

Large scale genetics in a small vertebrate, the zebrafish

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ABSTRACT The systematic isolation and characterization of mutants in *Drosophila* has enormously facilitated the analysis of molecular mechanisms underlying developmental pathways in the embryo. A similar approach is presently being used to study embryonic development of the zebrafish, which is becoming a mainstream model organism for vertebrate development. With its genetic versatility and sophisticated embryology, zebrafish offers the possibility to rapidly increase our knowledge of vertebrate development and add to what we have learned from other vertebrate model organisms.

KEY WORDS: *zebrafish, mutagenesis, vertebrate development, embryonic development, saturation screen*

Introduction

The mystery of our own origin and mode of development has for over 100 years stimulated interest in vertebrate development. Fundamental processes of early development appear conserved among the vertebrates. By studying a particular vertebrate model organism we can therefore draw conclusions about vertebrate development in general and ultimately come up with models about our own development. Genes encoding key functions in development show a high degree of conservation with each other. Once we have identified a developmentally important gene from one vertebrate we can easily clone the homolog from other vertebrates and study its function in the vertebrate which is the most practical for the selected approach. This review is about a mutational approach towards the study of vertebrate development using the zebrafish, *Danio rerio*, as a system.

Genes that may be important for vertebrate development have, so far, mainly been identified using two approaches. The first approach was based on the finding that many genes with important functions in the development of *Drosophila melanogaster* have conserved counterparts in vertebrates. Frequently whole families of vertebrate genes were isolated by molecular screening for homology to specific *Drosophila* genes. The obvious limitation of this approach is that vertebrate genes without homology to previously cloned genes will go undetected. The second approach is to isolate genes displaying interesting spatial or temporal expression patterns in the embryo. The function of these genes is studied by generating loss-of-function mutations using the powerful ES-cell based knockout technology in the mouse (Mansour *et al.*, 1988). Some of these knockouts display interesting specific defects in embryonic development, whereas others have disappointingly little or no effect. Genes whose function in development is not modulated at the level of expression may be missed by approaches that are based on differential gene expression patterns.

The spectacular progress in understanding the embryonic development of *Drosophila* is mainly due to a genetic approach (Nüsslein-Volhard and Wieschaus, 1980). Systematic mutational screens were carried out in which genes were identified based on the phenotype caused by a mutation in these genes. This approach proved to be very successful and allowed the identification of most if not all genes required for early development (Jürgens *et al.*, 1984; Nüsslein-Volhard *et al.*, 1984; Wieschaus *et al.*, 1984). The key feature for such saturation screens was the highly developed genetics of *Drosophila*. This includes a large collection of visible marker mutations in the adult which had been accumulated by geneticists over the years. The availability of balancer chromosomes enormously facilitated saturation screens covering particular chromosomes and the subsequent maintenance of mutant stocks. The detailed genetic maps and the cytological maps of giant polytene chromosomes have allowed rapid cloning of the mutated genes. In spite of the sophisticated genetic methods available in *Drosophila*, it is still not possible to create loss-of-function mutations by targeted gene disruption. Nevertheless, for many developmental biologists *Drosophila* is still the model organism of choice.

Until recently, a systematic mutational approach to study vertebrate development was not considered feasible. Amphibians and chicken, although the favorite experimental organisms of embryologists for many years, cannot be used for genetic approaches due to their size and generation time. The mouse with its long-standing tradition of genetic research and its powerful ES-cell based gene knockout technology has provided us with many insights into vertebrate development. However, due to the intrauterine development of its embryos and the small number of progeny obtained from one genetic cross, it is impractical

Abbreviations used in this paper: ENU, ethylnitrosourea; EMS, ethylmethanesulfonate; RAPD, random amplified polymorphic DNA; PCR, polymerase chain reaction; SSR, simple sequence repeats.

