

THE IDENTIFICATION OF GENES CONTROLLING DEVELOPMENT IN FLIES AND FISHES

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by

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INTRODUCTION

In the life of animals, complex forms alternate with simple ones. An individual develops from a simple one-celled egg that bears no resemblance to the complex structure and pattern displayed in the juvenile or adult form. The process of embryonic development, with its highly ordered increase in complexity accompanied by perfect reproducibility, is controlled by a subset of the animal's genes. Animals have a large number of genes. The exact number is not known for any multicellular organism, nor is it known how many and which are required for the development of complexity, pattern and shape during embryogenesis. To identify these genes and to understand their functions is a major issue in biological research.

Genes can be detected by mutations, changes which affect their function. Compared with other experimental approaches, mutations provide a uniquely powerful tool for studying the role of individual components in development: Primarily, only a single component, the gene product, is removed or altered, while the remainder of the organism is left intact. The function of a gene can be deduced from the mutant phenotype, the way the animal develops in the absence of the primary gene product. The mutant phenotype provides most useful information about a gene function. Genes with similar phenotypes are likely to have similar functions, and their products are likely to cooperate in a developmental process.

Mutations occur spontaneously at a low rate, but their frequency can be increased using X rays or chemicals which alter the DNA sequence. This property was first used to systematically score for mutations affecting processes of interest in bacteria and fungi (1-3). Mutations affecting developmental processes had been collected in *Drosophila melanogaster* more or less fortuitously, starting with the *Bithorax* mutation by Bridges in 1922 (4). In addition, a small number of embryonic mutants including *Notch* had been described in detail by D. Poulson and collaborators (summarized in (5)). In the seventies, more systematic approaches were started. In the nematode, *Caenorhabditis elegans*, screens were begun by S. Brenner for mutations which

alter the stereotyped pattern of postembryonic development (6). E. Lewis in the California Institute of Technology collected a number of mutations causing homeotic transformations in the adult and the larva of *Drosophila* (7). In 1979, Eric Wieschaus and I, at that time in the EMBL, Heidelberg, had developed the methods for the large scale screening for embryonic lethal mutations in *Drosophila*. The screening procedure focused on the segmented pattern of the larval epidermis (8). In this and subsequent screens, a number of new genes acting in the embryo and required for the formation of a morphologically normal larva were discovered (9-11). Similar screens in several laboratories have led to the identification of genes that are expressed by the maternal genome, and whose products, laid down in the unfertilized egg, control the expression of the zygotic genes (12-16). The subsequent phenotypic analysis and molecular cloning of many of these genes, and investigation into the interactions of their products, resulted in a rather complete general picture of the mechanisms establishing the anteroposterior and the dorsoventral axis of the early embryo (17-19). These mechanisms now provide a useful paradigm for the development of complexity from a simply shaped egg cell.

Drosophila is a fly and as such has rather special properties. It is no more or less "special" than a worm or a frog, but in many respects very different from those animals. Therefore it was not clear a priori to what extent the results obtained in *Drosophila* could be generalized, and how much we could learn from them for an understanding of the development of other animals, in particular vertebrate species. Knowledge of vertebrate embryogenesis had been collected predominantly in experiments in frogs (20) and chickens, but only to a very small degree using genetic approaches. Because of the small size and the turgor under which *Drosophila* embryos develop, transplantation of tissues as done in frog and chicken is hardly possible in *Drosophila*, and the power of systematic mutant searches for the analysis of complex processes, although highly desirable, cannot readily be applied to most vertebrates. Therefore, the description and understanding of the development of animals from the two phyla-arthropods with *Drosophila*, and vertebrates with frogs and chicken, mice and man - were on such different levels, that for a long time a comparison seemed almost pointless. However, the development of recombinant DNA technology and with it the cloning of genes on a large scale allowed a comparison of genes from different organisms on the basis of DNA (or protein) sequences. Two most important findings emerged from these studies:

1. The biochemical function of *Drosophila* gene products, in many cases, could be deduced from a comparison of their amino acid sequences with that of related and well characterized proteins from other organisms, such as mammals, bacteria, or yeast. This revealed that many of the components controlling development are members of well known classes of proteins, such as transcription factors, protein kinases, secreted signalling molecules or receptors.

2. In many instances, the similarity between *Drosophila* and vertebrate proteins was found not to be restricted to their biochemical properties, but to extend to a true homology of function in a developmental process. This homology is apparent both in similar expression patterns and in the phenotypes obtained by eliminating the gene function by homologous recombination in mice. These studies lead to the surprising conclusion that the basic features of body organization, such as specification along the anteroposterior axis, and polarity of gastrulation, are apparently conserved in organisms of different animal phyla (21, 22). This conservation suggests the existence of a common basic body plan, originating from common ancestors, the first bilaterally symmetrical organisms, in evolution.

The investigation of genes discovered by their homology to *Drosophila* genes is now one of the most successful approaches to an understanding of the genetic control of vertebrate development. Although the elegant method of homologous recombination in the mouse allows the introduction of mutations into the chromosomal copy of any previously cloned gene (23), there is no way of predicting which genes are indispensable in development and will therefore give an informative phenotype in knockout mice. An important reason for the success of the homology approach is based on the fact that the *Drosophila* genes in question represent a small fraction of the animal's genes, that were selected, through mutagenesis experiments, for being crucial and indispensable in development. Their vertebrate homologs often also have unique functions as revealed in the mouse (24).

Approaches based on the homology between invertebrate and vertebrate genes focus on conserved properties and therefore select against the features which make these animals different. Vertebrates have acquired specific structures and novel mechanisms during evolution. In order to identify genes affecting such functions, it is necessary to do mutagenesis screens directly in a vertebrate organism. Therefore, several laboratories have established methods to use the zebrafish as a model organism to analyze the genetic control of embryonic development in a vertebrate (25-28).

