

## A genetic screen for mutations affecting embryogenesis in zebrafish

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### SUMMARY

Systematic genome-wide mutagenesis screens for embryonic phenotypes have been instrumental in the understanding of invertebrate and plant development. Here, we report the results from the first application of such a large-scale genetic screening to vertebrate development.

Male zebrafish were mutagenized with N-ethyl N-nitrosourea to induce mutations in spermatogonial cells at an average specific locus rate of one in 651 mutagenized genomes. Mutations were transmitted to the F<sub>1</sub> generation, and 2205 F<sub>2</sub> families were raised. F<sub>3</sub> embryos from sibling crosses within the F<sub>2</sub> families were screened for developmental abnormalities. A total of 2337 mutagenized genomes were analyzed, and 2383 mutations resulting in abnormal embryonic and early larval phenotypes were identified. The phenotypes of 695 mutants indicated involvement of the identified loci in specific aspects of embryogenesis. These mutations were maintained for further characterization and were classified into categories according to their phenotypes. The analyses and genetic

complementation of mutations from several categories are reported in separate manuscripts. Mutations affecting pigmentation, motility, muscle and body shape have not been extensively analyzed and are listed here. A total of 331 mutations were tested for allelism within their respective categories. This defined 220 genetic loci with on average 1.5 alleles per locus. For about two-thirds of all loci only one allele was isolated. Therefore it is not possible to give a reliable estimate on the degree of saturation reached in our screen; however, the number of genes that can mutate to visible embryonic and early larval phenotypes in zebrafish is expected to be several-fold larger than the one for which we have observed mutant alleles during the screen. This screen demonstrates that mutations affecting a variety of developmental processes can be efficiently recovered from zebrafish.

Key words: zebrafish *Danio rerio*, mutagenesis, genetic control, embryogenesis

### INTRODUCTION

Genetic analysis of vertebrates, especially mice, has traditionally focused on traits that are expressed postnatally, and do not lead to embryonic lethality (Lyon and Searle, 1989). Zygotic-effect mutations leading to intrauterine embryonic lethality are difficult both to identify and to analyze in mammals. Therefore, there have been few mutations found to interfere with the establishment of the body plan and early steps of organogenesis. This situation has changed since targeted gene disruption makes it possible to follow the segregation of mutant alleles using molecular analysis, and to determine whether a given embryo is homozygous for the induced mutation (Capecchi, 1989). This technology has made a tremendous impact on our understanding of vertebrate development (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Joyner et al., 1991; Carpenter et al., 1993; Urbanek et al., 1994; Wurst et al., 1994). Two important approaches

remain very difficult in mice. First, forward genetics to identify novel genes involved in embryogenesis is prohibitively expensive. Second, experimental and embryological studies of mutations acting early in embryogenesis are greatly inhibited by the intrauterine mode of development.

It is exactly the combination of the last two approaches that has been the experimental basis for the current detailed understanding of invertebrate and plant development. Large scale, systematic genetic screens led to the characterization of genetic pathways that pattern the *C. elegans*, *Drosophila* and *Ara-bidopsis* embryos (Hirsh and Vanderslice, 1976; Nüsslein-Volhard and Wieschaus, 1980; Mayer et al., 1991).

Zebrafish (*Danio rerio*), a small tropical freshwater teleost, was recognized as a genetic system in which similar approaches would be feasible (Streisinger et al., 1981). The high fecundity, short generation time and rapid development of the externally fertilized, translucent embryos make it an excellent vertebrate genetic model system (reviewed by Driever et al., 1994). Devel-

opment of the zebrafish embryo (Kimmel et al., 1995) has been studied in detail, from pre-gastrula and gastrula stages (reviewed by Driever, 1995; Kuwada, 1995; Solnica-Krezel et al., 1995; Woo et al., 1995) through organogenesis (Schmitt and Dowling, 1994; Stainier and Fishman, 1994), lending a solid base of knowledge for the detection and interpretation of mutant phenotypes. Prior to our screen, nine zebrafish mutations affecting embryogenesis have been published and their effect on development studied in detail, providing important insights into mechanisms of vertebrate development (Grunwald et al., 1988; Kimmel et al., 1989; Felsenfeld et al., 1990; Westerfield et al., 1990; Hatta et al., 1991; Halpern et al., 1993; Abdelilah et al., 1994; Stainier et al., 1995; Talbot et al., 1995).

Chemical mutagens are preferred over gamma- or X-rays for genome-wide screens, since they predominantly induce lesions limited to single genes (Singer and Grunberger, 1983). N-ethyl-N-nitrosourea (ENU), which had been demonstrated previously to be the most potent mutagen of the mouse germ line (Russell et al., 1979), can induce mutations in the proliferating germ line of male zebrafish with high efficiency. Specific-locus rates ranging from one newly induced allele per 300 to 2000 mutagenized genomes were achieved for four different pigmentation loci. Methods have been developed to induce and recover mutations in zebrafish on a large scale, with the goal of saturating the genome for mutations affecting embryogenesis (Mullins et al., 1994; Solnica-Krezel et al., 1994; Riley and Grunwald, 1995).

Here, we report the results of one of the two first large-scale screens for embryonic visible mutations in a vertebrate (for details of the other, parallel screen see Haffter et al., 1996). Our screen is based on the following concepts. (1) Detailed visual inspection of living zebrafish larvae during several stages of embryonic and early larval development will identify mutants deficient in genes that are required for patterning and differentiation of the embryo. The translucent zebrafish embryo is ideally suited for such a study, since from fertilization to late organogenesis, development can be observed *in vivo*. (2) Recessive embryonic lethal mutations can be efficiently recovered in a two-generation breeding scheme (Haldane, 1956). (3) The analysis of large F<sub>3</sub> egg clutches (20-90 embryos) makes it possible to distinguish polygenic or non-genetic defects from single-gene mutations, which will segregate in a Mendelian fashion.

