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The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*

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SUMMARY

In a large-scale screen, we isolated mutants displaying a specific visible phenotype in embryos or early larvae of the zebrafish, *Danio rerio*. Males were mutagenized with ethylnitrosourea (ENU) and F_2 families of single pair matings between sibling F_1 fish, heterozygous for a mutagenized genome, were raised. Egg lays were obtained from several crosses between F_2 siblings, resulting in scoring of 3857 mutagenized genomes. F_3 progeny were scored at the second, third and sixth day of development, using a stereomicroscope. In a subsequent screen, fixed embryos were analyzed for correct retinotectal projection. A total of 4264 mutants were identified. Two thirds of the mutants displaying rather general abnormalities were eventually discarded. We kept and characterized 1163 mutants. In complementation crosses performed between mutants with similar phenotypes, 894 mutants have been assigned to 372 genes. The average allele frequency is 2.4. We identified genes involved in early development, notochord, brain, spinal cord, somites, muscles, heart, circulation, blood, skin, fin, eye, otic vesicle, jaw and branchial arches, pigment pattern, pigment formation, gut, liver, motility and touch response. Our collection contains alleles of almost all previously described zebrafish mutants. From the allele frequencies and other considerations we estimate that the 372 genes defined by the mutants probably represent more than half of all genes that could have been discovered using the criteria of our screen. Here we give an overview of the spectrum of mutant phenotypes obtained, and discuss the limits and the potentials of a genetic saturation screen in the zebrafish.

Key words: zebrafish, ENU mutagenesis, 'Tübingen' mutants', vertebrate development

INTRODUCTION

Multicellular organisms contain a large number of genes. The exact number is not known for any organism, nor can we say how many and which are absolutely required for survival, shape and pattern of the adult, juvenile or embryonic forms. Using modern methods of recombinant DNA technology, attempts are being made to clone and eventually sequence the genomes of several organisms such as *Caenorhabditis elegans, Drosophila melanogaster, Arabidopsis thaliana*, the mouse and *Homo sapiens*. Although it is likely that each gene has a function important for the organism, be it ever so subtle, from a large number of studies it emerged that in early pattern-forming processes and morphogenesis of the organism only a small fraction of the genes have indispensable and unique functions. The identification of these genes is a major issue in modern biological research.

Three types of information are most important to elucidate the function of a gene: the loss-of-function phenotype, the structure and biochemical properties of the protein product, and the distribution of the gene products within the organism. In vertebrates, a number of genes essential for development and pattern formation have been identified on the basis of their protein product or expression patterns. Mice mutants for any previously cloned gene can be created by homologous recombination in ES cells (Mansour et al., 1988), thus allowing in vivo analysis of gene function. This reverse genetic approach has the advantage that the gene corresponding to a mutant phenotype is already cloned, but a considerable disadvantage is that we cannot easily predict which genes have an indispensable function in the vertebrate embryo and thus will show a phenotype when mutant. So far, therefore, the most successful approach for identifying genes with indispensable functions in morphogenesis and pattern formation in the vertebrate

embryo is based on their surprising degree of homology to genes of invertebrates, i.e. *Drosophila melanogaster* and *Caenorhabditis elegans*. In invertebrates, a large collection of such genes have been identified by mutations.

Mutant searches have been carried out, with more or less systematic attempts at saturation, in a number of organisms for different phenotypic traits. Screens have been done for zygotic mutants showing a phenotype in the larval cuticle in *Drosophila melanogaster* (Jürgens et al., 1984; Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1984; Wieschaus et al., 1984), for various cell lineages in the post embryonic development of *Caenorhabditis elegans* (Brenner, 1974), for embryonic development in *Arabidopsis thaliana* (Mayer et al., 1991), and maternal mutants in *Drosophila* (Gans et al., 1975) and *C. elegans* (Kemphues et al., 1988). In these screens, a large number of lines originating from individuals treated with mutagenic agents were inbred for several generations. Homozyous progeny obtained were scored for the presence of morphological changes indicative of abnormal development. Using this approach, a large number of mutants with important functions in many developmental processes were identified. Groups of genes sharing particular phenotypic features helped to define pattern-forming pathways. For example, systematic searches for mutants affecting vulval development in *C. elegans* led to a detailed understanding of signaling processes and cellular interactions (Horvitz and Sulston, 1980). Mutants affecting axis determination and segmentation in the *Drosophila* embryo led to the elucidation of a small number of largely independent pattern-forming pathways (Nüsslein-Volhard and Wieschaus, 1980; St Johnston and Nüsslein-Volhard, 1992).

The advantage of mutagenesis is that it selects for the relatively small fraction of genes with unique and at least partially non-redundant functions. Their identification does not depend on preconceived ideas and models, although the ability to recognize a significant phenotype is dependent on the visibility of the structure affected, and is not always unbiased by models of interpretation. Genes with redundant functions go undetected, however (see Nüsslein-Volhard, 1994, for further discussion).

In vertebrates, systematic mutagenesis has been difficult so far because of long generation times, large space requirements, and laborious handling required in maintenance and breeding of these animals. Nevertheless, a small and valuable collection of mutants affecting early development in mice and zebrafish has accumulated. Because of the high degree of homology between genes of different vertebrates, a gene from one organism provides easy access to its homolog in another vertebrate. From gastrulation onwards, the early development of vertebrates is remarkably conserved. Therefore, the only essential requirement for the organism of choice is the possibility of doing large-scale mutagenesis. We have chosen to perform saturation mutagenesis screens in the zebrafish, *Danio rerio*, because of the many advantages it has as an embryological and genetical system (Streisinger et al., 1981). The most important property of the zebrafish is that the embryos are optically clear and are produced in large numbers. This allows screening for a large number of phenotypic traits by visibility with the dissecting microscope. For genetical analysis and stock keeping it is of advantage that eggs can be fertilized in vitro and sperm can be frozen. Furthermore, mutations can be induced with high efficiency. A random amplified polymorphic DNA map of the zebrafish genome has been constructed, on which a number of mutants and many cloned genes have already been mapped (Postlethwait et al., 1994).

Screens have been carried out before in the zebrafish, predominantly using X-rays as the mutagen. In these screens, the mutant embryos were detected in haploid progeny of females heterozygous for a mutagenized genome in part of the germline (Kimmel, 1989). Although elegant in principle, such screens have the disadvantage that specific phenotypes must be recognized against a high background of retarded and often abnormal development associated with haploidy (Streisinger et al., 1981). Frequently, the recovery of the mutant presents severe problems. We decided to screen diploids, although much more laborious, because the identification and recovery of mutants of many phenotypic classes can be performed with more consistency and reliability (Mullins et al., 1994).

In this paper we describe the isolation of 1163 mutants with defects in more than 372 genes of the zebrafish. The common feature of these genes is that they are indispensable for the development of a morphologically normal fish larva. Most of them display a specific and uniform visible phenotype in mutant embryos or larvae. The genes are required, as judged from the phenotypes, in a large number of processes in morphogenesis, pattern formation, organogenesis and differentiation. A preliminary account of the mutagenic treatment and the screening procedure has been published (Mullins et al., 1994). The genes and their properties are described in the accompanying papers (this issue).

MATERIALS AND METHODS

Fish strains

The wild-type strain used in mutagenesis was Tübingen. It originated from a local pet shop and was inbred for several generations. For outcrossing the mutant fish, the AB strain from the Oregon laboratory, a Tübingen:AB hybrid strain, and a strain homozygous for *leo*^{t1} and *lof*^{dt2} called TL, were also used. This latter strain was obtained from a dealer and kept by raising mixed eggs from different egg lays of particularly well-laying females. *leo*^{t1} is a recessive mutation causing spotting in adult fish, also known as *tup*, or *Brachydanio frankei*. *lof*^{dt2} is a dominant, homozygous viable mutation causing long fins. Both are described in more detail by Haffter et al. (1996).

Media

Embryo medium: (E3 medium) 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$. To suppress growth of mold, the medium was supplemented with 10^{-5}% methylene blue. For anesthetizing, a 0.2% solution of 3-aminobenzoic acid ethyl ester (Sigma), containing Tris buffer, pH 7, was used (Westerfield, 1993).

Fish raising and keeping procedures; mutagenesis

The zebrafish breeding conditions and the mutagenesis protocol have been described by Mullins and Nüsslein-Volhard (1993). A detailed account of the keeping and raising methods has been described recently (Brand et al., 1995), and is available upon request. Adult male fish were mutagenized with 3 mM ENU by placing them into an aqueous solution for three 1 hour periods within 1 week. 3 weeks after the ENU treatment, males were crossed to wild-type females at weekly intervals and the progeny originating from mutagenized premeiotic germ cells obtained were raised. The progeny from each mutagenized male were kept separate such that the origin of individual mutations could be traced back to the founder male. Strings of

mutations originating from the same mutational event in the spermatogonia of the paternal founder fish have probably not been recovered, at least not in a significant number, since of the 59 genes with two alleles, in only five cases did both alleles come from the same founder male. The families were labeled with letters (ta-tz, ta200-tz200) corresponding to the founder male, and a consecutive number.

Screening procedure

For screening, about 15 pairs of fish per family, and about 100 families per week were set up for egg lay in one afternoon (Tuesday) of the week as described (Brand et al., 1995; Mullins et al., 1994). The following morning eggs from successful matings were collected using a tea strainer, and 2-3× 40 fertilized embryos were sorted within 6 hpf (hours postfertilization) into 60 mm Petri dishes in E3 medium. From about 300 families no successful matings were obtained, mainly because the fish showed an extreme sex ratio (mostly male families). The parents of successful matings were kept until the final evaluation of the retinotectal screen (Baier et al., 1996). Fish that had not laid on the first day were checked again on the second day after set up. The number and quality of the fish varied within the screen, and not all matings were successful. Fish of unsuccessful matings by the second day (40-80% of the crosses set up) were returned to their tank, and more crosses of their family, if necessary, were set up 2 weeks later. In general, not more than eight crosses of one family were evaluated with care, the average being 4.7. Table 1 summarizes the data of the mutagenesis experiment.