In this lecture I would like to discuss the *Drosophila* screens and their most important results, but also their limitations. I will compare them with the results of a large scale screen for mutations affecting development and pattern in the zebrafish recently performed in my laboratory in Tübingen (29).

THE *DROSOPHILA* MUTANT SCREENS

As a model organism for genetical studies of development, *Drosophila* has a long tradition and is by far the most well established organism available (30). Also, it proved to be quite well suited for studying embryology. Some properties of *Drosophila* are listed in Table 1. Its small number of chromosomes made possible the development of many genetical tools such as balancer chromosomes carrying multiple inversions which prevent recombination, visible markers that allow the scoring for the absence or presence of par-

Table 1: Properties of *Drosophila melanogaster* and *Danio reio* for genetical and embryological research

	DROSOPHILA	DANIO
adult size	4mm	40 mm
space requirement	1000 / liter	10 / liter
generation time	14 days	3 months
life time	6 weeks	1-2 years
egg size	0.15 x 0.5 mm	0.8 mm
embryonic development	24h	48h
fecundity	50 eggs / day / female	200 eggs / week / female
number of chromosomes	4	25
advantages for genetical research	polytene chromosomes marker mutants balancer chromosomes conditional lethals	in vitro fertilization haploid development homozygous fish freezing of sperm
advantages for embryological research	mechanical hatching cuticle preparations external fertilization	external segmentation synchronous development optically clear embryo

ticular progeny, and conditional lethal or sterile mutations making selection systems possible. These tools were invaluable in systematic mutagenesis screens for mutations causing lethality or sterility (Figure 1). In combination

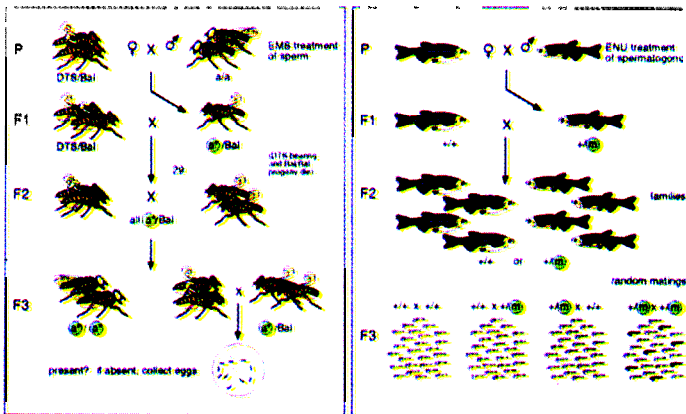


Figure 1: Crossing schemes for isolating mutants with embryonic visible phenotype in *Drosophila melanogaster* (left) and *Danio reio* (right). In *Drosophila*, eye colors and wing shape are markers useful for the distinction of carrier animals from noncarriers, which inevitably are produced in the crosses required for inbreeding. They also are useful for determining whether a lethal mutation was induced (absence of white eyed progeny in the F3). Marking and selecting systems, i. e. balancers, recessive mutations and dominant temperature sensitive mutants useful for selection, are available for each major chromosome, but it is not easy to do screens for the entire genome at once, because the multitude of markers significantly affects viability. *Drosophila* larvae hatch mechanically, a process that is very sensitive to perturbations. A large fraction of lethal mutations prevent hatching, although most of them do not visibly affect the embryonic pattern. Mutant embryos can be separated as unhatched from their normal siblings. In the zebrafish, the large number of chromosomes makes the development of marking systems impractical. Instead, the entire genome is scored at once, and in each generation, carriers must be recognized by the production of mutant progeny with a visible phenotype. Mutations causing lethality without a visible mutant phenotype cannot readily be detected. In the F3, only a quarter of the crosses will yield mutant embryos (25% of all embryos), as only half of the F2 fish share a particular mutation, but there is no way to distinguish carriers from noncarriers.

with the giant chromosomes that provide a physical measure for the number of genes, it is possible, in *Drosophila*, to determine rather accurately the total number of genes essential for survival and fertility. About 5000 genes mutate to lethality. The number of genes required for fertility is less well defined, but is probably not more than one thousand. However, the total number of genes, defined as transcription units, is much larger, about 20000. This means that in *Drosophila* the majority of the genes do not have indispensable functions. A large fraction, about one third of all lethal mutations, cause a failure of the embryo to hatch (embryonic lethality), however, only about ten percent of the embryonic lethals show an easily detectable and specific morphological phenotype in the unhatched differentiated larvae.

The *Drosophila* larva displays a clear axial organization with many landmarks of position and polarity, which are provided by the external cuticle shed by the larval epidermis (Figure 2). This larval skin is derived from a