Here and in the accompanying manuscripts (Brockhoff et al., 1995; Abdelilah et al., 1996; Neuhauss et al., 1996; Pack et al., 1996; Schier et al., 1996; Solnica-Krezel et al., 1996; Stainier et al., 1996; Stemple et al., 1996; Weinstein et al., 1996; Malicki et al., 1996a,b), we present 577 mutations that were found to affect specific aspects of early zebrafish development.

## MATERIALS AND METHODS

### Fish stocks and fish keeping

Fish husbandry and genetic crosses were performed as previously described (Solnica-Krezel et al., 1994), except that the fish water is now based on reversed osmosis water (Ionpure Reversed Osmosis system, Millipore), with the addition of 157 mg/l calcium sulfate (Plaster of Paris), 20 mg/l sodium bicarbonate and 100 mg/l Instant Ocean salt mix (Aquarium Systems, Mentor, Ohio). The pH in the recirculating water system equilibrates to about 6.9, due to acid production in tanks and filters.

The mutagenesis was performed using fish of the 'AB' line (Chakrabarti et al., 1983). The AB line was maintained in our facility by inbreeding, and fish stocks were tested every generation for the absence of embryonic- or larval-lethal mutations.

### Mutagenesis and breeding of F<sub>2</sub> families

The screen was designed as a two-generation breeding screen with analysis of mutant phenotypes in F<sub>3</sub> embryos (Solnica-Krezel et al., 1994). Adult male AB zebrafish were incubated at weekly intervals for 1 hour each in solutions of the mutagen ENU. Nine sets of males were mutagenized using ENU at concentrations between 2 mM and 3.5 mM, and the dose was repeated two to six times (see Table 1). About 240 out of 266 males survived the mutagenic regimens. Mutations were recovered from mutagenized proliferating germ line, such that mutations were fixed in the G<sub>0</sub> male. The mutagenized males were bred several times with females heterozygous for four unlinked pigmentation mutations to determine the efficiency of mutagenesis (Table 1; and Solnica-Krezel et al., 1994). Between 4 weeks and 2 months after mutagenesis, males were bred with AB females, except for the NB 11 set, for which about half of the progeny was obtained from crosses with AB/HK hybrid females. The F<sub>1</sub> generation comprised 17,000 progeny of G<sub>0</sub> males from 346 crosses. Survival of F<sub>1</sub> larvae to adulthood was low, and ranged between 10% and about 70% for individual families.

From August 1992 to March 1994, up to 50 F<sub>2</sub> families were generated every week. Only crosses with more than 60 larvae with inflated swim bladders by 5 dpf (days postfertilization) were kept and up to 125 larvae were raised. 2312 families from F<sub>1</sub>×F<sub>1</sub> crosses, and 487 from F<sub>1</sub>×AB were raised, representing a total of 5111 mutagenized genomes. Of these 2799 F<sub>2</sub> families, 594 were discarded before the age of 4 months, since the number of fish had dropped below 20, which made efficient screening impossible. Of the 2205 F<sub>2</sub> families raised to adulthood, 397 could not be screened because either they contained fish of one sex only, or no productive crosses could be obtained. Thus, 1808 F<sub>2</sub> families (317 from F<sub>1</sub>×AB, 1491 from F<sub>1</sub>×F<sub>1</sub> crosses) representing 3299 genomes, were screened. The overall screen is depicted in Fig. 1.

At the time the screen was designed, the only possibility for mapping of mutations appeared to be based on a RAPD map (Postlethwait et al., 1994), so care was taken to perform the mutagenesis and generate F<sub>1</sub> and F<sub>2</sub> families in an AB-strain inbred background. With the availability of an SSR-based map, however, mapping of mutations recovered from outbred backgrounds has become feasible (Knapik et al., 1996).

Since specific locus rates had been determined for each of the separate mutagenic regimes, we were able to compare the specific locus rate (Solnica-Krezel et al., 1994) with the number of lethal mutations induced per mutagenized genome (Table 1), and found that the correlation was good.

### Organization of the screening procedure

For each F<sub>2</sub> family, up to 25 single pair crosses were set up in breeding traps, in the late afternoon. Starting at noon on the next day, eggs were harvested into Petri dishes, and parents of successful crosses were transferred pairwise into containers with about 1.5 l of fish facility water (disposable plastic containers, 1.9 l, Fisher). Each container with F<sub>2</sub> parents, as well as the Petri dish with their progeny, was given a unique label for later reidentification. Fish were stored in these containers for up to 7 days without feeding and survived as well as fish in tanks. If fewer than six crosses with more than 20 fertilized embryos each were obtained, the F<sub>2</sub> family was crossed again 1 or 2 weeks later before fish were discarded. A minimum of 20 and up to 90 fertilized embryos from each cross were sorted, at 6-12 hours postfertilization (hpf), into Falcon 6-well tissue culture plates, 30 embryos per well.

