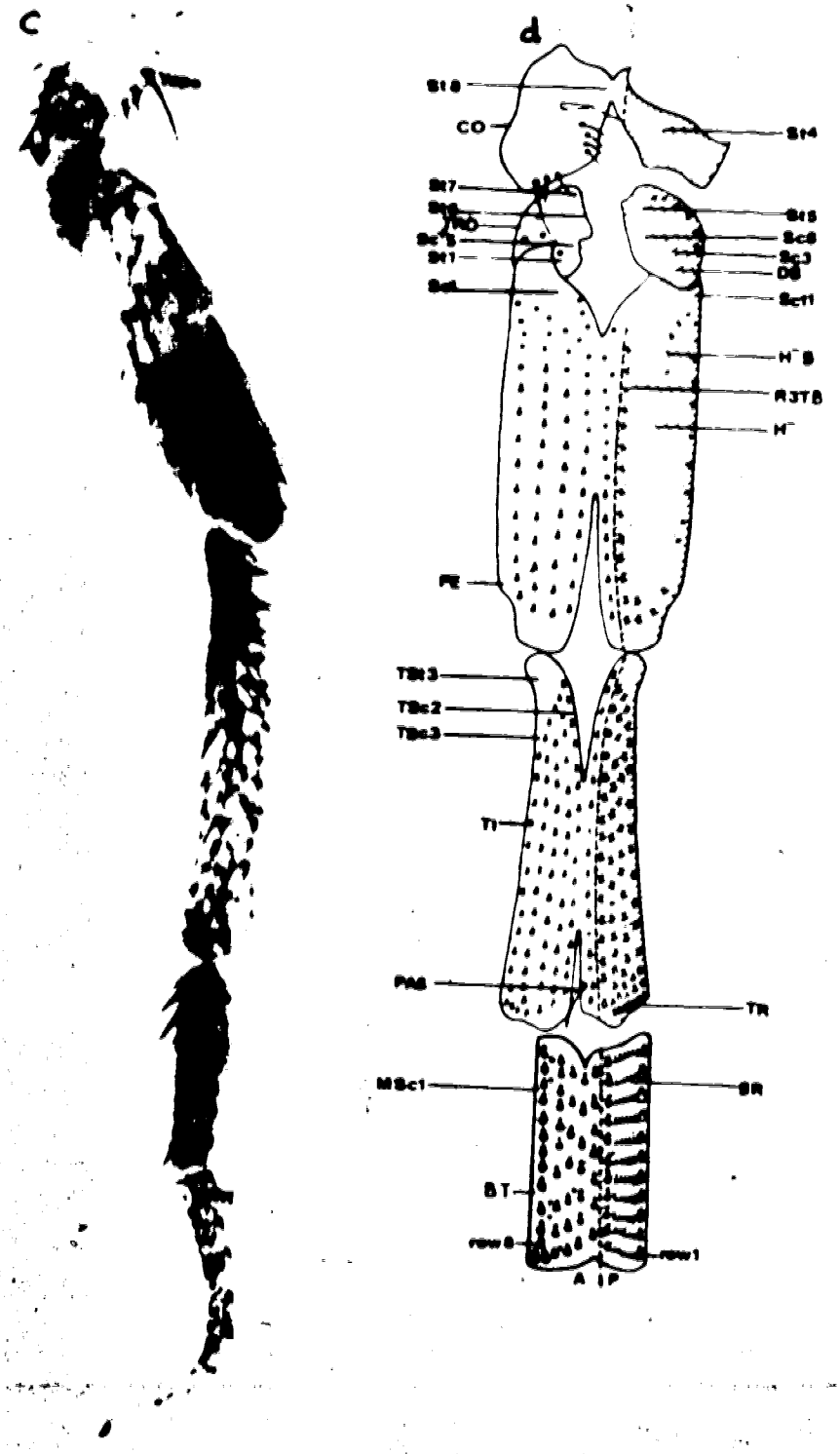
Larvae raised on standard medium in the half-pint bottles at appropriate ages were irradiated in a "Gamma-cell 220" machine with a ^{60}Co Gamma-ray source to induce mitotic recombination (Becker, 1957). At the time of irradiation, the Gamma-ray dose rate was 2.5 Kr per minute. The dosage given to the larvae was controlled by varying the time of exposure using the automatic timer of the machine. No attempt was made to correct any radiation dose absorbed by the glass. The radiation absorbed by the glass should not be greater than 1% (Girton, 1979).

Immediately after irradiation, the cultures were transferred to a 29degrees C incubator for 48 hours and then returned to a 22degrees C incubator for completion of development. The heat pulse treatment was used to induce pattern duplications in the metathoracic legs. Females of the genotype *w sn³ /(1)ts726/Dp(3;1)mwh⁺, y v f³⁶ /(1)ts726;mwh bx³⁴/mwh bx³⁴* were collected and prepared for microscopic examination as described above.

As shown in Figure 6; mitotic recombination within different intervals along the X-chromosome will give rise to single or twin spots (Stern 1936, 1968). A list of all the kinds of clones one would expect in the experiment is also shown in Figure 6. In a similar

Figure 5. Photograph and map of the wild-type mesothoracic (a-b) and metathoracic (c-d) legs. See Table 4 for explanation of symbols (Leg maps are from Steiner, 1976). R1 = row 1.


a
b
SP
TH
THA
S18
ST
CO
S14
BH
CoP
Sh7
Sh6
Sh5
Sc8
Sc5
TRD
Pt8
Sc3
St1
Sc1
Sc11
R3Sb
H
FE
TSt3
TSc2
TSc3
TI
ATtip
AB
PTop
PAB
MBc1
BTR2
HT
row8
row1


c
d
SI8
CO
SI4
SI7
SI6
SI5
Sc6
Sc5
SI1
Sc4
Sc3
DS
Sc2
Sc11
H¯B
R3TB
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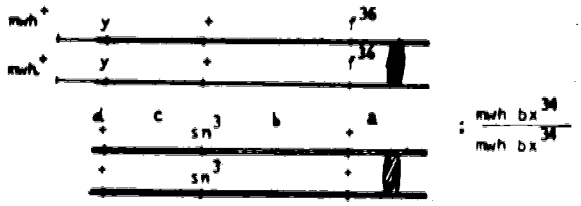
TR
MSc1
SR
BT
row8
row1
A
P



Position of the Mitotic Recombination Event	Clone Phenotype
a	yf-mwh sn twin
b	y-mwh sn twin
c	y-mwh twin
d	mwh single
a + b	f single
a + c	f-sn twin
a + d	yf-sn twin
b + c	sn single
b + d	y-sn twin
c + d	y single

Figure 6. Mitotic recombination events and kinds of spots expected. Most events would occur at the heterochromatic region to give a yf-mwh sn twin spots (Becker, 1974). Simultaneous events greater than double are not listed as double events are already extremely rare. Recombination in chromosome II.I does not change the cell genotype.

experiment Becker (1974) has shown that the majority of the mitotic recombination events occur between *forked* and the centromere as most of the observed clones are twin spots for yellow-forked and singed-multiple wing hair cuticular markers. One must be aware that all the clones are scorable only in regions of the fly where bristles and trichomes exist and also that certain mutations do not affect both bristles and trichomes. For instance, one cannot distinguish a multiple wing hair bristle or a yellow trichome from a wild-type one. Therefore, it is not unlikely that a twin spot could be scored as a single spot, which would lead to the underestimation of clonal frequency.

III. RESULTS

In this chapter are presented the results of experiments in which pattern duplications are used as a tool to study the involvement of the *bithorax* complex in determination. The first step was to examine the compartment specificity of the homeotic transformations caused by the *bithorax* and the *postbithorax* mutants in the metathoracic leg. The next step was to investigate the effects of these mutations on pattern duplication. Finally a cell lineage analysis of *bithorax* duplications was performed to establish the origin of these duplications and to look at the behavior of anterior and posterior cells which may be differently determined in the *bithorax* metathoracic disc.

Morphology of *bithorax*^{3} and *postbithorax* metathoracic legs

The effects of *bithorax* and *postbithorax* have been demonstrated to be compartment specific in the haltere disc (Morata and Garcia-Bellido, 1976). In the *bx*^{3} fly, only the anterior haltere is replaced by a set of anterior wing structures while in the *pbx* homozygotes only the posterior haltere structures are transformed into the posterior wing. Interestingly, the transformed structures found come from either anterior or posterior compartments in the wing as revealed by clonal restriction analysis (Morata and Garcia-Bellido, 1976). In addition, *bx* homozygous clones induced by somatic recombination in the haltere define an anterior-posterior compartment restriction line and differentiate only anterior wing structures. Similar clones had no effect in the posterior haltere. On the other hand, homozygous *pbx* clones differentiated posterior wing structures only when induced in the posterior haltere and defined a similar anterior-posterior compartment restriction line (Morata and Garcia-Bellido, 1976). Therefore the evidence is strong that *bx* and *pbx* are indeed compartment specific in the dorsal metathorax.

It may be the case that the effects of *bx* and *pbx* are also specific to the anterior and the posterior compartments in the ventral metathoracic disc (Morata and Lawrence, 1977), but in no case has extensive evidence in support of this conclusion been presented. Therefore a detailed analysis of *bx*^{3} and *pbx*-transformed metathoracic legs was carried out to establish the sets of markers that are affected and to confirm that the transformations are indeed limited to the anterior or the posterior compartments as in

the haltere.

If the effects of *bx* and *pbx* do indeed correspond respectively to the anterior or the posterior compartments, one would expect in the most extreme *bx* homozygotes the replacement of all anterior metathoracic landmarks by anterior mesothoracic landmarks and a substitution of posterior metathoracic markers by posterior mesothoracic ones in the *pbx* mutant. Table 5 shows that in all 20 cases examined, only anterior mesothoracic and posterior metathoracic landmarks were present in bx^3 homozygotes. The absence of sternal bristle in one case was probably due to a developmental defect rather than incomplete expressivity of bx^3 for this marker, as many additional legs were subsequently examined for this marker and it was present in all cases. In *pbx* homozygotes, only anterior metathoracic and posterior mesothoracic markers were found in the 20 metathoracic legs examined. Posterior tibial spur (PTsp) was the only marker which lacks complete expressivity as it is present only 19 times. This same marker is present in only 80 percent of the mesothoracic legs in the Oregon-R strain however. In no case, in the absence of PTsp was posterior transverse row (TR), which is located at an analogous position to PTsp in the metathoracic leg, present. Failure to present a set of anterior metathoracic markers in Table 5 is due to the fact that no discrete anterior markers are present on the metathoracic leg. Therefore, identification of the anterior metathoracic compartment is based on the absence of anterior prothoracic and mesothoracic markers. It is noteworthy however, that the number and the arrangement of chaetal elements in different segments of mesothoracic and metathoracic legs are not identical and that the anterior metathoracic pattern was found whenever anterior mesothoracic markers were absent.

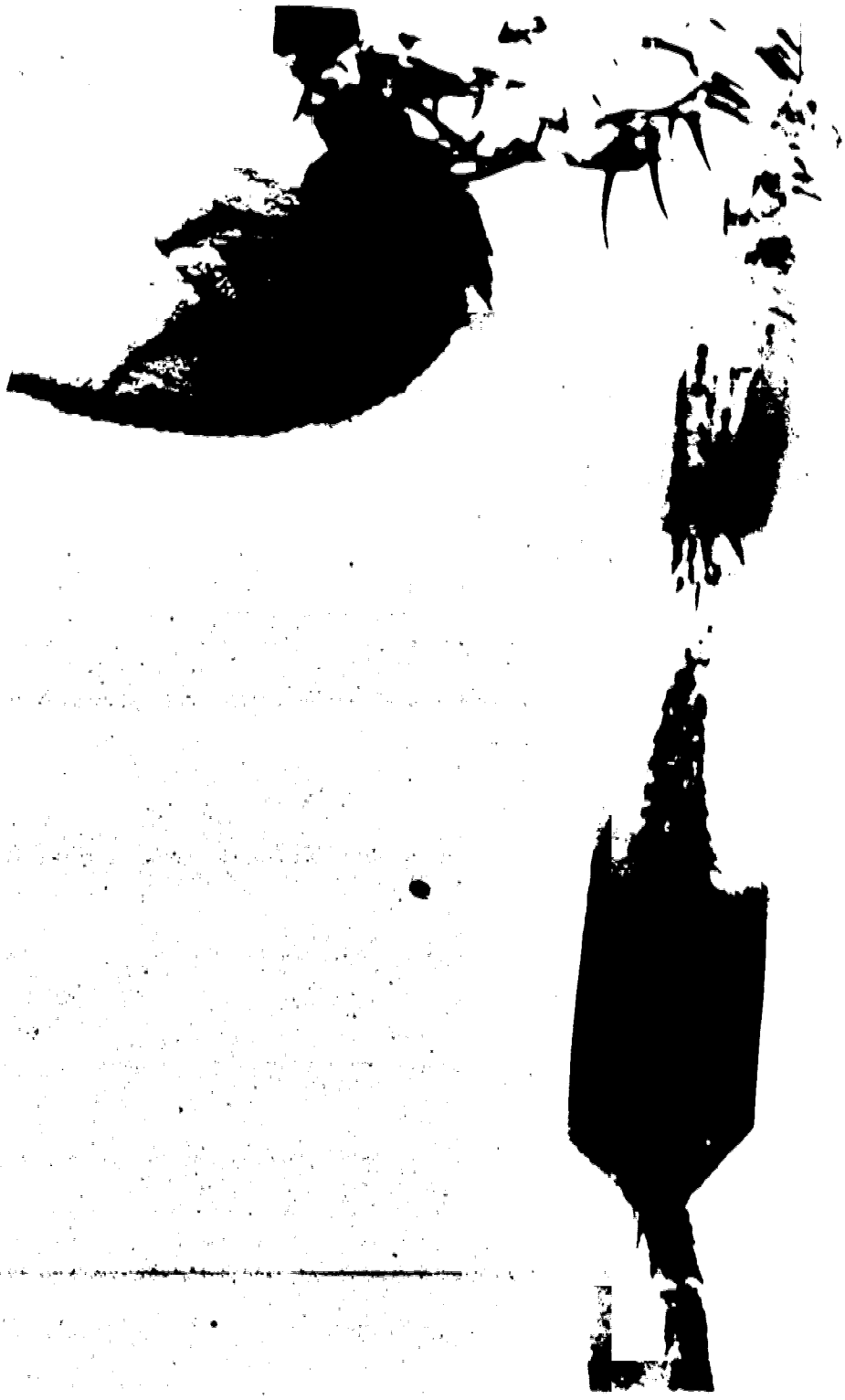
In double mutants homozygous for both bx^3 and *pbx*, two perfect pairs of mesothoracic legs were present as expected. Figure 7 shows both compartments of bx^3 and *pbx* transformed metathoracic legs. In conclusion, the observations are consistent with the idea that in the ventral metathorax, the effects of bx^3 and *pbx* are specific to either the anterior or the posterior compartment as in the haltere. In this thesis, we will be dealing specifically with the metathoracic leg disc throughout.

Table 5. Unduplicated control metathoracic legs from flies cultured at 22°C. Incidence of compartment specific markers in unduplicated legs of three genotypes

Kind of leg	Genotype	Sample size	Landmarks[1]																	
			Anterior Mesothorax						Posterior Mesothorax								Posterior Metathorax							
			ST	SP	ThB	EB	ATsp	AB	Cop	BH	PrBR2	H'	R3SB	PTsp	BTR2	STR2	Yapo	DBR2	H	HB	R3TB	TR	BR	STR
Unduplicated L2	Oregón-R	20	20	20	20	20	20	20	20	20	20	20	20	16	20	20	0	0	0	0	0	0	0	0
Unduplicated L3	Oregon-R	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	20	20	20	20	20	20
Unduplicated L3	*sbd[1]bx[1]/sbd[1]bx[1]*	20	19	20	20	20	20	20	0	0	0	0	0	0	0	0	20	0	20	20	20	20	20	20
Unduplicated L3	*pbx/pbx*	20	0	0	0	0	0	0	20	20	20	20	20	19	20	20	0	0	0	0	0	0	0	0

[1]Symbols for morphological markers are defined in Table 4

Figure 7. The *bithorax*^{3} and *postbithorax* transformed metathoracic legs. AW = anterior wing, PW = posterior wing, AH = anterior haltere, PH = posterior haltere, See Table 4 for explanation of marker symbols.