Embryos were scored at three successive time points (the second, third and sixth day after collection) corresponding to the pharyngula period, the hatching period, and the swimming larva (Kimmel et al., 1995), for abnormalities visible under a dissecting microscope at maximal 80× magnification. During the screening period, the egg lays were kept at 28°C. At each time point, after a general inspection, at least 12 embryos or larvae of each egg collection were aligned and inspected using a checklist (see Figs 1 and 2 for the structures on the checklist at various developmental stages). Embryos of the pharyngula period (24-48 hpf) were examined for abnormalities in the shape and morphology of the developing eyes, brain, notochord, spinal cord and somites. After hatching (third and sixth day screen), embryos were checked for motility (see below), and were then anesthetized before further scoring. In addition to the structures scored earlier, the development of the cardiovascular system and the fins were scored in embryos of the hatching period. At day 6 of development, particular attention was given to late developing organs such as the jaw and gill arches, the gut, the liver, the eyes, the otic vesicles, the pattern and

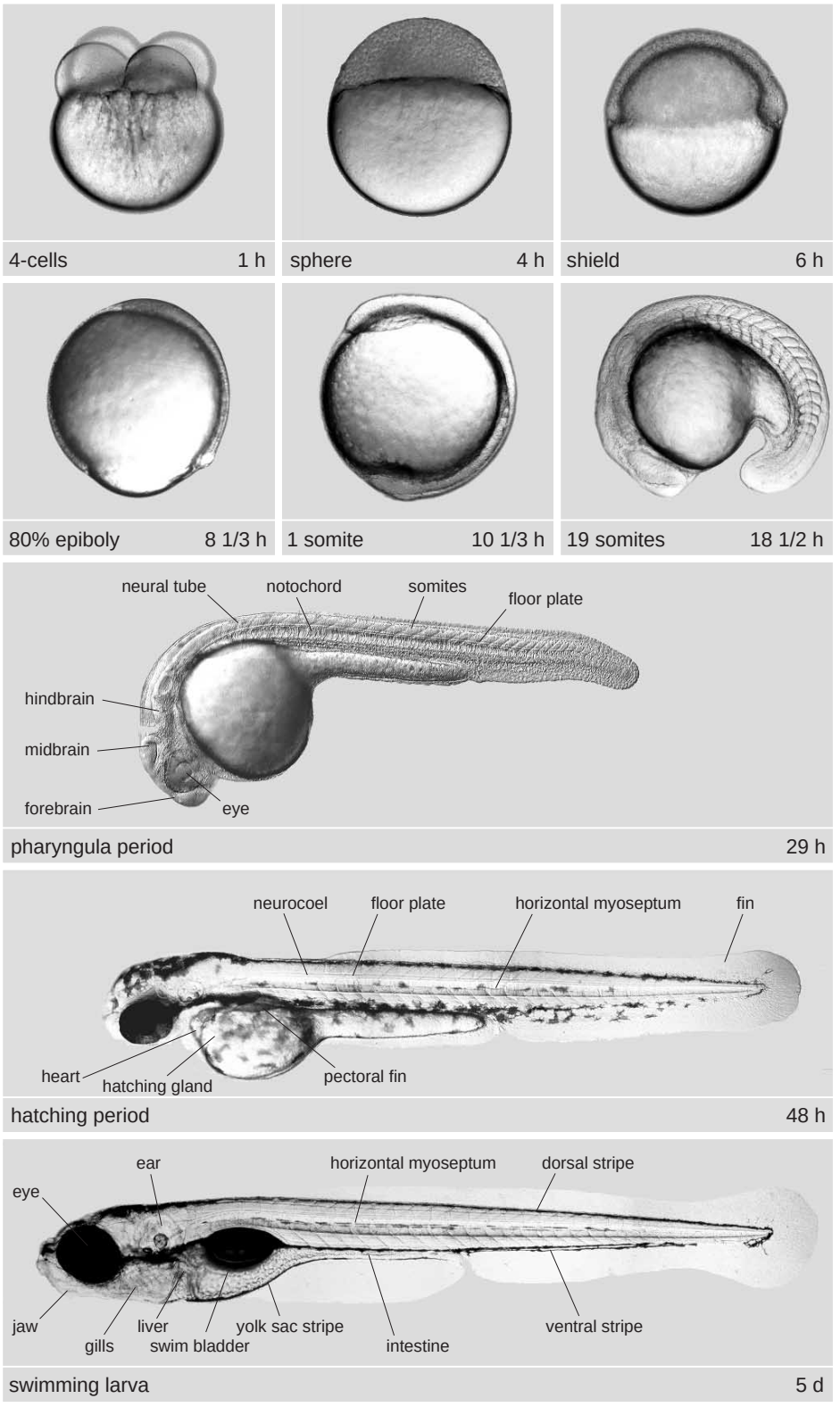
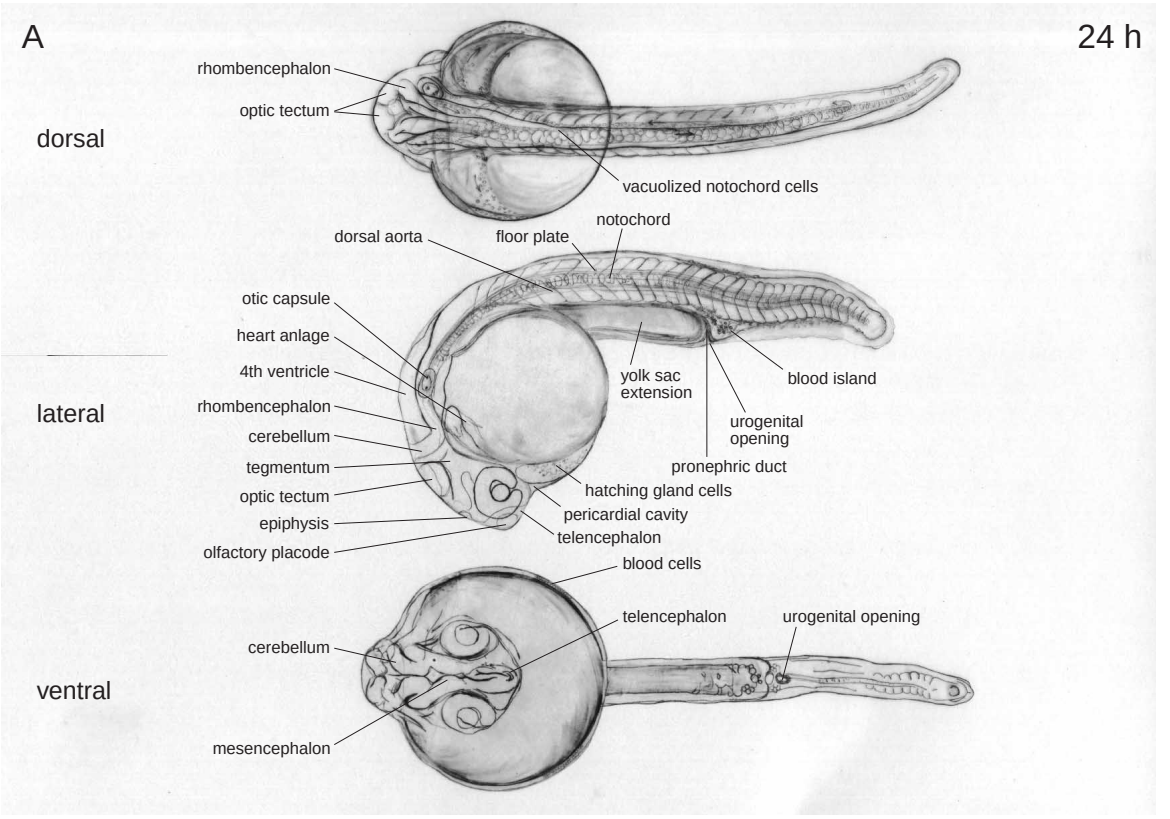


Fig. 1. Living embryos of relevant stages during the first 24 hours of development and of the approximate age during the three screening periods. The structures that were on the checklist are marked.



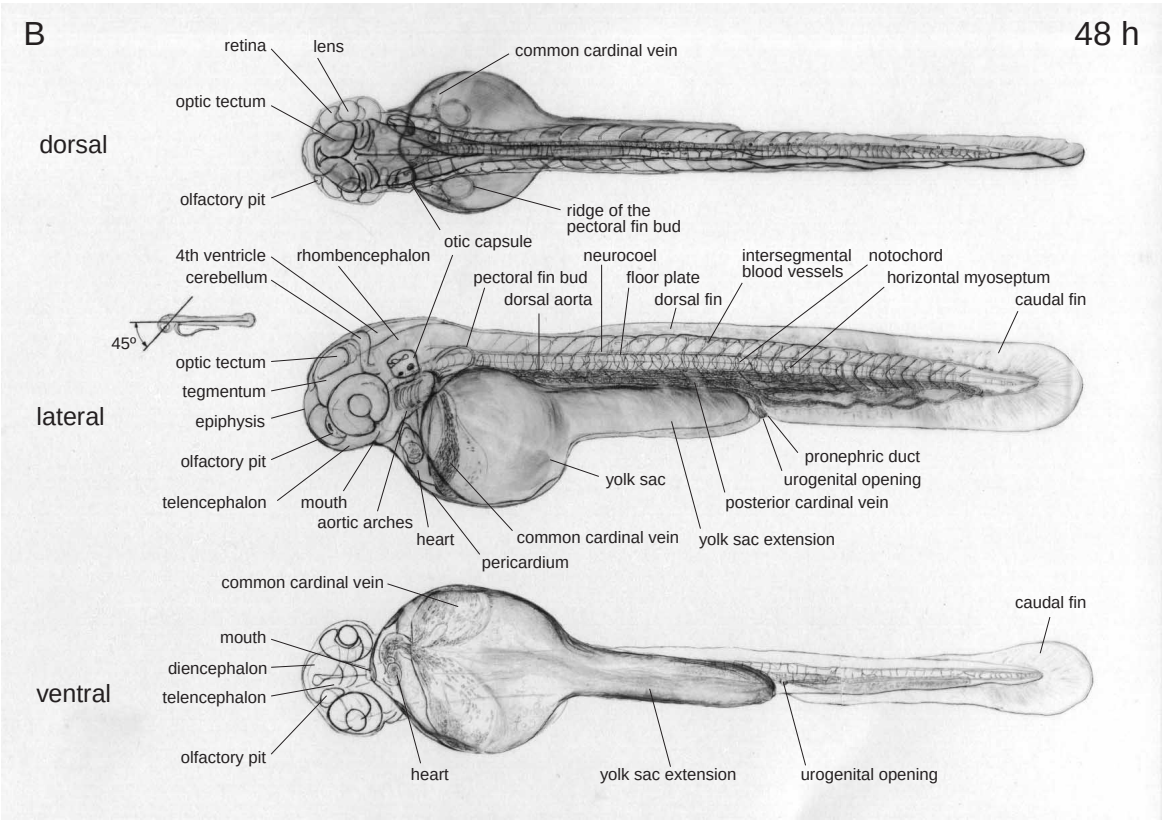
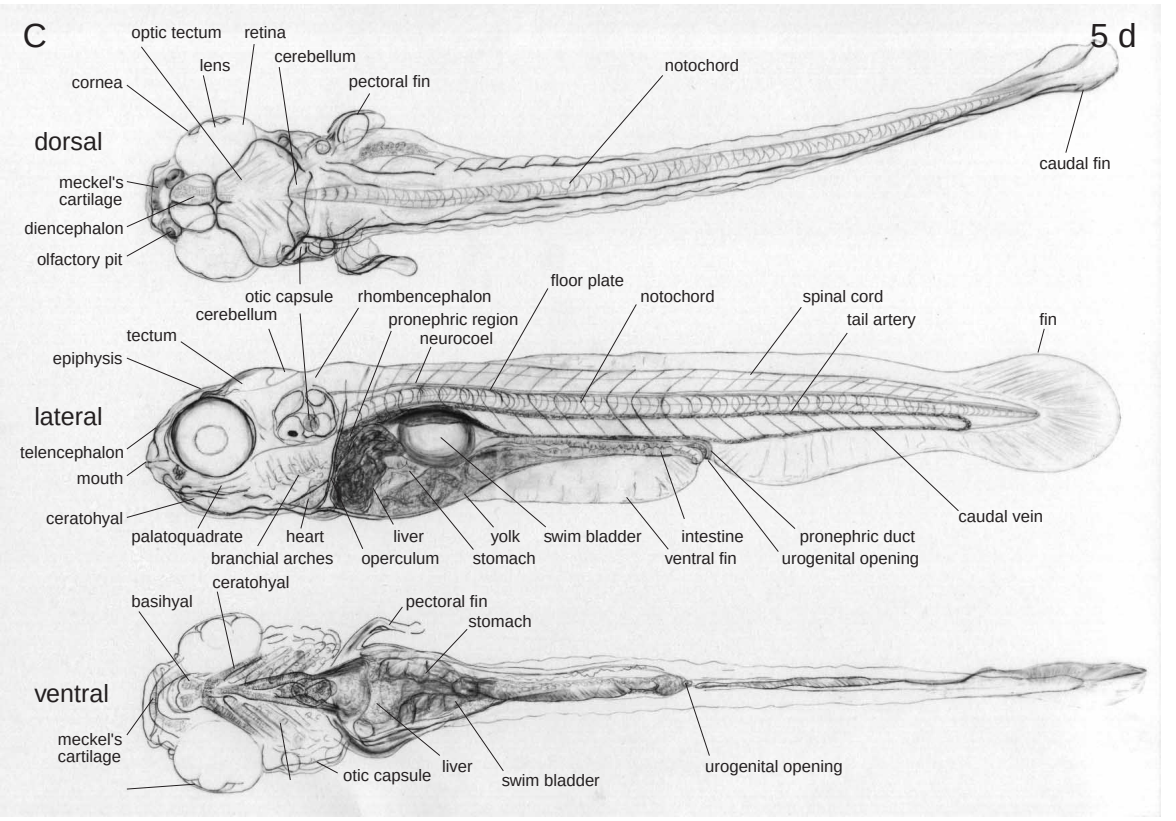


