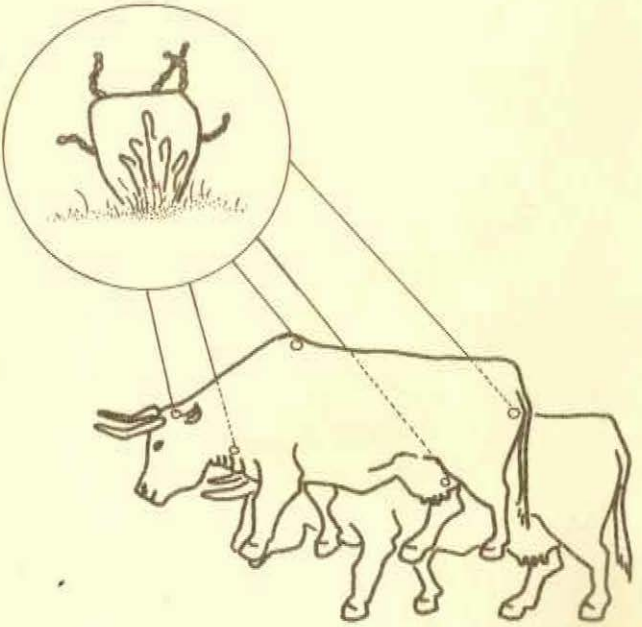
MOLECULAR EVENTS IN INSECT PATTERNING AND MORPHOGENESIS



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THE INTERNATIONAL CENTRE OF
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The understanding of fundamental developmental processes at the molecular level in insects is advancing at a rate that would have seemed impossible only a few years ago. Nowhere is this clearer than in the study of the homeotic genes of *Drosophila*. Mutations in homeotic genes can cause quite clean transformations of one body part into another [1-3], as illustrated in Figure 1. The realms of action of these genes often appear to define segment or compartment borders in the fly, and these genes appear to be important in the specification and maintenance of the determined state of the different