Effect of *bithorax*^{1} on pattern duplication

As *bithorax* specifically transforms the anterior metathoracic leg into anterior mesothoracic leg, it was interesting to find out if the determined state is inherited when the *bithorax* disc duplicates.

Metathoracic leg duplications were generated by subjecting larvae of ages 0 to 120 hours (120 hour egg laying period) from a *y v f l(1)ts726/FM7;sbd*^{1} *bx*^{3}*/TM2* stock to a 48 hour, 29degrees C heat pulse as described in Materials and Methods (page 23). The *TM2* balancer chromosome also carries a dominant pseudoallele, *Ubx*^{130} of the *bithorax* complex which behaves like a null allele of the *bithorax* and *postbithorax* loci. Homozygous *sbd*^{1} *bx*^{3} individuals could be distinguished from the *sbd*^{1} *bx*^{3}*/TM2* ones by the stubbloid phenotype of the former. Flies hemizygous or homozygous for *y v f l(1)ts726* in combination with homozygous *sbd*^{1} *bx*^{3} or *sbd*^{1} *bx*^{3}*/TM2* were prepared for scoring under the compound microscope. In this section, only results of *bx*^{3} homozygotes will be dealt with and results on *bx*^{3}*/TM2* will be presented later. For a control, a sample of heat pulsed unduplicated legs of the same genotypes from the same experiment was scored. Markers were scored in original and duplicate parts of each duplicated leg. The member with symmetry appropriate to its side of the fly was designated as "original" (orthodrome) and the other member with opposite symmetry was designated as the "duplicate" (antidrome). The results of clonal analysis experiments (Girton, 1979) support this classification. Also, the duplicate can usually be distinguished from the original by its smaller size or incompleteness. In interpreting the results it is important to note that in pattern duplications a part of the pattern is often deficient due to cell death caused by the heat pulse. Therefore classification of pattern duplications into different categories with respect to the effect of *bx* depends on the markers that remain.

The incidence of each marker in the *bx*^{3}*/bx*^{3} heat pulsed unduplicated and duplicated metathoracic legs is shown in Table 6. Note that in the anterior compartment of both the original (O) and duplicate (D) mesothoracic markers were always found. This was also the case in the heat pulsed *bx*^{3}*/bx*^{3} unduplicated control legs. However, in the posterior compartment of the duplicate, in addition to the presence of metathoracic markers, mesothoracic structures were also found. In contrast, no unambiguously

Table 6. Incidence of each marker present in the heat-treated *bx*[1] and *bx*[1] *pbx*[1] unduplicated and duplicated metathoracic legs

Kind of leg	Genotype	Sample size	Landmarks[2]																					
			Anterior Mesothorax						Posterior Mesothorax									Posterior Metathorax						
			ST	SP	ThB	EB	ATsp	APB	CbP	BH	PrBR2	H'	R3SB	PTsp	BTR2	STR2	Yapo	DBR2	H	HB	R3TB	TR	BR	STR
Unduplicated L2[2]	+/+	20	20	20	20	20	20	20	20	20	20	20	--	20	20	20	0	0	--	0	--	0	0	0
Unduplicated L3[2]	+/+	20	0	0	0	0	0	0	0	2	0	0	-	0	0	0	20	10	--	20		20	20	20
Unduplicated L3	*bx*[1]/*bx*[1]	20	20	20	20	20	20	20	0	0	0	0	--	0	0	0	20	0	--	20	--	20	20	20
Duplicate L3																								
original	*bx*[1]/*bx*[1]	23	2	19	21	23	23	23	0	1	0	0	--	0	0	0	18	1	--	21	--	22	17	21
duplicate	*bx*[1]/*bx*[1]	23	1	17	18	22	23	23	6	4	6	10	--	16	13	13	7	0	--	11	--	4	4	5

[1]Symbols for morphological markers are defined in Table 4

[2]Data reproduced from Table 8

mesothoracic markers were found in the posterior compartment of the original or of the heat treated controls. The only exception with the wild-type control legs was that of the hairy island bristle (BH-) which appeared invariably in the posterior mesothoracic leg. A similar bristle was also found in 2 out of 20 cases in the wild-type posterior metathorax. Therefore this marker lacks complete specificity and care should be taken when interpreting the results for this marker. The metathoracic BH- is much smaller in size as compared to the mesothoracic one and the one in the *pbx* transformed metathoracic leg. Single distal bristle (DB) in the trochanter in the posterior metathoracic compartment, which has not previously been used as a marker, showed incomplete penetrance. It was found in 10/20 cases in the wild-type controls but never in the bx^3/bx^3 unduplicated controls.

The bx^3/bx^3 pattern duplications could be classified into three types. In all three kinds, the original member was the same as in the bx^3/bx^3 metathoracic control legs, that is the anterior compartment (A) was transformed to the anterior mesothoracic pattern (AMS) while the posterior compartment (P) remained metathoracic (PMT). This kind of pattern will be referred to as an AMS-PMT pattern and the same kind of nomenclature will be used throughout. On the other hand, three kinds of pattern were observed in the duplicates. The first kind, also designated AMS-PMT, is a duplicate of the original (AMS-PMT) in mirror image symmetry. The second type, designated AMS-PMS, has both the anterior and the posterior compartments of the duplicate completely transformed to mesothorax. The last type, designated AMS-P(MS&MT), again has the anterior compartment totally transformed to anterior mesothorax but the posterior compartment contains a mixture of both mesothoracic and metathoracic markers. In such mixed posterior compartments, usually only one or two markers different from the rest were present. Note that no difference exists between the anterior compartments of all three kinds of duplicate, all of which have their anterior landmarks entirely transformed to the anterior mesothorax. Examples of three different kinds of *bithorax* pattern duplications are illustrated in Figure 8.

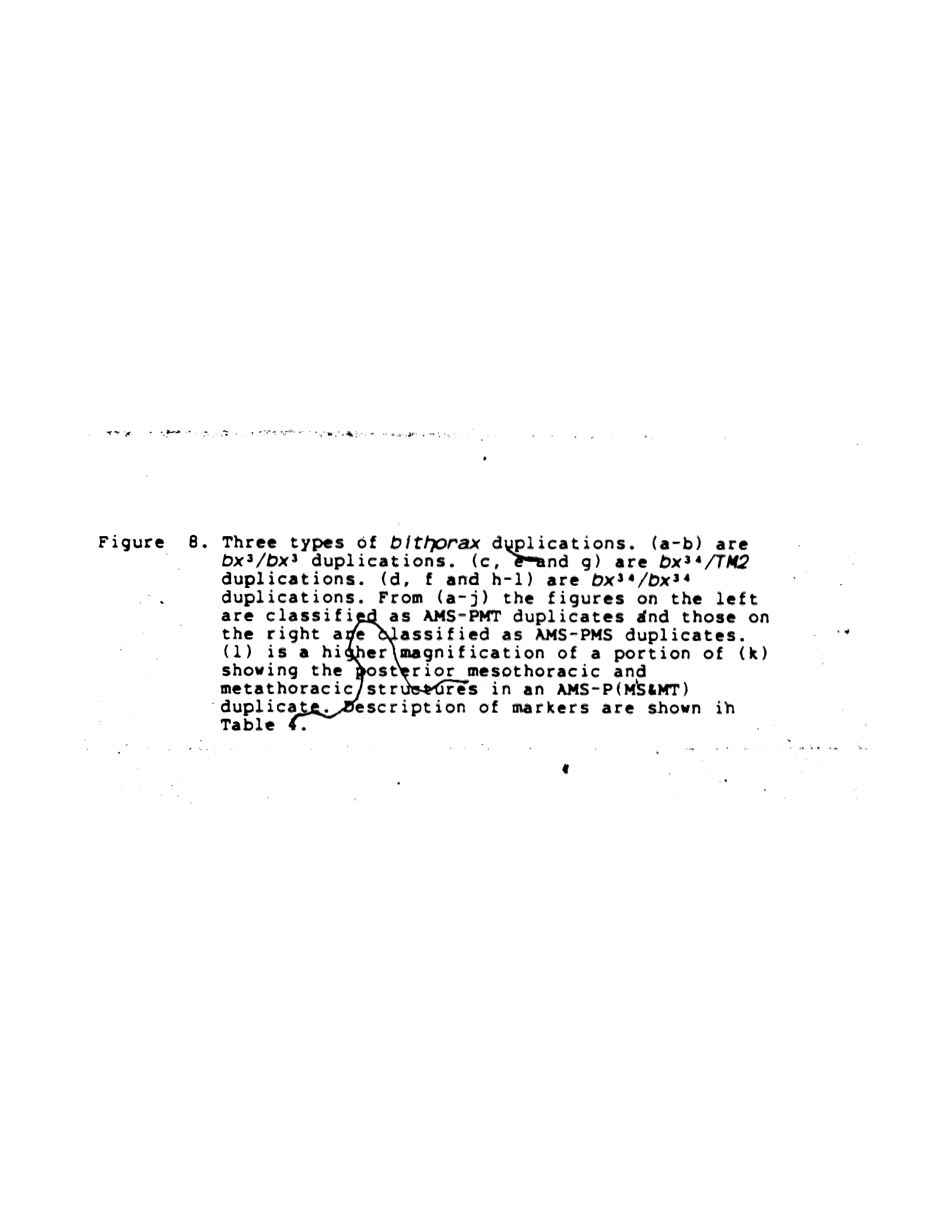


Figure 8. Three types of *bithorax* duplications. (a-b) are bx^3/bx^3 duplications. (c, e and g) are $bx^{34}/TM2$ duplications. (d, f and h-l) are bx^{34}/bx^{34} duplications. From (a-j) the figures on the left are classified as AMS-PMT duplicates and those on the right are classified as AMS-PMS duplicates. (l) is a higher magnification of a portion of (k) showing the posterior mesothoracic and metathoracic structures in an AMS-P(MS&MT) duplicate. Description of markers are shown in Table 4.

Pattern duplication in *postbithorax* metathoracic legs

The *pbx* (AMT-PMS) and *bx* (AMS-PMT) mutant transformations are analogous in that they both affect only one compartment of the metathoracic leg disc. Therefore it was interesting to see if the *pbx* mutation had any effect on pattern duplication in a cell lethal system. It was also interesting to find out if the effect was analogous to that of *bx*. Such an analysis would enable one to determine if an analogy also exists in the underlying function of bx^+ and pbx^+ in metathoracic disc determination during normal pattern formation as well as pattern duplication.

Pattern duplications were induced using the same procedure as for the *bx* experiments. In the current experiment, a *y v f l(1)ts726/FM7,pbx/TM2* stock was used. Since the *pbx* homozygotes and *pbx/TM2* flies both showed full penetrance and very similar expressivity in both the haltere and the metathoracic leg, it was difficult to tell the two genotypes apart. For this reason, no attempt was made to distinguish them in the following analysis. Only flies homozygous or hemizygous for *y v f l(1)ts726* and carrying either *pbx/pbx* or *pbx/TM2* were used for these studies.

As shown in Table 7, in the heat-pulsed unduplicated *pbx* controls from the same experiment, only mesothoracic markers were found in the posterior compartment of the metathoracic leg. The bristle pattern and morphology in the anterior compartment remained substantially unchanged. Most anterior mesothoracic compartment specific landmarks like ST, Sp or ThB were not observed, but EB (11/25 cases) and AB (7/25 cases) sometimes appeared. These may perhaps be ascribed to a weakly dominant *bx* effect of Ubx^{130} in *TM2* in certain genetic backgrounds since these markers were never observed in a sample of 20 *pbx/Df(3)bxd*^{109} individuals and the only mesothoracic marker found was BH- in the posterior compartment (11/20) of *+/TM2* metathoracic legs. In addition, it is known that *Ubx* homozygous clones are cell viable and transform both compartments of metathorax to mesothorax completely. Therefore it is important to refer to the appropriate controls when interpreting the results for these two markers.

Fifty duplicated metathoracic legs were obtained. Table 7 indicates that only one kind of pattern was present. The anterior compartment was always metathoracic and the posterior compartment mesothoracic (AMT-PMS) in both the original and the duplicate. EB, which is an anterior mesothoracic marker was found in 12/50 duplicates as

Table 7. Incidence of each marker present in the heat-treated *pbx* unduplicated control and duplicated metathoracic legs

Kind of leg	Genotype	Sample size	Landmarks[1]																						
			Anterior Mesothorax						Posterior Mesothorax									Posterior Metathorax							
			ST	SP	ThB	EB	ATsp	AB	Cop	BH	PrBR2	H'	R3SB	PTsp	BTR2	STR2		Yapo	DBR2	H	HB	R3JB	TR	BR	STR	
Unduplicated L2[2]	+/+	20	20	20	20	20	20	20	20	20	20	20	--	20	20	20		0	0	--	0	--	0	0	0	
Unduplicated L2	+/*TM2*	20	20	20	20	20	20	20	20	19	20	20	--	20	20	20		0	0	--	0	--	0	0	0	
Unduplicated L3[2]	+/+	20	0	0	0	0	0	0	0	2	0	0	--	0	0	0		20	10	--	20	--	20	20	20	
Unduplicated L3	+/*TM2*	20	0	0	0	0	0	0	0	11	0	0	--	0	0	0		20	9	--	20	--	20	20	20	
Unduplicated L3	*pbx/pbx* & *pbx/TM2*	25	0	0	0	11	0	7	25	25	25	25	--	22	25	25		0	0	--	0	--	0	0	0	
Unduplicated L3	*pbx/Df(3)bxd*[k49]	20	0	0	0	0	0	0	20	20	20	20	--	20	20	20		0	0	--	0	--	0	0	0	
Duplicate L3 original	*pbx/pbx* & *pbx/TM2*	50	0	0	0	19	0	4	34	34	48	50	--	37	50	50		0	0	--	0	--	0	0	0	
duplicate	*pbx/pbx* & *pbx/TM2*	50	0	0	0	12	0	0	34	34	49	50	--	32	50	50		0	0	--	0	--	0	0	0	

[1] Symbols for morphological markers are defined in Table 4.

[2] Data reproduced from Table 8

compared to 19/50 originals. AB was present in 4/50 cases in the original member only. Other anterior mesothoracic landmarks were not observed in the duplicates. Therefore one can conclude that indeed only one kind of duplicate is produced in *pbx* flies and that this mutant has no effect in the anterior compartment of the duplicate in the cell lethal system. An illustration of an AMT-PMS duplicate is shown in Figure 9.

Pattern duplication in the wild-type

Intensive earlier analyses of pattern duplications in the mesothoracic leg using the same cell lethal system had not revealed any transformation of the duplicates to prothoracic or metathoracic leg by transdetermination or phenocopy (Russell, 1974; Russell, Girton and Morgan, 1977; Postlethwait, 1978; Girton, 1979). However, partial transformation of the duplicate to wing in extremely low frequency had been reported (Postlethwait, 1978). In addition, treatment with agents like ether or heat shock at early embryonic stages are known to cause *bx* and *pbx* phenocopies (Gloor, 1947; Capdevila and Garcia-Bellido 1974, 1978; Bownes and Seiler, 1977). For these reasons, a number of pattern duplications were analyzed in the wild-type metathoracic leg.

The wild-type metathoracic leg duplications and unduplicated controls were obtained by screening a number of *y v f l(1)ts726*/Y males heat-treated as described in the earlier experiments (Russell, Girton and Morgan, 1977).

Only one kind of metathoracic duplication pattern was found (Table 8). In both compartments, the metathoracic state was unaffected after heat pulse treatment and the process of duplication. The original member of each duplicated leg was always entirely metathoracic and the duplicate was a mirror image copy of the original with the metathoracic pattern maintained. In all twenty-four duplications examined, as shown in Table 8, no mesothoracic structures were found. An example of the wild-type metathoracic leg pattern duplication is shown in Figure 10.

The effects of other *bithorax* alleles on pattern duplication

The *bithorax* Complex consists of at least eight tightly linked loci all of which map in the region of two doublets (89E1,2-3,4) located on the right arm of the third chromosome. Interestingly, all the loci are involved in the development of either

Figure 9. A *postbithorax* metathoracic leg duplication.