Fig.2a,b. For legend see p. 6.



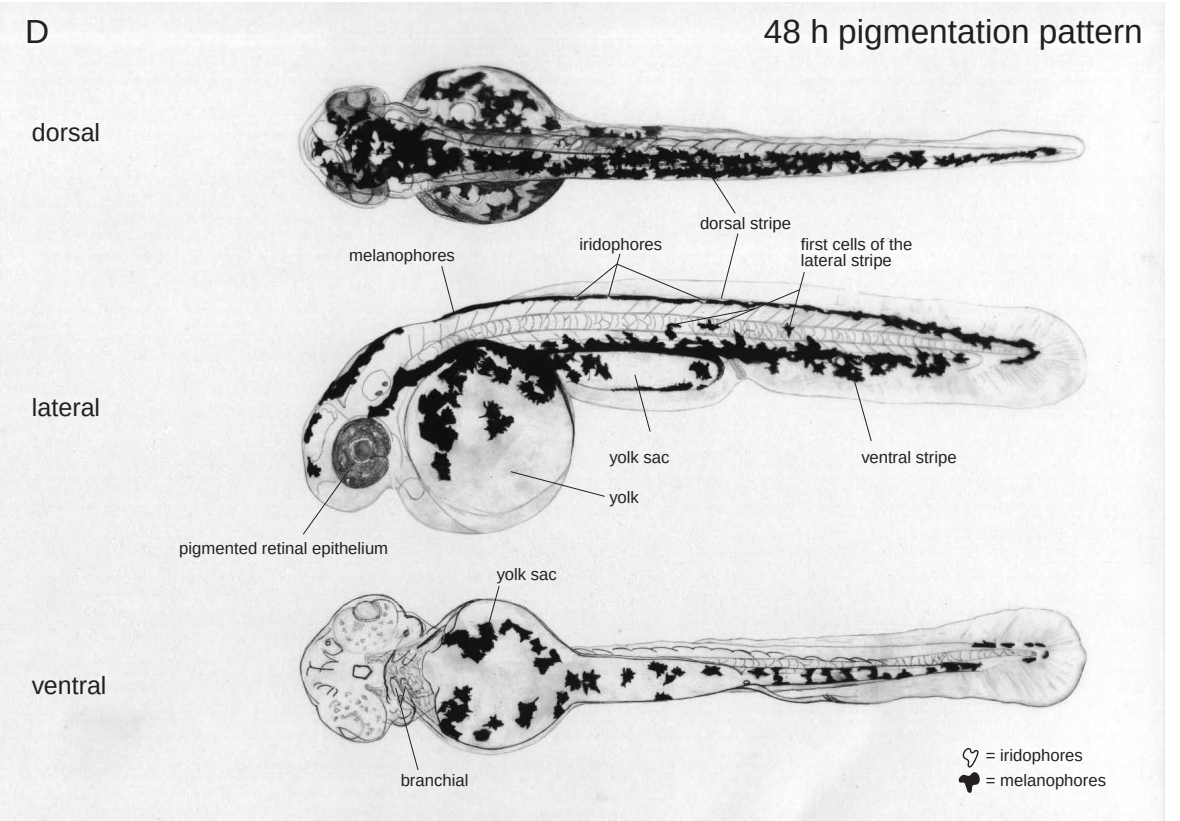


Fig.2c,d. For legend see p. 6.

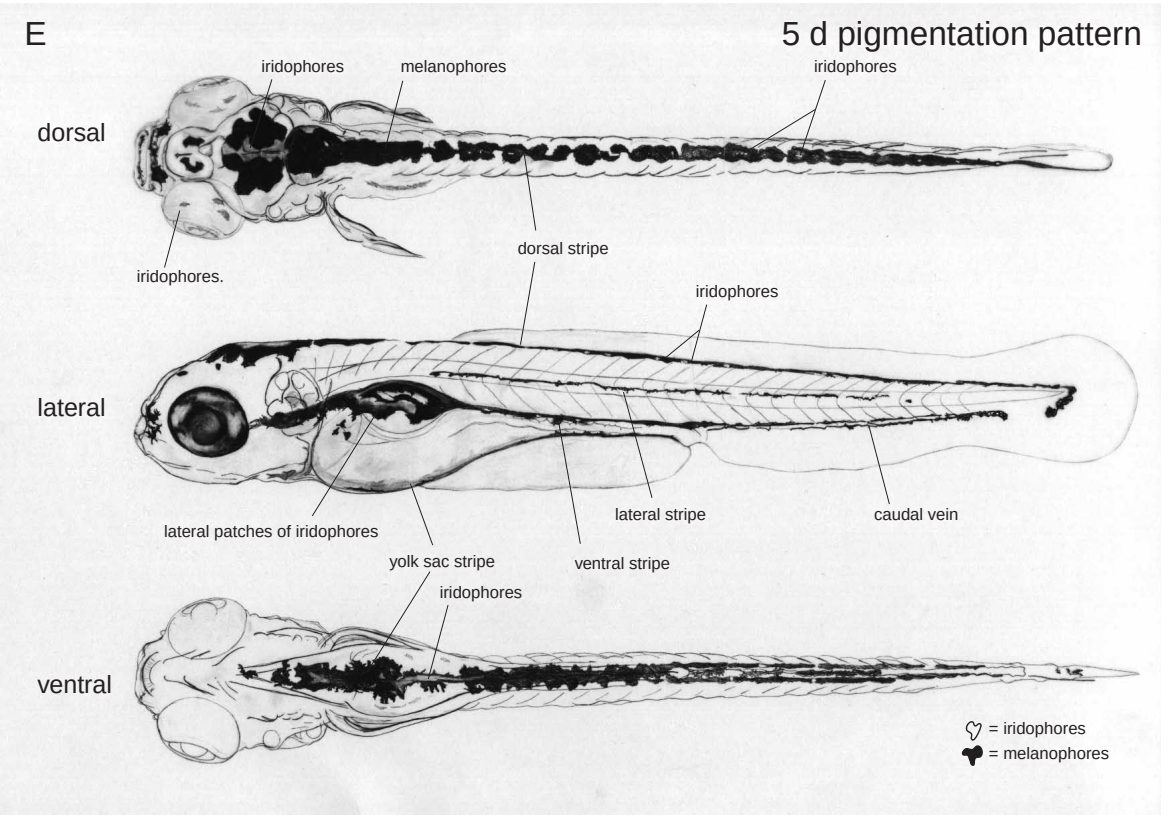


Fig. 2. Drawings of embryos at 24 hours (A), 48 hours (B,D), and 5 days (C,E) of development. For clarity, the melanophore pigmentation pattern is omitted from B and C. It is depicted in D and E. Most of the structures that can be seen in the living embryo with a compound microscope are marked.

size of the melanophores, and the intensity of pigmentation of xanthophores, melanophores and iridophores. We also checked whether the embryos had an air-filled swimbladder. The larvae were also inspected with incident light to examine iridophore pigmentation and muscle striation. To assay for motility, the larvae were swirled into the center of the dish and observed as they moved from the center. The response to touch was tested. During each screen, the general appearance of mutant embryos or larvae was noted, and it was recorded whether they showed signs of retardation (beady eyes, bent body, unconsumed yolk by day 5) or slow death (degeneration, edema, enlarged heart cavity).

The evaluations were recorded in a score sheet, and each phenotype recognized in a family was described separately. As expected, often a particular mutant phenotype occurred in more than one cross of a family. Often, more than one mutation was identified in different crosses within a family, and these were distinguished by consecutive letters added to the family name. In several cases two independent mutations occurred in one cross, and three different phenotypes were found (the singles and a double). In almost all such cases, the frequency of the doubles was 1/4 of that of the singles, as expected for independent segregation. In rare cases, the low frequency of singles indicated linkage between the two mutations. After the last screen, the larvae were fixed and subjected to a screen for mutations affecting the retinotectal projection (Baier et al., 1996).

Table 1. Mutagenesis of zebrafish

	Number (average)	Number (range)
Mutagenized males	49	
Families per male	63	0-181
Families raised	3075	
Families scored	2746	
Successful crosses	14357	20-60%
Crosses per family	4.7	0-18
Genomes screened*	3857	
Mutants identified	4264	
Mutants per family	1.4	0-8
Mutants per genome	1.1	

*Summarized over the numbers calculated for each family, starting from two heterozygous parental fish using the formula: $2x(1–0.75^n)$. n is the number of crosses scored per family.

