FROM MOLECULAR PATTERNS TO MORPHOGENESIS THE LESSONS FROM DROSOPHILA

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by

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INTRODUCTION

During development, cells in an embryo face two major tasks. First they must be programmed to form specific parts of the body, and second they must realize those fates by altering their shape, position and patterns of gene expression. Both the determination of cell fate and the corresponding alterations in form occur progressively during development. The organism that results from this process contains a wide variety of different cell types and functions, arranged in a complex spatial pattern. In Drosophila this final pattern is achieved about 20 hours after fertilization, when the embryo has formed a larva of 40,000 cells. The most obvious external feature of this larva is a segmentally repeated pattern of hairs and denticles secreted by the underlying epidermis. Beneath these hypodermal cells are a complex array of muscles also arranged in a segmentally repeated fashion. Internal to this somatic musculature are other mesodermal derivatives, as well as equally complex structures and organs of endodermal origin. By following the development of individual cells, it has been possible to trace the precursors for each structure in the differentiated larva back to the earliest cellular stages in the embryo (Poulson, 1950, Campos-Ortega and Hartenstein, 1985). In Drosophila this stage (the "blastoderm") consists of an oblong embryo about half a millimeter in length, with about 100 cells along the future AP axis and 40 along the DV axis. The epidermis is formed by cells on the lateral side of the embryo, the muscles and other mesodermal derivatives are formed from a stripe of cells on the ventral side of the blastoderm, and the endodermal cells from two groups of precursors at the anterior and posterior ends of the embryo.

At the blastoderm stage, all these cells are similar in size and morphology. A few minutes later, at the onset of gastrulation, the mesodermal precursors initiate cell shapes changes that carry them into the interior of the embryo (reviewed in Leptin, 1995). Similar changes in cellular morphology then internalize the endodermal cells. These morphogenetic movements are the first of many examples where cells in an embryo change their shape in

response to the developmental programs that control their fate. The large size of cells at gastrulation and the rapidity of these changes give gastrulation a particularly strong visual impact. The cellular mechanisms underlying these movements, however, utilize many of the same cytoskeletal and adhesive structures found in all cells. The pathway between a particular cell fate decision and the mechanisms controlling cell form have not yet been worked out in detail in any organism.

The past 15 years have seen remarkable advances in our understanding of how the changing pattern of gene activity in the *Drosophila* embryo directs cells to their final fates. These advances have been produced by an unprecedented combination of molecular biology and classical genetics. At this point many of the genes that control cell fate have been cloned and their basic cellular roles are understood. In principle, this information provides an ideal starting point to address how cell fate is translated into cell form. This has proven more difficult, largely because the genetic strategies that work so well in elucidating cell fates have met unanticipated obstacles in the elucidation of cell form. In today's talk, I would like to review the general picture that has emerged from our studies on how genes control cell fate in the *Drosophila* embryo, and examine how the activity of those genes might be related to the constantly changing form of the embryo.

THE HEIDELBERG MUTAGENESIS SCREENS.

It was to address both cell fate and cell form that Christiane Nüsslein-Volhard and I began the mutagenesis experiments honored in Stockholm this week. In the 1970's when we were finishing our graduate studies in Base1 and Tübingen, it was clear that development, like all processes in living cells, depended on genes and gene products. Not only was the relationship between DNA, RNA and protein known, but the analysis of several bacterial operons made it clear that gene activities could be controlled temporally and spatially. Almost everyone believed that such temporal and spatial control might explain the sequence of events giving rise to embryonic pattern. The analysis of such genes seemed formidable, however. Molecular cloning was in its infancy and there were no obvious tools for analyzing single genes in higher eukaryotes. What made the matter worse was the enormous diversity of genes and gene products present in an embryo. RNA complexity experiments estimated the number of different RNA species to be between 10,000 and 50,000 (reviewed in Davidson, 1986). These RNAs were expressed at varying levels and in a changing pattern as the embryo progressed through various stages. How could one sort out which gene products were essential for particular steps in development, how could the relevant genes and proteins be identified? To Christiane Nüsslein-Volhard and me, the genetic techniques that had been established in Drosophila over the preceding sixty years seemed to offer the best approach.