Table 8. Incidence of each marker present in the *bx*' *pbx*' unduplicated control and duplicated metathoracic legs

Kind of leg	Genotype	Sample size	Landmarks[1]																						
			Anterior Mesothorax						Posterior Mesothorax								Posterior Metathorax								
			ST	SP	Thb	EB	ATsp	AB	Cop	BH	PrBR2	H'	R3SB	PTsp	BTR2	STR2	Yapo	DBR2	H	HB	R3TB	TR	BR	STR	
Unduplicated L2	+/+	20	20	20	20	20	20	20	20	20	20	20	--	20	20	20	0	0	--	0	--	0	0	0	
Unduplicated L3	+/+	20	0	0	0	0	0	0	0	2	0	0	--	0	0	0	20	10	--	20	--	20	20	20	
Duplicated L3																									
original	+/+	24	0	0	0	0	0	0	0	0	0	0	--	0	0	0	23	3	--	24	--	23	22	21	
duplicate	+/+	24	0	0	0	0	0	0	0	0	0	0	--	0	0	0	23	3	--	22	--	17	13	10	

[1]Symbols for morphological markers are defined in Table 4

Figure 10. A wild type metathoracic leg duplication.

mesothorax, metathorax or abdominal segments. In view of the genetic complexity of the Complex, pattern duplications were analyzed in three additional genotypes to rule out the possibility that the observed effects in the bx^3 homozygotes might be allele specific.

The procedure used for making the duplication was the same as that described above for bx^3/bx^3 except that a *y v f /(1)ts726/FM7;bx*^{34}*/TM2* stock was used. The bx^{34} homozygotes could be distinguished from $bx^{34}/TM2$ individuals by scoring the haltere where the *bx* transformation is much more strongly expressed over *TM2* (Figure 11). In addition, sbd^2 $bx^3/TM2$ pattern duplications obtained from the previous experiment were examined. In each experiment a sample of unduplicated metathoracic legs of each of the above genotypes was also scored to provide a control.

The results, shown in Table 9, indicate that all three genotypes bx^{34}/bx^{34}, $bx^{34}/TM2$ and sbd^2 $bx^3/TM2$ gave qualitatively similar results. In each case only anterior mesothoracic and posterior metathoracic structures were found in the original member. However, in duplicates, while only mesothoracic markers were found in the anterior compartments, both mesothoracic and metathoracic landmarks were observed in the posterior compartments. The duplications of all three genotypes can also be classified into just three types with AMS-PMT patterns in the original and AMS-PMS, AMS-PMT or AMS-P(MS&MT) patterns in the duplicate. Thus, as far as the duplication pattern is concerned, all four genotypes analyzed gave qualitatively similar effects. Examples of the different kinds of duplicated legs are shown in Figure 8.

The penetrance was complete and the expressivity quite constant for the homozygotes carrying the weaker *bithorax* allele bx^{34}. 'Sternal bristle' (ST) was the only mesothoracic marker that was only occasionally expressed (5%) in the bx^{34} homozygotes. The expressivity of bx^{34} was much increased in the haltere when balanced over *TM2*, but not much change was detectable in the metathoracic leg as compared with bx^{34} homozygotes. ST was found in 80% of $bx^{34}/TM2$ legs and in addition, BH- was also present in 25% of the legs. Not much difference in expressivity between bx^3/bx^3 and $bx^3/TM2$ was found. The penetrance for both genotypes was 100% and expressivity was essentially complete in both the dorsal and ventral discs. (The only exception was that BH- was found in 65% of the $bx^3/TM2$ legs.)

Figure 11. The expressivity of haltere to wing transformation in (a) $Bx^{34}/TM2$ and (b) Bx^{34}/Bx^{34}. AW = anterior wing, PH = posterior haltere, TRC = triple row chaetes.

Table 9. Incidence of each marker[1] present in the three heat-treated *bx* genotypes (duplicated and unduplicated metathoracic legs), and *bx*$^{+}$ *pbx*$^{+}$ unduplicated control

Kind of leg	Genotype	Sample size	Landmarks[2]																												
			Anterior Mesothorax						Posterior Mesothorax										Posterior Metathorax													
			ST	SP	ThB	EB	ATsp	AB	Cop	BH	PrBR2	H'	R3SB	PTsp	BTR2	STR2	Yapo	DBR2	H	HB	R3TB	fR	BR	STR								
Unduplicated L2'	+/+	20	20	20	20	20	20	20	20	20	20	20	--	20	20	20	0	0	--	0	--	0	0	0							
Unduplicated L2'	+/*TM2*	20	20	20	20	20	20	20	20	19	20	20	--	20	20	20	0	0	--	0	--	0	0	0							
Unduplicated L3'	+/+	20	0	0	0	0	0	0	0	2	0	0	--	0	0	0	20	10	--	20	--	20	20	20							
Unduplicated L3'	+/*TM2*	20	0	0	0	0	0	0	0	11	0	0	--	0	0	0	20	9	--	20	--	20	20	20							
Unduplicated L3	*bx*$^{1+}$/*bx*$^{1+}$	40	1	40	40	40	40	40	0	0	0	0	--	0	0	0	40	8	--	40	--	40	40	40							
Unduplicated L3	*bx*$^{1+}$/*TM2*	44	35	44	44	44	44	44	0	13	0	0	--	0	0	0	44	22	--	44	--	44	44	44							
Unduplicated L3	*bx*^{1}/*TM2*	20	20	20	20	20	20	20	0	13	0	0	--	0	0	0	20	2	--	20	--	20	20	20							
Duplicate L3																															
original	*bx*$^{1+}$/*bx*$^{1+}$	32	1	32	32	32	32	32	0	1	0	0	--	0	0	0	27	5	--	--	--	32	32	-							
original	*bx*$^{1+}$/*TM2*	21	2	19	20	21	21	21	0	11	0	0	--	0	0	0	13	12	--	--	--	21	21	-							
original	*bx*^{1}/*TM2*	18	2	15	14	14	18	18	0	5	0	0	--	0	0	0	16	1	--	17	--	18	18	15							
duplicate	*bx*$^{1+}$/*bx*$^{1+}$	32	1	27	27	32	30	30	7	4	3	--	--	9	10	-	16	0	--	--	--	20	20	-							
duplicate	*bx*$^{1+}$/*TM2*	21	2	16	16	18	17	17	4	7	4	--	--	7	7	-	9	2	--	--	--	10	10	-							
duplicate	*bx*^{1}/*TM2*	18	2	15	14	14	16	16	3	5	2	5	--	3	6	3	13	0	--	12	--	9	10	6							

[1] Symbols for morphological markers are defined in Table 4

[2] Data reproduced from Tables 7 and 8

Frequency of different kinds of duplication in the four *bithorax* genotypes

Although the kinds of pattern duplications produced in the four *bithorax* genotypes were similar due to different degrees of expressivity among the alleles, one might expect the frequency of the three kinds of duplicates to differ

The incidence of each kind of duplicate classified by genotype is presented in Table 10. The results show that the frequencies were very similar in bx^{34}/bx^{34}, $bx^{34}/TM2$ and $bx^{3}/TM2$, but that in bx^{3}/bx^{3} the distribution of duplication types may be different. Statistical testing using a contingency chi-squared test confirms that significant heterogeneity with respect to the incidence of each class of duplicate does exist among the four genotypes ($X^2(6)=17.86$, $0.01>p>0.005$). When the same test was done after excluding bx^{3}/bx^{3} data, no significant heterogeneity among the three remaining genotypes was found ($X^2(4)=7.10$, $0.2>p>0.1$). Therefore, results from these three genotypes were pooled and another contingency chi-squared test was done. The result of this test (Table 11) shows that the frequency of the three kinds of pattern duplication is not independent of genotype, that is, there is a significant difference between bx^{3}/bx^{3} and the other three genotypes ($X^2(2)=15.04$, $p<0.005$).

When the weaker allele, bx^{34} was used, 63% of the duplicates in the homozygotes were of AMS-PMT type and the remaining 37% differentiated at least one PMS marker. However, the frequency of AMS-PMT duplicates fell significantly to 22% with the remainder differentiating at least one PMS marker when the more extreme allele, bx^{3}, was homozygous. Among duplicates of all four genotypes, mixed posterior compartments (P(MS&MT)) occurred at a much lower frequency, only 11 out of 94 cases (12%) were obtained.

Kind of duplicate with respect to the developmental stage at initiation of the duplication

In order to find out whether each kind of duplicate might be formed only at some particular stage of development, two kinds of analysis were done. The first involved a comparison of left and right duplicate patterns from the same fly and the second a systematic experiment to produce duplications at different stages of development.

Table 10. Incidence of type of duplicate classified by genotype

Type of Duplicate	Genotype			
	bx^{1+}/bx^{1+}	$bx^{1+}/TM2$	$bx^{1}/TM2$	bx^{1}/bx^{1}
AMS-PMT	20	12	11	5
AMS-PMS	11	8	5	11
AMS-P(MS & MT)	1	1	2	7
Sum	32	21	18	23

Table 11. 3 x 2 Contingency X^2 test for heterogeneity in type of duplicate frequency among different genotypes

Genotype	Type of duplicate: AMS-PMT	AMS-PMS	AMS-P(MS & MT)	SUM
bx¹/bx¹	5	11	7	23
bx¹⁺/bx¹⁺ & *bx¹⁺/TM2* & *bx¹/TM2*	43	24	4	71
Sum	48	39	11	94

Contingency $X^2(2) = 15.04$; $p < 0.005$

The first analysis made use of data from flies analyzed in the previous sections. In those experiments, the duplications were generated by heat treatment of larvae from 0 to 120 hours old. If the kind of duplicate formed were strictly dependent on stage of development at the time of the heat treatment, one would not expect to find two different kinds of duplicates in a single individual which had both left and right legs duplicated. Among all the flies of the four genotypes examined, 10 had both legs duplicated. The results are given in Table 12. In only three cases were both left and right duplicates similar in type. The results show that all three kinds of duplicates can be formed at least at some developmental stage at which duplicates are initiated.

Further evidence comes from the analysis of duplications induced in two specific intervals during development. Homozygous *bx*^{34} larvae aged either 54±6 hours or 78±6 hours (12 hour egg laying period) were subjected to a 29degrees C, 48 hour heat treatment to produce pattern duplications. The results are shown in Table 13. Note that all three kinds of duplicate were found in both treatments. This confirms that the kind of duplication pattern produced is independent of the developmental stage at which it is initiated. A contingency chi-squared test shows that the frequency of each kind of duplicate is insignificantly different between the two heat treatment times ($X^2(2)=2.54$, $0.5>p>0.25$).

Size of marker deficiency in relation to type of pattern duplication in *bithorax* flies

Use of the cell lethal system in generating the duplication has the disadvantage that we do not know the compartmental derivation of the cells that form the duplicate. However, it has been shown in a number of studies that a pattern duplication is usually if not always associated with a morphological deficiency (Russell, 1974; Arking, 1975; Simpson and Schneiderman, 1975; Russell, Girton and Morgan, 1977; Postlethwait, 1978). The deficiency generally extends a variable distance from the medial edge of the leg disc (see Figure 12) and thus may either intersect only the anterior compartment, or both compartments. It has been assumed that the observed limit of the deficiency represents the free edge of disc epithelium created by cell death and that cells from this free edge form the duplicate part of the pattern (Russell, Girton and Morgan, 1977; Clark and Russell, 1977; Postlethwait, 1978). Thus, from the size of the morphological

Table 12. Incidence of left and right duplication pattern in individual *bithorax* flies

Genotype	Combined left and right posterior duplicate pattern			
	PMS & PMS	PMS & PMT	PMS & P(MS & MT)	PMT & PMT
bx^{1+}/bx^{1+}	0	3	0	2
$bx^{1+}/TM2$	0	2	0	0
bx^{1}/bx^{1}	1	0	1	0
$bx^{1}/TM2$	0	1	0	0
Sum	1	6	1	2

Table 13. Incidence of type of duplicate classified by the time at which the duplication is initiated

	Duplicate type			
Time(hr.)	AMS-PMT	AMS-PMS	AMS-P(MS & MT)	SUM
54 ± 6	39	12	1	52
78 ± 6	49	21	5	75
Sum	88	33	6	127

Contingency $X^2(2) = 2.54$; $0.5 > P > 0.25$

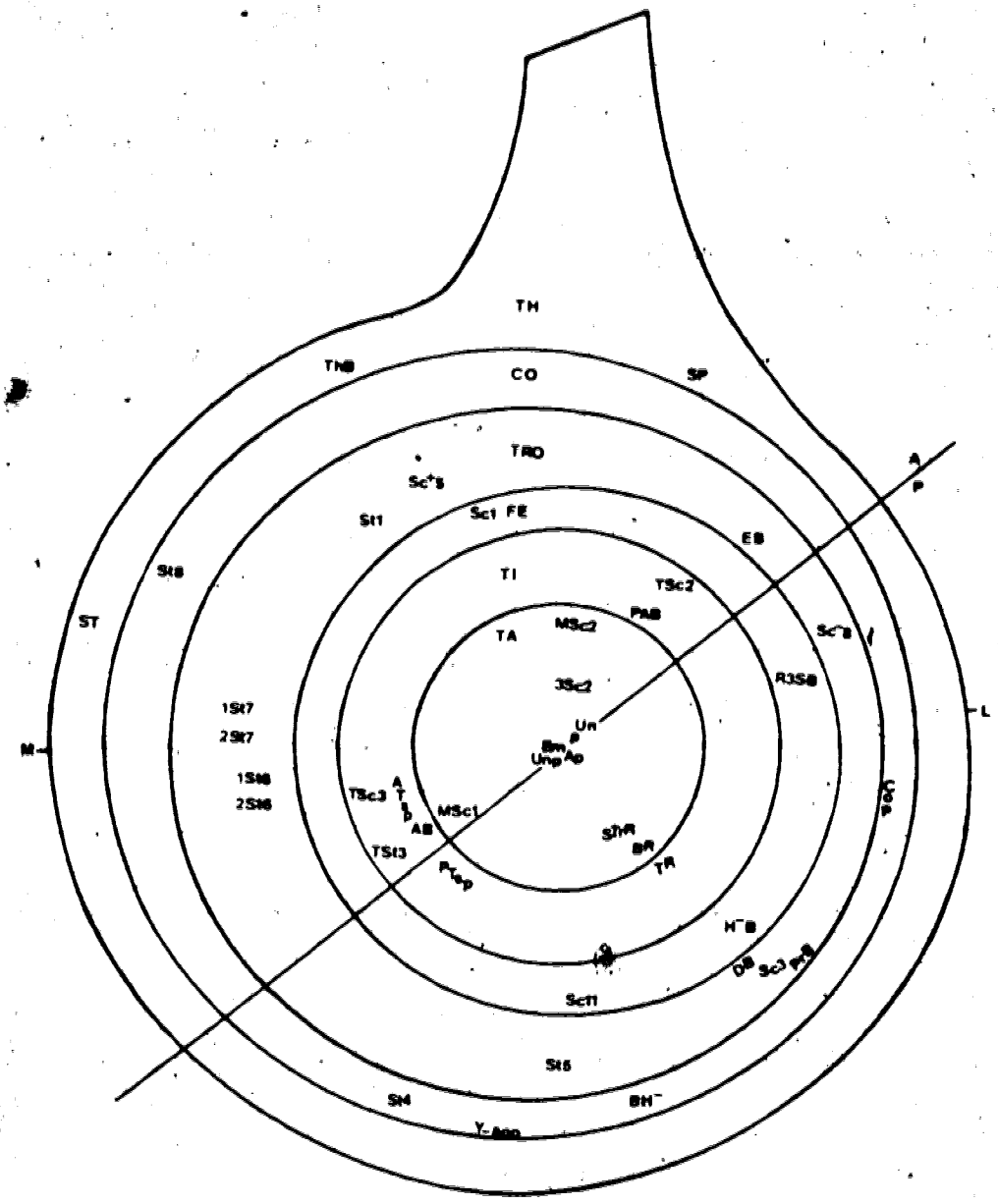


Figure 12. A leg-disc map with both mesothoracic and metathoracic landmarks. A description of landmarks is given in Table 4. New landmarks are placed on the map according to the bristle row and segment in which they are located (After Schubiger, 1971; Steiner, 1976; and Girton, 1979). A = anterior, P = posterior, M = medial, L = lateral.

deficiency, one can gain some idea about the likelihood that a duplicate originated from cells of either the anterior, the posterior or both compartments of the original disc.