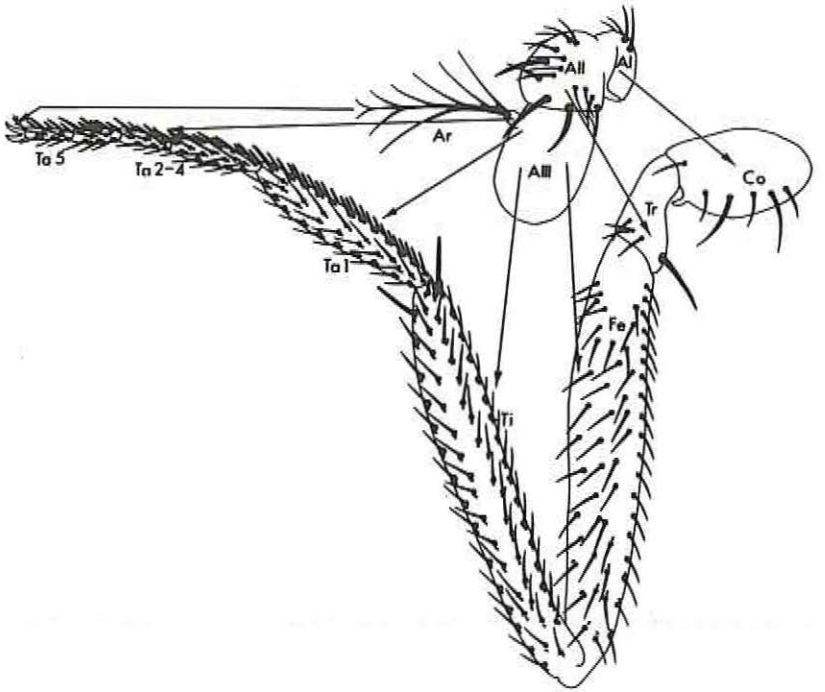


Figure 1

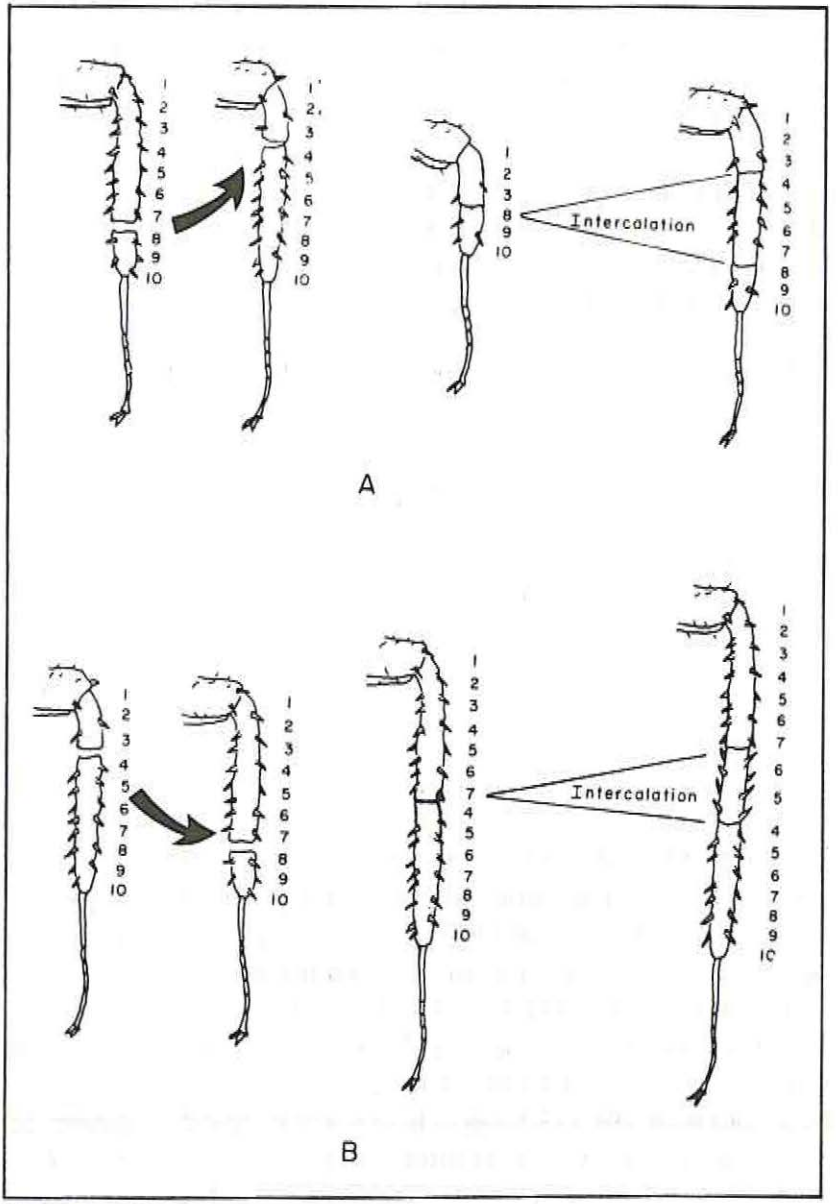
segments[4-7]. Recent advances in nucleic acid cloning techniques, combined with a renewed burst of developmental genetics work in *Drosophila,* make one optimistic that we may understand the functions of these genes at the molecular level in the not too distant future. (See, for example, references 8-10).

While we are rapidly moving toward a molecular understanding of what makes one segment different from another, we are still far from understanding the developmental patterning processes that are shared by the different segments. Returning to Figure 1, we can see that there is a very well defined and reproducible pattern to the bristles and other structures on the antenna and leg. Moreover, there appears to be a point-by-point correspondence between the pattern elements in the two structures. This correspondence can be shown with precision by examining structures that are only partially transformed, and has been observed in a number of cases of homeotic transformation of different body parts in the fly [1-3] These and other data indicate that there is a two-dimensional positional field that is repeated homologously in each body part, in this case each imaginal disc derivative.

At this point, I would like to discuss some of the characteristics of these positional fields, and the approach that we are taking to try to understand, at the molecular level, how these fields are maintained and how the cells within a field co-ordinate their activities during patterning and morphogenesis.

POSITIONAL FIELDS IN INSECTS

Initially, I will simplify things by considering patterning in one dimension, during regeneration in the legs of cockroaches. In a series of elegant experiments, Bohn and his followers showed that legs of larval cockroaches can be amputated and grafted together at different levels, and the regulative properties of the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1 2 3 4 5 6 7 8 9 10
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Intercalation
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B

Figure 2

various graft/host combinations assayed at subsequent molts (Figure 2). For example, if a distal leg, cut at the level of the distal tibia, is grafted onto a stump severed in the proximal tibia, regeneration ensues to produce a tibia of normal size and pattern (reviewed in ref. 11). More informative is the result when proximal tibia is grafted onto a distal tibia stump. Again regeneration is stimulated, generating all of the intermediary positional values at the new proximodistal discontinuity, even though these values already are present on both sides of the graft site.