The particular mutagenesis strategy we adopted depended on two facts established about the *Drosophila* genome in the early 1970s. First, mutagenesis experiments involving small, cytologically defined chromosomal regions (Judd et al., 1972) suggested that, regardless of the total molecular complexity of the *Drosophila* genome, only about 5000 genes present in the DNA were essential for viability under laboratory conditions. This number set an upper limit on the genes essential for embryonic development. Second, a

Table 1: *Results of Heidelberg Mutagenesis Experiments.* **Numbers are totals for experiments on thr X chromosome and the two major autosomes. The number of mutations in the various classes have been corrected using the Poisson distribution to account for those lines containing multiple mutations.**

Results of mutagenesis screens

Total lines established and tested	26978
Lethal mutations	18136
Mutations causing embryonic lethality	4332
Mutations causing embryonic phenotypes	580
Complementation Groups (Genes)	139

detailed analysis of flies monosomic or trisomic for various genomic regions (Lindsley et al., 1972) suggested that although flies are normally diploid, a single copy of most genes was sufficient for viability. This meant that most loss-of-function mutations would be recessive and could therefore be maintained in heterozygous stocks. These two observations suggested that the requirements for gene activity during *Drosophila* embryogenesis could be surveyed in large scale (but limited) mutagenesis experiments. If transcription of a gene was essential for embryonic development, homozygous embryos should develop abnormally when that gene was eliminated. If the gene played a specific role in patterning or cell fate, most structures in the affected embryo would be normal; the defects limited to specific regions of the body or specific tissues. Based on these defects, it should be possible to reconstruct the normal role of each gene. In that view, all that was required for a saturating survey of development was to make enough mutant lines, and to be able to recognize defects by looking at embryos.

Because flies are diploid and most mutations are recessive, the mutagenesis scheme we settled on involved establishing inbred lines from single individual carrying mutagenized chromosomes (Fig. 1). In two generations, this

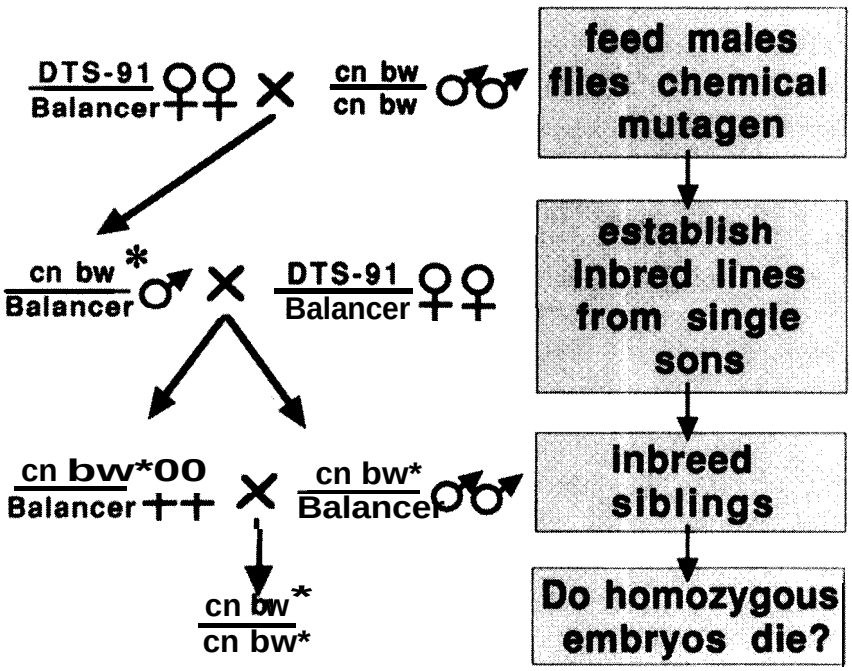

Figure 1. *Mutagenesis scheme used in Heidelberg to identify second chromosome mutations causing lethality in homozygous embryos.* Similar crossing schemes were used for the third chromosome and the X chromosome.

inbreeding produces homozygotes whose development can be compared with their heterozygous siblings. To obtain mutations in most or all genes in the genome, we had to establish enough mutagenized lines to hit most genes several times. Although Christiane and I carried out preliminary mutagenesis runs ourselves, the final experiments were extremely labor intensive and were completed with the help of Gerd Jürgens and Hildegard Kluding (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984; Jürgens et al., 1984; Wieschaus et al., 1984). Over the course of a year (from fall of 1979 to summer of 1980), we established 27,000 inbred lines. These lines contained an estimated 18,000 independently induced mutations, each causing lethality at some point during the life cycle of the animal (Table 1). Only about a quarter of these lethal mutations prevented homozygous embryos from hatching to the larval stage, and only about 2. 5% caused visible alterations in the external morphology of the embryo. These 580 mutations could be assigned by complementation tests to one of 139 different genes. The relative smallness of this final number was an important result, since it meant that each gene could be characterized in some detail. By examining mutant embryos from the various stocks, Christiane and I were able to establish a general picture of how gene activity in the embryo directs the development of individual cells.