Therefore, *bithorax* duplications from the previous experiments were scored for the presence or absence of a series of leg markers (see Table 4). Deficiencies were then classified as affecting either anterior, posterior, or both compartments. Since a large number of uniformly distributed markers were scored (see Figure 12), it is reasonable to assume that if only anterior structures are deficient, the duplicate would be formed from only anterior cells. Likewise, one might expect both anterior and posterior cells to participate in formation of the duplicate if the region of marker deficiency extended into both the anterior and the posterior compartments of the disc.

In all four *bithorax* genotypes analyzed previously, only two kinds of marker deficiency were found. The first kind lacks only markers of the anterior compartment, while the other kind also lacks posterior markers. However, AMS–PMS, AMS–PMT and AMS–P(MS&MT) duplicate patterns were found among both of these deficiency classes. Examples plotted on a *bithorax* metathoracic leg-disc map are shown in Figure 13. The results of this classification for duplications pooled over the four genotypes are shown in Table 14. (See Appendix 1 for details of each genotype and justification for pooling.) Note that each of the three duplicate classes can be found either when the anterior compartment only is deficient or when both compartments are deficient. The kind of duplicate is statistically independent of the kind of deficiency (contingency $X^2(2)=1.88$, $0.5>p>0.25$). This suggests that the origin of the duplication blastema may not be important in determining the kind of duplicate formed. The observation that 48 percent of the duplicates have both compartments deficient and yet only 12 percent of the duplicates are of the AMS–P(MS&MT) kind (see Table 10) is also consistent with the hypothesis that the kind of duplicate may be independent of compartmental origin. There is of course no way at this point to rule out the possibility that in the case where both the anterior and the posterior compartments are deficient, only the anterior cells are involved in forming the duplicate. In addition, when only the anterior markers are observed to be deficient, this does not necessarily imply that the posterior cells do not contribute to the duplicate. For example, cell death may occur in the peripodial membrane which contributes no landmarks to the adult leg.

Figure 13. Two kinds of marker deficiency in the original member of the *bithorax* metathoracic leg duplications plotted on leg-disc maps. (a) *bithorax* metathoracic leg-disc map, (b) and (c) are duplications with anterior and both compartments deficient respectively, classified as AMS-PMT duplicates, (d) and (e) are duplications with anterior and both compartments deficient respectively classified as AMS-PMS duplicates.

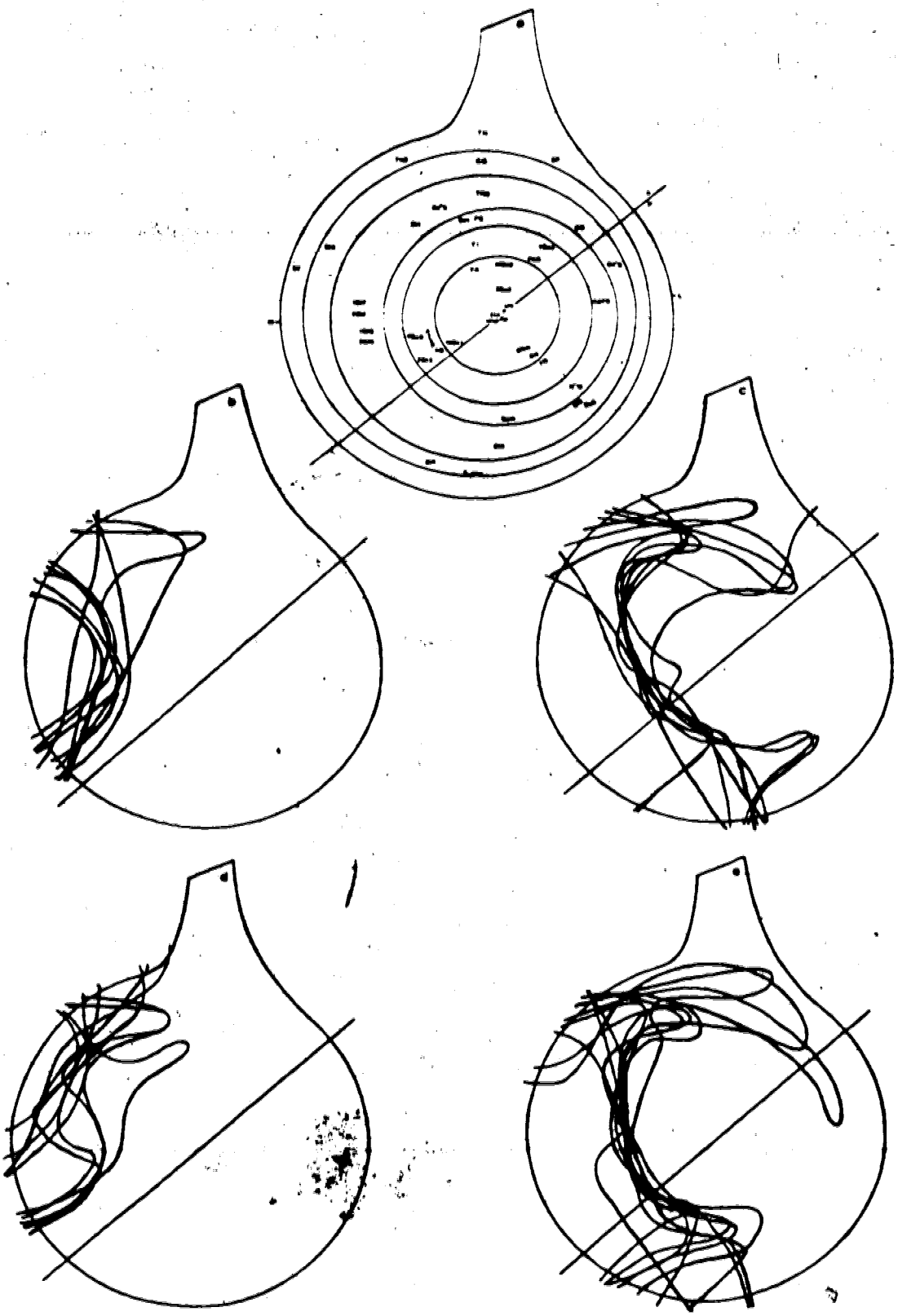


Table 14. Incidence of type of duplicate classified by the size of marker deficiency in the four *bithorax* genotypes

	Size of Marker Deficiency		
Duplicate type	Anterior only	Anterior & posterior	SUM
AMS-PMT	23	25	48
AMS-PMS	21	14	35
AMS- P(MS & MT)	4	7	11
Sum	48	46	94

Contingency $X^2(2) = 1.88$; $0.5 > P > 0.25$

Marker deficiency in *postbithorax* and wild-type metathoracic leg duplications

When *bithorax* (AMS-PMT) metathoracic legs duplicate, they sometimes give rise to posterior mesothoracic structures in the duplicates. However, no analogous effect was observed in the anterior compartment of the *postbithorax* (AMT-PMS) duplicates. Failure to find any effect of *pbx* in the anterior compartment of the duplicate does not necessarily imply that the mechanism by which *pbx* causes its transformation is not analogous to *bx*. It could be that the region of cell death never extends into the posterior compartment of the *pbx* metathoracic leg disc. As a result, the posterior *pbx* transformed cells might not have any opportunity to participate in formation of the duplicate.

An analysis of the *pbx* pattern duplications shows however that 19 have both anterior as well as posterior markers deficient and 23 have only anterior markers deficient. The remaining 8 duplications have no detectable marker deficiencies and the duplicates are essentially complete for all markers. It is quite likely that all these 8 cases have only anterior compartment deficient as no distinct marker is present at the sternal bristle (ST) region which was found to be most frequently deficient in the *bithorax* duplications as well as the mesothoracic leg duplications (Russell, Girton and Morgan, 1977). The two kinds of marker deficiency plotted on *pbx* metathoracic leg-disc maps are shown in Figure 14. Statistical analysis shows that the incidence of deficiency in either only anterior or both compartments is not significantly different ($X^2(1)=0.16$, $0.75>p>0.5$) from that found in the *bx* experiments (see Table 15). Therefore, failure to differentiate anterior mesothoracic markers is most likely not ascribable to the exclusion of posterior *pbx*-transformed cells from formation of the duplicate, but due instead to some difference between the effects of *bx* and *pbx* in pattern duplication in a cell lethal system.

As with *bx* and *pbx* duplications, wild-type ones also exhibit marker deficiencies. Among 13 duplications which were scored, the marker deficiencies could again be classified into those with only the anterior compartment deficient (4 cases) and those with both compartments deficient (9 cases). Figure 15 shows the two kinds of marker deficiency plotted on wild-type metathoracic leg-disc maps. Note that the marker deficiency patterns exhibit a remarkable similarity to the *bx* and *pbx* ones. Overall, the

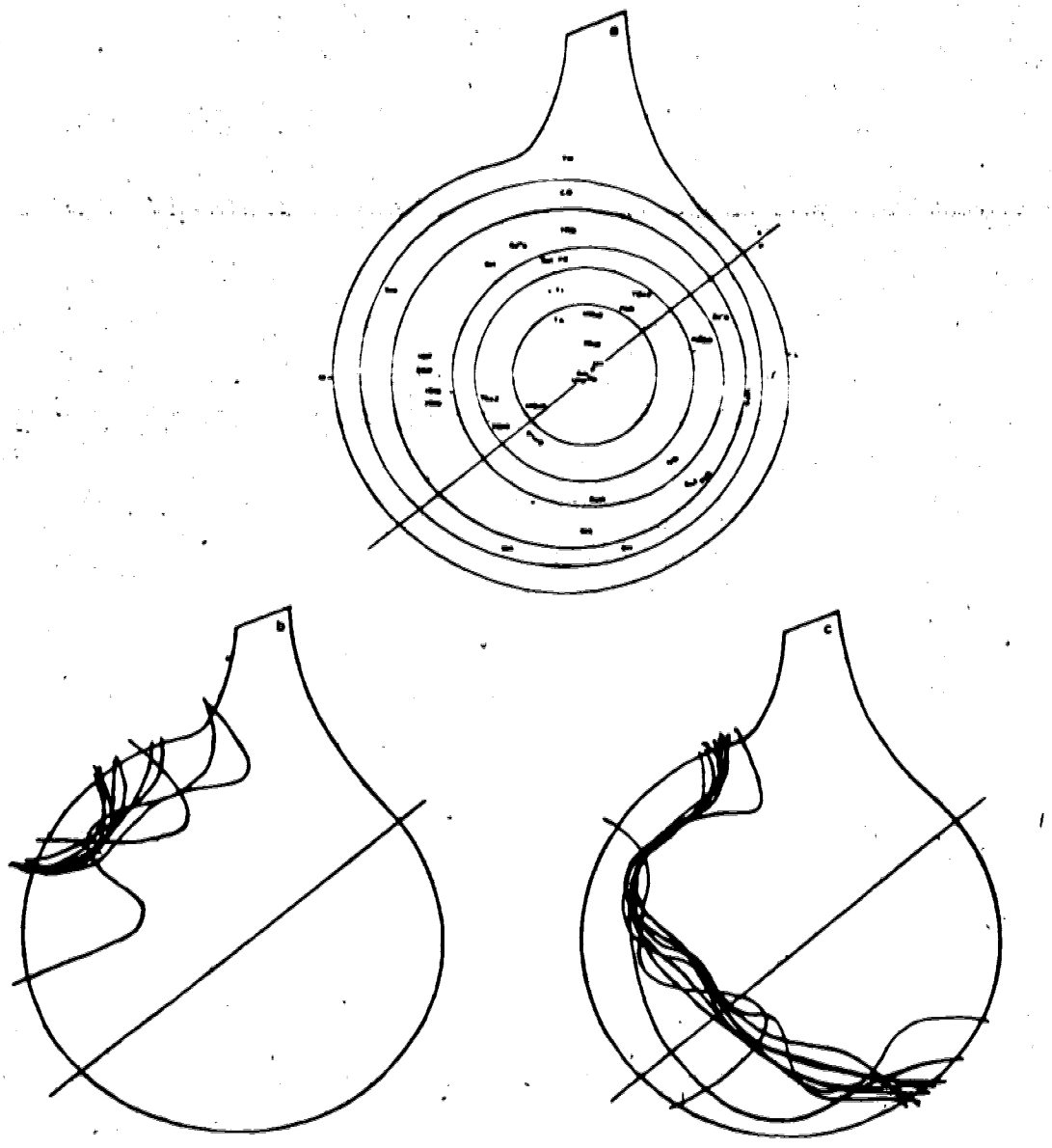


Figure 14. Two kinds of marker deficiency in the original member of the *postbithorax* metathoracic leg duplications plotted on leg-disc maps. (a) a *postbithorax* metathoracic leg-disc map, (b) and (c) are duplications with anterior and both compartments deficient respectively.

Table 15. Comparison of marker deficiency in *bx*' and *pbx*' duplications

Genotype	Size of Marker Deficiency: Anterior only	Anterior & posterior	SUM
bx	48	46	94
pbx	23	19	42
Sum	71	65	136

Contingency $X^2(1) = 0.16$; $0.75 > P > 0.5$

[a] *bx*^{34e}/*bx*^{34e}, *bx*^{34e}/*TM2*, *bx*'/*bx*' and *bx*'/*TM2* combined data

[b] *pbx/pbx* and *pbx/TM2* combined data

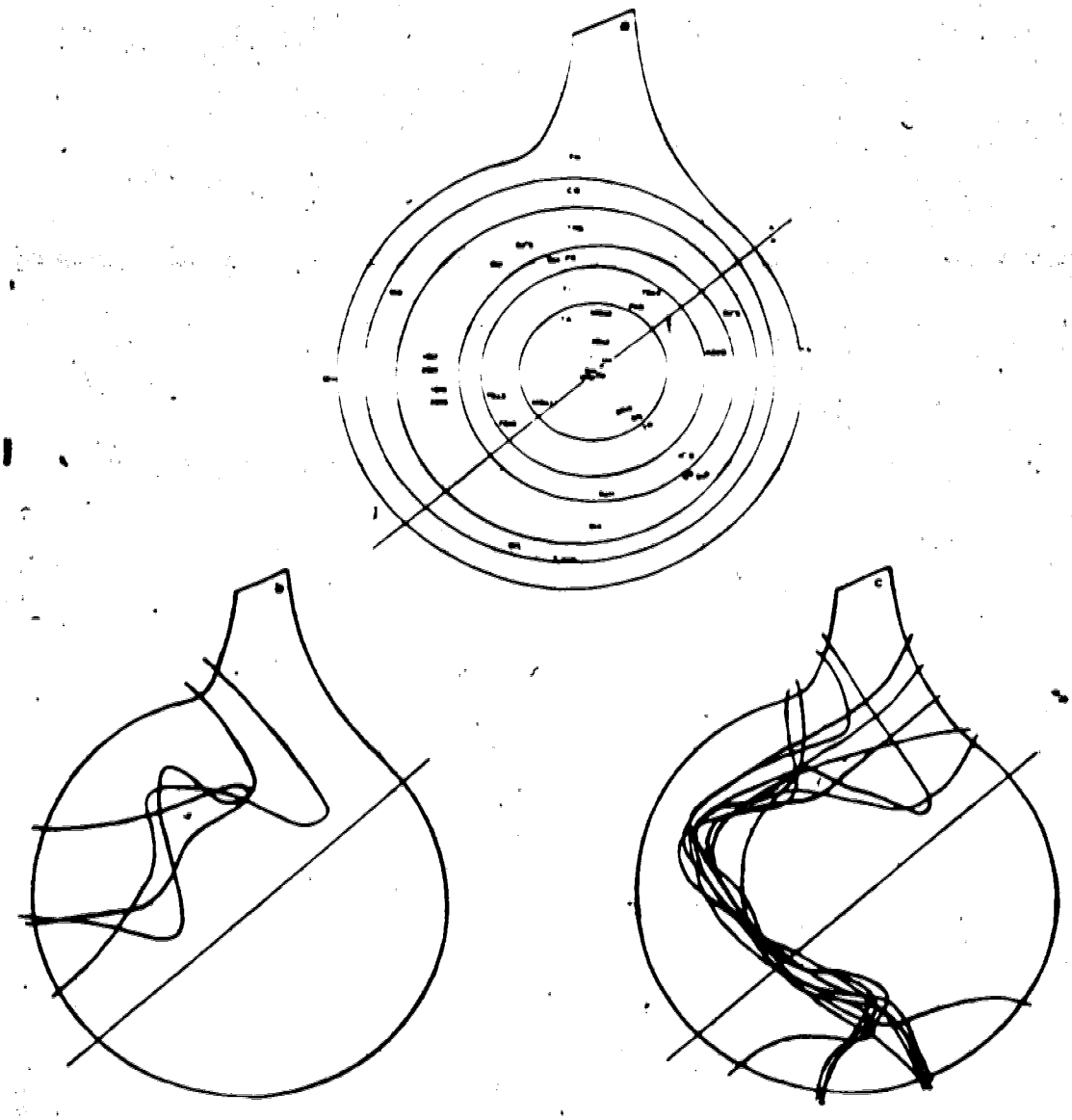


Figure 15. Two kinds of marker deficiency in the original member of the wild-type metathoracic leg duplications plotted on leg-disc maps. (a) is a wild-type metathoracic leg-disc map, (b) and (c) are duplications with anterior and both compartments deficient respectively.

analysis suggests that cells from anterior and posterior compartments both have the opportunity to participate in formation of the duplicate in all genotypes, and that differences between the effects of the mutants cannot be explained on this basis.