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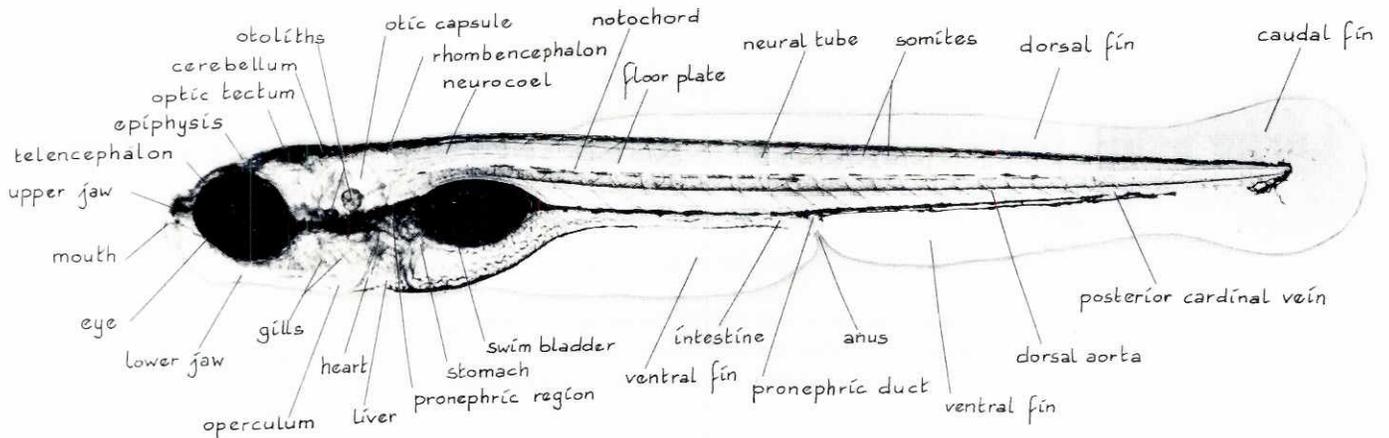


Fig. 1. 5-day-old zebrafish embryo. Tissues and organs that are visible under a simple dissecting microscope are indicated.

to perform large scale saturation screens for mutations affecting early development.

The zebrafish is becoming an increasingly popular model organism for studying vertebrate development (Kimmel, 1989; Mullins and Nüsslein-Volhard, 1993; Driever *et al.*, 1994). It has many properties that make it an ideal organism for systematic mutational approaches. It has a short generation time of 2-4 months and can be kept at a high density with relatively little maintenance. One pair produces on average 200 progeny at weekly intervals, which greatly facilitates genetic analysis. Embryonic development is rapid and synchronous. External fertilization provides easy access to the embryos throughout development. In addition, zebrafish embryos are large and transparent. This allows all tissues and organs to be observed during development under a simple dissecting microscope without prior manipulations of the embryo (Fig. 1). This includes the major subdivisions of the brain, the neural tube, floorplate, notochord, somites, heart, jaw, gills, eyes, otic vesicles, fins, blood, liver, gut and pigmentation.

A number of genetic methods have been developed by G. Streisinger and his colleagues (Kimmel, 1989). These include genetic tricks such as the production of haploid embryos and gynogenetic diploid embryos, whose genome is fully derived from the maternal genome (Streisinger *et al.*, 1981). Both techniques were used by laboratories based in Eugene, Oregon, to isolate a significant number of interesting mutations following γ -ray mutagenesis, demonstrating the mutability of the zebrafish genome (Kimmel *et al.*, 1989; Felsenfeld *et al.*, 1990; Westerfield *et al.*, 1990; Hatta *et al.*, 1991; Halpern *et al.*, 1993). A method allowing successful regeneration of frozen sperm was also developed (in Westerfield, 1993). This will in the near future become a very important feature of zebrafish genetics, since a large number of genetic stocks can be kept without continuous turnover.

In addition to the genetic versatility, zebrafish also offers excellent experimental techniques, which were mainly developed by C. Kimmel and his collaborators in Eugene (Oregon) (Kimmel and Warga, 1986, 1988; Kimmel *et al.*, 1990). This includes a detailed fate map of the early zebrafish embryo and sophisticated embryology such as lineage tracing and transplan-

An increasingly important reason for studying zebrafish as a model organism is purely economical. The breeding and maintenance of zebrafish is relatively cheap, when compared to other vertebrate organisms. Increasing economical considerations are likely to contribute towards zebrafish becoming a mainstream vertebrate model organism in the future.