Criteria for keeping mutants

On average 1.1 mutations with a recessive phenotype clearly visible in 25% of the embryos/larvae were found per mutagenized genome, confirming the result of a small-scale screen (Mullins et al., 1994). A total of 4264 mutants were tentatively identified by scoring 14357 successful crosses resulting from 2746 families (fish from 329 families did not give eggs) (Table1). Mutants were classified into one of four large classes of phenotypes. (A) general abnormality: degeneration, retardation, necrosis; (B) well defined specific deviation from normal morphology; (C) abnormal motility, or touch response; and (D) abnormal retinotectal projection.

Because of the large numbers, it was impossible for us to keep all identified mutants. Mutants with phenotypes characterized by rather general abnormalities (Class A) were therefore not kept. These mutants displayed any of four phenotypes, occurring with about equal frequency (Mullins et al., 1994): (1) degeneration of the entire embryo, observed during the hatching period-screen; (2) degeneration of the brain, usually followed by general necrosis of the entire body; (3) degeneration, associated with an enlarged heart cavity and reduced

circulation during the hatching period; (4) retardation, judged by underdevelopment of the jaw, the liver and gut, small eyes, and the presence of unconsumed yolk on day 6 of development. All these phenotypes affect the entire body of the fish and do not show any specific visible defects that are restricted to a limited set of body regions or organs. Furthermore, they comprise large numbers of mutants with similar phenotypes, which are difficult to distinguish. This makes it practically impossible to perform comprehensive complementation tests. Mutants with late phenotypes (affecting jaw and arches, liver, gut, eyes and ear, pigmentation of iridophores and xanthophores), were only kept if they did not show signs of severe retardation. Several phenotypes, especially those affecting late events, were difficult to detect or distinguish in a background of general degeneration or retardation. For instance, small eyes and small jaw and gill arches are features often associated with the general retardation phenotype, and we kept mutants with small eyes and arches only if other late features such as the development of the liver and gut appeared well advanced, or if associated with another specific phenotype such as pigmentation or fin defects. Mutants with fin necrosis were only kept if the embryos developed a swimbladder. This also applied to mutants that were morphologically normal but had expanded melanophores (black fish). Mutants with a curved body axis were kept only if the curvature was pronounced until day 6 of development. A number of egg lays showed variable abnormalities, notably anterior defects, which were rarely consistent and often occurred with frequencies deviating from the 25% expected for a zygotic mutant. In almost all such cases, in the rescreen, the egg lays were normal.

Rescreen, outcross and complementation

Parents of a total of 2092 independent mutant progeny were kept and subjected to a second screen. This included all mutants with distinct and specific phenotypes of classes B, C and D. In addition, we rescreened cases in which many embryos had lysed by the pharyngula period, which might be due to a mutation affecting the first 24 hours of development. We also rescreened mutants on which a clear decision could not be made.

In the rescreen, the embryos were scored during early somite stages, twice during the pharyngula period, and once at all subsequent days until the swimming larva period. Careful counts of mutant embryos were carried out and each screen was performed by at least two observers. Often, pictures of mutant embryos were taken as a permanent record and, if appropriate, the phenotype was described with the help of a compound microscope. After the rescreen, about 30% of the candidate mutants were discarded, and the mutants to be kept were classified using a three star system. The rescreen protocol was used for the subdivision of the mutants into phenotypic groups for further analysis. 22 mutants were lost before retesting. 513 mutants were either not confirmed or reassigned to class A, and discarded. 1474 mutants were confirmed during the rescreen, but 83 could not be rescreened because one of the parents had died, or because the pair did not produce eggs any more. The data are summarized in Table 2.

Table 2. Mutants recovered

Mutants	Number	Percentage
Total identified	4264	100
Kept for rescreen	2070	49
Not confirmed or lost in rescreen	513	12
F_3 raised	1557	35
Not confirmed in F_3	221	5
Lost in F_3	237	6
Newly discovered in F_3	102	2.5
Confirmed and characterized	1163	27
Mutants total	1163	=100
Assigned to genes	894	77
Unresolved	269	23

From these 1557 mutants, an F_3 generation was raised. In general one, and in particularly valuable mutants, both, of the parental fish were crossed to wild-type fish. Although the incidence of carrier fish in the F_3 generation would have been higher following the raising of viable fish from two heterozygous parents, in practice the viability and vigor of fish resulting from inbreeding was very much reduced compared to outbred fish. This was also true in the next generation, and therefore outcrossing to independent wild-type strains was usually performed. In the F_3 generation, heterozygous carrier fish were identified by sibling matings and used for further characterization of the mutant phenotype as well as for complementation crosses with other mutants. In the course of identification of carrier fish in the F_3, 121 previously unnoticed mutants were found, 102 of which were kept. About 221 mutants were lost, and 237 were classified as general abnormalities and discarded. In the end, 1163 mutants were kept (Table 2).

For the further analysis of mutant phenotypes, carrier fish were identified by scoring random crosses between siblings from the F_3 outcrosses. As a backup, sperm of mutant carriers was frozen as described by Westerfield (1993), with the modifications described by Driever et al. (1996). For the establishment of complementation groups, crosses were performed between mutants with similar phenotypes. The methods for the analysis of the mutant embryos are indicated in the individual papers in which the mutants are described in more detail.

RESULTS

Mutagenic treatment and screening

For large-scale mutagenesis, it is desirable to induce point mutations with a potent mutagen that mutagenizes genes on a random basis. For zebrafish, as for mice (Russell et al., 1979), ENU had the highest mutagenicity of the agents tested, inducing apparent point mutations at a rate of about one mutation causing a visible phenotype in the embryo or larva per treated haploid genome (Mullins et al., 1994). Alleles of previously known pigmentation mutants were induced at a rate of one to three per thousand treated genomes. Other mutagens such as X-rays, EMS, or insertion of DNA proved to be by one to several orders of magnitude less efficient at inducing recoverable mutations (Mullins et al., 1994; Solnica-Krezel et al., 1994, Gaiano et al., personal communication). The procedures used in the present screen have been described previously (Mullins et al., 1994; Materials and Methods section).

49 treated males provided the mutagenized genomes for inducing the 1163 mutants described in this issue (Fig. 3). Single pair matings were performed between F_1 fish and 40-70 F_2 progeny from each mating were raised. They constitute a family of sibling fish that share a gene pool of two mutagenized haploid genomes (one from each parent). In order to detect the induced mutations, crosses between F_2 siblings were carried out and the resulting embryos scored for the occurrence of mutant phenotypes. As only 50% of the fish in a family share a particular mutation, on average every fourth cross yields embryos homozygous for a mutation originating from one of the F_1 parents of that family. The probability of detecting such a mutation increases with the number of crosses. On average 4.7 crosses per family were evaluated from a total of 3075 families. This corresponds to 1.3 mutagenized haploid genomes on average screened per family, amounting to a total of 3857 mutagenized haploid genomes screened (Fig. 3; Table 1).

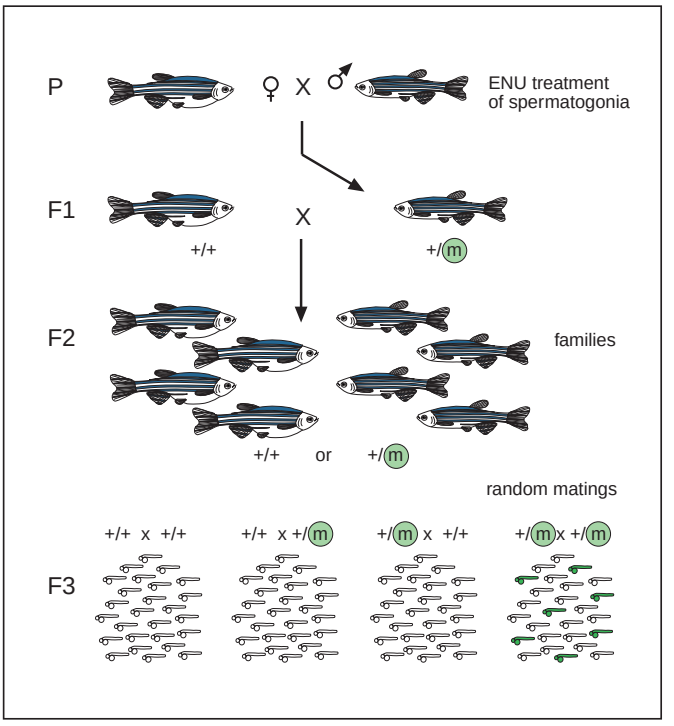


Fig. 3. Crossing scheme. Males mutagenized with ENU were mated to wild-type females. The F_1 progeny raised from matings 3 weeks after the mutagen treatment (resulting from treatment of spermatogonia) was heterozygous for one mutagenized genome. An F_2 generation was raised from sibling matings. A mutation **m** present in one of the F_1 parents was shared by 50% of the fish in the F_2 family. Eggs were collected from a number of matings between sibling fish. If both parents were heterozygous for **m,** expected in 1/4 of the crosses, 1/4 of the eggs will be homozygous and show the mutant phenotype. In this scheme, for simplicity, only one F_1 fish is heterozygous. In our experiment, both were heterozygous for a mutagenized genome. Therefore, the number of mutagenized genomes per family was two. The number of mutagenized genomes screened per family depends on the number of crosses (see legend to Table 1).

Complementation analysis

The mutants were assigned to one of several large groups according to the most prominent phenotypic feature. Within each group, several smaller subgroups were established in order to facilitate complementation tests among mutants. Several mutants were assigned to more than one group or subgroup, and a more careful analysis of phenotypic features was performed, depending on the phenotype. Crosses were performed between pairs of mutants with similar phenotypes. In general, not all possible complementation crosses within each group were done, and often only one or two members of one complementation group were tested against further mutants. In most cases, members of one complementation group showed the same or a very similar phenotype. In at least 40 complementation groups, alleles with different allelic strength could be identified. Many complementation groups have a unique phenotype, while in other cases a small number of genes share a similar or identical phenotype. In these cases, complementation testing presented no problem and the numbers of alleles per gene are reasonably high (see Table 5). Frequently, mutants display more than one phenotypic feature, often in seemingly unrelated traits, and therefore in several instances different alleles of genes were initially assigned to different groups. Examples are *chameleon* and *you too* (Brand et al., 1996b; van Eeden et al., 1996b), with a somite and a neural tube phenotype, *boxer* and *dackel* (Schilling et al., 1996; Trowe et al., 1996; van Eeden et al., 1996a), with a jaw, a pectoral fin, and a retinotectal pathfinding phenotype, and *bashful* (Odenthal et al., 1996a; Karlstrom et al., 1996) with a brain, a notochord and a retinotectal projection phenotype. For that reason we expect that in a few instances allelism will be assessed between what are now described as independent genes, following more extensive analysis and mapping. Difficulties were also encountered in cases where rather large numbers of mutants share similar phenotypes, with few clear cut and easily displayed criteria to distinguish between them, for example mutants with notochord degeneration (Odenthal et al., 1996a), circling behaviors (Granato et al., 1996), curly body (Brand et al., 1996b), and jaw defects (Piotrowski et al., 1996; Schilling et al., 1996). In many of these, it has not yet been possible to complete the complementation analysis.