These and other experiments have led to the idea that there are gradients of positional information in the cockroach leg, and host/graft combinations that disrupt the gradient stimulate intercalary regeneration to smooth the discontinuity in the gradient, seen experimentally as a replacement of any structures normally found between the juxtaposed graft and host tissue. The fact that local interactions are important in sensing positional discontinuities can be inferred from the polarity of the regenerated tissue and the size of the regenerated appendage when proximal tissue is grafted onto a distal stump. The polarity of the regenerated tissue is reversed relative to the surrounding leg tissue, and the leg is longer than normal, both consistent with the notion that local growth is responsible for smoothing the positional gradient, as opposed to a general respecification of positional values throughout the tibia. Or, in the parlance of developmental biologists, the regeneration proceeds by epimorphosis rather than by morphallaxis.

The above concepts appear to hold when looking at patterning in two dimensions as well. Although two-dimensional patterning can be studied in cockroaches, I will change systems here, because the cockroach is not a very good organism for attempting to understand patterning processes at the molecular level. For this, one wants a system that offers genetic and

molecular genetic tools, and so I turn to *Drosophila,* and in particular *Drosophila* imaginal discs.

In mature third instar *Drosophila* larvae, just before pupariation, each wing imaginal disc is comprised of a flattened epithelial sac of about 50,000 cells. The side of the sac that will make the most of the adult dorsal mesothoracic structures is a highly columnar epithelium that is folded in a characteristic way, providing numerous morphological landmarks. Discs can be fragmented by precise cuts, and the determined states of the different fragments assayed by injecting them into larvae that are about to pupariate. The fragments undergo metamorphosis along with their hosts, and by carefully examining the implant-derived cuticular structures a detailed fate map of the disc can be constructed[12] (Figure 3). Fragments, or combinations of fragments, can also be cultured (in the abdomens of adult females) before injection into host larvae; in this way the regenerative potential of the disc tissue can be tested.

Numerous experiments, involving many variations on this general scheme, suggest that the one-dimensional rules that explained regenerative behaviour of the cockroach leg can be applied to the two-dimensional disc[13]. Specifically, the disc is an autonomous and complete positional field with regard to pattern regulation. Local discontinuities in the positional field stimulate growth and intercalary regeneration. In general, regeneration fills in positional gaps via the shortest route regardless of the respective polarities of the regenerated and surrounding tissue. (It should be noted that this relationship between growth and pattern probably is not confined to regeneration. It appears that the normal signal for growth cessation is the formation of a complete two dimensional pattern [14].)

Finally, unlike the segmental state of determination, which is very stable and influences how a cell will respond to its position in the field, the positional specification of a cell appears to