Genetic screens

Genetic screens in diploid organisms such as zebrafish pose the problem that recessive mutations must be bred to homozygosity to uncover their phenotype. The ability to make haploid embryos in zebrafish makes it possible to circumvent this problem (Streisinger *et al.*, 1981). Haploid embryos are obtained by fertilizing eggs *in vitro* using UV-inactivated sperm. 50% of the haploid embryos derived from a female heterozygous for a specific mutation will reveal the phenotype of this mutation (Fig. 2). The major advantages of this method are that it can be done with a small fish facility and mutant carriers are identified directly among the F1 female fish. However, the major disadvantage of this method is that haploid embryos display a rather high background of abnormal development and die around five days after fertilization. This makes it impossible to screen for mutations causing subtle defects or mutations affecting organs or tissues at a later stage in development. Mutations identified in haploid screens have to be recovered from the F1 carrier before further analysis of the mutant can be performed.

With the development of efficient systems to raise and maintain large numbers of independent lines of zebrafish, it has become feasible to perform large scale mutational screens utilizing standard crossing schemes to produce homozygous diploid embryos (Fig. 3, Mullins *et al.*, 1994; Solnica-Krezel *et al.*, 1994). In such a scheme, the spermatogonial cells of G0 founder males are mutagenized and the males are then outcrossed to wildtype females, resulting in a large number of F1 fish that are all heterozygous for a different and unknown set of newly induced mutations. From single pair matings between F1 fish, F2 families are raised. Half of the fish in such an F2 family are heterozygous for a particular mutation. Sibling crosses among F2 fish will match two carriers of a specific mutation in a quarter of the matings, and the mutant phenotype will be dis-

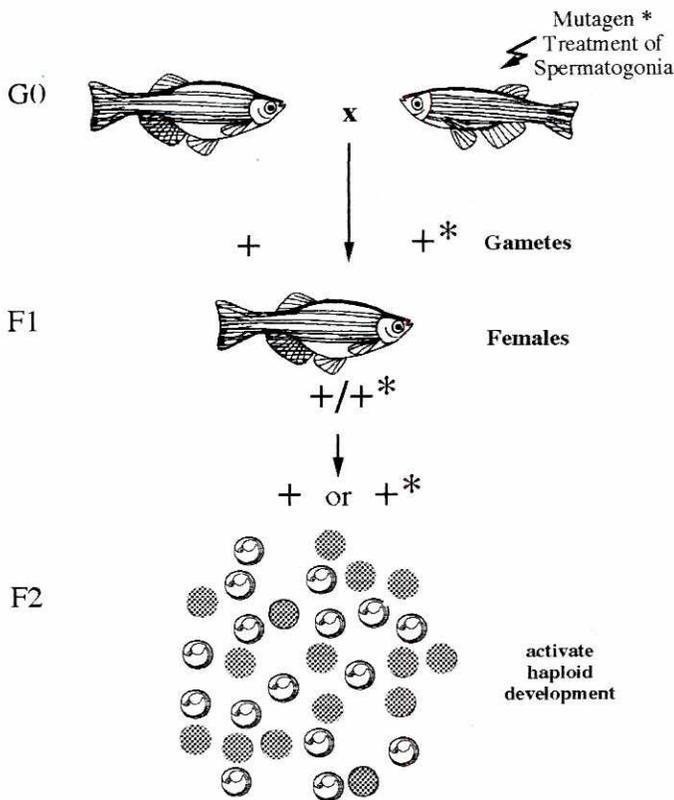


Fig. 2. Crossing scheme for the isolation of embryonic mutations (indicated by an asterisk) using haploid embryos. G0 founder males are mutagenized and outcrossed to wildtype. Haploid embryos are derived from F1 females by fertilizing eggs in vitro using UV-inactivated sperm (Streisinger *et al.*, 1981). 50% of the haploid embryos will reveal the phenotype of a mutation carried by the F1 female. If a mutation was induced, 50% of embryos show a mutant phenotype.

played in 25% of the F3 embryos. Our laboratory in Tübingen (Germany) and the laboratory of Wolfgang Driever in Boston are presently carrying out such screens. These screens are aimed at saturating the genome for zygotic mutations affecting early development. The sole criterion for keeping a mutant is a distinct and specific phenotype visualized under the dissecting microscope.

Zebrafish has 25 chromosome pairs (Endo and Ingalls, 1968), and the entire genome is screened at once, since we cannot predict which part of the genome is made homozygous in a particular cross between F2 siblings. An F2 family contains the two mutagenized genomes from its F1 parents, but only 25% (i.e. 0.5 mutagenized genomes) are made homozygous in each sibling cross of F2 fish. On average, 1.2 mutagenized genomes are screened in 3 successful sibling crosses of one F2 family. It can therefore be estimated that it takes approximately 800 F2 families for every 1000 mutagenized genomes to be screened.