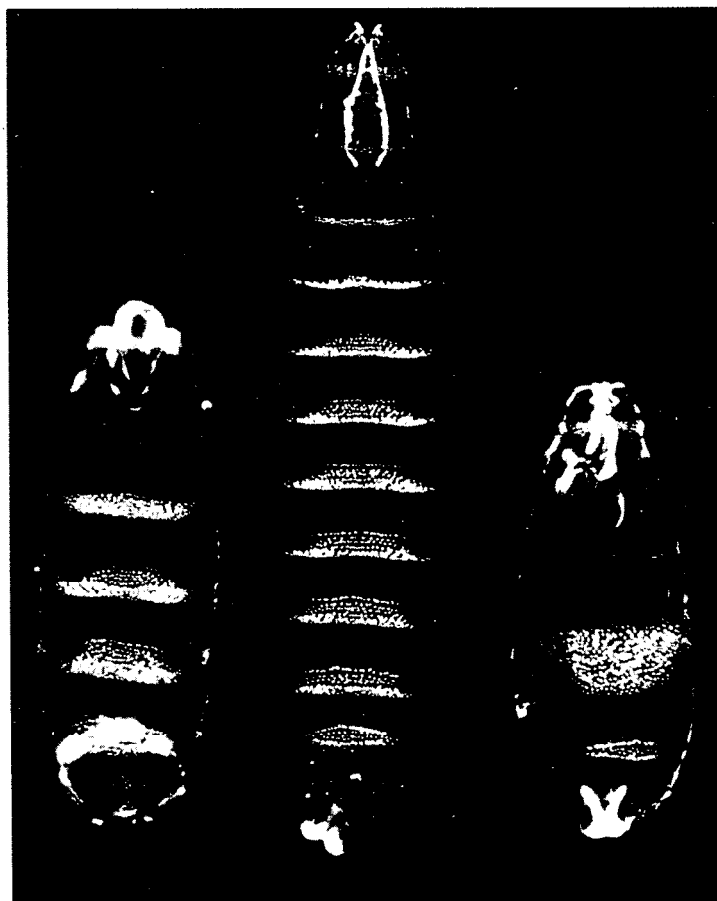


Figure 2: The cuticular pattern of segmentation mutants. The picture shows the central view of a normal larva (middle), and a mutant *pawed larva* to the left, and *knirps* to the right. *paired* is a pair rule gene, and mutants produce approximately half the normal number of segments, every other segment is deleted. *knirps* belongs to the gap class of segmentation genes, most of the abdominal segments, marked by the prominent denticle belts seen in the normal larva, are deleted. Anterior up

large portion of the embryonic fate map, while the remainder of the blastoderm largely gives rise to internal organs which are less visible in the living embryo. However, while for the skin an excellent and simple fixation technique is available, at the time of the mutant screens there were no efficient methods to allow visualization of the internal organs, which are hidden, by the opaque yolk. This problem has been overcome now by the development of molecular probes and antibodies, and a number of screens have used these for the detection of mutants, albeit every screen has been for a rather limited range of phenotypes. The properties and advantages of *Drosophila* for the genetical analysis of embryonic pattern formation are summarized in Table 1.

In the Heidelberg screens (9-11), we collected mutants detectable by a distinct and significant deviation from normal patterning of the cuticle. These mutants, after complementation tests, defined about 130 genes, distributed randomly over the three major chromosomes. Using similar scoring criteria, screens for maternal mutants, defining about 30 genes, were performed in several laboratories (12-16). Without molecular markers, most of the mutant phenotypes could not readily be analyzed and interpreted at the time. We used rather pragmatic criteria both for screening and characterization of the mutant embryos. By similarity in phenotype, several groups of genes were recognized that probably were affecting the same or related developmental processes. Subsequent phenotypic and genetic analysis, followed by the molecular cloning of the genes, frequently supported this notion. The groups of genes identified in the mutant screens are shown in Table 2.

In our screens, we put great emphasis on completeness, i. e. on the saturation of the genome with mutants detectable by our criteria. Several lines of evidence, such as allele frequency and comparison with phenotypes of deletion mutants, supported the notion that the majority of genes which mutate to a phenotype visible in the larval cuticle had been discovered in our screens. As an additional generation of inbreeding is required, the screens for maternal mutants were more demanding and difficult than those for embryonic lethals. Therefore, it is likely that the degree of saturation for the maternal genes is not as high as that for the zygotically expressed genes. While the screens were successful in the identification of many important genes, and screening more lines would not have significantly increased the number of genes discovered, we were aware of a number of severe intrinsic limitations of our approach:

Genetic redundancy: At the time, the viewpoint held amongst most *Drosophila* geneticists was that genes not mutating to lethality or sterility were almost not worth studying. There was the tacit assumption that the great majority of all genes were essential for survival or fertility. Concern about redundancy and duplicated genes was a later issue, as it depended on the physical isolation of a gene by cloning, whereas in "precloning times" a gene was only detectable by a mutation causing a phenotype. However we took redundancy as a theoretical possibility into account. Among the segmentation genes, a number of cases of gene duplications have been fortuitously dis-

Table 2: The *Drosophila* gene classes

CLASS	subclass	genes	cloned	vertebrate homologs
MATERNAL	anterior	4	4	0
	posterior	8	6	2
	terminal	6	4	1
	dorsoventral	12	10	3
ZYGOTIC	gap	9	7	1
	pair rule	8	8	4
	segment polarity	13	8	6
	homeotic	11	9	
	head morphology	10	4	0
	dorsalized		4	3
	ventralized	6	5	2
	dorsal holes	8	6	3
	others	8	2	0
	neuralized	6	6	2
	midline	5	5	
	epithelia	4	2	2
	cell cycle	6	4	0
	denticles and hairs	4		0
	pigmentation	5		
	tracheae	2	0	0
	hyperactive	4	1	0

covered during the molecular analysis. As methods of reversed genetics are not generally applicable in *Drosophila*, the proportion of redundant genes cannot readily be determined. It is not at all clear why some genes are duplicated and others not.

Maternal and zygotic contribution: Another limitation we were more concerned about was that genes whose products were required both maternally and zygotically would be difficult to detect in mutant screens. This concern applied in particular to the maternal mutants: in cases of additional functions in the embryo, mutations in genes with important maternal functions might be lethal zygotically and therefore cannot be discovered in maternal screens. A number of such cases have been found. In addition, in cases where the product is provided maternally, the zygotic phenotype may not represent the complete lack of function. To this day, the genes required both maternally and in the zygote are not easily accessible to investigations of function.