In all species, many of the gene products present in the embryo are supplied by the mother during oogenesis. These include most RNAs and proteins needed for normal cell functions during early stages, as well as certain inhomogeneities that are used to define initial axes. In our experiments, homozygous embryos are derived from mothers that have a wild-type allele. Consequently, the Heidelberg mutagenesis screens missed all maternally supplied gene products. The 139 genes identified represent only those genes whose products must be supplied by the embryo's own transcription. We therefore explained the large number of the RNA and protein species in the embryo as largely derived from maternal supplies.

The fact that zygotically required transcripts were relatively rare suggested that they might play specialized roles in development. One possibility was that zygotic transcription allows particular gene products to be expressed in one cell and not in its immediate neighbors, at particular times and not others. This would contrast with the ubiquitous distribution generally observed for maternally derived transcripts. In this view, the embryo would use transcription to elaborate differences between adjacent cell populations, differences that would ultimately account for the variety of cells fates and cell behaviors observed in the embryo after the blastoderm stage.

THE HEIDELBERG MUTANT COLLECTION: A SURVEY OF ZYGOTIC GENE ACTMTIES.

One historical coincidence that contributed to the significance of the Heidelberg screens was the almost simultaneous development of molecular techniques that allowed genes to be cloned based on their genetic position. After publication of our screen, a number of *Drosophila* labs began a concerted effort to clone the genes identified by the mutations. This effort has continued over the past ten years and is still going on today. Of 139 lines that produce phenotypes, 20 were single hits, had relatively nondescript phenotypes and were never given names or further characterized. Most were eventually lost. Of the remaining 119, an informal survey of the *Drosophila* database suggests that 75 have been cloned and their expression patterns examined in embryos. These molecular analyses, coupled with phenotypic descriptions begun in Heidelberg have generated an increasingly detailed view of the kind of functions supplied by zygotic transcripts in the *Drosophila* embryo.

In general, the greatest insights into developmental mechanism came when a number of different Heidelberg genes could be grouped together because they affected the same developmental process. Sometimes, as in the segmentation series (Nüsslein-Volhard and Wieschaus, 1980) peculiarities of the phenotype of individual genes suggested regulatory hierarchies between members of the group (Fig. 2). In other cases (e. g., *armadillo* and *wingless,* Riggleman et al.), similar phenotypes pointed to unanticipated relationships between previously characterized proteins.