Cell lineage analysis

The effect of *bithorax* in the posterior compartment of the duplicate could mean that the duplicate was sometimes derived from the *bithorax* transformed anterior cells. These cells might have some finite chance of following either the mesothoracic or the metathoracic developmental pathway when entering the duplication blastema. The marker deficiency analysis above suggests that the anterior *bithorax* cells can form all three kinds of duplicate. However this evidence is only indirect as it depends on a particular model for pattern duplication which is still controversial (Jurgens and Gateff, 1979).

In order to investigate the origin of the duplicates more rigorously, individual cells were labelled by somatic recombination so that the influence (if any) of a cell's compartmental status on the type of duplicate formed could be assessed. The experimental design was to induce labelled clones in *bithorax* discs after the anterior-posterior compartmental restriction had been established but before the initiation of pattern duplication and to follow their fates in the duplicates. In these studies, bx^{34} homozygotes were used because they gave a much higher frequency of survival after combined X-ray and heat pulse treatments than other genotypes tested. Moreover, this genotype was known to give all three kinds of duplicate regardless of the time of heat treatment as required for this analysis (see Table 13 above). A detailed description of the experimental procedure can be found in materials and methods section (page 26). Larvae aged between 48 and 72 hours (24 hour egg laying period) after egg deposition were irradiated with 1500R of gamma-rays to induce mitotic recombination (this dose was used to ensure a relatively high frequency of clones per duplication), and were then immediately shifted to 29degrees C for 48 hours to induce pattern duplications. Thus, labelled clones were induced before the recruitment of cells into the duplication blastema. Previous studies suggest that such clones would be expected to cross the anterior-posterior compartmental restriction line in the duplicate (Girton, 1979). Four hundred and fifteen duplicated metathoracic legs were obtained from amongst about

2500 pharate adults dissected.

The kinds of clone detected in the duplications are shown in Table 16. The majority (90%) of clones recovered were twins ascribable to recombination proximal to *forked*. Since only clones labelling both original and duplicate (OD clones) will give us the information we require, twin spots were treated in this analysis as single clones to maximize the probability that both the original and the duplicate would be labelled. The frequency of duplicated legs labelled by clones was 153/415. If the frequency follows a Poisson distribution, 121 of the 153 should be due to single events.

Distribution of clones in the duplicated legs

The clones were classified into those that marked only the original (O), the duplicate (D) or both members (OD) of the duplicated leg and all three classes were found (Table 17). Note that among the duplications with clones, the majority of the clones (61%) were found to label both members of the duplicated leg. Only a small percentage (4%) were found to mark the duplicate only, and 35% labelled only the original member. OD clones usually formed a contiguous patch with the larger labelled area in the duplicate. In the original, the larger clones marked several leg segments and one or two longitudinal bristle rows. However, in the duplicates, such clones usually marked larger numbers of bristle rows and had basically the same clone shape and bristle rows marked as in the original. An example of such an OD clone is shown in Figure 16. A contingency chi-squared test (Table 17) indicates that the distribution of three types of duplicates classified by clonal labelling patterns is not significantly heterogeneous ($X^2(6)=4.15, 0.75>p>0.5$).

Incidence of clones classified by compartment and type of duplicate

In order to determine if the origin of the duplicate has any influence on the kind of pattern differentiated, each clone was classified as labelling either the anterior, the posterior compartment, or both, in the original as well as the duplicate members of the duplication. The results of this classification are shown in Table 18.

Of the sixteen possible labelling patterns, all but two were found. These were (P+A') and (AP+P'), where the "prime" indicates a duplicate compartment. The presence of

Table 16. Incidence of different types of labelled clones in the duplications induced in females of the genotype *Dp(3:1)mwh⁺ y v f³⁶ /(1)ts726/w sn³ /(1)ts726; bx³⁴/bx³⁴*

Clone Phenotype	No. of Clones
y f & *mwh sn³* twins	143
y & *mwh sn³* twins	3
y & *mwh* twins	6
sn³ single	1
Total	153
Total No. of duplications scored	415

Table 17. Incidence of O, D, OD, and no clones classified by duplicate phenotype

	Duplicate type			
Location of clone	AMS-PMT	AMS-PMS	AMS-P(MS & MT)	SUM
O	40	11	2	53
D	5	1	1	7
OD	72	15	6	93
No clone	186	61	15	262
Sum	303	88	24	415

Contingency $X^2(6) = 4.15$; $0.75 > P > 0.5$

Figure 16. An OD clone in a *bithorax* duplication. (---) boundary between the original (O) and the duplicate (D) portions, (....) outline of the clone, f = yellow-forked, s = singed-multiple wing hair, y = yellow.

Table 18. Incidence of each kind of clonal labelling pattern in the *bithorax* duplications[1]

Original	Duplicate			No. of each kind of duplicate		
P	A	A'	P'	AMS-PMT	AMS-PMS	AMS- P(MS & MT)
	+			32	9	1
	+	+		21	5	1
	+	+	+	40	8	3
	+		+	4	0	0
+				6	2	1
+		+		0	0	0
+			+	3	1	2
+			+	2	1	0
		+		3	1	0
		+	+	2	0	0
			+	0	0	1
+	+			2	0	0
+	+	+		1	0	0
+	+	+	+	1	0	0
+	+		+	0	0	0
Sum				117	27	9

[1] The symbol "+" in the column for a particular compartment means that this compartment was labelled with one or more clones. No attempt was made to distinguish single from multiple clones.

D clones with both the anterior and the posterior compartments labelled is consistent with earlier results which show that compartmental commitments may be lost during pattern duplication and regeneration (Haynie and Bryant, 1976; Szabad, Simpson, and Nothiger, 1979; Girton, 1979). In only 4 cases out of 153 were both compartments marked in the original. These cases might be due to multiple independent mitotic recombination events occuring simultaneously, or to intercalary regeneration caused by cell death. Assuming each instance to be due to two independent clones, one can estimate the frequency of A and P clones (as well as A' and P' clones) from the number of compartments without clones. The expected number of multiple events estimated from the product of these frequencies was seven (Table 19), and this is not significantly different from the observed number. This is important because only those OD clones confined to one compartment in the original will be useful for our purpose of determining the origin of the duplicate.

Fate of anterior *bithorax*-transformed cells

When *bithorax* metathoracic discs duplicate, both mesothoracic as well as metathoracic structures are found in the duplicates. Therefore, the main aim of the cell lineage analysis was to find out whether the anterior *bithorax*-transformed metathoracic cells could form all three kinds of posterior duplicates. The presence of clones labelling the anterior compartment of the original and the posterior compartment (AP') of the duplicate in all three classes of duplicates would be good evidence for this hypothesis. This kind of clone could indeed be found in the three kinds of duplicate (see Table 18). Figure 17 shows such clones in both AMS-PMS and AMS-PMT duplicates. The positions of five such clones in an AMS-PMS duplicate and nine in an AMS-PMT duplicate plotted on leg maps are shown in Figure 18. Note that the distribution and the shapes of the clones are not very different in the two kinds of duplications.

The possibility exists however that PMS duplicate compartments originate strictly from anterior transformed cells (AMS) and PMT duplicates from posterior non-transformed cells (PMT). Some of the clones observed that appear to label both anterior compartment of the original and posterior compartment of the duplicate in AMS-PMT duplicate would then in reality be multiple independent clones. The expected

Table 19. Incidence of clones in the four compartments among the 415 *bithorax* duplications from the data of Table 18

	Compartment			
	Original		Duplicate	
	A	P	A'	P'
No. with one or more clones	128	22	92	68
No. with no clones	287	393	323	347
Observed frequency with one or more clones	0.31	0.05	0.22	0.16

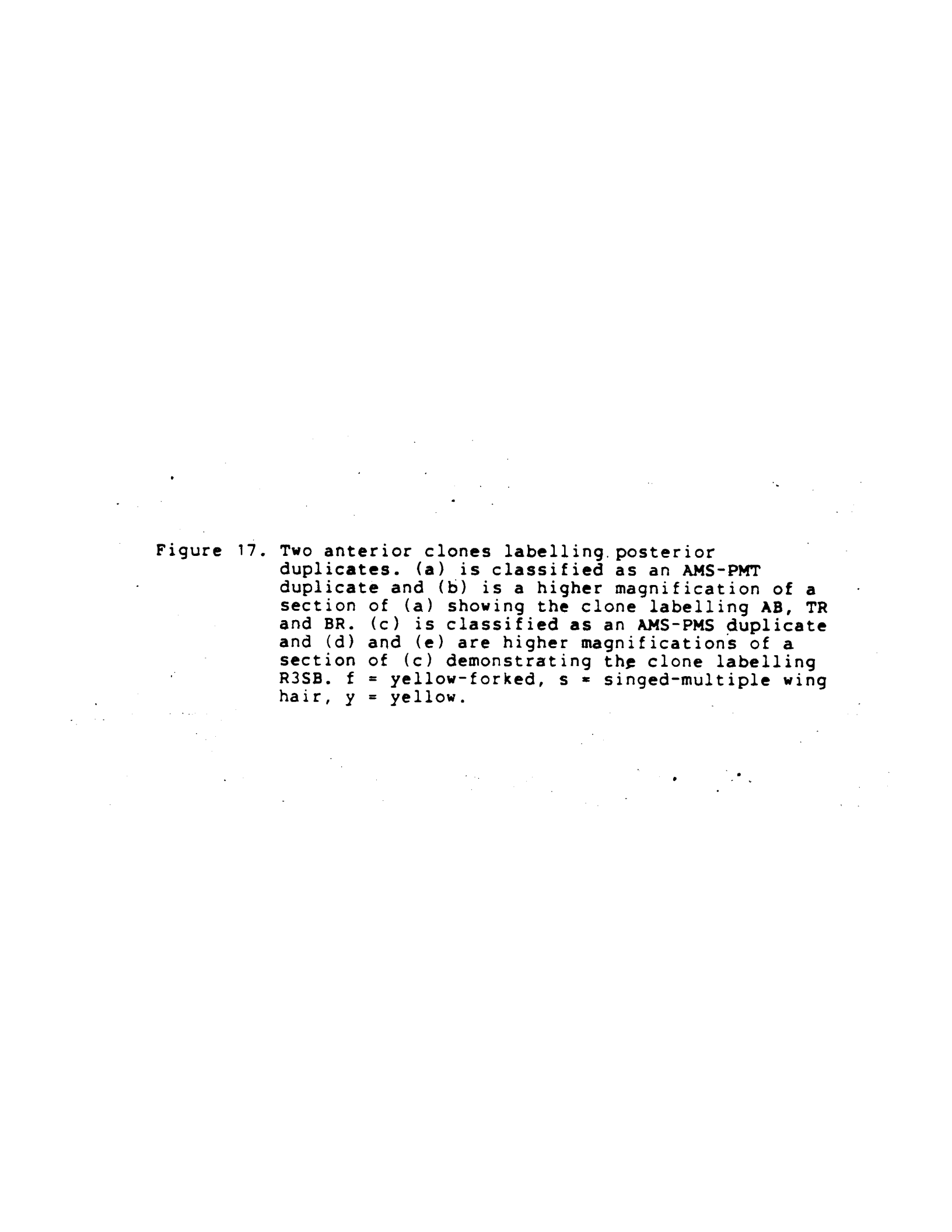
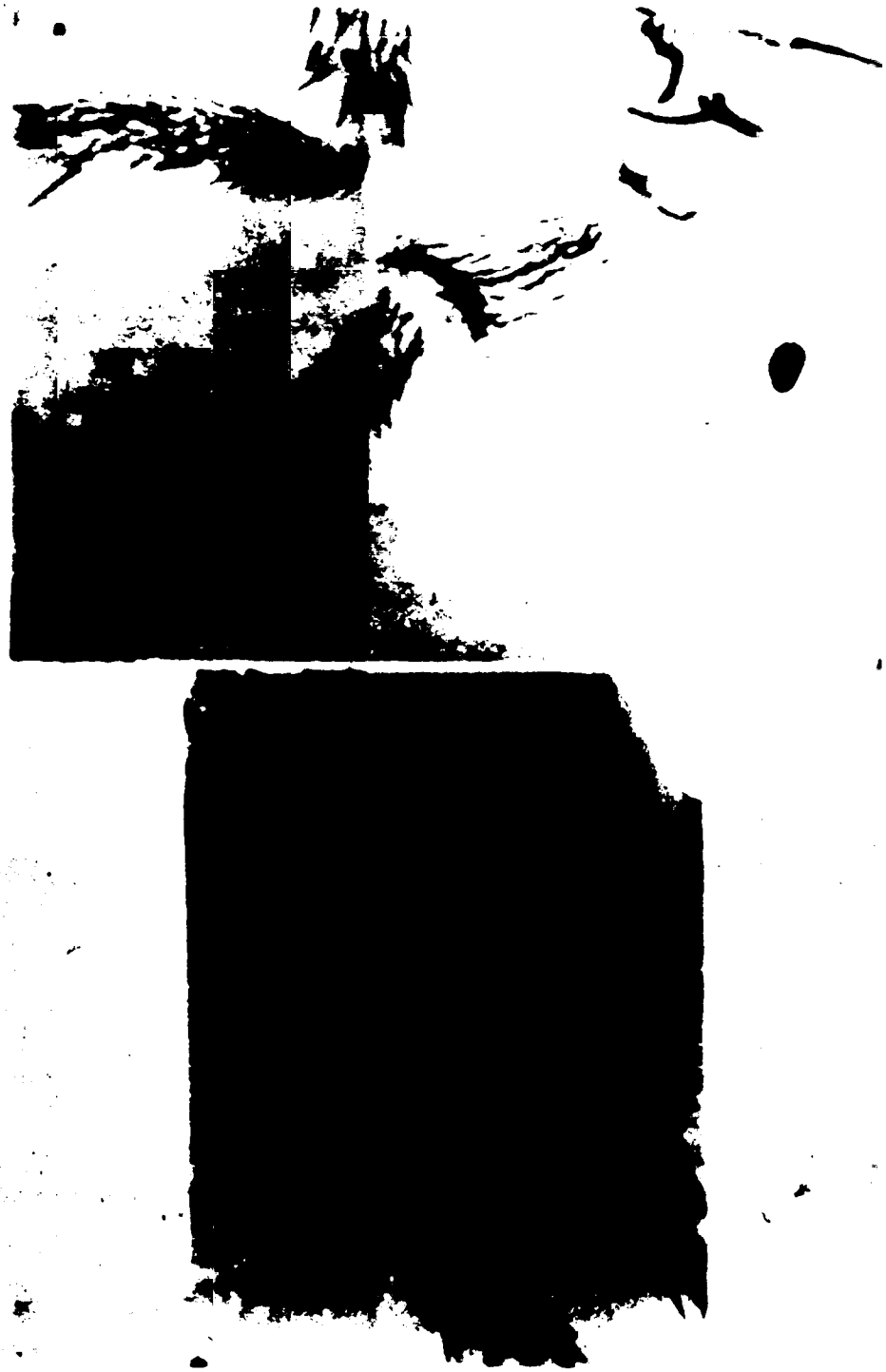


Figure 17. Two anterior clones labelling posterior duplicates. (a) is classified as an AMS-PMT duplicate and (b) is a higher magnification of a section of (a) showing the clone labelling AB, TR and BR. (c) is classified as an AMS-PMS duplicate and (d) and (e) are higher magnifications of a section of (c) demonstrating the clone labelling R3SB. f = yellow-forked, s = singed-multiple wing hair, y = yellow.