As soon as specific phenotypes were observed, parental fish were transferred from containers to facility 11 tanks and fed. The same pair was crossed again 1-2 weeks later, and phenotypes were analyzed in more detail and documented photographically. If the phenotype was confirmed, and segregated in about one quarter of the embryos, inter-

**Table 1. Mutagenesis conditions and the recovery of embryonic and larval lethal mutations**

Mutagenesis	Conditions, ENU treatment	Specific locus rate	Genomes screened	Lethal mutations	Lethals per genome
NB4	6×1 hour, 3 mM	1:735	315.2	393	1.25
NB5	6×1 hour, 3 mM	1:495	373.3	390	1.04
NB6	2×1 hour, 3 mM	1:2040	173.5	85	0.49
NB7	4×1 hour, 3 mM	1:578	281.6	381	1.35
NB8	4×1 hour, 2.5 mM	1:1298	175.1	196	1.11
NB9	4×1 hour, 2 mM	1:1351	245.9	209	0.85
NB10	4×1 hour, 3 mM	1:719	226.6	251	1.11
NB11	4×1 hour, 3.5 mM	1:719	176.3	205	1.16
NB12	4×1 hour, 3 mM	1:385	369.8	273	0.74*
NB total		1:651	2337	2383	1.02

The specific locus rate for NB4 to NB11 was determined for the pigmentation loci golden, brass, sparse and albino, and is reported in Solnica-Krezel et al. (1994).

\*Screening for NB12 was less efficient since a nematode infection affected survival of fish.

esting and specific mutations were assigned an allele number ( $m$ ). For preservation of mutations, usually three sperm samples were frozen from the heterozygous  $F_2$  male (see below). Further, heterozygous fish were outcrossed with an 'AB' strain fish, and sometimes with a 'HK', 'Tübingen' or 'AB-*gol*' strain fish. The outcrossed  $F_3$  progeny was raised, and segregation of the mutation in the  $F_3$  as well as  $F_4$  generation was determined.

We tried to screen at least six eggclays per  $F_2$  family to optimize the chance of finding a mutation against the effort involved and facility space required. The probability of finding a mutation in a family is  $P=(1-0.75^n)$ , where  $n$  is the number of successful crosses. For  $n=6$  (4;8),  $P$  gives 0.82 (0.68;0.9), respectively. Thus, by screening a minimum of six crosses for a family, we would lose at most 18% of the mutations. The rationale behind analyzing at least 20 embryos per cross was twofold. First, the probability of finding at least one mutant among 20 embryos if both parents are heterozygous is 99.6%. More importantly, on average there are five mutant embryos of a given mutation among the 20 embryos, thus allowing the segregation of a mutant phenotype to be determined during the initial screening.

The actual number of mutagenized genomes analyzed per  $F_2$  family was calculated according to the following equation:

$$G = (1 - 0.75^n) \times a,$$

where  $G$ =genomes analyzed;  $n$ =number of crosses with more than 20 embryos;  $a$ =number of mutagenized genomes per family:  $a=1$  for  $F_1 \times AB$ - and 2 for  $F_1 \times F_1$ -derived  $F_2$  families).

### Visual screening of embryonic and early larval development of $F_3$

Each cross was analyzed under a dissecting microscope (Wild M5 with transmitted light base; 5-50× and 10-100× optics) at five stages during embryonic and early larval development. At each time point, phenotypes were documented on a protocol form, and the percentage of mutant embryos noted.

(1) At 6-12 hpf, morphology of the gastrula was examined during sorting of fertilized embryos. Progress of epiboly, formation of the embryonic shield and quality of the yolk cell were recorded.

(2) At 24-36 hpf the major focus of screening was the central nervous system (CNS) morphology. During later stages of development, the morphology of the brain is more difficult to screen, since the brain becomes more compact and obscured by pigmentation. The following were analyzed: shape of diencephalon, telencephalon, epiphysis, tectum, cerebellum, midbrain-hindbrain border and hindbrain; size of brain ventricles; shape of the spinal cord, presence of floor plate; shape of eyes and otic vesicle. In addition, the shape and proper differentiation of notochord and somites, the overall shape of the body and yolk, and the presence of a touch response, were noted.

(3) At 48-60 hpf the major focus was on morphology and function

of the cardiovascular system. By this time, the heart and vasculature are fully functional. Screening for cardiovascular defects was performed at this early stage because pericardial and general edema and/or secondary cardiovascular defects can obscure the phenotype at 3 dpf or later. Morphology of the heart, beat rate, blood flow and the major vessels were analyzed. Additionally, pigmentation and body shape were evaluated.

(4) At 72-84 hpf the following features were analyzed: general body shape and pigmentation; morphology of the brain, eyes, ears and spinal cord; formation of jaw and branchial arches; form and function of heart, blood and the circulatory system, presence of edema; and differentiation of notochord, somites, muscle, pectoral and tail fins. Furthermore, touch response and motility of the larvae were tested by tapping them with a needle at one side of the trunk. Wild-type larvae respond with a typical fast-start escape response (Eaton and Bombardieri, 1978). Normally, by 72 hpf all larvae are hatched. The percentage of non-hatched larvae was recorded, and larvae that did not hatch were tested for motility by tapping the chorion, or dechoriation.

(5) After 5 dpf morphological analysis and tests performed at 3 dpf were repeated. Following observation of touch response and motility, fish were anesthetized for detailed analysis of late differentiating structures. Specifically, the morphology of the jaw and branchial region was inspected. Number and location of lateral line organs was analyzed. Presence of intestinal organs (gut, liver, pancreas) and anus was verified, and larvae were inspected for formation of edematous kidney cysts. Inflation of the swim bladder was scored for crosses with mutant phenotypes. In a subset of mutagenized lines (corresponding to about 500 mutagenized genomes), a detailed screen of the morphology of gut, liver and pancreas was performed utilizing a Wild M10 dissecting scope and 50-100× magnification (Pack et al., 1996).