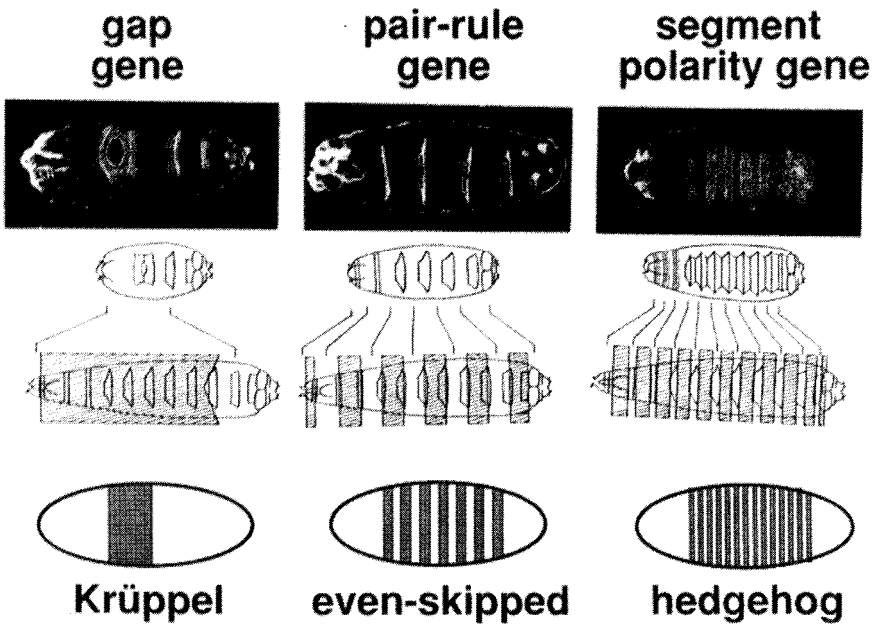

Figure 2. *Phenotypes of Mutations affecting segmental pattern.* The size and spacing of the pattern deletions in mutant embryos at the end of development allows extrapolation back to requirements for gene activities at blastoderm stage. Segmentation genes can be grouped into threi: classes: mutations in gap genes show large contiguous deletions in the pattern, mutations in pair-rule genes show pattern deletions spaced at double segment intervals, and mutations in segment polarity genes cause pattern deletions in each segment. One example is shown for each class.

One of the most striking features of the Heidelberg mutant collection was the restricted nature of the phenotypes. For a given line, certain cell types or regions are affected in the mutant embryo, other cell types and regions are normal. This phenotypic specificity was reinforced by molecular data once the various Heidelberg genes had been cloned. Most genes show highly regulated patterns of expression such that transcripts accumulate only in those regions and cell types where they are needed. Defects associated with a given mutant stock were generally different from those of other stocks, and expression patterns show a corresponding diversity. The two observations together suggest that most of the genes play unique roles at morphologically distinct steps in development.

Mutants usually affect areas of the embryo, rather than specific cell types. These areas generally do not correspond to obvious subdivisions and could not have been predicted based on morphological considerations alone. Obvious examples of these regional specificities are provided by the genes affecting segmentation: one could not have predicted the domains of gap gene expression, the existence of pair rule phenotypes, or the precise structures duplicated in segment polarity mutants. Because the region affected by a particular gene ultimately gives rise to a variety of different cell types, the genes seem to define positions or spatial coordinates, rather than tissues or organs.

Genes often are used several times again during development. Most segmentation genes, for example, are used later in the nervous system and in other tissues as well (e.g. *Krüppel* in Maipighian tubules, Knipple et al., 1985). This pleiotropy is not limited to genes controlling segmental pattern. Many of the genes controlling dorsal-ventral patterning at the blastoderm stage are used again at specific times in development. Even a gene like *twist* that ultimately seems specific for muscle differentiation, shows a more complicated, broader expression pattern at the blastoderm stage, suggesting a role in determining ventral fates in general, rather than specific mesodermal identities. These features make it difficult to relate early expression patterns of particular genes to final aspects of differentiation. Again, these observations are consistent with a role for the genes in programming regions or fates, rather than defining differentiated cell types per se.

More that half of the 75 cloned genes encode transcription factors, judging from the presence of DNA binding motifs, or their homology to known vertebrate or yeast transcription factors (Fig. 3). Their DNA binding properties might allow them to control expression of many different target