Mutagenesis

Efficient mutagenesis methods and high mutation rates are prerequisites for performing large scale mutational screens. In order to recover mutations affecting single genes it is important

that a point mutagen is used. A point mutagen also offers the advantage that besides complete loss-of-function mutations, partial loss-of-function and gain-of-function alleles can be isolated. Such mutations have proven very useful in many other organisms such as yeast, *Drosophila* and *C. elegans* for elucidating the function of the mutated gene.

In zebrafish, ethylnitrosourea (ENU) was found to be the most efficient chemical mutagen (Mullins *et al.*, 1994; Solnica-Krezel *et al.*, 1994). Males were treated by repeatedly placing them into aqueous solutions of ENU at a sublethal dose. Four known mutations affecting pigmentation were used to assess the mutation rate resulting from this treatment, which was found to be between 1/1000 and 1/300. These mutation rates were similar to those produced by EMS in *Drosophila*. Using the lower mutation rate of 1/1000, it can be estimated that to find at least 1 mutation in 95% of all genes, 3000 mutagenized genomes would have to be screened.

Two important considerations are worth mentioning. Firstly, it is important that germ cells are mutagenized at premeiotic stages during spermatogenesis to avoid mosaicism in the germline of the F1 progeny (Jenkins, 1967; Mullins *et al.*, 1994; Solnica-Krezel *et al.*, 1994). A point mutation induced in one DNA strand of premeiotic germ cells is fixed in both DNA strands

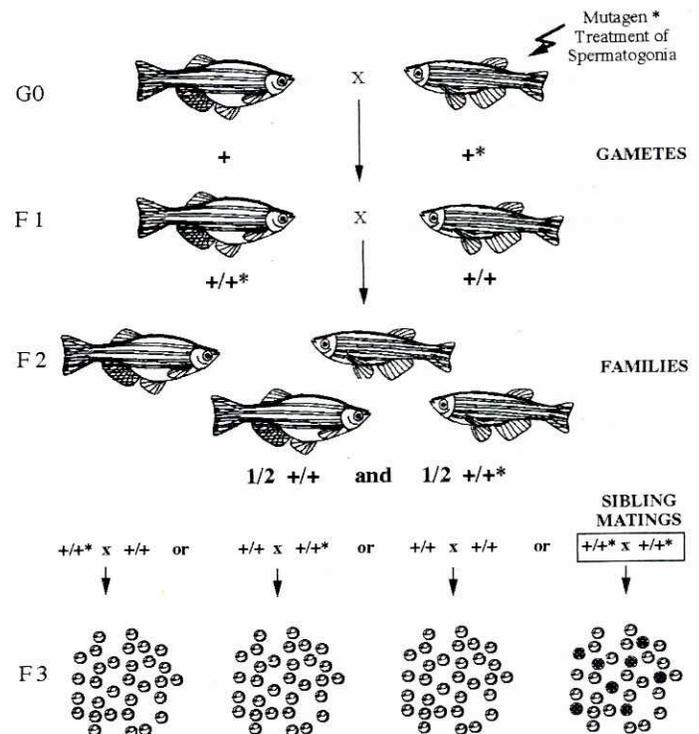


Fig. 3. Crossing scheme for the isolation of embryonic mutations (indicated by an asterisk) using diploid embryos. G0 founder males are mutagenized and outcrossed to wildtype. The resulting F1 fish are all heterozygous for an unknown set of newly induced mutations. F2 families are obtained from single pair matings between F1 fish. Sibling crosses among F2 fish will match two carriers of a specific mutation in a quarter of the crosses, which will then display the mutant phenotype in 25% of the F3 embryos (Mullins *et al.*, 1994; Solnica-Krezel *et al.*, 1994). If a mutation is induced, 25% of the matings show a phenotype in 25% of the embryos.