C
D
O
A'
R1

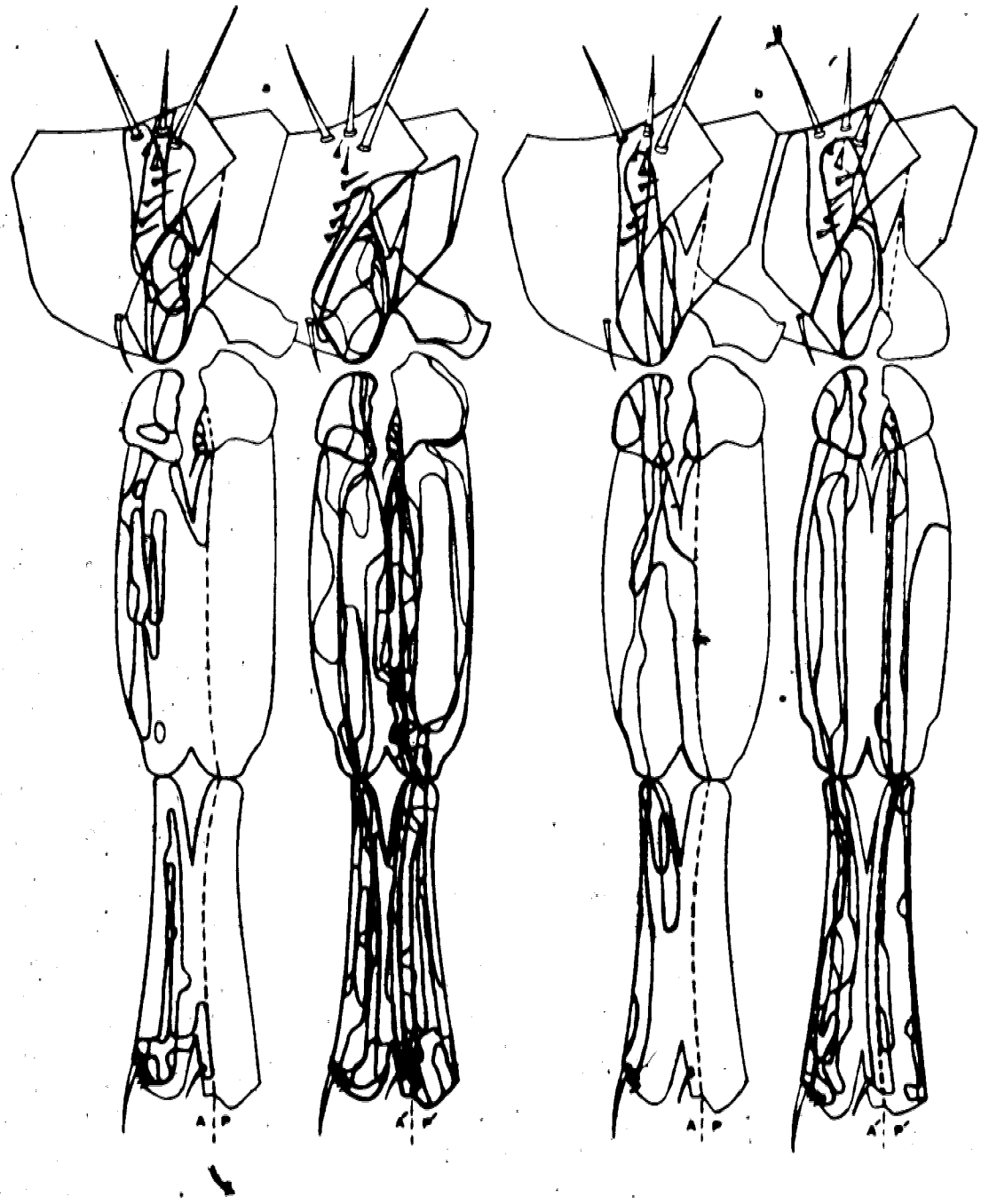


Figure 18. AA'P' clones in the *bithorax* duplications plotted on a metathoracic leg map (Steiner, 1976). (a) represents AMS-PMT duplicates, (b) represents AMS-PMS duplicates.

number of duplicated legs with the anterior compartment of the original and the posterior compartment of the duplicate marked by independent clones, can be estimated from the data in Tables 18 and 19. As shown in Table 20, this calculation yields an estimate of 21 for the expected number of multiples. A total of 55 cases in which clones marked the A compartment as well as the P compartment were observed. Since 44 of these were found in AMS-PMT duplicates, not all 44 can be accounted for by the 21 expected multiple clones. The results therefore clearly suggest that when anterior *bithorax*-transformed cells enter the duplication blastema, they can form not only anterior mesothoracic structures, but also either mesothoracic or metathoracic posterior structures.

Posterior markers differentiated by the anterior clones in the duplicate

Even though the anterior clones in the original can contribute to both anterior and posterior compartments in all three types of duplicates, one cannot be absolutely sure that the anterior *bithorax*-transformed cells can actually differentiate both posterior mesothoracic and metathoracic structures, as those clones may not have included any distinctive marker in a mosaic posterior duplicate for the assessment of their determined state. Therefore, the landmarks differentiated by all the AP clones in the posterior compartment of the duplicate were individually scored. The results of this analysis are shown in Tables 21 and 22. An example of the mesothoracic and the metathoracic structures differentiated by two clones is shown in Figure 17. Among the 55 anterior original clones which apparently extended into the posterior compartment of the duplicate, 33 differentiated at least one posterior metathoracic structure and 8 differentiated at least one mesothoracic structure (see Table 22 for kinds of stuctures differentiated). The other 16 do not include any distinct landmarks. Note that the 33 definitive posterior metathoracic clones still cannot all be explained by the 21 expected multiples. Therefore, the evidence is strong that the anterior *bithorax*-transformed cells have some finite chance of forming either the mesothoracic or the metathoracic structures after entering the duplication blastema.

Table 20. Incidence of AP' clones classifed by duplicate type from the data of Tables 18 and 19, and calculation of the expected incidence of multiple clones marking the A and P' compartments.

Duplicate Type	Incidence of AP' Clone
AMS - PMT	44
AMS - PMS	8
AMS - P(MS & MT)	3

Calculation:

Total no. of duplications scored = 415

Observed total no. of duplications with A and P' compartments marked = 44 + 8 + 3 = 55

Observed frequency of A compartments with one or more clones = 128/415 = 0.31

Observed frequency of P compartments with one or more clones = 68/415 = 0.16

Hence, expected number of duplications with A and P' compartments labelled = 0.31 x 0.16 x 415 = 20.58

Table 21. Incidence of clones which differentiated at least one posterior mesothoracic and/or metathoracic structure

Clonal Origin	Kind of Posterior Compartment in Duplicate		
	PMT	PMS	P(MS & MT)
Anterior	31	6	2[1]
Posterior	5	1	2[2]

[1]Clones include both meso- and metathoracic structures.

[2]One clone differentiated both meso- and metathoracic structures, and the other differentiated only metathoracic structures.

Table 22: Markers differentiated by the twin clones in the posterior compartment of the duplicates

Class	Posterior Metathorax						Posterior Mesothorax							Clonal Origin: Anterior			Clonal Origin: Posterior		
	DBR2	R3TB	H'B	TR	BR	STR	BH	Pr BR2	R3SB	H'	PTsp	BTR2	STR2	A'	B'	C'	A'	B'	C'
1		+	+	+	+	+								3					
2		+	+	+	+									1					
3		+	+		+	+								1					
4		+	+	+													1		
5		+		+	+									2					
6		+	+											1			1		
7		+		+										1					
8		+			+									2					
9		+												4					
10			+	+	+	+								1					
11			+	+	+												2		
12			+														1		
13				+	+	+								4					
14				+	+									1					1
15				+		+								1					
16					+	+								4					
17					+									4					
18						+								1					
19							+			+					1				
20								+	+	+	+	+	+		1				
21								+				+	+		1				
22									+						1				
23										+					1				
24											+	+	+		1			1	
25		+									+	+	+			1			
26			+	+	+	+				+						1			
27			+		+				+	+									1
Sum														31	6	2	5	1	2

AMS - PMT
AMS - PMS
AMS-P(MS & MT)

The fate of posterior metathoracic *bithorax* cells

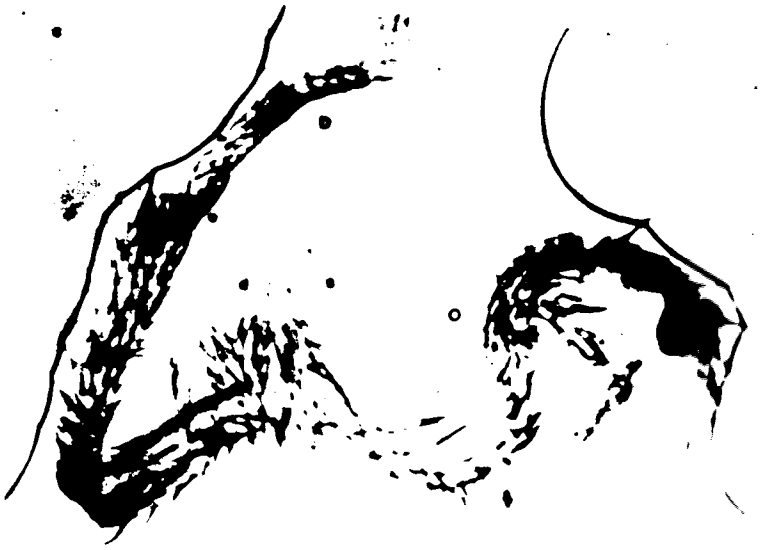
Clonal analysis data in Table 18 suggests that posterior *bithorax* non-transformed metathoracic cells also sometimes contributed to the duplication blastema in this study using the cell lethal system. It is interesting therefore to examine the fate of these cells in the pattern duplications. This analysis should provide us with further information about the stability and the heritability of the determined state in *bithorax* cells forming a duplication blastema.

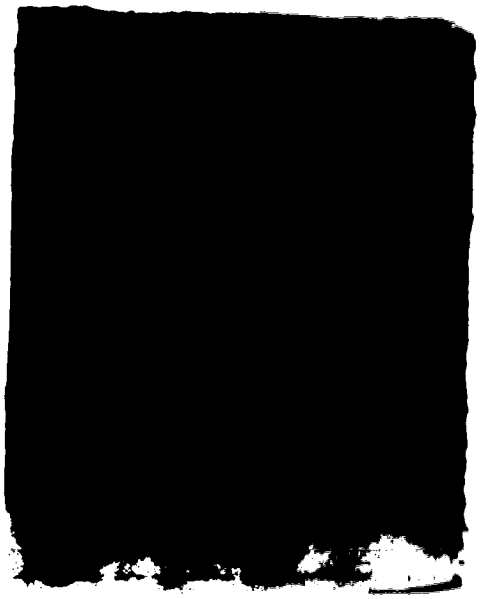
Only 9 posterior original clones extending into the duplicate were obtained. Six of these were observed to cross the A'P' boundary while the other three marked only the P' compartment in the duplicate (see Figure 19). The nine clones were found in all three kinds of duplicates (Table 18). The expected number of duplications with multiple independent clones simultaneously marking the P compartment of the original and the P' compartment of the duplicate can be estimated from data in Tables 18 and 19. As shown in Table 23, this expected number was estimated to be three and is nearly sufficient to account for the two PP' clones observed in AMS-PMS and the two in AMS-P(MS&MT) duplicates. So these data do not rule out the heritability hypothesis. However, some of the observed PP' clones are probably true single clones as they are contiguous across O and D. In addition, the assumption that each AP or A'P' clone is due to two independent events will bias the estimation of expected multiples upwards as the clones were induced before the anterior-posterior compartmental restriction in the duplicate is established (Girton and Russell, 1981). Moreover, it is unlikely that all three of the expected multiples would fall by chance into the AMS-PMS and AMS-P(MS&MT) classes. Thus, the results do not prove, but are consistent with, the possibility that the behavior of *bithorax* posterior non-transformed cells is similar to that of transformed anterior cells in forming mesothoracic as well as metathoracic structures in pattern duplication.

Posterior markers differentiated by posterior original clones in the duplicate

To confirm that the P clones apparently extending into the P' compartment of the duplicate could include both posterior mesothoracic and metathoracic structures, the markers differentiated by these clones were scored. The results are summarized in Table 21. Seven clones differentiated at least one posterior metathoracic structure and two

Figure 19. Two posterior clones labelling posterior duplicates. (a) is classified as an AMS-PMT duplicate and (b) is a higher magnification of a section of the duplicate illustrating the clone labelling BR. (c) is classified as an AMS-PMS duplicate and (d) and (e) demonstrate landmarks R3SB and BTR2 labelled by the clone. f = yellow-forked, s = singed-multiple wing hair, R1 = row 1.





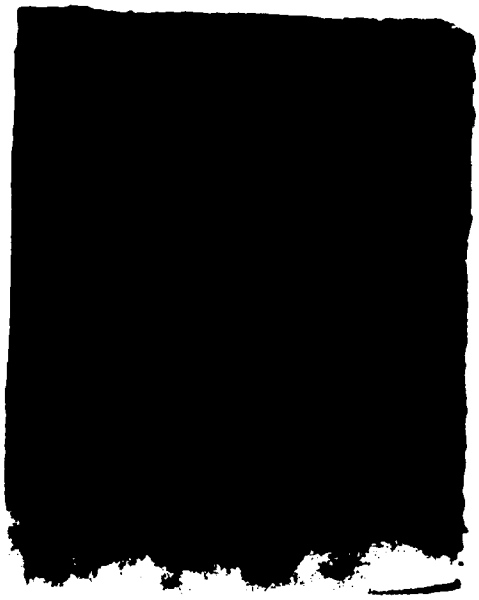


Table 23. Incidence of PP' clones classified by duplicate type from the data of Tables 18 and 19, and calculation of the expected incidence of multiple clones marking both P and P' compartments

Duplicate Type	Incidence of PP' Clone
AMS–PMT	5
AMS–PMS	2
AMS–P(MS & MT)	2

Calculation:

Total no. of duplications scored = 415

Observed total no. of duplications with P and P' compartments marked = 5 + 2 + 2 = 9

Observed frequency of P compartments with one or more clones = 22/415 = 0.05

Observed frequency of P' compartments with one or more clones = 68/415 = 0.16

Hence, expected number of duplications with P and P' compartments labelled = 0.05 x 0.16 x 415 = 3.32

clones differentiated at least one posterior mesothoracic structure (also see Table 22 for markers differentiated) Examples are shown in Figure 19 Both these clones appear to be continuous from the P into the P compartment suggesting that they are both really single clones It is rather surprising that posterior non-transformed *bithorax* cells appear to be able to form posterior mesothoracic as well as metathoracic structures after entering the duplication blastema

The origin of mixed posterior compartments in the duplicate

The cell lineage analysis suggests that the mixed posterior compartments of the duplicates may be derived from cells from a single compartment of the original member of the duplicated leg since cells from either compartment seem to be able to form both kinds of structure Tables 21 and 22 show that two of the four clones originating from the anterior compartment and one of the two clones originating from the posterior compartment differentiated both mesothoracic and metathoracic structures In each case the clonal label was found in a single continuous patch suggesting these cases may well be true single clones An example is shown in Figure 20 These clones provide further support for the idea that in *bx* duplications the state of determination may not be clonally inherited

One can estimate the probability that anterior or posterior cells are both included in a duplication blastema from the proportions of OD clones marking either A or P but not both compartments of the original member Among 91 such clones obtained (see Table 18), 82 labelled the anterior compartment and 9 labelled the posterior compartment of the original If we assume independent recruitment of cells from these two sources, knowing the average number of cells which make up the duplication blastema, one can estimate using the binomial probability distribution the expected number of duplicates formed from both transformed anterior and non-transformed posterior *bithorax* cells The expected number of duplicates of mixed origin is calculated as (1-(probability all anterior + probability all posterior))N where N is the total number of duplications obtained Assuming that 7 - 22 cells form a duplication blastema in this cell lethal system (Girton, 1979, Girton and Russell 1980), this calculation suggests that from 52% to 90% of all the duplicates may be of mixed origin In the cell lineage analysis shown in Table 18,

Figure 20. An anterior clone which differentiated both mesothoracic and metathoracic landmarks, R3TB (b) and BTR2 and PTsp (c) in the same duplicate. f = yellow-forked, s = singed-multiple wing hair.