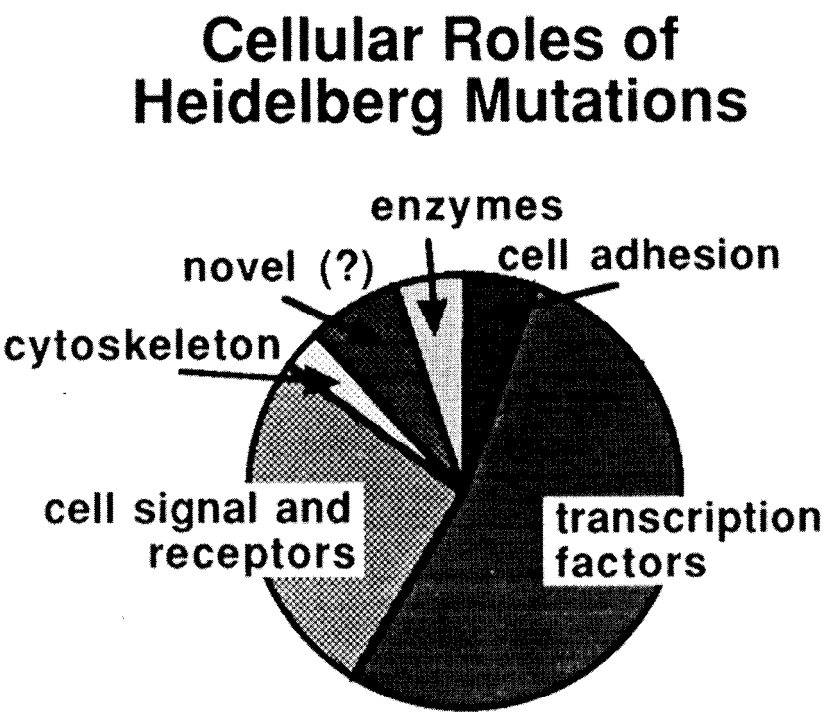

Figure 3. *Cellular Function of Heidelberg Mutations.* Based on the sequence of 75 cloned genes, most of the loci identified in Heidelberg encode transcription factors, or cell signals and receptors.

genes. If used at various stages of development and in different combination with other genes, they might therefore affect a variety of different developmental pathways. Most of the cloned genes which are not transcription factors are cell surface signals or receptors. Some of these signaling factors may

have immediate consequences on cell behaviors (see below), but most of this signaling ultimately feeds back on positional identities not directly related to final differentiated states.

GENES MISSED IN THE HEIDELBERG SCREENS: THE CONTROL OF CELL FORM

During the period when patterns of cell fate are being established, the embryo undergoes dramatic morphological changes. The final stages of cleavage occur when the embryo is still a syncytium. Once the number of nuclei have reached 6,000, they stop dividing and cell membranes are pulled down between individual nuclei. This process of cellularization involves a major reorganization of the embryonic cytoskeleton and takes about an hour. When it is completed, the embryo consists of 6,000 individual cells that are still morphologically identical. They are molecularly different however, due to expression of many of the 139 genes and transcription factors described in the previous section. At the onset of gastrulation, these differences are translated into changes of cell shape that result in the invagination of mesoderm or endoderm during gastrulation. Overall, the 90 minutes from completion of cleavage through early gastrulation represents the major period of morphological transformation that occurs in the *Drosophila* embryo.

What is the relationship between these morphological changes and the genes that we identified in the Heidelberg screens? When embryos from each of the 139 mutant stock were examined, we found that even the earliest acting mutants showed no morphological defects before the onset of gastulation. This suggested that the products of zygotic transcription were irrelevant for the early morphological events of cleavage and cellularization, and were only required when cells in individual regions of the embryos began their regionally specific cell shape changes. One way to test this was to block all new RNA synthesis by injecting early embryos with drugs that inhibit RNA polymerase. To our consternation, work from several labs (Arking and Parente, 1980, Edgar et al., 1986) indicated that such treatment causes embryos to become abnormal at the onset of cellularization, an hour before the earliest defects produced by any of the Heidelberg mutants. These experiments provided the first suggestion that the Heidelberg screens may have missed early acting, morphologically important genes.

To identify such genes, we developed a system of translocation crosses to generate embryos deficient for defined chromosomal regions(Wieschaus and Sweeton, 1988; Merrill et al., 1988). Using this system, it was possible to work our way through the entire genome in a small number of crosses, examining deficiency embryos at early stages to detect relatively subtle deviations in morphology. These experiments identified seven regions (or genes) which taken together account for the defects observed in drug treated embryos. These genes were identified because of their effects on early morphology rather than viability or overall pattern at the end of embryonic devel-

opment. They therefore represent a different sampling of the transcriptional requirements than the Heidelberg loci. Three of these seven genes have now been cloned (James and Vincent, 1986; Rose and Wieschaus, 1992; Schejter and Wieschaus, 1993). Unlike the Heidelberg genes, they do not encode transcription factors or components of a cell signaling system. Instead all three are cytoplasmic proteins associated with the cytoskeleton (Schejter and Wieschaus, 1993, Postner and Wieschaus, 1994).

Genes affecting morphology during gastrulation also appear to have been missed in the Heidelberg screens (Leptin, 1995). For example, the two Heidelberg genes absolutely required for mesodermal programming are *twist* and *snail*. Both have been cloned and have been shown to encode transcription factors (Boulay et al., 1987, Thisse et al., 1988). Immediately before gastrulation, these genes are expressed in the nuclei of mesodermal precursors on the ventral side of the blastoderm. When either gene is mutated, no cells are programmed to mesoderm and no ventral furrow forms. Thus, *twist* and *snail* not only direct blastoderm cells to a mesodermal cell fate, they have an immediate read-out in terms of mesodermal cell behavior. Since they are transcription factors, they must do this by controlling the expression of other genes.