TABLE 1
 FREQUENCY OF MUTATIONS ISOLATED PER SCREENED
 HAPLOID GENOME

Mutagen	Number of haploid genomes screened	Number of mutations isolated	Mutations per haploid genome
ENU	74	90	1.2
EMS	44	1	0.02
X-rays	103	9	0.09

G0 males were mutagenized using either ENU, EMS or X-rays and outcrossed to wild-type. Following the standard crossing scheme shown in Figure 3, F3 progeny were screened for mutations with phenotypes that are visible at embryonic stages. The frequency of mutations per haploid genome screened are much higher for ENU than for EMS or X-rays (Mullins *et al.*, 1994).

during DNA replication and thus does not produce mosaic progeny. Mosaicism among the F1 fish would result in fewer fish within an F2 family carrying a specific mutation which would cause an enormous drop in the efficiency of screening. Mutagenized males are therefore outcrossed three weeks or longer after the mutagenic treatment, by which time no mosaicism among the F1 progeny is observed. A potential problem of mutagenizing premeiotic germ cells are spermatogonial clones, which would result in an identical mutation being isolated from two separate F2 families that derive from the same mutagenized founder male. In zebrafish this is very unlikely to happen, since fertilization occurs with a vast excess of sperm (Streisinger *et al.*, 1981) and the number of spermatogonial stem cells is estimated to be between 500 and 1000 (Mullins *et al.*, 1994). As a precaution, large numbers of F1 fish are nevertheless obtained from a reasonably high number of mutagenized males.

X-rays and γ -rays also produce high mutation rates in zebrafish (Chakrabarti *et al.*, 1983). In contrast to ENU, however, mutations induced by X-rays or γ -rays are much more difficult to recover, resulting in a lower frequency of recoverable mutations (Mullins *et al.*, 1994). This indicates that large deletions or chromosome breaks are induced. Deletions are often desired, because breakpoints serve as physical landmarks which facilitates cloning of a mutated gene. However, in saturation screens, point mutations are favored over deletions, because only mutations affecting single loci allow unambiguous association of specific phenotypes with single genes.

In a small-scale pilot screen, the efficiencies of ENU, EMS and X-rays at inducing recoverable mutations in zebrafish were compared to each other (Table 1). ENU yielded about 1.2 mutations per haploid genome, whereas only 0.02 and 0.09 mutations per haploid genome could be recovered after mutagenesis with EMS or X-rays respectively. This demonstrated that ENU is a very potent mutagen for recovering mutations in zebrafish.

The outcome of saturation screens

In *Drosophila*, saturation screens identified most if not all genes with unique and indispensable function in embryonic

development (Jürgens *et al.*, 1984; Nüsslein-Volhard *et al.*, 1984; Wieschaus *et al.*, 1984). They represent only a small fraction of all the essential genes in the fly. The same should be true for the large-scale screens presently carried out with zebrafish. In a small-scale pilot screen, 70% of the identified mutants were found to display rather general defects (Mullins *et al.*, 1994). These genes are likely to encode proteins or genes that are required in many cell types. The remaining 30% of the mutants had specific defects in embryonic development or organogenesis. The number of mutations causing late lethality was found to be similar within the order of magnitude to the number of embryonic and early larval mutations (P.H. and F.v.E., unpublished data). This suggests that the fraction of genes with specific and unique functions in early development is about 15% of the total of all the essential genes.