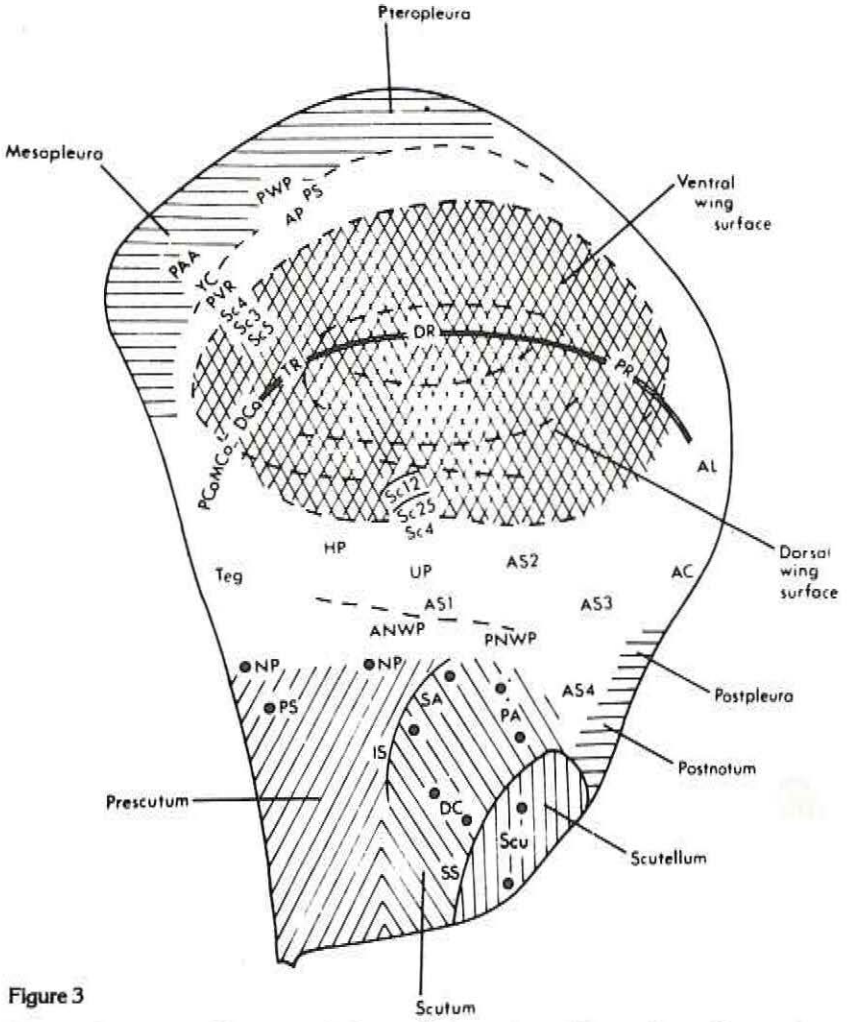


Figure 3

depend on continuous intercellular signalling. A cell can be induced to participate in a regenerative event if its neighbours are experimentally altered at any time during larval life, and cells may send positional signals even under circumstances in which they themselves are unable to participate in regeneration (for example, following a lethal dose of irradiation).

DEVELOPMENTAL COMPARTMENTS IN IMAGINAL DISCS

It is possible to examine cell lineages in developing imaginal discs by genetic means. If developing embryos or larvae are X-irradiated, recombination is induced at a low frequency in somatic cells. In an animal that is heterozygous for some recessive cell autonomous marker,somatic recombination can generate a homozygous marked daughter. Typically, the chromosome in question is constructed so that a dominant *Minute* mutation that causes slow growth is eliminated from the marked daughter by the same recombination event, and the new genetically marked clone has a growth advantage relative to its neighbours. Thus, because the clone can grow very large, clonal analysis using *Minutes* allows one to probe for developmental restrictions that would not be evident from a simple fate map.

When the patterns of marked clones are examined in the wing, the first thing that becomes clear is that the lineages of the cells are not precisely fixed. For example, clones of various sizes and shapes can all include the same region in different wings. However, lineage restrictions are observed in some well-defined areas [15,16] (Figure 4). The most striking of these sepa-

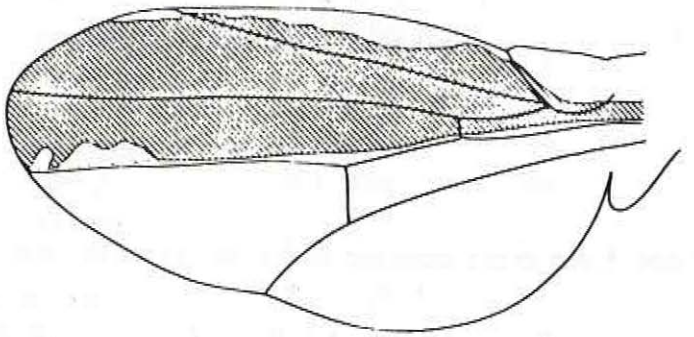


Figure 4

rates the wing into anterior and posterior compartments. Clones made after the blastoderm stage never cross the anteroposterior border in wild type wings, even though they may encompass over 90% of one of the compartments. Moreover, the compartment border was defined, until very recently, only by this lineage restriction. No morphological manifestation of the border has been detected, either in adult wings or in imaginal discs.

Although the mechanism whereby the anterior and posterior cells remain segregated is unknown, we do have some information concerning the genetic basis of this compartmentalization. A number of observations have led to the hypothesis that the *engrailed* locus is important in determining a cell's compartmental identity[17,18] Specifically, it is thought that an anterior ground state is converted to a posterior state of determination by the activity, at least in part, of the *engrailed* gene. For example, mutations of *engrailed* produce a variety of pattern disruptions in posterior compartments, but are without phenotype in anterior compartments. Recent experiments in which DNA from the *engrailed* locus was hybridized to RNA directly on tissue sections support the notion that the *engrailed* gene is expressed only in posterior compartments [19]