AXIS DETERMINATION IN DROSOPHILA

A large fraction of the genes identified in both the zygotic and the maternal screens affect either the anteroposterior or the dorsoventral axis. The pattern of the larva is composed of a series of segments that change their character from anterior to posterior. At both ends, nonsegmented terminal structures are located. The pattern also displays a clear dorsoventral dif-

ference, although the ventralmost (mesoderm) and dorsalmost (amnioserosa) structures of the embryonic fate map are not represented in the epidermal pattern.

The anteroposterior axis: We defined three classes of zygotic segmentation genes, which we called gap genes, pair rule genes and segment polarity genes (8). In mutant embryos of the gap genes, large unique regions are deleted. In pair rule mutants, deletions in the pattern affect homologous regions in every other segment (Figure 2), while every segment shows a defect in mutants of the class of segment polarity genes. The phenotypes suggested that the process of segmentation involves at least three levels of spatial organization: Processes beginning during oogenesis define the large unique regions requiring the gap gene function, the gap genes in turn control a repeat pattern with the periodicity of double segments. Finally, the individual segments are established as developmental units in response to the action of the pair rule genes (8).

The subsequent molecular analysis of the properties and function of the segmentation genes was carried out in many laboratories. In these studies, the method to make transgenic flies using P element induced germline transformation, developed by Alan Spradling and Gerald Rubin, opened up a rich repertoire of powerful approaches (31). Many of the segmentation genes are transcription factors that control the expression domains of other segmentation genes, either within the same or of the downstream class. The pattern of expression of many segmentation genes in first approximation corresponds to the deletion pattern displayed in cuticle preparations (18). During early embryogenesis, a series of such molecular prepatterns, composed of the expression domains of transcription factors, which are the products of the segmentation genes, is formed. The gap genes are expressed in large unique regions early in embryogenesis, their expression patterns being controlled by maternally provided transcription factors (18). The earliest metameric pattern, that of the pair rule genes, has 7 stripes that are determined one by one by the action and interaction of a particular combination of gap gene products (32,33). The pair rule genes finally control the pattern of more than 14 stripes preceding the formation of the morphological pattern, the segments (34) (Figure 3).

The identification and analysis of the maternal genes affecting the segmentation pattern revealed that the anteroposterior axis is controlled by three groups of genes, each independently determining a subset of the pattern, the segmented anterior or posterior, or the unsegmented terminal regions of the embryo (35-37). Although the molecular processes by which the three groups function are largely different, they share some common features: Spatial signals are produced that are localized at the anterior or posterior egg pole. In each group, a series of molecular interactions finally results in the formation of a transcription factor gradient with the highest concentration at the site of the localized signal, spanning a substantial portion of the egg length (Figure 2) (38). These transcription factor gradients

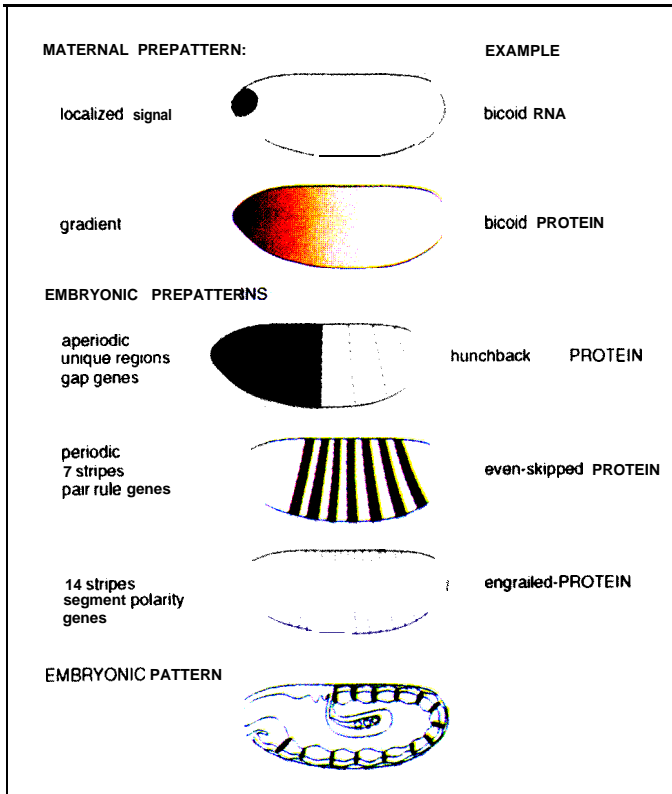


Figure 3: The hierarchy of genes establishing the anteroposterior pattern in *Drosophila melanogaster*. In every tier, the distribution of the protein product of one representative gene of its class is shown in color. Anterior is left, ventral at the bottom in each picture.

determine the expression of the gap genes in a concentration dependent manner, thereby defining the first subregions of the embryo.

The dorsoventral axis: Two classes of zygotic genes have been identified, mutation of which cause a dorsalized or ventralized phenotype (39). In *Drosophila*, the mesoderm is formed during gastrulation in the ventral region of the egg, its formation depends on the expression domain of two zygotic transcription factors. The refinement of the dorsoventral pattern on the dorsal side involves a long range signalling process, unlike the series of prepatterns of transcription factors observed along the anteroposterior axis (40). The maternal control of the dorsoventral pattern is achieved by only one group of genes that establish a nuclear localization gradient, with its maximum at the ventral side, of a transcription factor that functions both as a repressor of dorsally expressed genes and an activator of ventrally expressed genes (17, 19). The first zygotic expression pattern divides the embryo into at least three domains from dorsal to ventral.