Mutations that lead to extensive cellular degeneration, and/or block circulation, before 72 hpf (when the hatching period begins; Kimmel et al., 1995), are called embryonic lethal. Mutations that result in defects manifest between 72 hpf and 5 dpf are classified as early larval mutations. Most of the latter are also lethal, as indicated by a failure to inflate the swim bladder and/or to feed.

### Maintenance of stocks and mutant alleles

Mutant alleles are maintained as outcrossed stocks and/or frozen sperm samples. Care was taken to have one to three samples frozen from the original heterozygous AB male(s) isolated during the screen. Up to six additional samples were frozen from the first and second generation outcrosses into AB, Tübingen or HK strain backgrounds. About 2900 sperm samples have been processed this way. More than half of the mutations reported from this screen were initially maintained as frozen samples only, and subsequently recovered by in vitro fertilization for further characterization of the mutant phenotype.

The protocol for cryopreservation of sperm was modified from that

described by Westerfield (1994). The detailed protocol is available, for brevity of printed space, at our WWW server sites 'http://zebrafish.mgh.harvard.edu' or 'http://zebrafish.uni-freiburg.de'.

**Genetic nomenclature and availability of mutations**

Mutant alleles and genetic loci are named according to the nomenclature rules for zebrafish (Mullins, 1995). Allele designations include the letter *m* indicating our laboratory as the source of the mutation, and a number, consecutively assigned to mutations as they were recovered during the screen.

If complementation within a phenotypic class of mutations has been completed, locus names have been assigned to complementation groups. Locus names and three-letter abbreviations are used in the text. Therefore, each locus reported from this screen should represent a separate gene. However, if alleles at a locus express unrelated phenotypes, complementation would not have been performed and separate names assigned. Genetic mapping of mutant alleles will resolve these rare problems. All mutations for which complementation has not been performed or not completed are referred to by their allele designations only.

All mutations reported by allele designation in this and the accompanying manuscripts are available on request. Requests should be directed to 'Driever@helix.mgh.harvard.edu'. Since mutant stocks will soon be also maintained at other sites, in the future the address where a specific mutation can be obtained will be made public on the Oregon (http://zfish.uoregon.edu) or MGH (http://zebrafish.mgh.harvard.edu) or Freiburg (http://zebrafish.uni-freiburg.de) zebrafish www Internet servers.

Due to limited space and resources, for most of the mutations we will only be able to send out batches of larvae from outcrosses of heterozygous fish. Mutant stocks that are not currently maintained in our laboratory will require us to schedule the recovery of the mutant allele from frozen sperm, raise the mutant stock in our facility, identify the heterozygous fish, replenish the depleted number of frozen sperm samples, and prepare a cross for shipment. Thus it might take several weeks to more than 6 months before stocks carrying a mutation can be shipped, depending on availability of heterozygous fish in our facility. A stock center for the zebrafish community is being planned to expedite this process.

**RESULTS**

**Design of the genetic screen**

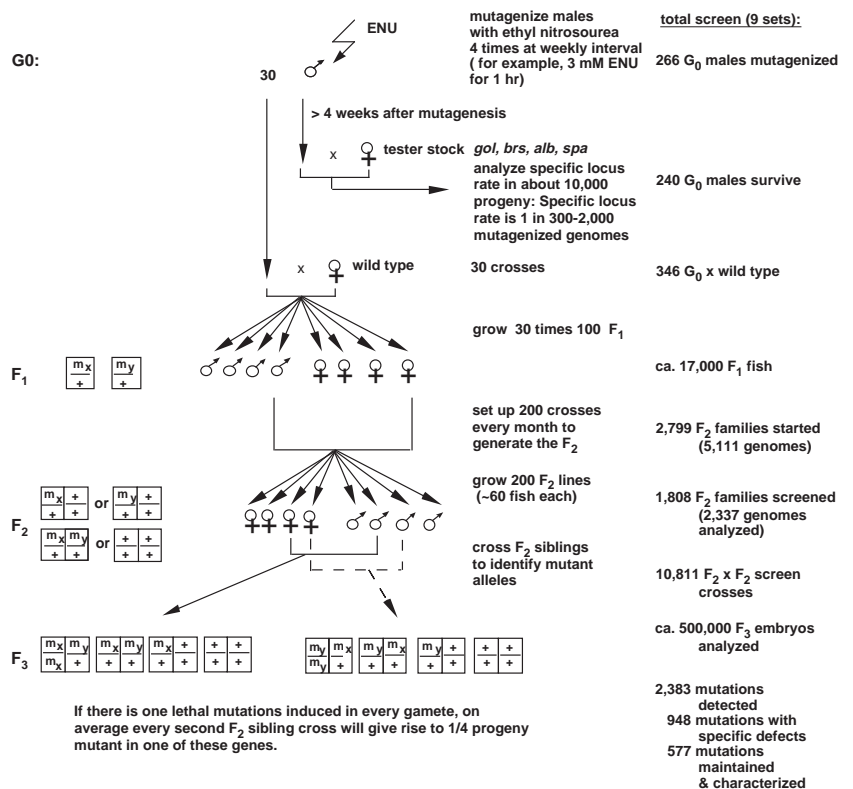
Our screen was designed to recover zygotic-effect mutations in genes involved in a broad range of developmental processes, namely establishment of the embryonic axes; early pattern formation and regionalization of the germ layers during gastrulation; differentiation of mesodermal derivatives; brain and sense organ formation; and finally, various aspects of organogenesis. The rationale for designing the screen to study such a wide range of developmental processes was threefold. Firstly, a broad screen can help answer important questions about the vertebrate genome, for example: Which developmental processes are sensitive to mutations? What type of embryonic-lethal and early larval lethal phenotypes can be observed? Secondly, we reasoned that the resources and time necessary to perform a screen for mutations affecting a single aspect of development would be similar to one of broader design. The same number of fish would have to be raised and the same number of crosses performed to screen the required

number of mutagenized genomes. Moreover, we wanted to establish a genetic resource that would attract researchers to exploit the advantages of the zebrafish system and use a genetic approach to study aspects of development that are difficult to investigate in other systems.