That anteroposterior compartmentalization is unambiguous appears to be general to most if not all of the cuticular segments, and apparently defines units for the expression of many of the known homeotic genes, such as those of the bithorax complex[4-7]. This compartmentalization event therefore appears to be temporally and functionally similar to the process of segmentation. The relationship of these compartments to two-dimensional patterning within the discs, however, remains unclear. Even more obscure is the significance of the other lineage restrictions seen in the developing wing. These post-embryonic restrictions arise near the end of larval life and are

seen typically along morphologically defined structures, in contrast to the anteroposterior boundary. For example, the best characterized of the late lineage restrictions is seen at the wing margin. It has been hypothesized that this dorsoventral restriction results from a compartmentalization event that is analogous to the anteroposterior compartmentalization[20,21] although a genetic basis dorsoventral event has not been found.

A MOLECULAR APPROACH TO PATTERNING IN IMAGINAL DISCS

There are numerous models that attempt to explain patterning in imaginal discs and other developing systems, but there is very little information concerning the molecules involved. Genetic analyses have proven to be extremely powerful in the identification and analysis of genes involved in the establishment and determination of segments, but standard genetic approaches have not been very helpful in identifying components that are involved in two-dimensional patterning in imaginal discs. This may be because the patterns result from an integration of many determinative and morphogenetic processes, in contrast to the relatively small number of genes that control segment identity. Also, because the patterning process is repeated homologously in each segment, any mutation that disrupts patterning will probably produce a grossly abnormal embryo, without clean transformations of particular defined body parts. Such a phenotype could be difficult to distinguish from other, non-patterning mutants.

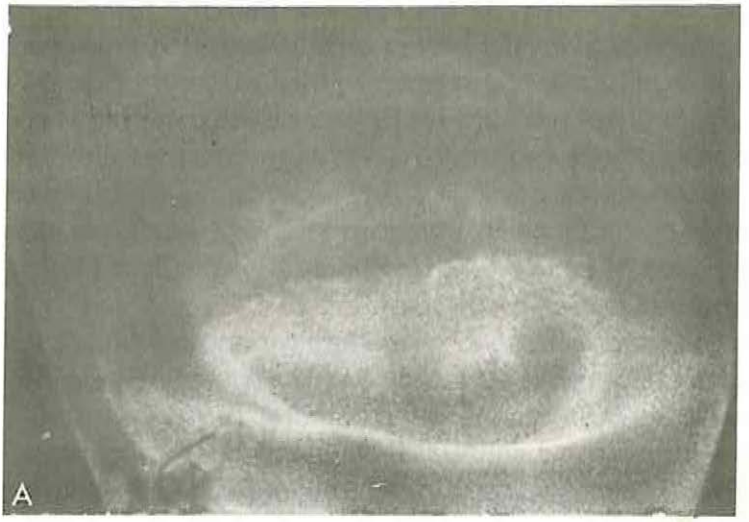
We therefore chose to try to identify gene products involved in patterning, using a strategy that is conceptually similar to a genetic screen. Instead of generating mutants, though, we make monoclonal antibodies against *Drosophila* tissues, and screen the antibodies by immunofluorescence on imaginal discs. Because the disc epithelium is composed of only one

cell type, any antigen that is present on one region of a disc but not another is unlikely to be a simple differentiation antigen and is a good candidate for a molecule involved in two-dimensional patterning processes.

POSITION-SPECIFIC CELL SURFACE ANTIGENS

One group of antigens that we have defined in this way shows particularly interesting patterns of expression on imaginal discs[22,23] Because the expression of the antigens depends not on the type of adult structure that a disc cell is destined to make, but rather on the position of the cell in the epithelium, we have called them the Position-Specific (PS) antigens.

On mature third instar wing discs, PS1 antigens are found primarily on the surfaces of dorsal cells and PS2 antigens are seen primarily on ventral cells (Figure 5). (I define the co-ordinates of the disc-dorsal, anterior, etc. — with respect to the locations of the structures in the adult that a disc region will make at metamorphosis). A third class of antibody, PS3, recognizes