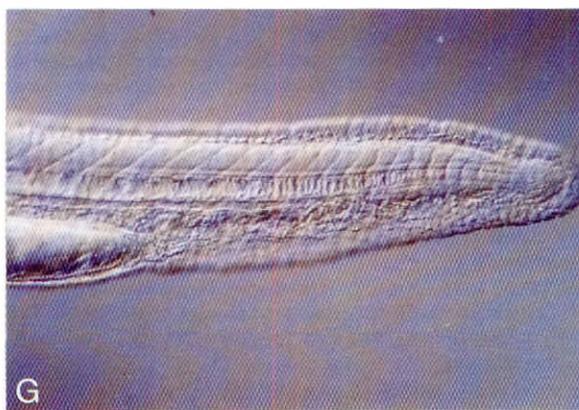
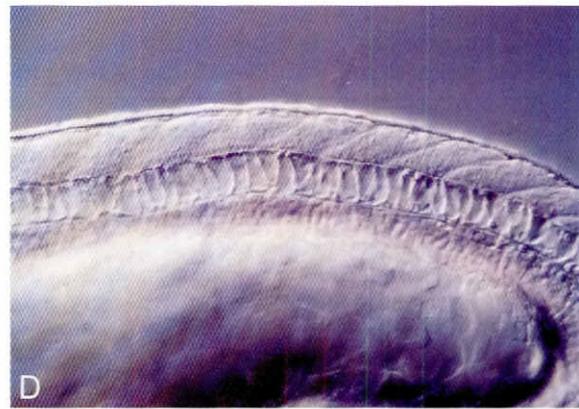
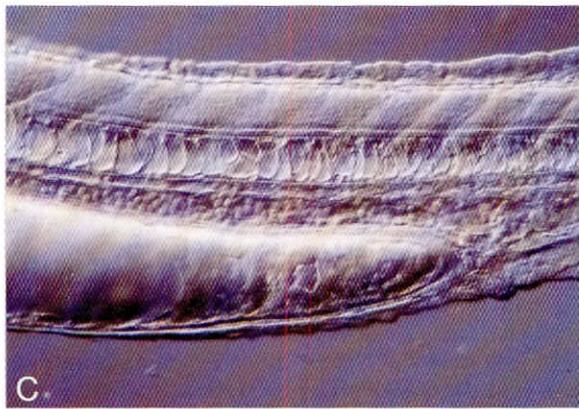
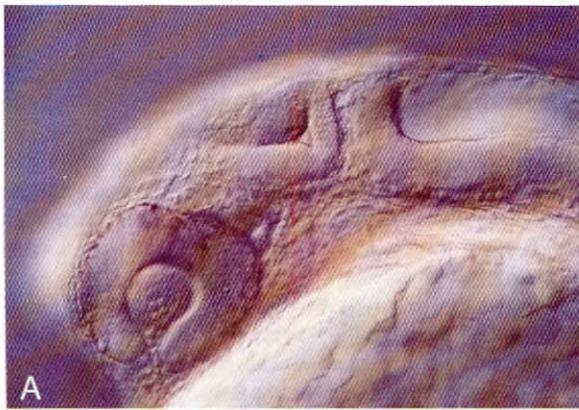
In a saturation screen often whole groups of genes involved in a specific developmental process are identified. This allows one to study the functional relationship of these genes to each other. Establishing epistatic relationships within such a group of mutants allows ordering these genes in a developmental pathway. Double mutants will not only be useful in establishing epistatic relationships, but also reveal redundant functions carried by separate genes. Double mutant analysis in zebrafish is enormously facilitated by the large number of progeny produced from a single mating.

Most genes encoding redundant functions will be missed in these screens. A prominent example of redundant function is in myogenesis, where Myf-5 and MyoD can in part substitute for one another (Rudnicki *et al.*, 1993). Both genes need to be mutated to show a complete loss of skeletal muscle. Many genes with interesting expression patterns have disappointingly little or no visible effect on development when mutated in the mouse. In many cases, this could be due to redundancy and the functional counterpart still remains to be identified. The targeted gene-knockout technology in the mouse and the random mutational approach carried out in zebrafish are therefore complementary approaches towards identifying developmentally important genes.

Some genes that form part of a developmental pathway encode proteins that are required in many cell types, and will be missed in screens for specific loss-of-function phenotypes. However, mutants identified in saturation screens serve as entry points into developmental pathways, allowing the gaps to be filled using biochemical approaches. The mutants will thereby serve as valuable tools in elucidating the function of biochemically characterized proteins in a specific developmental process. Whole mount antibody staining and *in situ* hybridization techniques, which are well established in zebrafish (Westerfield, 1993), will greatly facilitate such experiments.

Due to the transparency and accessibility of zebrafish embryos, elegant cell lineage tracing and transplantation experiments can be carried out (Kimmel and Warga, 1988). Such techniques allow one to study the migration of individual cells or groups of cells in living mutant embryos and to study cell-autonomous or non-autonomous requirements of mutated genes. The power of these techniques was demonstrated when

Fig. 4. Examples of mutations with specific defects in the development of zebrafish embryos. All embryos shown are 24 hours-old. (A) Wildtype. (B) *cyclops* mutant with partially fused eyes (Hatta *et al.*, 1993). (C) Wildtype. (D) *cyclops* mutant showing the absence of a floor plate (Hatta *et al.*, 1993). (E) Wildtype. (F) *no tail* mutant which lacks a differentiated notochord, has no tail and abnormally shaped somites (Halpern *et al.*, 1993). (G) Wildtype. (H) *spade tail* mutant accumulates trunk somitic mesoderm precursor cells in the tail (Ho *et al.*, 1990).