MOLECULAR MECHANISMS OF PATTERN FORMATION

Despite the limitations of the mutant screens discussed above, the collection of genes within each phenotypic group and thus of components of a developmental pathway appears to be relatively complete. In several instances, the molecular interactions of the components within and between pathways have been elucidated in some detail. These examples show how complex patterns can develop from a small set of independently localized signals. A principal mechanism by which spatial complexity is increased is based on concentration gradients of morphogens, molecules that elicit different responses at different concentrations. An example of this mechanism has been discovered by studying the maternal control of the expression pattern of the gap gene *hunchback* by the morphogen Bicoid (41-43). Bicoid is the transcription factor determining the anterior pattern, it is distributed in a concentration gradient with its maximum at the anterior pole of the egg and controls the transcription of several target genes in a concentration dependent manner. Morphogen gradients can be formed by diffusion of the protein translated from a localized mRNA source, as in the case of the anterior and posterior maternal gradients (Figure 2) (35, 37). In other cases, gradients are apparently produced by diffusion in the extracellular space (44). Their spatial distribution is communicated to the interior of the egg cell via a ligand-receptor based signal transduction mechanism (45). In the case of the dorsoventral axis, this mechanism results in the formation of a gradient of nuclear localization of the initially equally distributed transcription factor Dorsal (46, 47). During segmentation, the series of transcription factor patterns with increasing spatial complexity develops by concentration dependent transcriptional activation and repression, involving combinations of transcription factors (18). The determination of the final molecular prepatter depends on short range signalling between adjacent cells (48, 34). This pattern- immediately precedes the first morphological changes occurring during segment differentiation.

LIMITATIONS OF THE *DROSOPHILA* SCREENS

The mechanisms by which axes are established in *Drosophila* are among the best understood examples of pattern formation to date. Because of some special features of early development in the *Drosophila* embryo, they may however not always have an immediate parallel in other animal classes. This restriction applies in particular to the processes requiring diffusion in a syncytial embryo, as in the case of the formation of the Bicoid gradient. On the other hand, examples such as the formation of gradients by diffusion in the extracellular space, signal transduction, and the establishment of a nuclear localization gradient, as in the case of Dorsal, may prove to have analogies in pattern forming processes in multicellular tissues. The genetic analysis of pattern formation in *Drosophila* teaches us about the logic of pattern formation in a complex system, it also led to the discovery of some basic mech-

anisms which allow an increase in complexity during the development of multicellular organisms. On the other hand, many aspects of animal development, in particular the formation of organs and their functions, have not been studied as thoroughly in *Drosophila*. The reason for this restriction is the fact that we relied on the epidermal pattern when scoring mutant phenotypes in our screens. Obviously, this limitation was very apparent to us, although we tried to use it to our advantage. It limited, with rigid criteria, the number of genes to be concerned with. However, many mutations could not be recovered by the scoring of the cuticular pattern only. To this day, a systematic screen for mutants with defects in inner organs in *Drosophila* has not been performed, however, a number of genes with particular functions in inner organs were identified in special screens using specific molecular probes (49), or by other means such as the very powerful enhancer trap screens (50, 51). A crude classification of the *Drosophila* genes discovered in the screens is presented in Table 2. To this day more than 60% of them have been cloned.

Many *Drosophila* genes have been shown to have homologs in vertebrates. This homology is not restricted to amino acid sequence and to their biochemical function, but extends to the biological role played in development. This remarkable conservation came as a great surprise. It had been neither predicted nor expected. One of the first indications came with the discovery of the *Hox* clusters in vertebrates, and the conservation of the colinearity of their expression domains with the chromosomal location (52, 22). This was followed soon by the isolation of homologs in vertebrates of other homeobox containing genes, and it now seems that a large fraction of the *Drosophila* genes that we found in our screens have vertebrate homologs. It is difficult to give an estimate of this fraction, because not in all cases have homologs been looked for, however, it may be significant that among the maternal genes very few are known to have vertebrate homologs so far (Table 2). This may reflect the very great differences observed in very early development between vertebrates and invertebrates. A large fraction of the vertebrate homologs of *Drosophila* genes have been shown to play an important role in pattern formation in the mouse. Important patterning pathways appear to be conserved, such as the signalling pathway involving TGF- β like growth factors, and homologs of *hedgehog* and *wingless* (21, 53, 54). Most of the components of such pathways have been detected by phenotypes visible in the segmented pattern of the *Drosophila* larva. In the vertebrate body organization they are not always required in this context. For example, genes involved in segmentation in *Drosophila* also participate in patterning of the brain and the limbs in *Drosophila* as well as in the vertebrate. Although the collection of the *Drosophila* pattern genes provides a rich source for the study of some aspects of vertebrate development, this sample is strongly biased, probably providing but a small fraction of the genes controlling patterning in a vertebrate embryo. In order to obtain a more complete collection of those genes, a direct screen for mutations in a vertebrate organism is required.

THE ZEBRAFISH SCREEN

The zebrafish, *Danio rerio*, was selected as a model with potential for genetical research by the late George Streisinger (28). In the past 20 years a number of useful methods have been developed for the breeding and genetic analysis, as well as for the investigation of embryonic development of the zebrafish (25-27). It is interesting to compare flies and fishes regarding their properties for genetic and embryological research. Obviously, the long generation time and space requirement for rearing of zebrafish make large scale genetic screens difficult. In addition because of technical and logistical problems in the running of large numbers of aquaria, working with fish is more costly and labor intensive than with flies. On the other hand; compared to mice, the more traditional vertebrate organism used in genetical research, zebrafish offers the advantages of extrauterine development, and high fecundity. In fish, in vitro fertilization, haploid development of embryos until the larval stage, diploidisation, and the freezing of sperm samples is possible (Table). These techniques are invaluable in the maintenance and analysis of a large number of mutants, and are not available for flies. The greatest advantage of the zebrafish, however, lies in the nature of its embryonic development. Zebrafish eggs are fertilized synchronously, they develop rapidly outside the maternal organism, and, most importantly, they are initially completely transparent. This allows the observation of the development of many tissues and organs directly in the living embryo and at subsequent stages without the necessity of fixing, clearing and staining (55) (Figure 4).