153 duplications were obtained. One should therefore expect between # duplicates to be of mixed origin. Given strict cell-heredity of determined [illegible] these should display an AMS-P(MS&MT) phenotype. But the actual observe[illegible] mixed duplicates was only nine. This implies, if our assumptions are correc[illegible] that all the cells in a single duplication blastema tend to assume the same state of determination (either mesothorax or metathorax) regardless of their compartmental origin.

On the other hand, it may be that the assumption of independent recruitment of cells into the duplication blastema is incorrect. According to French, Bryant, and Bryant's model (1976), cells are recruited from the cut edge of the disc. In our duplications, the cut edge would intersect both compartments of the originals in about 50% of all cases (Table 14). This is also inconsistent with the strict cell-heredity of determined states hypothesis in pattern duplication. It may be that other parameters, such as the opposition patterns generated during wound healing process, may also play an important role in determining the origin of blastema cells. Even so, the results of this analysis lend some support to the surprising conclusion from the clonal labelling experiment that in the duplicates, a single clone from either compartment of the original can differentiate either mesothoracic or metathoracic posterior structures or occasionally both.

IV. DISCUSSION

Mechanism of duplication in the cell lethal system

Pattern duplication can be generated using either surgical techniques (Schubiger, 1971, Bryant, 1971, Strub, 1977a,b, Bryant, 1978 for review) or mutants such as the temperature-sensitive cell lethal system employed here (see Girton and Bryant, 1980 for review). Two mechanisms have been suggested by which pattern duplicates can be formed. The first proposes that pattern duplications develop by respecification of existing cells to form two incomplete patterns in mirror image symmetry after the developing system is perturbed. This is a morphallactic type of process since no extra cell divisions are required (Morgan, 1901). It was proposed by Jurgens and Gateff (1979) on the basis of experiments using a temperature-sensitive lethal, *l(1)ts mad*, which is an allele of *suppressor of forked* and therefore an allele of *l(1)ts726*. Clones induced before initiation of pattern duplications by heat treatments were all confined to either the original or the duplicate part of the pattern. Moreover, no clone was found to transgress the anterior-posterior compartment boundary in either original or duplicate part. In addition, they were unable to find evidence of cell death in the heat treated leg discs using the toluidine blue staining technique (Simpson and Schneiderman, 1975). The authors argue that these results are compatible only with a morphallactic duplication mechanism.

The second kind of model proposes that the initial stimulus causing pattern duplication is the extirpation of a part of the disc at some stage in development. Some of the remaining cells are assumed to form a blastema which proliferates to form the duplicate part of the pattern. Since cell division is involved, this is an epimorphic process of pattern regulation. This model is based on precise surgical experiments such as those of Schubiger (1971) and the interpretations of Bryant (1971), Schubiger (1971), and Postlethwait and Schneiderman (1973). Schubiger (1973) showed that pattern duplication in this system depends on cell proliferation since it does not take place if the disc fragments are cultured in sugar-fed adult hosts where proliferation does not occur. Secondly, Dale and Bownes (1980) labelled mitotic cells of regenerating or duplicating disc fragments using tritiated thymidine and found that the most heavily labelled region of the disc, which presumably corresponds to the region most active in cell division, lies in a

small area near to the cut edge. They concluded that pattern regulation is epimorphic and the blastema contains a small number of cells. This view has recently been strengthened by clonal analysis in surgical disc fragments (Szabad, Simpson and Nothiger, 1979; Abbott, Karpen and Schubiger, 1981). Clones induced before initiation of duplication or regeneration may mark both the original or the new material. Moreover, analysis of partial duplicates or regenerates showed that markers are regenerated or duplicated in a specific sequence with those closer to the cut edge appearing first. This mechanism of pattern regulation was central to the development of the "Polar co-ordinate" model for disc positional information of French, Bryant and Bryant (1976) which is now the most widely accepted view.

Mutants at a great many loci (Russell, 1974; Arking, 1975; Simpson and Schneiderman, 1975) selected as cell-autonomous lethals cause pattern duplications when heat treated during larval life. The epimorphic mechanism outlined above has been proposed to account for pattern duplication caused by heat treatments in temperature-sensitive cell lethal systems (Russell, Girton and Morgan, 1977). In the *l(1)ts726* system, several lines of evidence suggest that pattern duplication induced by heat pulse treatment of individual animals carrying this mutation is due to localized cell death which mimics a surgical cut and produces a disc fragment which duplicates *in situ*. Firstly, extensive analysis of head, mesothoracic and prothoracic leg duplications showed that the pattern duplication is almost always accompanied by a pattern deficiency (Russell, 1974; Russell, Girton and Morgan, 1977; Postlethwait, 1978). In the mesothoracic leg duplications, the medial fate map elements were found most frequently deficient and least frequently duplicated, while the lateral portion was found to be most often duplicated rather than deficient. These results are in good agreement with the results obtained from the surgical technique for the prothoracic leg disc (Schubiger, 1971; Strub, 1977a,b). Secondly, histological studies of the heat pulsed discs showed that localized cell death occurred. However, the regions of cell death could not always be correlated with the cuticular marker deficiency (Clark, 1976; Clark and Russell, 1977). Thirdly, clonal analysis of the duplications demonstrated that single clones induced before the induction of pattern duplications often mark both the original and the duplicate members and that clone sizes are consistently larger in the duplicates (Girton, 1979;

Girton and Russell, 1980) Similar results have been obtained in the present study (Table 18) These results would suggest that the duplicates are derived from the original via epimorphic regulation

These findings apparently contradict those of Jurgens and Gateff reviewed above This is surprising since *l(1)ts726* and *l(1)tsmad* are functional alleles of the same locus At present no resolution to the paradox is possible but the weight of the evidence from different systems seems to favor the epimorphic model at present In this thesis the results shall be interpreted according to this view

If epimorphic regulation is indeed involved an issue of importance in the present study, is the origin of the cells that form the duplication blastema Two alternatives have been suggested These cells may either be ordinary disc cells recruited from the cut edge of the disc epithelium (as proposed in the polar co-ordinate model), or they may come from a special population of 'reserve cells' set aside in imaginal discs for the purpose of pattern regulation in case of injury (Schubiger, 1971) There is no direct evidence demonstrating the existence of reserve cells and a number of experimental results argue in favor of the alternative 'cut edge' hypothesis First, the autoradiographic study of Dale and Bownes (1980) mentioned above demonstrates active cell division at the cut edge Therefore, if reserve cells exist, their number must be large and their distribution even in the disc, or else they would have to migrate to the cut edge so that regulation is made possible regardless of where the cut falls Migration of cells is not supported by the clonal data as clones tend to form single contiguous patches Also, clones induced just before blastema formation are rarely confined to either the original or the new material as one might expect if there were large numbers of reserve cells (Girton and Russell, 1980, Abbott, Karpen and Schubiger, 1981) If ordinary disc cells in fact form the duplication blastema in the *l(1)ts726* system, then it is important to know about their compartmental origins The present clonal analysis suggests that roughly 90% of clones labelling duplicates originate from the anterior compartments and 10% from the posterior of the original disc (Table 18)

In order to give a proper interpretation of the results it is also important to know that heat treatment does not interfere with the determination system in the duplicates In a large number of prothoracic and mesothoracic leg duplications analyzed, no

transformations between legs, due to either phenocopy or *in situ* transdetermination, have so far been reported (Russell, Girton and Morgan, 1977, Postlethwait, 1978, Steiner, Kollier-Wiesinger and Nothiger, 1981) Moreover, the same applies to the twenty-four *l(1)ts726.bx⁻ pbx⁻* metathoracic leg duplications analyzed here. Hence the *l(1)ts726* system is reliable in generating duplication phenotypes without additional transformations.

The role of *bithorax* and *postbithorax* in determination during normal development

One of the main purposes of the work presented here is to assess the role of *bithorax* mutants in determination. This is achieved by looking at the heritability of the determined state in *bx*-transformed cells when they participate in forming duplications.

The *bithorax* mutants transform the anterior metathoracic leg into anterior mesothoracic leg. Gene dosage studies suggested that the *bx* and *pbx* mutants represent a loss of gene function (Lewis, 1963). The transformation could be the result of defective embryonic or imaginal positional information leading to failure in the activation of a selector gene in the anterior metathorax. According to this idea, *bx⁻* acts as an activator gene for a selector gene for the establishment of the anterior metathoracic compartment. A second possibility is that *bx* mutants cannot maintain the metathoracic state activated by the embryonic field, and hence *bx⁻* is itself a selector gene for the anterior metathoracic compartment. A third possibility is that *bx* mutants cannot interpret (i.e. express) their anterior metathoracic determined state at differentiation. Fourthly, it may be that the phenotype results from all or a combination of the above possibilities. Failure to interpret the determined state can be distinguished from the other two possibilities when *bx*-transformed anterior cells duplicate across the compartment boundary.

When *bithorax* metathoracic leg discs duplicate, the anterior duplicate compartments are always mesothoracic while the posterior compartments can contain mesothoracic, metathoracic, or a mixture of mesothoracic and metathoracic structures. The formation in some cases of posterior mesothoracic elements suggests that the *bx*-transformed determined state is heritable when duplication across the compartment occurs and therefore that *bx⁻* is important in the determination of anterior metathoracic

leg rather than in the interpretation or expression of the determined state in normal development.

The question then is whether *bx*$^{+}$ is involved in the establishment or maintenance of the anterior metathoracic determined state. Although it is at this point hard to distinguish these two hypotheses, two lines of evidence suggest that the latter is the more probable. Firstly, the anterior-posterior compartments appear to be established independently in the embryo before the formation of a clonally distinct metathoracic leg disc. If *bx*$^{+}$ were required only in the establishment of the compartment, one would expect *bx* clones induced after this event to behave non-autonomously. The wild-type gene would no longer be required. Morata and Garcia-Bellido (1976) found that *bx* clones induced as late as the third instar larval stage differentiated autonomously. In addition, in *bx*^{3} *pbx* transformed halteres, the number of cells and clonal growth parameters were found to be very similar from the earliest stages to those in the normal wing. These results suggest that *bx*$^{+}$ must function continuously in development to prevent transformation and therefore are consistent with the maintenance hypothesis. Secondly, phenocopies of *bx* induced with ether at the cellular blastoderm stage are not only clonally heritable, but also dependent on the number of copies of *bx*$^{+}$ in the genotype (Capdevila and Garcia-Bellido, 1978). These results were interpreted to mean that ether interferes with the initial activation of the *bx*$^{+}$ gene at the blastoderm stage. This interpretation is also consistent with the idea that *bx*$^{+}$ is required in the maintenance of the metathoracic state, or in other words, maintenance of a memory of position in the embryonic field.

The *pbx* mutant transforms posterior metathorax into posterior mesothorax. Hence it may be involved in the establishment, maintenance, or expression of the determined state. In order to find out if the *pbx* mutant affects any of these functions, pattern duplications were induced in the metathoracic leg discs (same rationale as for *bx*). All of the 50 duplications obtained could be classified as AMT-PMS duplicates (Table 7). This shows that the *pbx*$^{+}$ gene is necessary in this system for differentiation of the posterior metathoracic compartments. Thus, *bx*$^{+}$ and *pbx*$^{+}$ could be considered analogous selector genes for anterior and posterior metathoracic compartments.

The function of *bithorax* during pattern duplication

The activation of *bx*$^{+}$ and *pbx*$^{+}$ genes appears to be independent during normal development as each locus is compartment specific in its effects. Transformation of the posterior compartment in *bx* duplicates implies that *pbx*$^{+}$ is not activated in these compartments. On the other hand, when *bx*$^{+}$*pbx*$^{+}$ discs duplicate, the posterior compartment is always non-transformed and therefore *pbx*$^{+}$ must have been active. It follows that *bx*$^{+}$ is involved (directly or indirectly) during pattern duplication in the activation of *pbx*$^{+}$ to establish the posterior metathorax state. Such an interaction between *bx*$^{+}$ and *pbx*$^{+}$ genes evidently does not occur in normal development. Hence the involvement of *bx*$^{+}$ in the establishment of the posterior metathoracic state is only revealed in pattern duplication and can therefore be considered as a latent function of this locus.

Consistent with the above interpretation, it was found that the frequency of transformation depended upon the allelic state at the *bx* locus. *bx*^{34} homozygotes gave 37% transformed posterior compartments while in *bx*^{3} homozygotes 78% of all duplicate posterior compartments were transformed (Table 10). This is as expected if there is some *bx*$^{+}$ activity threshold level above which *pbx*$^{+}$ can be switched on as *bx*^{3} is closer to the amorphic condition than *bx*^{34} (Lewis, 1963).

The duplicate phenotype in relation to compartmental origin

Although the formation of AMS-PMS duplicates of metathoracic legs strongly suggests that the posterior duplicates are sometimes derived from the anterior *bx*-transformed cells, there are several possible alternatives with respect to the cellular origin of the duplicates that may influence their phenotypes. The first possibility assumes strict heritability of the transformed (anterior) or non-transformed (posterior) states of *bx* cells when they enter the duplication blastema. This would imply that AMS-PMS duplicates are derived entirely from anterior *bx*-transformed cells, while AMS-PMT and AMS-P(MS&MT) may be derived from both anterior and posterior *bx* cells. A second hypothesis would be that the kind of duplicate formed is independent of the developmental history of cells involved, but dependent upon the allelic state of the *bx* gene alone. To distinguish between these models, a cell lineage study was done using

clonal analysis. Clones were induced after the establishment of the normal anterior-posterior boundary in the metathoracic leg, but prior to the heat-treatment used to initiate duplications. The clones showed that both anterior and posterior cells can participate in forming the duplicates and that their compartmental commitments are lost during pattern duplication (Table 18). A number of earlier workers have obtained similar results in regenerating and duplicating wing discs (Szabad, Simpson and Nothiger, 1979), mesothoracic (Girton and Russell, 1980) and prothoracic legs (Abbott, Karpen and Schubiger, 1981) using either surgical techniques or temperature-sensitive cell lethal systems to induce the duplications. Jurgens and Gateff (1979) however reported that compartmental restrictions were always maintained in both the original and duplicate throughout the process of pattern duplication.

In the present results, 44 of the 55 clones which originated in the anterior, and also labelled the posterior compartment in the duplicate, were found in duplicates classified as AMS-PMT. This suggests anterior transformed mesothoracic cells can form metathoracic posterior structures after entering the duplication blastema. The alternative hypothesis which assumes strict cell-heredity of the determined state can only be upheld if all 44 "clones" represent unrecognizable multiple inductions. The 21 multiple clones expected (see Table 22) on the basis of independent events cannot account for all 44 of the clones actually observed.

In four of the nine posterior clones also marking the posterior duplicate, two were in AMS-PMS duplicates, and two in AMS-P(MS&MT) ones. The expected three multiples (see Table 23) could account for these results without assuming a change of state, but the results are equally well explained if posterior cells can also form both kinds of posterior compartment. Taken together, the clonal analysis strongly supports the second hypothesis, that the kind of duplicate formed is independent of the previous developmental history of the cells that form the duplicate but dependent upon the allele at the *bx* locus that they carry.

Also consistent with the above interpretation are the observations that two original-anterior clones and one original-posterior clone differentiated both posterior duplicate mesothoracic and metathoracic structures within a contiguous patch (Table 22). The markers outside the clones were all metathoracic. This suggests that the clonal origin

of mixed posterior duplicate compartments can be either anterior or posterior original cells.

Stability of determined states and determinative decisions in the *bithorax* disc in pattern duplication

It has been proposed that the basis for heritable maintenance of determined states may be positive feedback control at a series of selector gene loci. For instance, each selector gene product might interact with its own promotor to maintain its own transcription once switched on. This idea is embodied in the model suggested for the *bithorax* complex by Hayes *et al* (Hayes, Girton and Russell, 1979). In this case both *bx*$^+$ and *pbx*$^+$ products are assumed to be capable of feedback at a common promotor. This idea was devised to account for the effects of *bx* on *pbx*$^+$ activation in pattern duplication reported in the present work. A prediction of this model is that the decision between mesothorax and metathorax in the duplication blastema would depend on the previous compartmental status of the cells involved.