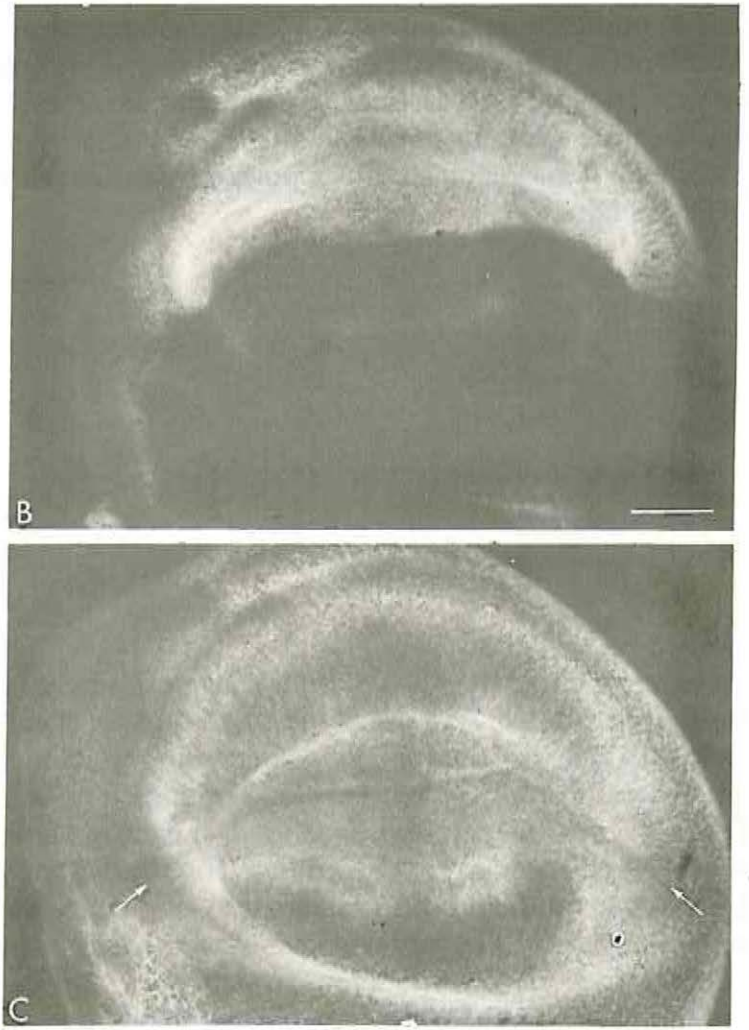


Figure 5

both the PS1 and PS2 antigens, as well as at least one other component. The PS antigens are all-oligomeric complexes of large glycoproteins [24] (approximately 92 to 135 or more

kilodaltons, Figure 6). These complexes appear to share at least one common component, probably recognized by the PS3 antibodies, and also possess components that are unique to the PS1 and PS2 classes. So far, we cannot rule out the possibility that the different specificities are generated by post-translational modification of similar polypeptides, for example by glycosylation.

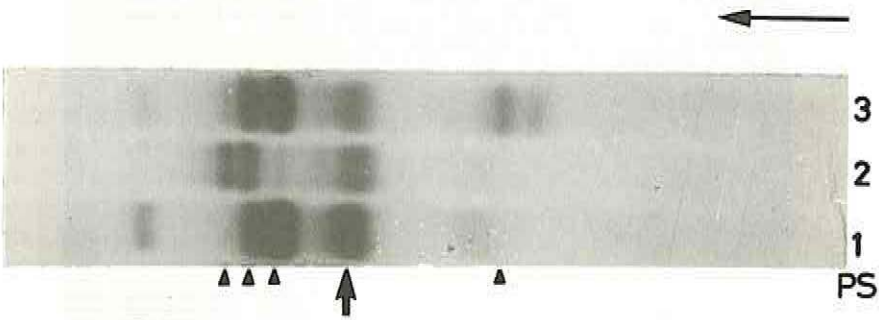


Figure 6

It should be noted that the PS patterns are extremely reproducible, even in detail. Moreover, the patterns are not altered by genetic changes that do not change patterning as defined by other means. For example, the *Minute* mutations that slow cell growth leave the patterns unchanged, and the patterns are similar in related species of *Drosophila*[25] Thus the PS antibody patterns appear to be reliable markers for the regional identity of undifferentiated cells, and we have used them to examine the development of some patterning mutants.