Of the 1163 mutants isolated and kept from the screen, 894 (77%) have been tested in complementation with most other mutants with similar phenotypes. These mutants define a total of 372 genes, 150 of which have more than one allele. 222 of the genes are defined by only one allele. 269 mutants are yet to be characterized further (Table 3). They are listed as unresolved in the publications. The allele frequencies observed are not random (Table 4). While a small number of genes have high allele frequencies (11 genes with more than ten alleles, the maximum allele number being 34), many genes are represented by only one allele. The average allele frequency varies between 1 and 7.5 in different phenotypic groups (Table 5), with an overall average of 2.4.

Table 3. Allele frequencies: an overview

	Number	Percentage
Mutants kept in F_3	1163	100
Mutants assigned to genes	894	77
Mutants in unresolved groups	269	23
Mutants with more than one allele	672	75
Single mutants	222	25
		=100
Total number of genes	372	100
Complementation groups (>1 allele)	150	40
Genes with one allele only	222	60

Table 4. Allele frequencies: genes

n alleles	*n* genes	*n* mutants	*n* alleles	*n* genes	*n* mutants
1	222	222	11	3	33
2	61	122	12	2	24
3	28	84	13	0	0
4	15	60	14	1	14
5	12	60	15	1	15
6	9	54	16	0	0
7	7	49	17	1	17
8	3	24	22	1	22
9	3	27	23	1	23
10	1	10	34	1	34
Sum	361	712	Sum	11	182

The mutant phenotypes

The mutants cover a wide range of phenotypes (Table 5). The earliest effects are seen in embryos with mutations in a small number of genes, such as *half baked* and *lawine*, affecting epiboly. In homozygous embryos, the embryo retracts at about 80% epiboly, followed by lysis of the yolk cell (Kane et al., 1996a). The establishment of the major body axes is affected in genes described by Mullins et al. (1996) and Hammerschmidt et al. (1996a). *snailhouse*, *swirl* and *piggy tail* mutants show different degrees of partial dorsalisation, while *dino* and *mercedes* embryos appear ventralised. In *dino*, the ventralisation is already detectable before the onset of gastrulation, and the blastula fate map appears distorted, suggesting a role of *dino* in both mesodermal dorsalisation and neural induction. In most of these early acting genes, the phenotypes, although principally zygotically determined, are influenced by a dominant maternal effect, and one mutant with a dominant maternal phenotype has been isolated. Mutations in one group of genes cause developmental arrest in an early somite stage, or rapid necrosis after somitogenesis. These genes may be involved in cell adhesion or control of the cell cycle (Kane et al., 1996b).

Several genes are required for the development of the axial mesoderm. The notochord is absent in embryos mutant in four genes, *floating head, (flh) no tail (ntl), momo* and *doc*. While *ntl* and *flh* were known previously (Halpern et al., 1993), *momo* and *doc* are new. In all these mutants the notochord of the trunk is not formed, and the separation of the trunk somites into a dorsal and ventral portion by the horizontal myoseptum does not occur (Odenthal et al., 1996a). Six genes affect notochord differentiation and three of these, *bashful, grumpy* and *sleepy*, are also required for normal brain morphology and axonal pathfinding (Odenthal et al., 1996a; Karlstrom et al., 1996). In mutants of another group of genes, the notochord is bent or wavy (Odenthal et al., 1996a).

Somite formation is partially or completely abolished as a result of mutations in four genes (van Eeden et al., 1996b). The phenotype is already apparent at early somitogenesis, when in *fused somites* somitic boundaries in the paraxial mesoderm are not formed. Later, the fish develop severe abnormalities in the vertebrae, but move almost normally. A small group of genes is required for the subdivision of the somites into a dorsal and ventral part, and mutant larvae fail to display a horizontal myoseptum (van Eeden et al., 1996b). Mutations in two of these, *chameleon* and *you too*, result in defects in the floor plate and spinal cord (Brand et al., 1996b).

A large number of genes are required for the formation of a brain with normal gross morphology. The forebrain and eye anlage are transformed to structures normally derived from a more posterior region of the brain in the *masterblind* mutant (Heisenberg et al., 1996). In some mutants, the eyes appear closer together. In most but not all mutants of this group, this 'cyclopia' is correlated with a midline defect, i.e. reduction or absence of the prechordal plate, floor plate and a smaller spinal cord (Brand et al., 1996b). Examples are *cyclops* (Hatta et al., 1991), of which two alleles have been isolated in our screen, and *schmalspur*. The midbrain and the cerebellum are affected in mutants of two genes. While the tectum is also reduced in *no isthmus* mutants, it appears enlarged in *acerebellar* mutants (Brand et al., 1996a). A number of mutations affect the formation of several brain regions in various ways. The *white tail* mutation (Jiang et al., 1996) causes the formation of supernumerary neurons in various brain regions, for example extra Mauthner neurons, and other neurons are present. We have also investigated the cause of neural degeneration of the brain, which is often associated with later general necrosis. As this phenotype is rather frequent (we classified about 600 such mutants), a representative collection of 32 mutants has been investigated (Furutani-Seiki et al., 1996). They display symptoms of apoptosis. Although these 32 mutants fall into several classes with distinct phenotypes, in only a few cases do the mutations affect a single specific region of the brain.

The morphology of the otic vesicle is affected in the mutants investigated by Whitfield et al. (1996). Several genes are required for the formation of the otoliths; in *einstein* mutants, one of the two is absent in each vesicle, while in *keinstein*, both otoliths are absent, and in *rolling stones* the otoliths are loose. The ear size may be much reduced (*van gogh*) or enlarged (*lauscher*). Ear defects are often associated with other defects such as lack of the pelvic fin in adults (*stein und bein*) or jaw development (*van gogh*).

Neural crest derivatives are affected in a large number of mutants. Mutations in more than 80 genes required for pigment cells, their morphology, pattern and pigment formation, have been identified. The patterning and distribution of pigment cells is a phenotypic feature affected by mutations in nine genes (Kelsh et al., 1996). Eight genes are essential for the formation of melanin, and mutations affect both the non-crest-derived pigmentation of the retina and that of the melanophores. One of these genes, *sandy*, has been identified as the candidate to encode the tyrosinase gene (Haffter et al., 1996; Kelsh et al., 1996). Mutations in about 17 genes affect the xanthophores. Several of these produce associated phenotypes, a retinotectal phenotype (*esrom, tilsit and tofu*), or a recessive maternal effect lethal phenotype (*yobo*; Odenthal et al., 1996b). All neural crest-derived pigment cells are absent in the mutant *colourless*, while different combinations of pigment cell types are lacking or unpigmented in mutants of another 24 genes described by Kelsh et al. (1996). Mutations of 13 genes alter the shape and size of the melanophores. Often the mutant phenotypes include specific additional features such as small eyes, defective ear or arch development (Kelsh et al., 1996).

The formation of the jaw and gill arches occurs late in embryogenesis. It is affected in about 100 mutants in a rather specific manner. A number of genes identified are required for cartilage differentiation, such as *geist* and *hammerhead*. The lower jaw is defective in *sucker*, while *lockjaw* mutant embryos do not develop branchial arches (Piotrowski et al., 1996; Schilling et al., 1996).

The development of a number of internal organs can be followed readily in the fish embryo and early larva. Mutations affecting blood formation are described by Ransom et al. (1996). In *moonshine* and *yquem* mutants, no red blood cells are present. Other mutants have reduced numbers of blood cells, or the cells are paler in colour. The heart and circulation is abnormal in mutants of at least 30 genes studied by Chen et al. (1996). The heart may be enlarged (*santa)* or duplicated (*miles apart, casanova*), suggesting failure of fusion of the initially paired anlage, or the heart beat may be abnormal (*breakdance*). *kurzschluss* mutations affect circulation in trunk and tail, while circulation is misrouted in *bypass* mutants. We