The early embryogenesis of zebrafish is very different from that of flies. A period of rapid synchronous cleavages results in a three-dimensional mass of cells, unlike the two-dimensional blastoderm formed in the *Drosophila* embryo. Gastrulation in the fish involves several morphogenetic movements, such as involution of mesendodermal regions and dorsal convergence (55). Although appearing very different, these movements might be related to the invagination of mesodermal and endodermal anlagen, and ventral condensation and extension of the germ band in the *Drosophila* embryo. They result in a multilayered embryo, the pharyngula or phylotypic stage, displaying the typical vertebrate body organization (Figure 4).

While in the *Drosophila* embryo fate mapping studies allow the construction of two-dimensional maps of the early gastrula with distinct and clearly separated anlagen, in the fish the fate of cells within particular gastrula regions is much less precisely predictable. The fish gastrula is multilayered. Large regions from which different organs or tissues later develop are overlapping and superimposed. This difference may reflect quite distinct developmental mechanisms determining fate of cells in the fish and fly embryo. A comparison of a fly and fish fate map reveals further dramatic differences between the relative size occupied by the anlagen for various organs: for

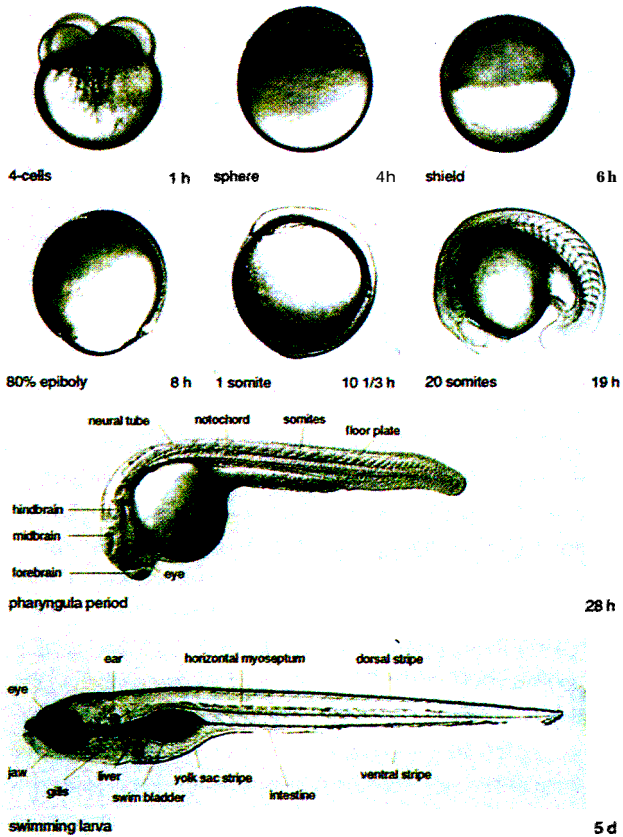


Figure 4: Embryonic development of the zebrafish. Photographs of live embryos at different ages. With the exception of the swimming larva, the embryos were dissected out of the chorion for photography. In the early embryos, dorsal is right. In the pharyngula and swimming larva, dorsal is up and anterior to the left. Characteristic structures scored in the mutant screen are indicated. Stages are according to (55).

example in the fish the brain anlage occupies a very large fraction of the gastrula, while the epidermis is rather inconspicuous, compared to its dominance in the fly map. To a certain extent, the sizes of the anlagen reflect the importance and visibility of the structures that can be scored in a mutant screen.

The crossing scheme used to make mutants with phenotypes visible in the embryo or early fish larva is displayed in Figure 1. For the fish, ENU treatment of spermatogonia has been used with a similar efficiency of mutagenesis as that obtained by EMS treatment of sperm in the fly (about 1 mutation per gene per 1000 genomes) (26). In contrast to the fly screens, in the fish no markers can be used to help distinguishing carrier from non carrier fish in the F2. Therefore, in order to detect a mutation induced by the treatment of parental males, a number of crosses have to be set up, of which only 25% on average yield progeny that are homozygous for a mutation. As fish hatch enzymatically, most homozygous mutant embryos also hatch, and therefore, with few exceptions, cannot be selected as nonhatchers, a useful way to iden-

tify mutant embryos in the *Drosophila* screens. Mutant fish embryos can be recognized by their phenotypes only. The lack of markers and preselection is compensated somewhat by the fact that the entire genome is scored at once, and not chromosome by chromosome. In the case of the fly, three separate screens, using a total of more than 20000 inbred families, were performed, to obtain about 600 mutants in 130 genes (9-1 1). In the fish 3100 families were screened, with an average of 4.2 crosses, corresponding to the screening of 3800 genomes (Figure 1) (29). As the breeding of such a large number of fish families is necessary to achieve a reasonable degree of saturation for any given phenotypic trait, this screen was done as a collaborative effort of 12 scientists. We screened as many different phenotypic traits as could be efficiently scored. We isolated a total of 1200 mutants, of which to date about 900 have been characterized both phenotypically and genetically. They define 350 genes, of which 150 have more than one allele (29).