For example, flies bearing a particular combination of alleles at the *apterous* locus display an abnormal distribution of bristles and other structures that normally are seen only at the wing margin. Multiple copies of the bristle rows are seen in the mutant and margin structures are often found in circles or tufts far removed from the normal margin. This phenotype could result

from either of two types of abnormality. The first possibility is that the tendency for cells to form margin structures is increased, so that margin forms ectopically in regions with positional values that normally would not induce wing margin. Another possibility is that the overall specification of positional values is aberrant. This second explanation is shown to be correct upon examination of the mutant discs with the PS antibodies; as shown in Figure 7, the entire dorsoventral patterning of mutant discs is disrupted[26]

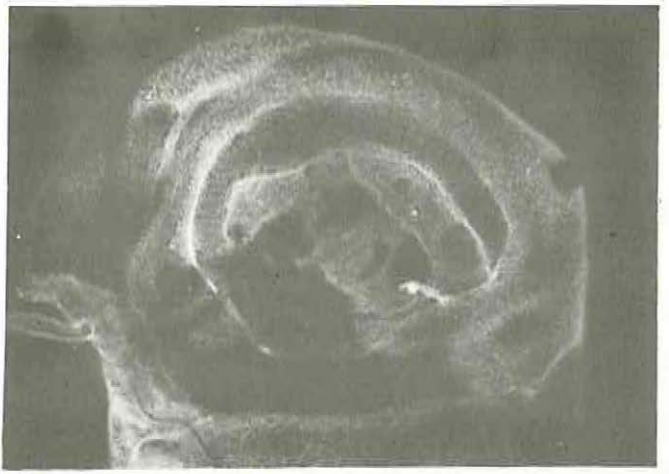
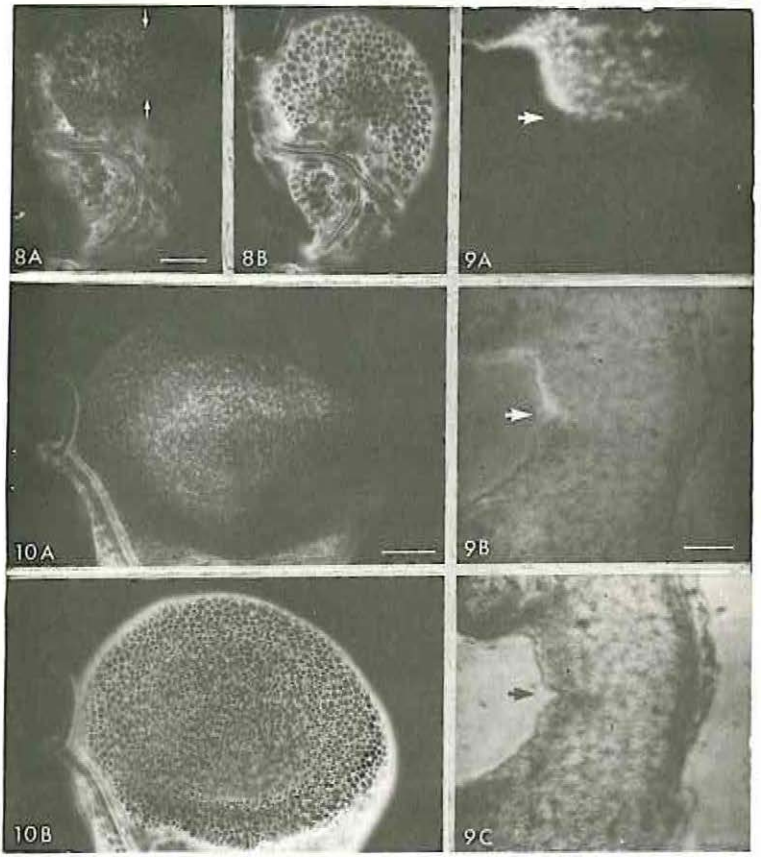


Figure 7

In a similar set of experiments we also examined wing discs from *engrailed* mutants. As described earlier, *engrailed* mutations produce phenotypes in adults only in posterior compartments, however there was doubt as to whether the phenotypes resulted from a tendency for posterior cells to transform to anterior identity or from cell death and unusual regeneration. Our findings of clear transformations in discs at early stages of development combined with clonal analysis data, allowed us to

rule out the cell death explanation, confirming the original transformation hypothesis[27].

So what are the functions of the PS antigens? The correlation in the mature wing disc between PS antigen expression and the dorsoventral lineage restriction is very striking, although not absolute; some PS1 antigen can be detected ventrally and faint patches of PS2 antigen are seen in some dorsal regions. When other discs are examined, any general correlation with dorsoventral character immediately vanishes. While the patterns of



Figures 8, 9, and 10

PS antigen expression are non-uniform in other discs, the patterns are not homologous. Even in the wing disc, the patterns change with development. For example, in wing discs from second instar larvae the PS2 antigen shows a strong and sharp anteroposterior difference in expression, in addition to a dorsoventral variation (Figure 8).