Table 5. Genes identified in the Tübingen screen: phenotypic groups

Phenotypic group	Subgroup	Genes*	Genes (*n*)	Alleles (*n*)	Alleles/ gene	Un-resolved (*n*)	Reference
Early	Epiboly	*hab, ava, law, weg, (yob)*	4	4	1.0	0	Kane et al., 1996a
	Early arrest and necrosis	*zom, spb, spr, trl, kap, ban, ogr, plt, niv, ghl, hrp*	11	18	1.6	0	Kane et al., 1996b
Body axes	Dorsalised	*swr, snh, pgy, laf, sbn, mfn*	6	19	3.2	5	Mullins et al., 1996
	Ventralised	*mes, din*	2	4	2.0	0	Hammerschmidt et al., 1996a
	Others	*ppt, spt, tri, bib, udu, kgg, sam, sch, ind, mpt*	10	17	1.7	2	Hammerschmidt et al., 1996b
	Prechordal plate, hatching	*dns, zja, oep*	4	4	1.0	3	Hammerschmidt et al., 1996b
Mesoderm	Notochord formation	*flh, mom, doc, ntl, (din)*	4	9	2.3	0	Odenthal et al., 1996a
	Notochord differentiation	*hap, sny, dop, sly, gup, bal*	6	45	7.5	0	Odenthal et al., 1996a
	Undulated notochord	*qam, ctd, wat, ziz, kik*	5	13	2.6	0	Odenthal et al., 1996a
	Degenerating notochord, late notochord defects	*luc, blo, kon, (acc, beo, zim, tnt, sla, roc, baj, que, exp, pun, diw)*	3	4	1.3	18	Odenthal et al., 1996a
	Somite formation	*fss, bea, des, aei, (sap, wit)*	4	19	4.8	1	van Eeden et al., 1996b
	Somite patterning	*syu, you, yot, ubo, (ntl, mom, flh, doc, con, cho)*	4	7	1.8	0	van Eeden et al., 1996b
CNS	Forebrain	*mbl, kas, slb, (cyc)*	3	4	1.3	1	Heisenberg et al., 1996
	Midbrain-hindbrain	*ace, noi*	2	7	3.5	0	Brand et al., 1996b
	Hindbrain, a. o.	*snk, pac, nat, wit, atl, ele, ott, ful, bid, vip, win, wis, sbd*	13	26	2.0	0	Jiang et al., 1996
	Brain degeneration†	*(rec, aoi, mur, awa, yug)† (brd, dfd, van, blc, wei, tin, ger, ivy, sll, bch, fla, dns, stu)*	0	0	0	32	Furutani-Seiki et al., 1996
	Spinal chord, a. o.	*con, cyc, sps, bad, dtr, sur, igu, aqb, stb, slt, mol, smh, (yot)*	12	21	1.8	2	Brand et al., 1996a
	Curly tails	*spi, wir, cup, tig, lok, cos, sin, sic, vic, pir, hkn sen, snt*	13	58	4.4	31	Brand et al., 1996a
Organs	Blood	*mon, frs, weh, cha, ret, yqe, frx, zin, sau, cdy, cia, gre, mot, pnt, tbr, cab, ris*	17	32	1.9	2	Ransom et al., 1996
	Heart morphology	*sug, sco, cas, mil, fau, olp, san, loa, (nat)*	8	9	1.1	0	Chen et al., 1996
	Heart beat	*bre, tan, hip, plk, sth, sih, wea, web, tre, leg, quh, pip, str, sli, (hel,vip, hat, slp, jam, sky)*	14	18	1.3	4	Chen et al., 1996
	Circulation	*byp, kus, (syu, you, yot, con)*	2	3	1.5	0	Chen et al., 1996
	Liver, gut, kidney	*flo, gam, tip, lum, trp, stf, (lok, noi)*	6	6	1.0	0	Chen et al., 1996
	Eye	*bum, rne, kor, sri, hez, mic, dre, lep, uki, (cco, blc, pio, blr, fad, mlk, sbl, coo, bch, fla, ivy, van, mir, fdv, dfd, sll, flo, sug, sah, era, ser, bab, fac, dul)*	9	12	1.3	10	Heisenberg et al., 1996
	Otoliths	*eis, kei, men, hst, wup, sub, rst, bks, (clx, blc, cls, stw, nob)*	8	29	3.6	7	Whitfield et al., 1996
	Ear morphology and lateral line organ	*bge, hph, lau, lte, dog, eso, vgo, eps, spk, era, bxe, mtc, hps, (ace, cls, puz, sah, box, ful, dak, snk, ott, clx, wei, lep, dre)*	13	19	1.5	4	Whitfield et al., 1996
	Fins	*frf, nag, rfl, pif, fra, fyd, bla, ika, dak, med, krm, tut, sto, (mes, sub, box, lep, dre, fls, ubo, mon)*	13	61	4.7	5	van Eeden et al., 1996a
	Skin	*gsp, ddf, pen, bob*	4	9	2.3	1	van Eeden et al., 1996a
Pigment cells	Pigment cell number and pattern	*cls, shd, uns, spa, cho, los, snp, pde, slk, (sal, pfe, mom, tig, ful, flh, wit, mes, ubo, mon)*	9	32	3.6	1	Kelsh et al., 1996
	Melanin pigmentation	*gol, alb, sdy, mrd, bry, nkl, pew, led*	8	25	3.1	1	Kelsh et al., 1996
	Melanophore differentiation	*tdo, fad, lnd, sln, fdv, dfd, sah, (qam)*	7	21	3.0	5	Kelsh et al., 1996
	Melanophore shape	*sas, drp, ger, uni, obs, nir, zwa, mac, sum, tin, fam, pet, lak*	13	25	1.9	2	Kelsh et al., 1996
	Xanthophores	*pfe, kef, sal, esr, edi, yob, clx, til, bri, nob, qua, yoc, tof, fet, ric, tar, bst*	17	62	3.6	0	Odenthal et al., 1996b
	Iridophores	*lcl, tnd, hei, bed, dim, tod, mat*	7	8	1.1	1	Kelsh et al., 1996
	Two cell types affected	*cco, pio, pep, pup, pol, mlk, coo, ivy, pun, frk, van, mir, blr, cot*	14	40	2.9	10	Kelsh et al., 1996
	Three cell types affected	*wsh, blc, sbl, puz, bch, wei, sll, bli, stw, pch*	10	15	1.5	1	Kelsh et al., 1996
Jaw and gills	Flatheads, posterior arches	*fla, low, ser, bab, fac, dul, gap, (box, puz, pio, mlk, dak, pic, ger, sbl)*	7	11	1.6	32	Schilling et al., 1996
	Hammerheads, ant. arches	*hot, ham, hen, get, stu, jef, suc, dol, koi, hoo, pek, she, (sur, ppt, vgo, con, igu, yot, dtr, slb)*	12	22	1.8	22	Piotrowski et al., 1996

Table 5. *Continued*

Phenotypic group	Subgroup	Genes*	Genes (*n*)	Alleles (*n*)	Alleles/ gene	Un-resolved (*n*)	Reference
Motility	Reduced motility, muscle striation	*slo, fro, fub, tur, buf, fap, slw, sne, hem, dus, mah, slp, jam, sky, sap, sof, sml, ruz*	18	51	2.8	12	Granato et al., 1996
	Reduced motility, normal striation	*sop, nic, red, hat, hel, unp, sho, two, ali, far, mao, ste, cro, sla, slm, (ubo, syu, yot, flh, wis, wir, box, nor, zwa, sum, tdo, clx, luc, kon, sth, cls)*	15	25	1.7	21	Granato et al., 1996
	Abnormal motility	*acc, zim, beo, diw, que, baj, exp, spo, spc, twi, tnt, roc, way, her, (ntl, doc, mom, nev)*	14	35	2.5	17	Granato et al., 1996
Adults	Body shape	*lil, dml, stp, smf, (sps)*	4	4	1.0	0	Haffter et al., 1996
	Pigment pattern	*leo, obe, ase, (sal, pfe, spa)*	3	8	2.7	1	Haffter et al., 1996
	Eyes	*(bum, fdv, kor, rne, yob)*				0	Haffter et al., 1996
	Fins	*alf, lof, wan, fls, (frf, krm, mes, pif)*	4	4	1.0	0	Haffter et al., 1996
	Pigmentation	*(alb, bry, bum, drp, gol, mrd, obs, sdy, shd, tar, brs)*				0	Haffter et al., 1996
Retinotectal	Pathfinding	*ast, bel, blw, uml, (bal, esr, box, yot, con, til, dtr, igu, tof, gup, pic, sly, dak, cyc)*	4	7	1.8	0	Karlstrom et al., 1996
	Mapping	*box, nev, pic, blu, mio, woe, brd, (dfd, esr, noi, dak, ace, mao, til)*	7	23	3.3	14	Trowe et al., 1995
Total			372	894	2.4	269	

*Genes in brackets are dealt with in more detail in a paper describing a different class of mutants.
†This class of mutants was not kept systematically (Furutani-Seiki et al., 1996).

also isolated a small number of mutations affecting other internal organs such as the liver and the kidney (Chen et al., 1996).

Many mutants affect the outer morphology of the body of the fish larva or adult. At least 13 genes were identified that are required for the formation of the larval fins (van Eeden et al., 1996a). The pectoral fins are reduced in mutants in at least four genes such as *ikarus* (van Eeden et al., 1996a). With the exception of the pectoral fins, the adult fins are quite different from the larval fins in morphology and pigmentation, appearing only in the third week. Nevertheless a number of mutants with altered adult fin morphology were fortuitously isolated, such as *wanda*, *stein und bein* and *finless* (Haffter et al., 1996; van Eeden et al., 1996a). Often, mutants showing altered larval fin morphology are viable and the adults show no or only very mild mutant phenotypes (Haffter et al., 1996).

Dominant mutations causing visible phenotypes in the adult fish have also been identified (Haffter et al., 1996). Several lead to a short body and some show severe skeletal defects. Other dominant mutations cause alterations in the striped pigmentation pattern of the adult. Broader stripes are formed in heterozygous and homozygous fish mutant in the genes *asterix* and *obelix*. The stripes are interrupted in heterozygotes, and spotted in the homozygous mutants *leopard, salz* and *pfeffer* (Haffter et al., 1996). *salz* and *pfeffer* homozygous larvae lack xanthophores (Odenthal et al., 1996b; Kelsh et al., 1996). In several instances, mutants showing a pale pigmentation in the larvae reveal a related phenotype in homozygous adults, such as those affecting melanin production. These may be useful marker mutations (Haffter et al., 1996; Kelsh et al., 1996).

About 100 mutants were isolated with specific defects in the motility of the larva (Granato et al., 1996). Mutant larvae fail to respond to touch properly, or show reduced or abnormal spontaneous swimming or escaping behavior. The phenotype of 18 of these genes is associated with muscular defects visible with incident illumination, and in several cases (*sloth, buzz-off*) muscle abnormalities could be detected in sections. In another 29 mutants, no obvious morphological abnormality in the musculature could be found. The phenotypes show reduced or no motility (*sofa potato*), spastic and circling behavior (*techno trousers*), as well as defects in reciprocal inhibition of muscle contractions (‘*accordion*’ group, 7 genes). In a small number of mutants, defects in the outgrowth of motor neurons have been detected (*unplugged, diwanka*; Granato et al., 1996).

About 100 mutations affecting the establishment of the retinotectal projection were isolated in a screen performed on larvae fixed after day 5 of development (Baier et al., 1996). A number of genes were identified that affect the pathfinding of the retinal axons to the tectum (Karlstrom et al., 1996), and several others which are involved in the mapping on the tectum were characterized (Trowe et al., 1996). Although many of these mutants were isolated on the basis of the axonal pathfinding phenotype, in both groups the majority of mutants have additional visible phenotypes and many are also described in other contexts, such as *bashful, boxer* and *dackel*, and *esrom* (Odenthal et al., 1996a,b; Schilling et al., 1996). In two cases, a motility defect is associated with a retinotectal phenotype *(never mind, macho)* (Granato et al., 1996; Trowe et al., 1996).

Viability

In several mutants, homozygous embryos develop a swimbladder. These were classified as embryonic viable. In many cases, these could grow up to adults, which frequently, but not always, displayed a visible phenotype. Mutations in 79 genes are homozygous viable, and 19 semiviable (Haffter et al., 1996). Seven of the lethal genes also have viable alleles, indicating that the viable alleles are weak or hypomorphic. Mutations in 38 of the adult viable genes cause a recessive adult phenotype, while mutants in the remainder look normal as adults. Not all viable alleles have been tested for fertility, but one maternal effect mutant (*yobo*) was identified. Homozygous *yobo* larvae derived from heterozygous parents have

unpigmented xanthophores. Homozygous adult females produce embryos with a delay in early development and tail formation (Odenthal et al., 1996b).

Frequently, 25% or more normal-looking embryos did not develop a swimbladder and died eventually without being able to swim and feed. We did not assess the frequency of lethal mutations without any obvious phenotype visible in the embryos or larvae. These and mutants with later lethal periods are probably as frequent as those with visible phenotypes, but their frequency is difficult to determine with accuracy, as the development of a swimbladder may be inhibited by bad water conditions even in viable lines. In order to get a rough estimate of the number of lethal mutants induced, we determined the number of adult fish growing up from 70 F_2 larvae each of a total of 45 crosses. In about 50% of the lines, less than 80% of the fish grew up, while in control lines almost all survived to adulthood. This indicates that about one late lethal mutant was induced per haploid genome.