The different features of development of the fish embryo were scored at different times in development, as the structures and organs become visible with age (Figure 4). On the second day of development, the somites, notochord and brain were scored, while after hatching heart, blood, musculature, fins, eye, ear and other features can be studied. Fish of the larval stage, the latest time scored, almost one week old, have developed a complete set of internal organs, liver, gut, pancreas, kidney, can see and respond to stimuli, and are pigmented with three types of pigment cells. They still display a pigment pattern characteristic for the larval stage, and the fin structure does not yet completely correspond to that of the adult fish (Figure 4). There are structural features of the fish which are recognized with safety and ease, while others are less conspicuous and therefore abnormalities may not always have been detected. The large degree of complexity, which is far more than that displayed in the cuticular pattern of the fly larva, allows a much broader spectrum of organs and tissues to be investigated, but it also poses considerable demands on the skill and expertise of the experimenter. The recognition of a particular phenotype is greatly enhanced if an interpretation of the phenotype is available, or if it has been seen before. Many mutants have rather general defects, they have not been kept for analysis (29). Mutants with complex or subtle phenotypes resembling rather general defects are not always recognized as interesting, or interpreted correctly. This means that the degree of saturation varies among different phenotypic classes (Table 3). On average it is certainly less than that achieved in the fly screens.

According to their predominant phenotypic traits, the genes can be classified in a large number of groups with similar phenotypes. Classification of each mutant depends on the interpretation placed on its phenotype, and this in turn on the degree of phenotypic analysis that can be performed. In the fish, a number of molecular probes are available that serve as markers for particular regions at specific developmental times. The expression patterns of such markers were very useful in characterizing many of the mutant phe-

notypes. The classes of fish genes identified by the mutants are shown in Table 3. A small number of classes have a parallel among the classes of fly genes, while the majority of phenotypes are novel. The collection of the fish gene classes is so different from that of the fly genes that a comparison is possible only in selected cases. Regarding the most prominent fly gene classes, gastrulation and segmentation, however, a small number of fish genes might reveal similarities to fly processes.

Table 3: The zebrafish gene classes

CLASS	subclass	genes	alleles	alleles/ gene
EARLY	epiboly, arrest	11	16	1.5
	ventralized	2	4	2
	dorsalized	5	16	3
	others	13	19	1.5
MESODERM	notochord	15	67	4.5
	somites	8	26	3.2
CNS	brain	19	37	1.9
	midline	10	19	1.9
ORGANS	blood	9	23	2.6
	heart, circulation	22	28	1.4
	liver, gut	6	6	1
	muscles	18	63	3.5
	ear	8	11	1.4
	fins	19	46	2.3
NEURAL CREST	pigment pattern	13	60	4.6
	pigment cells	10	39	3.9
	jaw and gill arches	61	150	2.5
MOTILITY	reduced	17	30	1.8
	abnormal	15	50	3.3
		15	53	3.5

THE ZEBRAFISH GENE CLASSES

Gastrulation is affected in two gene groups, the phenotypes of which display a partial dorsalisation or ventralisation (56, 57). In *dino* mutant embryos, dorsal anterior structures are reduced, while ventral posterior structures are increased in size. This results in embryos with small heads and large tails. The effect is most pronounced in the structures derived from the ventral-most position in the fate map: the blood and ventral tail fin, that is increased in size in *dino*. Using molecular markers that are expressed before gastrulation in a polar manner, it is apparent that the fate map is distorted already at an early point in fish embryogenesis (Figure 5A). As suggested by a characteristic duplication of the ventral tail fin, mutants in the *mercedes* gene have a role in the same process (56) (Figure 5K). A phenotype complementary to that of *dino* and *mercedes* is displayed in mutants in a number of genes with a dorsalized phenotype. Swirl embryos, for example, have much enlarged



Figure 5: Zebrafish mutants. In the pairs of panels, a wildtype embryo is shown together with a mutant embryo, which is to the right or below the wildtype. Anterior is left and dorsal up except when otherwise noted. A *dino*, *fkd 3* in situ hybridization, shield stage. Optical section trough the germ ring, dorsal right (56). B *fused somites*, living embryos, 12 somites stage (65). C *floating head*, *myoD* in situ hybridization, the brown staining in the wildtype marks the notochord with *ntl* antibody, dorsal view (69). D *masterblind*, living, swimming larval stage (76). E *white tail*, *islet 1* antibody, 8 somite stage, dorsal view ('72). F *swirl*, *myoD* in situ hybridization, 8 somite stage, anterior up (57). G *you*, living, swimming larva, (65). H *diwanka*, *znp-1* antibody, pharyngula (78). I *van gogh*, living, swimming larva (79). J *leopard*, *obelix*, adult fin (29). K *mercedes*, swimming larva, view on tail fin (56). L *no isthmus*, brown: 4D9 (Eng) antibody, blue: *Krox 20*, 8 somite stage, dorsal view (80). M, N *fused somites*, M *myoD* in situ hybridization, 14 somites, dorsal view, N skeletal staining, adult. (65). O *sucker* alcian blue staining of cartilage, swimming larva (81).

pax-axial mesoderm, the forming somites encircle the entire embryo (Figure 5F), and ventrally derived structures such as blood and the ventral tail fin are

reduced or lacking entirely (57). These groups of genes are reminiscent of genes with dorsalizing and ventralizing phenotypes also observed in *Drosophila*. For genes of both groups, homologs in the frog and fish have been cloned and shown to be expressed at opposite regions of the blastula (58-61). These and other observations led to the revival of an old theory, postulating that the orientation of the dorsoventral axis in invertebrates is inverted compared to that of vertebrates (62-64). The fish genes are likely to provide components in these conserved pathways.