What are these downstream targets? If they produced phenotypes visible in the final cuticle, such genes would have been detected in the Heidelberg screens. One candidate for such a target is *folded gastrulation*. In *fog* mutant embryos, the cell shape changes of the ventral furrow are delayed and uncoordinated. Wild type cells in the ventral furrow express fog immediately before changing their shape (Costa et al, 1994). When ectopically expressed outside the ventral furrow primordium, *fog* is capable of eliciting those same initial cell shape changes, even in cells that lack *twist* or *snail* or any other factors normally associated with mesodermal fate (Morize et al, in prep.) All this makes *folded gastrulation* an excellent downstream target for *twist* and *snail,* one whose expression might account for the cell shape changes associated with mesodermal programming.

Folded gastrulation is the only gene detected in the Heidelberg screen (other than *twist* and *snail)* that affects ventral furrow formation. Because *fog* embryos make slow uncoordinated invaginations, whereas *twi* or *sna* embryos make no ventral furrow at all, *fog* cannot be the only target of *twist* and snail. Other genes not detected in Heidelberg must account for the difference between the *twi, snail* and *fog* phenotypes, and for the delayed mesodermal invagination that still occurs in a *fog* mutant. These target genes may not have been detected in Heidelberg because they may not be essential for mesoderm invagination, as long as the embryo has a wild type alleles of *fog*. They might however have been detected if mutant embryos had been scored directly for morphological defects during gastrulation itself. Given the large number of stocks examined, this was not possible in the Heidelberg mutatgenesis experiments. On the other hand, relevant chromosomal regions might be identified using translocation crosses, since only a small number of

such crosses are needed to. examine the effects of most chromosomal regions. Ongoing experiments of this kind in my lab suggest that there are regions that affect ventral furrow formation in ways that cannot be explained by any Heidelberg genes. Most of these morphological effects appear to be transient, similar to the case in *fog* mutant embryos, embryos that are dele-, ted for these genes eventually internalize their mesoderm. It is therefore possible that point mutations in genes responsible for these early morphological effects may not cause lethality or easily visible effects on final morphology.

THE RELATIONSHIP BETWEEN CELL FATE AND CELL FORM.

The efficiency with which mutagenesis screens identify mutations affecting cell fate but not cell form may point to a difference in the way the two processes are controlled. The existence of mutations affecting cell fate suggests that patterning functions are not redundant, each gene being absolutely essential for normal development. Having only single, non-redundant factors controlling cell fate might avoid ambiguity in determined states; in this view, a precise cell by cell pattern of gene expression may not be as important as having at least some cells unambiguously programmed to each fate. Once a cell choice has been made, it must be realized in morphological terms. At this point there may be an enormous advantage for redundancy, since multiple pathways might ensure that a particular fate once chosen is actually achieved. This redundancy need not be total; elimination of a single target pathway may causes subtle deviations in the process similar in magnitude to the defects observed in the ventral furrows of *fog* embryos. In this view, cell shape changes at gastrulation would be the summed effect of various targets, each of which would be necessary for the characteristic wild type morphology, but not for the final outcome itself.

Developmental biologist are a diverse lot; some are more interested in cell fate and patterning, others in morphogenesis. Those interested in pattern might focus on genes like *twist* and *snail,* since their expression at the blastoderm stage determines the ventral cells to become mesoderm (Fig. 4A). In

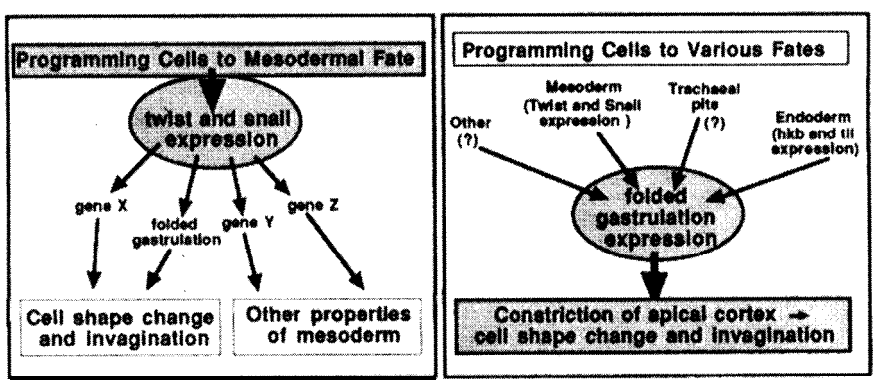

Figure 4. *Contrasting views of devolopment* **. Panel A emphasizes programming of cells to specific cell fates, whereas panel B emphasizes the cellular mechanisms that produce changes in morphology.**