A classic two-generation breeding scheme (Haldane, 1956) was employed in the screen. With this approach, the segregation of recessive mutations can be easily tested, and the F<sub>2</sub> parents of a cross producing mutant F<sub>3</sub> embryos are identified as heterozygous carriers for the mutation. We decided against an F<sub>1</sub> screen based on haploid embryos to avoid the obfuscation of mutant phenotypes by the abnormal development of haploid embryos. Additionally, we chose not to utilize the 'early pressure' approach to generate diploid gynogenic embryos, since the fraction of mutant embryos of the developing half tetrad F<sub>2</sub> would depend on the gene centromere distance, and bias against isolation of mutations in genes close to the telomeres (Streisinger et al., 1981; Streisinger et al., 1986; Driever et al., 1994).

We raised 2799 F<sub>2</sub> families, a total of about 224,000 larvae, representing 5111 mutagenized genomes. Only 1808 F<sub>2</sub> families finally produced successful crosses for F<sub>3</sub> embryos to be screened. More than 30,000 crosses were set up, 10,811 of which produced a sufficient number of fertilized embryos for screening. Nearly 500,000 F<sub>3</sub> embryos were visually analyzed at five different developmental stages for abnormalities in development. Based on the number of crosses analyzed for each family, we calculate that we effectively screened 2337 mutagenized genomes of the 3299 genomes represented in the 1808 F<sub>2</sub> families.

Based on the specific locus rates determined after ENU mutagenesis, we had previously argued that a screen of 1600 genomes would be sufficient to identify mutations in 87% of all genes of



**Fig. 1.** F<sub>2</sub> screen to identify embryonic lethal mutations in zebrafish.

average mutability. For genes of less than average mutability, we estimated that 5,000-10,000 genomes would have to be screened (Solnica-Krezel et al., 1994). Based on this estimate, initially F<sub>2</sub> families representing 5111 mutagenized genomes were generated. The number of genomes actually screened was still well above the number of genomes estimated to be necessary to saturate for mutations in loci that mutate at average rates.

Given that only a small number of mutagenized genomes (on average about 150) was screened from each mutagenized male, the chance that alleles at any of the identified loci represent clonal events is small (see also Discussion in Solnica-Krezel et al., 1994). For most loci with several mutant alleles, we can trace mutant alleles to different sets of mutagenized males (data not shown).

### Detection of mutant phenotypes

Detailed visual inspection of living zebrafish embryos at five stages during embryogenesis and early larval development was the main strategy to identify new mutations. Two additional small-scale screens were carried out in parallel. In one screen, F<sub>3</sub> crosses representing 241 mutagenized genomes were screened for abnormal optokinetic behavior. This screen was based on analysis of the optokinetic nystagmus (OKN), and led to the isolation of two mutations with absent or abnormal OKN, both being morphologically normal. An additional sixteen mutations with both morphological and OKN defects were identified. Results from this screen are published elsewhere (Brockerhoff et al., 1995).

In a second small-scale screen, we wanted to assess whether a visual inspection of brain morphology is sufficient to detect most abnormalities in organization of the zebrafish brain. Embryos from crosses representing 101 mutagenized genomes were screened for abnormal distribution and levels of acetylcholine esterase (AChE) activity in the zebrafish brain at 24 hpf. At 24 hpf, AChE is expressed in a characteristic pattern in early neuronal clusters of the fore-, mid- and hindbrain (Ross et al., 1992); the rhombomeric organization of the hindbrain is especially well visualized. Our pilot screen did not reveal any specific mutation that was not already detected by morphological criteria (two mutations affecting brain development, and five mutations affecting neuronal survival were recovered per 100 genomes; Table 4). Frequently reduced AChE levels were encountered in 25% of embryos due to delay of embryonic development. In all

cases, live siblings maintained for visual inspection indicated a mutation-induced delay of development that was not specific to the CNS. Thus, the effort to perform a AChE screen was not considered an efficient way to recover subtle CNS mutations.

### Classes of mutations isolated during the screen

During the screen, 2383 mutations that lead to abnormal embryonic and early larval development were found (Table 2). Thus, about one mutation with a visible phenotype was induced per mutagenized haploid genome. Due to lack of good cytogenetics in zebrafish, we were not able to determine whether all of these mutations represent lesions in single zygotic genes, or if some of them are caused by chromosomal abnormalities. However, we applied the strict criterion that only mutant phenotypes segregating at 25% were considered for our statistics. Further, since we screened on average more than five crosses per F<sub>2</sub> family, we determined that a given such mutation was recovered on average from one of four F<sub>2</sub> crosses (data not shown). Therefore, we consider it likely that most of the mutations presented here do not represent chromosomal rearrangements producing aneuploid embryos, or haploinsufficient maternal or zygotic effect mutations. Phenotypes were classified into four major categories.

(1) 494 mutations resulted in a widespread, apparently non-tissue-specific onset of degeneration between 1 and 6 dpf. In most cases, delay of developmental progress was detectable as early as 24 to 48 hpf. We assume that these genes are essential for general cellular survival.