DISCUSSION

Phenotypic criteria and assessment of the screening procedure

The simple and clear axial body organization and optical clarity in early developmental stages make it possible to score for a large number of different phenotypic traits. As the effort and time involved in the production of mutant lines is considerable compared to the time of screening, we did not restrict ourselves to particular developmental processes or organs, but aimed at discovering genes needed in many aspects of development, morphogenesis, organogenesis, differentiation and simple behavior. Our aim was to approach saturation, i.e. the recovery of one or more alleles of most genes that can mutate to a specific phenotype visible in the embryo or larva. With the exception of the screen for mutants affected in the retinotectal projection (Baier et al., 1996), we did not apply any aids other than that of a stereomicroscope in our primary screen. This obviously sets a limit to the detectability of developmental or structural abnormalities. Subtle deviations can only be found fortuitously in this way. Screens for less conspicuous phenotypes would require the help of staining with molecular probes, or the restriction to a small number of phenotypic traits that can be analyzed with more care. As the scoring success depends on the uniformity and consistency of the phenotype, a rather large number of progeny were scored in each cross, and the frequency of embryos or larvae that could be sorted upon displaying a particular phenotype was taken as an important aid for recovery of a mutant. In our screen, we used a checklist containing a large number of phenotypic traits, organs and structures (Figs 1, 2). We did not include the olfactory bulb, the lateral line, the pronephros or the pancreas, although these structures can be seen in the living embryo at high magnification. Using the criteria described, we isolated a large number of valuable mutants, which reached the limit of our capacity to keep and analyze them.

As the visibility of the various organs and tissues depends on the developmental age of the animal, we screened at three different times during the first 6 days of development. In zebrafish, embryos or larvae lyse very quickly after death, and many phenotypes cause early death of the animal. Therefore it is important to recognize the phenotype as early as possible. It was impracticable to screen within the first 24 hours after egg lay, so the detection of mutants affecting very early processes depended on the assumption that early defects would result in severe abnormalities displayed on the second day of development. They were detected in the rescreen of parents that had produced 25% or more eggs that lysed within the first day in the primary screen. Such cases were always kept and rescreened. Although in the majority of cases the early lysis could not be confirmed, a number of mutants with very early phenotypes were recovered, such as *lawine* (Kane et al., 1996a) and *swirl* (Mullins et al., 1996). Transient abnormalities in e.g. gastrulation or epiboly, with little consequence for later development could, however, only be fortuitously detected in our screen. Examples are *mercedes* (Hammerschmidt et al., 1996a; van Eeden et al., 1996a) *silberblick* (Heisenberg et al., 1996) and *dirty nose* (Hammerschmidt et al., 1996b).

There are several other examples of phenotypic features that are best seen during a short developmental time interval. The folding of the brain is most conspicuous between 26 and 32 hours of development. Among the brain mutants, a case of a transient phenotype is *atlantis* (Jiang et al., 1996), for which four alleles have been isolated, with strikingly abnormal folding of the brain during the pharyngula stage, and later normal development, leading to adult viable fish. Mutants affecting heart development are also best scored during the hatching period, as heart failure often leads to the development of enlarged heart cavities and edemas (resembling one of the phenotypic classes we have discarded), obscuring a primary, more specific phenotype (Chen et al., 1996). Scoring of a number of phenotypic traits such as the floor plate and somite shape was easier after the larvae had hatched on the third day of development. Others were scored after final differentiation had occurred. This applies to structures developing from the neural crest, such as the pigment pattern (Kelsh et al., 1996), the pharyngeal skeleton (Piotrowski et al., 1996; Schilling et al., 1996) and internal organs such as gut and liver (Chen et al., 1996). By this time, normal development of the larva has been dependent on a large number of processes, and if some of them were abnormal, it is likely that later events will also have been affected. Therefore, only the earliest effect of any specific mutation could be detected with reliability.

The majority of mutations detected in our screen lead to the formation of rather general abnormalities causing the slow death of the animal. We did not keep and analyze mutants with only such general defects. Because their phenotypes suggest a rather broad range of function in the maintenance and differentiation of many tissues in the body, they are of little value for the investigation of defined processes of development and differentiation. We kept mutants in which a specific phenotypic trait appeared before, but not after, the onset of general degeneration, and most of our mutants do not show general abnormalities. In summary, while we tried to be consistent in using the same cut off criteria during the duration of the screen, it is likely that some of the mutants that were kept and analyzed shared characteristics of others that were discarded. This means that for several phenotypic groups the allele frequencies are expected to be lower than average.

In other organisms in which systematic mutant searches have been carried out, a screen for lethal mutants often preceded the scoring of the mutant phenotype. This is possible

in organisms for which marker mutants and balancer chromosomes exist, allowing the scoring for the absence of a particular class of progeny in suitable crosses. In the mouse, a screen for lethal mutations that map in the region of the inversion associated with T mutations has been carried out (Shedlovsky et al., 1986). In *Drosophila*, the screens for zygotic mutants affecting the pattern of the larval cuticle were done for the first and second chromosome by prescreening for viability first (Nüsslein-Volhard et al., 1984; Wieschaus et al., 1987), while the screen for mutants on the third chromosome relied on the phenotype only (Jürgens et al., 1984). In *Drosophila* screens, in contrast to the zebrafish screen, the use of conditional lethal mutants and balancer chromosomes permits the selection of parental flies heterozygous for the same mutagenized chromosome, and thus only crosses yielding homozygous mutant progeny are scored. The lack of balancer chromosomes in the zebrafish makes it necessary to inspect eggs from multiple crosses per family, as was done in our screen. In zebrafish, the isolation and characterization of marker mutations has only just begun, and the large number of chromosomes (Endo and Ingalls, 1968) will make the use of special balancer chromosomes impracticable. Thus the only possible method of detection of mutants in zebrafish screens is direct detection of the mutant via its phenotype from multiple random crosses and this will probably remain the case for a long time. Because the entire genome is screened at once, rather than each chromosome in a separate screen, this can be considered as an advantage. Furthermore, the large number of linkage groups allows the independent segregation of multiple mutations which may be induced with high mutagen doses.

Phenotypic spectrum, complexity of phenotypes and groups of genes

In our screen, we have isolated and characterized mutants with alterations in more than 372 genes. While some have been described previously, most are new. We assume that most of the mutants represent a lack of function (or reduction of function) condition, as they have been induced with the point mutagen ENU. This is likely in cases where genes have more than one allele with the same phenotype, while in cases of the single mutants a fraction may be deletions, or translocations, or rare gain of function mutants. In two cases where a mutant allele from our screen has been sequenced (Talbot et al., 1995), a single amino acid change was detected, which probably eliminated the gene's function.

The genes identified share the common feature that each of them has an indispensable function in development. No other gene can completely take over another's role, although partial compensation may occur in cases of genes with overlapping function. In the mouse, as in *Drosophila*, several cases are known where single mutants display only a very mild phenotype while the phenotype of double mutants is much stronger. Examples in *Drosophila* are the *Polycomb* group of genes (Jürgens, 1985) and in the mouse the myogenic genes, *myoD* and *myf5* (Rudnicki et al., 1993). The study of the phenotypes of double mutants in zebrafish, which have only just begun, will reveal whether genes with overlapping functions have been identified in our screen.

The mutants show a broad range of phenotypes. Many phenotypes are restricted to a rather defined organ or tissue, or developmental process. Clearly, not all imaginable phenotypes have been found. Many genes produce quite unique phenotypes that are distinct from those of all other genes. Examples are *masterblind*, *colourless*, *unplugged* and *van gogh*. There are a number of cases where small groups of genes are responsible for the same or a very similar phenotype, such as those affecting early notochord development (Odenthal et al., 1996a; four genes), somite formation (van Eeden et al., 1996b; four genes), dorsalisation (Mullins et al., 1996; six genes), the *'accordion'* group (Granato et al., 1996; seven genes) and notochord differentiation (Odenthal et al., 1996a; two groups of three genes each). Such groups are not very large, and do not exceed seven. The similarities in phenotype suggest that the genes within the group participate in the same developmental process. The small number of similar genes may indicate that linear pathways are not long (or that many components went undetected). In the majority of cases, careful investigation of the mutant phenotypes using additional criteria revealed small but significant differences between genes, and indistinguishability is the exception rather than the rule. It is therefore likely that each of the genes has a quite unique function in its pathway, that the pathways are often branched, and that many genes participate in more than one pathway. By and large, the picture is similar to that obtained from the comprehensive screen for zygotic mutants in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980). Most genes identified in the *Drosophila* zygotic screens are responsible for distinct phenotypes, although many could be grouped by similarity in phenotype. The molecular analysis of most of them has now complemented the phenotypic analysis. In the majority of cases, genes producing similar, but not identical, phenotypes are participants in the same pathways and often interact. Examples are *wingless*, *hedgehog*, *engrailed* and *patched* in segmentation, *Notch*, *Delta*, *big brain* and *neuralized* in neurogenesis, and *decapentaplegic (dpp)*, *short gastrulation*, *tolloid*, *screw*, *schnurri*, *thick vein* and *punt* in dorsoventral pattern formation.

Many of the zebrafish genes have complex phenotypes in which more than one structure is affected. Often, closer inspection reveals a functional link. For example, the notochord lesions observed in the *accordion* group of mutants seem to be caused by the abnormal contraction of the body (Granato et al., 1996). Mutations affecting the establishment of the body axes may show reduction or overproduction of blood cells, and affect the tail fin, as the most ventrally derived body structures (Hammerschmidt et al., 1996a; Mullins et al., 1996). In such cases, analysis of different phenotypic traits helped in interpreting the mutant phenotype in the context of gene function. In other cases, complex phenotypes include seemingly unrelated phenotypic traits. Mutations in *moonshine* affect pigmentation as well as blood formation (Kelsh et al., 1996; Ransom et al., 1996), the ear is affected in the pigmentless mutant *colourless* (Kelsh et al., 1996; Whitfield et al., 1996), and in *no isthmus*, the brain and the pronephrous are affected (Brand et al., 1996a; Chen et al., 1996). Such findings were more or less expected. Many cloned genes are expressed in more than one region of the body and often display more than one phenotypic trait. Even entire pathways are used repeatedly to control the patterning of quite different organs or processes, such as the *en-wnt* pathway in segmentation and disc patterning in *Drosophila*, and in brain and limb morphogenesis in vertebrates. While the complexity of the phenotypes often makes

their interpretation difficult and ambiguous, it may be helpful in cases of groups of genes sharing seemingly unrelated phenotypes. Examples are *boxer* and *dackel*; mutations in both produce three similar phenotypes: defects in the gill arches, in retinotectal mapping and in pectoral fins (Schilling et al., 1996; Trowe et al., 1996; van Eeden et al., 1996a). The notochord, retinotectal pathfinding and brain morphology are affected in very similar ways in *bashful, grumpy* and *sleepy* (Odenthal et al., 1996a; Karlstrom et al., 1996). The strict correlation of these phenotypes suggests that the genes in the group cooperate, and that they do so in more than one pathway.