An interest in the mechanism of segmentation in vertebrates was an important incentive for the mutant screens in fish. In contrast to flies, which have a clearly segmented ectoderm, the mesoderm is the primary vertebrate tissue that is organized in a metameric pattern. We did not find many genes in the fish that have similarities to fly segmentation genes. The closest are mutants in which the somites, the earliest visible repetitive structures formed in the fish embryo after 10h of development, are fused. Five genes have a phenotype with fused somites (Figure 5 B, M, N) (65). The somite pattern in the fish embryo is much less complex than the segment pattern of the *Drosophila* larva. However, the vertebrae that develop in postlarval stages in mutant fish display characteristic mirror duplications (Figure 5N), albeit less striking and regular than the phenotypes of the segment polarity genes in the fly, with which they may be compared. No genes with obvious resemblance to gap or pair rule genes have been detected in the fish screens. Using molecular approaches, no clear homologs to the gap genes could be detected so far, and the homologs of the pair rule genes do not seem to be expressed in a pair rule fashion (59). The failure to find mutations resembling the gap and pair rule mutations in the fish may mean that the formation of metameric patterns differs between *Drosophila* and most other animals. On the other hand, it may be the result of redundancy.

Other mutations affect the dorsoventral subdivision of the somites by the horizontal myoseptum. Some of these also lack the notochord, while in others the notochord is apparently normal. The notochord is a vertebrate-specific structure. The gene *no tail*, a fish homolog of the mouse *brachyury* or *T* gene, is expressed in the notochord (Figure 5C) (66, 67) (a homolog in *Drosophila* is expressed in the hindgut (68)); and mutations in *no tail* as well as in three other genes required for notochord formation have been identified (Figure 5C) (69, 70). The structure of the somites in fish is thought to be dependent on signals derived from the notochord. The genes affecting somite subdivision by the horizontal myoseptum might represent members of these signalling processes (Figure 5G). Their products may be involved in producing or receiving the signals, respectively (65). For these genes, homologous processes in *Drosophila* are not obvious.

A large number of the fish genes affect structures and functions of the central nervous system and sensory organs (Table 3). This class is poorly represented in the fly screen. In our screen several aspects of CNS development have been scored. For the neurogenic genes of *Drosophila*, such as *Notch*

and *Delta*, homologs have been detected in the vertebrate by molecular means (71) and in our screen at least one gene with neurogenic features, *white tail*, has been found. Mutant embryos display a considerable multiplication of primary neurons at the expense of secondary neurons and certain types of glial cells (Figure 5E) (72). Another class of genes affect structures along the midline in all three germ layers. In the mutants, the eyes are often fused. Mutant embryos display a lack of structures derived from axial mesoderm and ventral regions of the spinal cord or brain. In some mutants the pathfinding of nerves across the midline is impaired. The most prominent and longest known representative of this class of genes is *cyclops* (73). The eyes of mutant embryos are fused anteriorly, and the floorplate, the ventral region of the spinal cord, is lacking. There are several new members of this class of genes including *diwanka* (Figure 5H) and *chameleon* (74). It will be interesting to see whether the mutations which delete the midline structures, the ventral portion of the neuroectoderm and head in *Drosophila*, such as *spitz* and others (75) are homologous to the fish midline genes.

The brain morphology is affected in a number of mutants of which only some will be mentioned here. The forebrain, including the olfactory placodes and eyes are deleted in the mutant *masterblind* (Figure 5D). The analysis with molecular markers revealed that the region which normally would develop forebrain, instead develops into more posterior structures, similar to a homeotic transformation (76). The midbrain-hindbrain boundary is absent in mutants of two genes, *no isthmus* and *acerebellal*. The *no isthmus* gene, (Figure 5L) which is also required for the formation of the kidney, is homologous to one of the *pax* genes, a family of genes first identified in the mouse by their homology to the *Drosophila* segmentation gene *paired* (77).

A large number of zebrafish mutant classes do not have parallels in our *Drosophila* mutant collection. They will only be summarized briefly here. Of particular interest regarding their potential for medical research are mutants affecting inner organs. A number of genes affecting blood formation and circulation as well as the heart, structures easily storable in the first two days of embryogenesis of the fish, have been identified. Mutants affected in organs developing later, such as liver, pancreas and kidney, are less well represented. Differentiation defects or degeneration of the musculature are displayed in embryos mutant for a number of genes. In addition, sensory organs such as eye and ear (Figure 5I), are affected in other mutants. A large number of genes are required for the formation of structures derived from the neural crest. The largest class are genes required for pigmentation, that is pigment cell formation and pigment pattern, and a number of genes affect portions of the jaw (Figure 50) or branchial arches, structures specific to vertebrates (Table 3). We also isolated mutants with specific defects in motility of the larva. Mutant larvae fail to respond to touch, show reduced or no motility, spastic or circling behaviour, as well as defects in the reciprocal inhibition across the midline of muscular contractions. In a small number of mutants, defects in the outgrowth of motoneurons were observed (78). In *Drosophila*,

our screens did not permit the recovery of motility mutants, although immobile larval phenotypes could be recognized in principle by an abnormal distortion of the larval cuticle in the unhatched embryo. Fortunately, a small group of 4 genes of *Drosophila* was detected in which larvae were hyperactive, sometimes turning around in the egg case (Table 2) (9-11). These phenotypes in addition showed defective head skeleton differentiation, to this day they are not analyzed in detail.

CONCLUSIONS

Genetical research on axis determination in the *Drosophila* embryo has provided perhaps the most complete understanding of pattern formation to date. The elegance and efficiency with which mutations can be isolated and genes can be manipulated in this organism will permit further important discoveries concerning a number of basic processes of cell and developmental biology. However, mechanisms in the fly are, in several instances, likely to be unique to this specialized organism. While the approach of using fly genes to find homologs in vertebrates is a very powerful one, it emphasizes the similarities between these clearly different organisms. To understand vertebrate development, genes specific to vertebrates must be investigated in addition to those common between flies and fishes. To find and elucidate these differences, the mutants discovered in the zebrafish will provide an important entry point from which fundamental biological and medical problems might be investigated with genetical tools.

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