(2) 587 mutations resulted in onset of retarded development between 2 and 5 dpf. The embryos developed a syndrome of defects, including small eye and brain size; under-differentiated and small jaw and gill region; short and underdeveloped pectoral fins; and delayed differentiation of internal organs. The trunk and tail region appeared to be largely normal. The extent of delay, and the degree to which different parts of the embryo were affected, were variable. We hypothesize that this group of mutations affects basic functions of all cells in the embryo. The late onset of the phenotype could argue that maternally derived gene products sustain development of the embryo for the first few days. We note that tissues most affected are late differentiating/proliferating tissues, while somitic muscle, for instance, is not initially affected.

(3) 351 mutations lead to degeneration of the entire CNS.

**Table 2. Categories of embryonic and larval lethal mutations detected in the screen**

Categories	Mutations	%
Widespread, apparently not tissue-specific; onset of degeneration 1-6 dpf	494	20.7
Normal early development, delayed development obvious only later than 24 hpf, late differentiating structures like jaw, pectoral fins, eyes, intestine very small and underdeveloped	589	24.7
Degeneration of CNS, not localized, onset 1-5 dpf. Death of embryo usually within two days	352	14.7
Abnormal late development, 'organogenesis': embryos develop normally during the first day, as judged by visual inspection	687	28.8
Abnormal development visually detectable during the first day of development: pattern formation and early differentiation	261	10.9
<b>Total number of mutations</b>	<b>2383</b>	<b>100</b>
Mutations maintained for further characterization after screen	695	29.2
Mutations reported here	577	24.2

Onset of degeneration was between 1 and 5 dpf. In most cases, degeneration spread within 24-48 hours to the rest of the body. These mutations could affect loci specifically required in the neural lineages, or, perhaps, the phenotypes reflect a more stringent requirement for metabolic gene products in the neural cell types.

Mutations from the above three categories were not maintained, since they did not appear to affect specific aspects of development and occurred too frequently to be maintained. However, all these mutations were characterized well enough during the screen to determine that they are very likely to be zygotic effect, recessive lethal mutations. Only mutations that segregate in 25% of the F<sub>3</sub> crosses were included in this count. During the first third of the screen, F<sub>2</sub> crosses were repeated to confirm segregation for these mutations. Frequently, the mutations were recovered in several (about every fourth) of the crosses within a given F<sub>2</sub> family.

(4) 261 mutations lead to region-, tissue- or organ-specific abnormalities during the first day of development, and 687 after the first day of development. Among these 948 mutations, 695 were maintained for further characterization. The other 253 mutations were of one of the following groups: (1) abnormal body curvature (bent toward dorsal, ventral or lateral) with no other visible morphological defects; (2) subtle deviations from normal pigmentation pattern or intensity of pigmentation; (3) mutations that were lost after phenotypes had been confirmed, and therefore neither outcrosses nor frozen sperm samples generated. For the first two groups, a subset of mutations was maintained (see below).

The 695 mutations maintained for further characterization are categorized in Table 4. Of the 695 initially characterized mutations, 114 were lost during attempts to recover the mutations, or were found to be non-specific, i.e. displaying a phenotype similar to one of the first three classes described above. Most misassigned mutations were in the eye and craniofacial groups. Often a developmental delay was initially misinterpreted as a region-specific defect. Many of the lost cardiovascular mutants were initially characterized as valve or functional phenotypes of variable penetrance, and difficult to recover from outcrosses.

### Complementation analysis and number of genetic loci

Complementation analysis was performed within phenotypically related groups of mutations. The number of crosses performed to isolate a sufficient number of heterozygous fish for allelism tests, and the actual complementation crosses, represented an effort comparable to the initial screen. Embryos from more than 17,000 crosses were analyzed to prepare and perform the complementation analysis reported in the above listed manuscripts.

Complementation within the phenotypic classes was completed for 331 mutations. These mutations define 220 genetic loci. On average 1.5 alleles per locus were recovered (Table 3).

### Miscellaneous mutations

Mutations affecting several aspects of zebrafish development have not yet been extensively analyzed. Classification of these mutations and their isolation numbers are listed in Table 5. More detailed information on these mutations is available on the World Wide Web at 'http://zebrafish.mgh.harvard.edu', including a brief description of each mutation, as well as several photographs depicting the mutant phenotype.

### Sources for updates on genetic information

Complementation of mutations isolated in this screen with those performed in other laboratories, further characterization of phenotypes and genetic mapping of mutant loci, will continuously increase the usefulness of these mutations in the study of vertebrate development. Under the lead of M. Westerfield and the University of Oregon computer science group, a searchable database will be established at the University of Oregon that will contain frequently updated information on zebrafish genetic resources (<http://zfish.uoregon.edu>).

In the meantime, updated and extended information will be available on the Internet/WWW at 'http://zebrafish.mgh.harvard.edu'. For mutations presented in this and the accompanying manuscripts, phenotypic descriptions and sets of microphotographs will be available. Further, a database will be established and routinely updated to search all published mutations by locus name and locus abbreviation, allele designation, and phenotype keywords.

## DISCUSSION

### Mutational analysis of vertebrate development

Our genetic screen demonstrates that it is possible to efficiently induce and recover mutations in various aspects of zebrafish development. Mutations affecting early developmental events, such as patterning of the embryonic axes and gastrulation movements (Solnica-Krezel et al., 1996), as well as mutations in genes controlling organ development (Stainier et al., 1996), were recovered. Moreover, since the screen was performed on living embryos and larvae at several stages of development, mutations affecting not only the form, but also functional aspects of early larvae, were identified. Examples include mutations affecting heart beat rate (Stainier et al., 1996), touch response or optokinetic behavior (Brockerhoff et al., 1995). Additionally, genetic pathways controlling the formation of certain structures, such as the notochord, may be described (Stemple et al., 1996).