The subsequent cell-lineage analysis reported above suggests that posterior *bx* cells which are already determined to be PMT can nevertheless form PMS duplicates. Although it may be argued that all these cases could be explained by multiple independent clone inductions, it is unlikely that this accounts for all the cases found since they would not all be expected to fall by chance into the critical PMS and P(MS&MT) classes. If posterior cells do indeed sometimes form PMS duplicates, this would indicate that *pbx*$^+$ can be switched from the on to the off state during pattern duplication. This is not what would be expected if *pbx*$^+$ product feeds back to maintain its own transcription as specified in the model of Hayes *et al*.

Instead, the decision between mesothoracic and metathoracic states seems to depend more on the allelic state of the *bx* locus than on the previous developmental history of the cells involved. This suggests the hypothesis that the posterior cells may change their states because of an interaction with anterior, transformed AMS cells. Consistent with this idea, the majority of the duplicates obtained have either entirely transformed (18%) or entirely non-transformed (76%) posterior compartments, as compared to the small number with mixed structures (6%). The average number of cells

which contribute to the formation of the duplication blastema in this system has been estimated as being between 7 and 22 (Girton, 1979, Girton and Russell, 1980). From the clonal analysis reported here (Table 21), it is clear that after entering the duplication blastema the determined state of these cells is normally clonally stable. Thus, among 47 clones labelling individual duplicate posterior compartments, 7 formed only mesothoracic markers, 37 formed only metathoracic markers, and only 3 formed both kinds of marker. If the state of determination of each cell in a duplication blastema consisting of 7 to 22 cells were independent, we would therefore expect a frequency of 1-[(probability (all mesothoracic cells) + probability (all metathoracic cells)] mixed compartments. This frequency as estimated from the multinomial distribution is greater than 80%. This is significantly in excess of the 6% observed (Table 18). This result strongly indicates that a co-ordinate decision is made by the cells comprising a given duplication blastema. Whether a co-operative decision is made, or whether the cells are subjected to a common external influence cannot be decided from the present results. This may be related to other situations where co-ordinate determinative decisions are made among cells related by physical proximity rather than clonal origin. Thus, co-ordinate determinative decisions in normal development also take place in groups of cells related by proximity rather than by descent (Nothiger, 1972 for review). Clonal analysis has demonstrated that transdetermination occurs in groups of adjacent cells which switch simultaneously to the same determined state (Gehring, 1967). Cell lineage analysis of an *Antennapedia* mutant has shown that the antennae to leg transformation is also made in cells related by proximity rather than by descent (Postlethwait and Schneiderman, 1971). The only difference in the case of the present *bx* duplicates, is that the co-ordinate decision must be made by cells which previously had different determined states. This is made possible perhaps by a de-determination step taken by cells when they enter the duplication blastema.

Effects of *bithorax* and *postbithorax* in the determination of haltere disc

The haltere to wing transformation caused by *bx* and *pbx* are also anterior and posterior compartment specific respectively in the two mutants (Garcia-Bellido and Santamaria, 1972; Morata and Garcia-Bellido, 1976). Adler (1978a) has published results

similar to the present ones, using a surgical technique on *bx* and *pbx* transformed halteres. He found that when cells from the transformed *pbx* posterior haltere compartment (wing) regenerated across the compartment boundary, they differentiated only anterior wing structures. This result is analogous to those reported above for *bx* metathoracic leg duplicates. However, the anterior *bx* haltere (wing) can only regenerate posterior haltere structures, which is analogous to the observed behaviour of *pbx* leg duplicates reported here. Adler interpreted his results as indicating that the mechanism of transformation in the two mutants is different. That is, *bx* affects the expression of determined state and *pbx* affects cell heredity of the determined state.

The results presented in the present work and Adler's results therefore appear to be in contradiction with respect to the roles of *bx* and *pbx* loci in dorsal and ventral metathorax determination. The difference is probably not due to the difference in techniques but it may be explained if there is a quantitative difference in the amount of bx^+ activity level required for establishment of posterior haltere and posterior third leg compartments in pattern duplication and regeneration. Gene dosage studies have demonstrated that existing *bx* alleles are all hypomorphs, as their phenotypes are more extreme when placed over a deficiency for the locus (Lewis, 1963).

A second hypothesis is that the difference is due to a dissimilar developmental function in the two discs of bx^+ and pbx^+, perhaps because of interaction with other homeotic loci active within them. Consistent with this hypothesis, there exist a number of mutants such as *Multiple-sex-comb (Msc)*, and *Extra-sex-comb (Sex)*, mutants of the *Antennapedia* complex (Kaufman, Lewis and Wakimoto, 1980), the recessive *extra-sex-comb (esc)* and *Polycomb (Pc)* which transform second and third legs into first leg with no homologous compartmental transformation in the haltere or wing (Hannah-Alava, 1958; for review and description, see Lindsley and Grell, 1968, and Ouweneel, 1976). Therefore, these loci might possibly interact with bx^+ and pbx^+ loci in the determinative decisions which establish compartments during pattern duplication.

Adler (1978b) also studied regulative behavior in the *bx* and *pbx* mosaic discs. He first analyzed the regulative behavior of wild-type haltere and wing fragments to define regions of homology between the two discs and found that the regulative behavior is very similar between them. Next he analyzed the regulative behavior of *bx* and *pbx*

transformed haltere discs and observed that the regulative behavior in these discs is also similar to that of the wild-type wing or haltere discs. In situations where a disc fragment contains both wing and haltere tissues, interaction between them can still occur to give pattern regeneration or duplication depending on the size of the fragment. However, the determined state of the duplicated or regenerated structures (mesothoracic or metathoracic) can be conserved or non-conserved, depending on the compartmental origin and determined state of the cells that form them. He concluded that wing and haltere discs not only contain homologous positional information systems, but also that they share the same positional information in the *bx* and *pbx* mosaic discs, and that they communicate in pattern regulation. This conclusion is consistent with the observation that in the *bx* third leg disc, anterior and posterior cells can both contribute to the formation of the duplicate. However, it is not clear from Adler's results if cells de-determine first and co-ordinately make a determinative decision as suggested above for the metathoracic leg disc.

Implication of *bithorax* duplicates in the light of models involving "combinatorial epigenetic codes"

Several experimental results suggest that pattern duplication may involve a reiteration of normal development. Firstly, by clonal analysis it has been demonstrated that the number of cells initiating a mesothoracic leg duplicate may be as few as 7-22. This is similar to the number set aside to initiate the leg disc in the embryo in normal development (Nothiger, 1972; Madhaven and Schneiderman, 1977; Girton and Russell, 1980). Secondly, clonal parameters such as cell division rate, and clone shape are also very similar to those found in normal legs (Girton and Russell, 1980). Finally, the compartment boundary is re-established in duplicates in the same location as in the normal appendage.

During normal development, a number of gene loci must be switched on or off. Through clonal analysis and developmental studies of homeotic mutants, it has been suggested that each compartmentalization event may represent a sub-division of a larger developmental unit into two smaller ones. The activation of a selector gene to control and maintain each determinative decision would be necessary at each step. At the end, each

compartment would be characterized by a combination of activities for a number of selector genes (Garcia-Bellido, 1975). Kauffman's binary switch epigenetic code suggests a similar combinatorial scheme (Kauffman, 1973, 1975). A schematic representation of this kind of model is shown in Figure 21. If pattern duplication indeed involves a reiteration of the normal development events, then some of these developmental steps must be reversible.

In the present context, the phenotypes of *bx* and *pbx* are inconsistent with their participation as selector genes in a simple combinatorial scheme for compartment development since neither mutation causes a complete transformation to mesothorax. Instead, the effects of *bx* and *pbx* are anterior or posterior compartment specific. In addition, genetic analysis has demonstrated that *bx* and *pbx* are two different loci (Lewis, 1963). Hence, these results are all consistent with the idea that the distinction between anterior and posterior metathorax is made by two separate loci rather than a simple on or off state of one selector gene, and that these same loci also code for the distinction between mesothorax and metathorax. Hayes *et al* have proposed an alternative model that can account for the observed metathorax to mesothorax transformations involving single compartments rather than the whole segment, caused by the two mutants (Hayes, Girton and Russell, 1979). According to this model, there is no selector gene corresponding to A in Figure 21.

Any model involving a combinatorial binary switch scheme for progressive disc determination would predict that a mutation in a selector gene which acts at a later stage should not affect a decision taken earlier in pattern duplication. If the mesothorax versus metathorax decision is encoded by a separate locus such as *Ubx* (gene A of Figure 21) rather than *bx* locus itself (gene B of Figure 21), one would not then expect a mutation in the *bx* locus to have any effect in the posterior compartment of the duplicate. Since *bx* has been shown here to influence determination in the posterior, it follows that the distinction between mesothoracic and metathoracic states must be encoded by the *bx* locus itself. This result is therefore inconsistent with the combinatorial scheme of determinative decisions as suggested by the model in Figure 21. It may however be argued that gene A in Figure 21 is *Ubx*, and that *Ubx* and *bx* are pseudoalleles

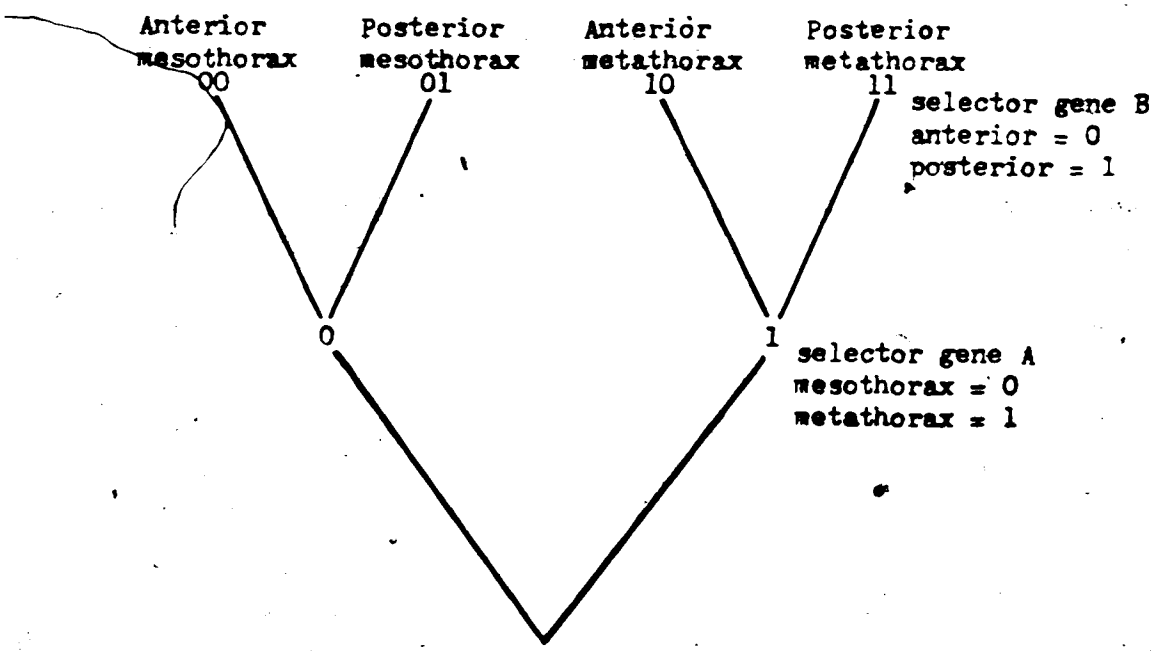


Figure 21. A combinatorial scheme showing how the selector gene and the binary switch epigenetic code models (Garcia-Bellido, 1975) and Kauffman, 1973, 1975), may be applied as an explanation for determination in the mesothorax and metathorax.

Consistent with the above conclusion, Morata and Kerridge (1981) have observed that *Ubx* homozygous clones induced any time before 17 hours of development transform anterior metathoracic to anterior mesothoracic leg and the posterior compartment of both mesothoracic and metathoracic legs to posterior prothoracic leg, while clones induced later than 17 hours cause transformation of both compartments of metathorax only into the corresponding mesothoracic compartments. These results are also inconsistent with the simple combinatorial scheme which would predict whole segment transformations.

It may therefore be concluded that during normal embryonic development, the decision between mesothorax and metathorax is not made by means of a combinatorial mechanism. The distinction between the two segments appears to be controlled by two different loci, *bx*$^{+}$ and *pbx*$^{+}$, which are activated independently in the metathorax. The observations are consistent with the alternative model (Hayes, Girton and Russell, 1979). But as discussed above, a prediction of this model was not borne out in the clonal analysis.

The requirement for *bx*$^{+}$ function in the establishment of the posterior duplicate may perhaps be necessary since the embryonic positional field which initially activates the two loci must have disappeared in the imaginal disc. Hence the re-establishment of determined states may depend on the selector gene(s) which act as a memory of determinative decisions taken in the embryo.

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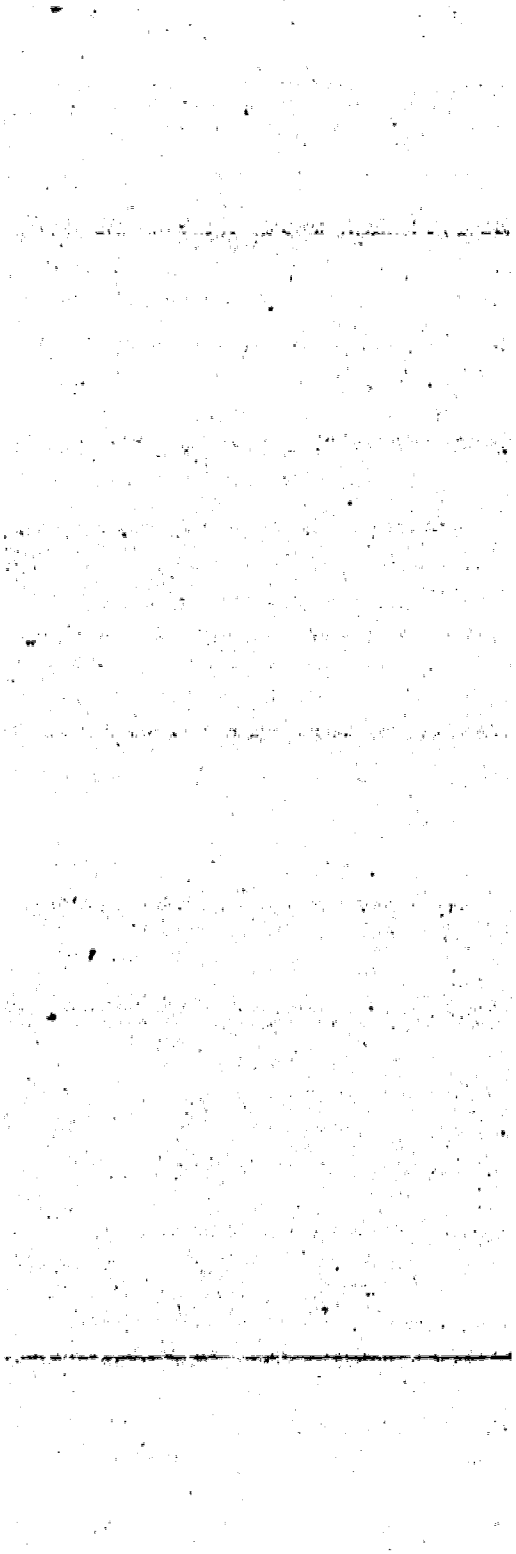
VI. APPENDIX



Appendix 1. Incidence of type of duplicate classified by size of marker deficiency (Anterior only (in parenthesis), and both anterior and posterior compartments), in various *bithorax* genotypes

Type of Duplicate	Genotype			
	bx^{34}/*bx*^{34}	*bx*^{34}/*TM2*	*bx*^{3}/*TM2*	*bx*^{3}/*bx*^{3}
AMS PMT	9(11)	4(8)	8(3)	4(1)
AMS-PMS	5(6)	1(7)	2(3)	6(5)
AMS- P(MS & MT)	1(0)	0(1)	1(1)	5(2)
Sum	15(17)	5(16)	11(7)	15(8)