Degree of saturation; number of genes with unique functions

The 372 or so new genes are probably only a fraction of all genes in the zebrafish that could have been detected with the criteria of our screen. To date it is difficult to estimate to what degree our screen has reached saturation for such genes. The large number of genes defined by only one mutant (which may get somewhat reduced upon further complementation analysis, but probably not substantially) indicates that many genes may have gone undetected by chance. The number of genes missed in the screen calculated from single mutant frequencies may be biased, however. Although many of the single mutations may be in genes with low hit frequency, a substantial fraction may also be gain of function mutations, or chromosomal aberrations such as deletions or translocations, causing phenotypes that are not easily recognized as allelic with lack of function mutations in the same gene. Although in many cases several mutations have been identified in the same family, and often in the same cross, it is unlikely that the collection contains many double mutations that have been mistaken for single hits. The large number of linkage groups in the zebrafish make independent segregation likely for the great majority of independent double hits. For the genes with more than one allele, it is likely that the common phenotype represents the lack of function condition of the gene. In at least 40 genes, alleles with different phenotypic strength have been isolated.

The allele frequencies are different for different phenotypic groups, ranging between 1 and 7.5 (Table 5). This variation reflects the ease with which a phenotype can be detected. For example, the notochord is very easy to see in the living embryo, and notochord genes have a rather high allele frequency (Table 5). Similarly, the mutations affecting one pigment cell type only are represented with rather high allele frequencies. In other cases, it is likely that mutants were either not always recognized, or not always kept because they were classified as general defects. This is probably the reason for the low allele frequency of genes affecting the heart, and the jaw and gill arches. We therefore believe that in some phenotypic classes, the degree of saturation is rather low, while in others, almost all genes have been detected in our screen.

About 75% (672/894) of the characterized mutants (58% of all mutants) fall into complementation groups with at least two alleles in our collection (Tables 3, 4), and this number will undoubtedly increase substantially when the data of the Boston and the Tübingen screen are merged by comprehensive complementation tests. Genetic mapping of the mutations using molecular markers will greatly aid the completion of our complementation data. The finding that, for the majority of our mutants, at least one additional independent allele is already known, means that continuation of the screen would mainly lead to the isolation of alleles of previously identified genes rather than to the discovery of new genes. We have investigated whether our mutant collection contains alleles of the genes described in detail by the Oregon laboratory. With the exception of *brass*, in all cases tested, alleles have been found. This sample includes *spadetail (2), no tail (3), floating head (2), sparse (9), albino (6), golden (2), cyclops (2)* and others (Kimmel et al., 1989).

Taking our data at face value, the average allele frequency of the 372 genes is 2.4, with a range between one and 34 alleles per gene. This number is much lower than that expected from our prescreen (average 2.3 hits per gene per thousand genomes (Mullins et al., 1994), predicting an average allele frequency of 9 in the 3857 genomes tested here. It is possible and even likely that the mutations we used for the determination of the mutagenicity were in genes with relatively high hit frequency, as these were among the first genes identified in the zebrafish. For the four genes for which the hit frequencies were determined in the prescreen we found 0 (*brs*), 2 (*gol*), 9 (*spa*) and 6 (*alb*). The average is 4.5. This number is also lower than expected from the prescreen. Several factors may have contributed to the observed discrepancy. We believe that the most relevant of them is that, given the large number of crosses that were screened by a large number of observers for many different phenotypic traits, phenotypes may have been overlooked more frequently than in the prescreen, in which we specialized on scoring only a small number of traits.

Assuming a Poisson distribution of frequencies, (i.e. equal mutability for all genes), the degree of saturation is high, approaching 90%. The calculation from the numbers of genes with one and two alleles each, however, gives a rather different picture, about 50%. Using this value we estimate that the total number of genes mutating to an indispensable and specific phenotype detectable with our criteria is less than 700. We would like to emphasize, however, that this number can only be taken as a very rough approximation, as the mutability is certainly not random, the single class is probably artificially enlarged, and the bias against discovery varies between phenotypes.

Limits of a genetic screen

Limits of detection are an important consideration, both for the allele frequencies and for the type of genes that could not be identified in our screen. Since we screened for obvious visible defects, mutations in genes with only subtle phenotypes would not have been detected. Allele frequencies might also be influenced in cases where only strong alleles had a significant phenotype while weak alleles could not be detected.

In genes where null mutations cause dominant lethality or semilethality, only weak alleles may have been detected. In the *Drosophila* screens, a number of important genes with dominant semilethal effects were identified, and predominantly weak alleles were recovered in the screens. All the gap genes, and many of the pair-rule genes, for example, are haploinsufficient, and the rate of survival depends on the genetic background. *twist, snail* and *decapentaplegic*, required in gastrulation in *Drosophila,* display dominant embryonic phenotypes. In the case of *twi* and *sna,* they do not affect viability; however *dpp* is haplolethal, and only weak alleles have been isolated in the *Drosophila* screen. Homologs of *dpp* exist in vertebrates

(*Bmp4*) and have important functions in gastrulation (Dale et al., 1992; Jones et al., 1992). Some of the mutants we isolated have dominant phenotypes in embryos, with adult viability often reduced compared to wild-type siblings, and they may represent haploinsufficient genes for which strong alleles have not been found (*you too*, *doc*, *dino*, *swirl*; Hammerschmidt et al., 1996a; Mullins et al., 1996; Odenthal et al., 1996a; van Eeden et al., 1996b).

Maternal effects may also modify or weaken an essential zygotic contribution of a gene in early developmental processes. As the females producing the eggs are heterozygous, a maternal product placed in the egg, in the form of RNA or protein, could postpone the requirement for the gene expressed in the zygote, and only mild or undetectable phenotypes might result. Many genes known to be expressed early in a spatially restricted manner have been shown to have maternal messages in the fish embryos (e.g. *snail-1*, *goosecoid*; Hammerschmidt and Nüsslein-Volhard, 1993). In reciprocal matings between alleles of different strength, or between mutants with similar phenotypic traits, several cases of dominant maternal effects were found, in particular in mutants of genes affecting the earliest processes detectable (*lawine* and other epiboly genes, *swirl*, *piggy tail*, and *dino*; Hammerschmidt et al., 1996a; Kane et al., 1996a; Mullins et al., 1996).

The most severe limitation of the detection of genes with important functions by mutational approaches is probably redundancy: completely or partially overlapping functions of two or more genes in the same process. In contrast to the *Drosophila* genome, the zebrafish genome, for many cases tested, contains large gene families, or series of genes with similar sequence and potentially similar function. Duplicated genes with partially overlapping functions also exist in *Drosophila*, but their number is probably much smaller than in most vertebrate genomes. Despite this finding, in *Drosophila* the fraction of genes displaying a visible and specific phenotype in mutants is also small, and lies in the range of 5% of all essential genes mutating to lethality (5000), and 1% of all transcription units (20,000). Redundancy might also be the reason why we did not find mutants with phenotypes obviously resembling those of the segmentation genes, in particular the gap genes of *Drosophila*. The gap genes control both segmentation and the spatially restricted expression of the homeotic genes in *Drosophila*. Homologs to those in vertebrates are the Hox genes, and to date the primary control of their expression is not known. The gap genes generally encode transcription factors with zinc fingers. A very large family of zinc finger genes exist in the zebrafish, and it is possible that the genes in question are duplicated and therefore not detectable in mutant searches.

Our data allow a very crude estimation of the number of genes essential for viability and/or normal development in the zebrafish. The number of genes mutating to a visible and specific phenotype is assumed to be 700 (see above). The number of mutations in genes with a visible but unspecific phenotype (class A) is about 2.4 times as large. Assuming equal hit frequency, we calculate a total number of genes mutating to an embryonic or larval visible phenotype of about 2400. Mutations causing death or later abnormal development can be determined from the fraction of progeny from an F_2 cross that do not grow up to adulthood. We estimated that the number of later lethals is similar to the number of visible embryonic and larval lethals. This estimate suggests a total number of roughly 5000 lethal genes in the zebrafish. This is about the number estimated for *Drosophila*, although the total number of genes in the zebrafish is probably larger than in *Drosophila*. The difference may reflect the higher incidence of duplicated genes in the fish.

Potentials of genetic screens

Despite the lower degree of saturation reached in the fish screen compared to the *Drosophila* screens, the number of genes identified in the zebrafish is much larger. This is because of the optical clarity of the fish, enabling more phenotypic traits to be scored in the living embryo. In *Drosophila*, the internal organs are not as easily visible as in the fish embryo, and scoring for visible phenotypes (rather than absence of particular progeny) requires staining with appropriate markers and/or clearing. Further, vertebrate-specific organs such as the notochord, fins, heart, eye, ear and neural crest derivatives, (jaw and branchial arches, pigmentation), could be scored in the fish. The investigation of the development of these organs, so far predominantly approached by descriptive approaches and rather difficult in vitro experiments, will be aided greatly by the availability of mutants.

The particular achievement of a systematic mutagenesis is the comparative completeness of the collection of genes with similar phenotypes. For many phenotypic traits, a number of distinct genes have been identified. The function of each has to be considered for approaching an understanding of the developmental process concerned. The number of genes with similar phenotypes allows an estimate of the complexity of the regulatory processes involved. Complex phenotypes or groups of genes may provide links between seemingly unrelated developmental pathways. With only a random assortment of individual genes identified on fortuitous grounds and known from different viewpoints, the assessment of the relative importance and role of individual functions remains elusive. Of unique value in this context is the possibility of defining pathways by groups of genes with similar phenotypes, the products of which interact, directly or indirectly. To date, there is no single more successful approach than saturation mutagenesis, as has been amply illustrated by the analysis of the genes identified in *Drosophila* and *Caenorhabditis elegans* in systematic mutant searches.

In *Drosophila*, the majority of the genes identified by mutations with a specific and visible phenotype have been cloned by now, and in many cases they have helped to elucidate novel pathways and developmental mechanisms. This demonstrates that the mutant screens have been extraordinarily powerful in selecting for genes with important and unique functions. It often seems that genes with important functions in *Drosophila* have homologs with similar, and similarly important, functions in the vertebrate. It is likely that many of the genes encode such factors already identified from the homology searches. While mapping of our mutants has only just begun, in a small number of cases segregation analysis involving cloned genes has been carried out. In two cases, *floating head* (Odenthal et al., 1996a; Talbot et al., 1995) and *no isthmus* (Brand et al., 1996a), identity between a gene cloned previously and from our collection of mutants could be shown. On the other hand, many of the genes we identified probably encode functions that are not related to those in

Drosophila, or the great morphological differences in many cases will preclude a recognition of similarities. The adventure of a mutant screen is the discovery of new functions. Their recognition and interpretation, however, requires careful investigation of the mutant phenotypes. In the accompanying papers we describe what we know about the genes we identified. While in some cases the relationship between well-described developmental processes and a mutant phenotype made the interpretation straightforward, in most cases we are still at the beginning of an understanding and interpretation of the function of the genes, and further analysis of the phenotypes with a variety of experimental means will be necessary. We expect that once the genes identified by mutations are mapped and eventually cloned, a number of novel mechanisms will be discovered, and important insights into previously untractable processes will be achieved.

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All mutants are available upon request.

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