A relatively small number of mutations has been isolated and shown to be involved in formation of the embryonic axes at the beginning of gastrulation (Solnica-Krezel et al., 1996). The same is true for anterior-posterior patterning of the brain (Schier et al., 1996). Whether this finding reflects a strong maternal contribution, or redundant genetic pathways, or the inability of this screen to isolate additional mutations involved

**Table 3. Distribution of frequencies at which alleles for individual loci have been recovered**

Alleles/locus	Loci	Number of mutations
1	164	164
2	31	62
3	13	39
4	6	24
5	3	15
6	1	6
7	0	0
8	0	0
9	0	0
10	1	10
11	1	11
Average=1.51	Total=220	Total=331

Only loci from phenotypic classes for which complementation has been completed are listed.

**Table 4. Phenotypic classes of mutations isolated during the genetic screen**

Class of mutations	Mutations				Loci <sup>2</sup>	Alleles per locus	References
	Isolated	Lost <sup>1</sup>	Non-specified	Confirmed			
Gastrulation, tail formation	38	10 <sup>3</sup>	3	25	14	1.8	a
Notochord	68	2	1	65	39	2.1	b
Brain development	44	3	4	37	21	1.8	c <sup>8</sup>
Early onset and neural degeneration	82	7	2	73	54	1.4	d
Late onset neural degeneration	33	–	–	(33) nd	nd	nd	e
Eye development	60	5	12	43	34	1.3	f <sup>6</sup>
Ear development	46	7	1	38	13	1.5	f <sup>9</sup>
Craniofacial development	59	6	5	48	34	1.4	g
Blood	13	–	–	13	9	1.4	h
Heart and vasculature	109	51 <sup>4</sup>	–	58	39	1.5	i
Gastrointestinal organs	9	3	–	9	6 <sup>5</sup>	1	j
Kidney	17	–	–	(17) nd	nd	nd	k
Fin development	43	nd	nd	(43) nd	nd	nd	l
Optokinetic response	2	–	–	2	2	1	m
Motility	24	1	–	23	nd	nd	n
Muscle and somite development	7	–	–	7	nd	nd	n
Pigmentation	59	5	–	54	nd	nd	n
General body shape	29	1	–	28	nd	nd	n
Total <sup>7</sup>	695	–	–	577	–	–	

<sup>1</sup>Lost either due to inability to freeze sperm prior to death of founder fish, or to inability to recover the mutant allele from an outcross obtained from a frozen sperm sample.

<sup>2</sup>Pending completion of complementation for about 5% of loci.

<sup>3</sup>Efforts are still undertaken to recover six potential maternal effect mutations from these ten.

<sup>4</sup>Data to differentiate between lost and nonspecific mutations not available.

<sup>5</sup>Three mutations were lost; however the phenotype was well enough documented to be reported.

<sup>6</sup>Not including *cyc*, *oep* and *boz*.

<sup>7</sup>Some mutations are listed in several groups, since the phenotypes are pleiotropic. Therefore, the sum of the numbers listed for the individual classes is in most cases larger than the total.

<sup>8</sup>Not including *sly*, *bal*, *gup*.

<sup>9</sup>Number of loci does not include 18 mutations affecting otoliths, for which complementation has not been completed.

n.d. not determined

References: a, Solnica-Krezel et al. (1996); b, Stemple et al. (1996); c, Schier et al. (1996); d, Abdelilah et al. (1996); e, M. Rodriguez et al. (unpublished); f, Malicki et al. (1996a,b); g, Neuhauss et al. (1996); h, Weinstein et al. (1996); i, Stainier et al. (1996); j, Pack et al. (1996); k, I. Drummond and W. Driever, unpublished; l, M.-A. Akimenko and W. Driever, unpublished; m, Brouckerhoff et al. (1995); n, this manuscript.

in these processes due to haplo-insufficient effects (see below), remains to be determined.

### Estimates on saturation and essential gene number

We have recovered new alleles in each of the six previously published zebrafish zygotic-effect genes, *ntl*, *flh*, *cyc*, *fub*, *clo* and *spt*. While it is tempting to use this fact as an argument that alleles in a large portion of all developmental control genes were recovered during the screen, it may indicate that most of the previously identified genes are mutational ‘hot spots’, and more effort is needed to saturate the genome for mutations affecting development.

Indeed, a statistical analysis of the distribution of allele number indicates that the 220 loci defined here represent only a small portion of all loci mutable to an observable developmental phenotype in the zebrafish genome. Since more than two-thirds of all loci are represented by only one allele it is impossible to provide a reliable estimate of the total number of genes required specifically for the control of development. The data on allele distribution reported here do not fit a Poisson distribution, which would require genes to be mutable at a similar rate. Fitting the data to a negative binomial distribution indicates that a large class of loci exists for which no alleles were recovered. It is possible that the number of developmental control genes is several-fold higher than the estimated 400

genetic loci (577 mutations, 1.5 alleles per locus average) for which alleles were recovered.