If a general correlation is to be drawn, it is that the expression of the PS antigens appears to be related to morphogenetic events in the disc epithelium. Moreover, these events appear to take place where there are discontinuities in the amounts of antigen on the surfaces of cells. At the presumptive wing margin, for example, the sharp and complementary PS1 and PS2 borders are characterized by discrete grooves in the surface of the epithelium (Figure 9). More subtle changes in PS antigen expression typically are seen where relatively gradual changes in disc morphology are occurring, such as the formation of the wing pouch (Figure 10).

FUTURE DIRECTIONS

As a result of these and other correlations, we hypothesize that the PS antigens are involved in cell recognition and/or adhesion processes important for morphogenesis. This hypothesis is being tested in two different ways. First, we are looking to see if the antibodies will interfere with defined cellular processes. For example, cell-cell adhesion can be quantitated using dissociated disc cells. In collaboration with Richard Fehon at the University of Washington, we are also examining adhesion in the presence of PS antibodies. Our second test is genetic; we will examine the effects of removing the structural genes for the PS antigens. Because animals that are lacking the antigens completely are likely to die as early embryos (based on the observation that the PS antigens are first expressed around the time of gastrulation), we will make use of the somatic recombi-

nation technique to generate mutant clones in phenotypically wild type animals. Combined with our knowledge of the spatio-temporal patterns of PS antigen expression, this approach should be very profitable.

In order to generate mutants, we will make use of the advanced state of *Drosophila* molecular biology. It is now possible to progress not only from a defined mutation to a protein product, but in the reverse direction as well. In *Drosophila*, any gene that can be genetically localized can be mutagenized by relatively standard (though, depending on the location, sometimes lengthy) genetic means. In order to localize a gene defined only by a protein, a number of alternative strategies can be employed. We will use our antibodies to purify enough protein to derive a portion of the amino acid sequence, probably at the amino terminus. Portions of this sequence, that correspond to relatively unambiguous nucleic acid sequences, can then be used to generate oligonucleotide probes, which can in turn be used to identify clones containing parts of our gene in DNA libraries[28]. These clones can then be used to localize the gene by *in situ* hybridization to polytene salivary gland chromosomes.

POSITION-SPECIFIC ANTIGENS AND LINEAGE RESTRICTIONS

Finally, I would like to consider some of the implications of our findings, and some recent data of others, on the question of imaginal disc compartmentalization. Remember that the dorsoventral border defined by the PS1 and PS2 antigen distributions appears to be coincidental with the dorsoventral lineage restriction defined by clonal analysis. Moreover, the timing of formation of the sharp PS boundary, and the associated epithelial grooves, is coincident with the establishment of the lineage restriction, at least within the limits of our experimental resolu-

tion. (The precise timing of the lineage restriction is difficult to ascertain for technical reasons.)

It is clear, however, that the dorsoventral line is just one of many morphogenetic processes with which the PS antigens are associated, and even in the wing disc, the antigens are not absolutely restricted to dorsal or ventral territories. The epithelial grooves that are observed along the dorsoventral border probably indicate an early step in the differentiation of the wing margin; consistent with this, O'Brochta and Bryant have recently reported that the grooves are contained in a band of non-dividing cells[29].

None of these morphological or physiological specializations are observed at the anteroposterior border. We must consider, then, the likely possibility that the dorsoventral lineage restriction arises not through a division of the disc into units with some fundamental developmental significance, but simply as a secondary consequence of disc differentiation and morphogenesis[30]. Hopefully, our continuing studies of the PS antigens will shed more light on this question.

It is a pleasure to acknowledge my collaborators, especially Michael Wilcox at the MRC in Cambridge and Michael Piovant, now at the CNRS in Marseille. The work in my laboratory was supported by the NIH, ACS, and the Monsanto Co.

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Dr. Brower obtained his doctoral training in molecular, cellular, and developmental biology at the University of Colorado, Boulder, USA, under the supervision of Professor J.R. McIntosh. His outstanding university career has been marked by a number of awards from such renowned bodies as the U.S National Science Foundation, the Boettcher Foundation, and the American Cancer Society. Since 1977 he has been involved in research and teaching in molecular biology at the University of Colorado, Boulder, USA; MRC Molecular Biology Laboratory at Cambridge University, England; and at the Developmental Biology Centre at the University of California, Irvine.

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