they were applied to a number of zebrafish mutants, three of which are shown in Figure 4. *cyclops* mutant embryos have no floorplate and are missing part of the ventral forebrain resulting in partially fused eyes (Hatta *et al.*, 1991). *no tail* mutant embryos lack a differentiated notochord, have no tail and abnormally shaped somites (Halpern *et al.*, 1993). *spade tail* mutant embryos do not form somites in the trunk due to aberrant cell migration during gastrulation leading to an accumulation of cells in the tail (Ho and Kane, 1990). These mutants have already given interesting insights into cell movements during gastrulation and the role of the floorplate and the notochord in dorso-ventral patterning. Many mutants with equally specific defects are expected to be found in the ongoing saturation screens.

How to clone the genes

A prerequisite for positional cloning of mutations will be a genetic map of the zebrafish genome. Two efforts towards generating such a map are under way. A RAPD (random amplified polymorphic DNA) map using random decamer primers to amplify arbitrary DNA sequences by PCR was made by the group of J. Postlethwait (Postlethwait *et al.*, 1994). This map takes advantage of the possibility of making haploid embryos, which circumvents the problem of identifying heterozygous individuals generally posed by the RAPD mapping technique. In the laboratory of H. Jacob, an SSR (simple sequence repeats) map using PCR primers homologous to unique sequences flanking CA repeats is being generated (Driever *et al.*, 1994). This mapping technique can be done using diploid embryos since it allows unambiguous distinction of heterozygous and homozygous individuals.

Both maps will be invaluable in mapping mutations and cloned genes to relative positions on the zebrafish genome. Some mutations will turn out to be in previously identified genes. An example for such a fortuitous match is exemplified by the *no tail* gene, which was found to encode the zebrafish homologue of the mouse *T/Brachyury* gene (Schulte-Merker *et al.*, 1994). Identifying closely linked molecular markers on a genetic map will provide a good starting point to undertake the positional cloning of mutations. A number of laboratories are currently constructing genomic libraries appropriate for carrying out chromosomal walks covering several hundred kilobases on the 1.6×10^9 base pair genome of zebrafish. The pufferfish, *Fugu rubripes rubripes*, has a normal vertebrate gene repertoire, but a much lower amount of "junk" DNA such as repetitive sequences, pseudogenes and introns (Brenner *et al.*, 1993). If gene order is conserved between the two fish species, this would dramatically simplify positional cloning, since chromosomal walks could be done in the four times smaller genome of the pufferfish.

Cloning of mutations is of course much easier if the mutated gene is tagged by an insertion. Insertional mutagenesis in zebrafish is not yet practicable due to the low efficiency of integration into the genome (Stuart *et al.*, 1988; Culp *et al.*, 1991). Recently, encouraging progress has been made by using retroviral vectors to infect zebrafish cells (Burns *et al.*, 1993; Lin *et al.*, 1994). High rates of integration allowing the isolation of insertion alleles by noncomplementation would enormously expedite efforts towards the cloning of mutated genes.

The future of zebrafish

Having a mutant and its affected gene at hand are major steps in analyzing a developmental process. *Drosophila* provides an excellent example of how having both stimulates research to understand the mechanisms underlying specific developmental programs. With its great embryology, zebrafish will become an even more attractive model organism to study vertebrate development when genes and their corresponding mutants are available.

The experience gained from systematic mutational screens will greatly improve the use of zebrafish as a genetic system. In addition to yielding mutants with interesting phenotypes in development, the screens will also produce a large collection of viable mutations with visible phenotypes in the embryo or the adult. These will serve as valuable genetic markers, and like the marker mutations in *Drosophila* will allow more sophisticated genetic experiments in zebrafish. More specialized screens involving particular assays directed at more specific aspects of development are feasible. An excellent example of such a specific assay is the screen presently being carried out in the laboratory of F. Bonhoeffer in Tübingen (personal communication), in which anterograde labeling of retinotectal axons is performed to visualize mutations affecting retinotectal projections in zebrafish.

Mutations identified twenty years ago in saturation screens in *Drosophila* still occupy an enormous number of scientists who are interested in developmental mechanisms at molecular levels. The potential offered by zebrafish as a genetic system is likely to stimulate its use as a model organism for vertebrate development.

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