this view, genes like *fog* would be just one of many downstream regulators. On the other hand, the cell shape changes that occur in mesodermal cells during ventral furrow formation are not restricted to gastrulation. Genes like *fog* are in fact expressed at other stages and in other primordia, not under control of *twist* and *snail*. Thus from the standpoint of the cellular morphology, genes likefigbecome the central players in a research program (Fig. 4B). *Fog* dependent cell shape changes may be activated in a variety of different developmental programs by a variety of different cell fate genes, but its role in cellular behaviour would be conserved.

CONCLUSIONS: THE POWERS AND LIMITATIONS OF THE GENETIC APPROACH

Mutagenesis experiments are a mixture of random and biased elements. Perhaps their strongest advantage is that we scientists have very little control over the kind of mutations produced. Mutations occur randomly throughout the genome, eliminating genes whether or not they have anything to do with the process of interest. This means that the production of mutants is usually not affected by biases about the kinds of molecules involved or the underlying mechanisms. Success depends on the experimenter's ability to sort through the mutations obtained and recognize and interpret phenotypes of interest. It is this second step that introduces the major bias; it depends on the individual's response to mutant phenotypes, on his or her ability to understand the nature of the defect and the proper context in which it can be described. Because we used a dual observation microscope, Christiane and I were often simultaneously confronted with particular phenotype for the first time. It was not always easy to agree about defects, or to decide why a particular stock was interesting. Because they were often the product of intense discussions, our final descriptions benefited from a dual input. However, Christiane and I were both primarily interested in spatial patterns and gross morphological aspect of embryogenesis. Mutations affecting finer aspects of cell differentiation were certainly produced in Heidelberg, but even if they had caused embryonic lethality, they would have been discarded.

In addition to the bias introduced by experimenters, genetic screens are inherently biased by the organism. *Drosophila* embryos develop rapidly and most gene products are supplied by maternal rather than zygotic transcriptions. Development is highly regulative and cell signaling pathways ensure that a constant final morphology is achieved even when starting situations are different. One obvious limitation of the Heidelberg screen is that even though we were interested in broad aspects of morphology and early cell shape changes, we scored only final differentiation, and there only cuticle pattern. Because the hypodermal cells that secrete cuticle represent a large fraction of the blastoderm, we assumed that it would be not be possible to cause morphological changes at gastrulation without altering final morphology. Our later experiments suggest that this may not be true. Thus although

we were not necessarily biased between mutations affecting cell fate and cell form, most genes actually detected altered fate, perhaps because effects on form in *Drosophila* are often more transient.

It is possible that different results would have been obtained had we chosen a different organism. One is curious whether nematodes, which show, extraordinarily constant lineages, would be more subject to subtle perturbations in the movement and positioning of cells than in *Drosophila.* Development in vertebrates on the other hand has been shown to be much more variable in terms of cell lineage and much more regulative in achieving constant final morphologies. In that respect, the ongoing screens for mutants in zebra fish and mice may produce a spectrum of genes more similar to that of *Drosophila.*

Of course, one of the important lessons from the Heidelberg experiments is that speculations have little predictive value. The phenotypes and genes actually obtained by a particular mutagenesis protocol will only be known once the experiments are done. The same lesson applies to the ongoing analysis of *Drosophila* development.In spite of enormous advances made in understanding cell fates, we do not yet know how those programs produce constant morphology and structure. The morphologies assumed by *Drosophila* cells are often very similar to those in vertebrates. Moreover, the molecules and therefore the underlying mechanisms have probably been conserved over the course of evolution. By bridging the connection between cell fate and cell form in *Drosophila, **we*** may therefore also reinforce the bridges that connect developmental studies in many different organisms.

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