Similar conclusions would apply to estimates of the total number of genes essential for embryonic and early larval survival. If one applies the same rate, 1.5 alleles per locus, to the total number of 2383 embryonic and early larval lethal mutations identified in the screen, one can estimate that they represent about 1600 essential loci. Again, the total number of essential genes would be expected to be several-fold higher. Such estimates are close to data obtained from mutational analysis of the mouse genome, for which estimates of the total number of essential genes range from about 5,000 to 10,000 (Carter, 1957; Shedlovsky et al., 1986).

These estimates are significantly larger than earlier estimates of the total number of loci yielding embryonic lethal mutations in zebrafish (Mullins et al., 1994; Solnica-Krezel et al., 1994). It appears that many genes mutate at rates significantly lower than the pigmentation loci used to determine the specific locus rates after ENU mutagenesis. One possibility is that the *albino*, *golden* and *sparse* loci used to determine the specific locus rates are mutational hot spots, which in turn could explain why they were the first genetic pigmentation defects characterized in zebrafish (Chakrabarti et al., 1983). The *brass* locus, which mutates at two- to fivefold lower rates, may in fact be more representative of the zebrafish genome. Further, the specific locus test is more

sensitive when compared to the F<sub>2</sub> screen, since even weak alleles can be recovered when *trans*-heterozygous with strong alleles. During the F<sub>2</sub> screen, a homozygote of a weak allele may result in a phenotype too subtle or penetrance too low to be recovered.

While saturation for loci mutable to visible phenotypes has not been reached, it is important to note that our screen indicates that a large number of developmental pathways, and many of their components, are amenable to genetic analysis. This holds true both for early, gastrulation phenotypes as well as for late organogenesis phenotypes. A large number of loci, probably in the thousands, can be defined by their phenotype during embryogenesis and early larval development.

### Limitations of this genetic screen

Several limitations on the recovery of developmental control genes are intrinsic to the design of the screen. Maternal-effect genes, which in *Xenopus* have been shown to play an important role in pattern formation (Kessler and Melton, 1994), would not have been recovered from our screen. A screen for maternal-effect genes would have required raising the F<sub>3</sub> generation, and phenotypes would only be expressed in F<sub>4</sub> embryos. Dominant lethal maternal-effect mutations would have been detected in the F<sub>2</sub> generation of the screen, but it is impossible to establish genetic lines bearing these mutations. Dominant lethal zygotic-effect mutations would be expressed in the F<sub>1</sub> generation, and heterozygotes eliminated. Haploinsufficient, or low-penetrance dominant mutations, would be eliminated to a large extent in the F<sub>1</sub> generation. A recent prominent example of such a phenotype in mice is the targeted disruption of the HNF-3 $\beta$  gene, for which a haploinsufficient effect reduces the viability of heterozygous carriers (Ang and Rossant, 1994; Weinstein et al., 1994).

Added limitations stem from the organization of the vertebrate genome. Genes are often represented in large families, with partially or completely overlapping developmental

functions. Therefore, mutations in individual genes might have only subtle or no phenotypes, while the developmental importance of a set of genes can only be uncovered by double or triple mutants. The functional redundancy of MyoD and Myf-5 in muscle determination is one such example (Rudnicki et al., 1993). In light of the growing evidence for genetic redundancy in the vertebrate, our screen is a first functional survey of a vertebrate genome for genes required uniquely for specific developmental processes. Our findings indicate that many loci are indeed essential and not part of completely redundant sets of genes. In the future, second-site non-complementation F<sub>1</sub> screens will be a powerful tool for unraveling genetic pathways.

### Prospects

#### (I) Future genetic screens

The main limitation of our screening strategy is the detection of subtle phenotypes. We tried to assess this problem by screening a subset of lines utilizing a simple histological stain for acetylcholine esterase activity. Those results indicate that we would not have missed many mutations affecting the overall architecture of the brain during the morphological screen. However, regional identity, neuronal connectivity and differentiation could only be assessed with more sophisticated sets of molecular markers, employing *in situ* or antigen expression analysis. Screens focused to specific aspects of development, and employing sets of markers to identify specific cell types, will undoubtedly identify a large number of additional mutations in novel genetic loci. A first such screen using immunohistochemistry to detect formation of dorsal root ganglia in zebrafish has recently been performed (Henion et al., 1996). The small size of the zebrafish embryo and early larvae make whole-mount immunohistochemistry as well as RNA *in situ* hybridization feasible even on the large scale needed for genetic screens.

We chose a two-generation breeding scheme for our screen,

**Table 5. Mutations isolated during the genetic screen and not studied in detail**

Categories	Mutations
Body shape	
Short bodies (viable)	<i>m607</i>
Early larvae have dorsally curved body	<i>m451, m737, m763</i>
Dorsal/ventral curvature	<i>m502</i>
Early larvae have ventrally curved body	<i>m71, m72, m85, m130, m177, m252, m408, m415, m439, m542, m570, m575, m577, m593, m609, m611, m648, m679, m695, m705, m742, m746, m773</i>
Lateral line organs:	
Number of lateral line organs reduced or missing in trunk and tail	<i>m 583</i>
Motility	
No touch response	<i>m92, m167, m355, m418, m434, m493, m519, m654, m624</i>
Reduced or abnormal touch response	<i>m127, m154, m246, m385 m386, m4421 m455, m522, m588, m596, m665, m723, m749</i>
Non-motile	<i>m237</i>
Muscle/somites:	
Abnormal shape or differentiation	<i>m143, m195, m260, m446, m505, m558, m774</i>
Pigmentation	
Pigmentation lighter or no dark pigment	<i>m26, m65, m104, m106, m164, m192, m261, m272, m279, m324, m334, m356, m374, m396, m397, m398, m449, m457, m460, m556, m589, goI<sup>m592</sup>, m621, m682, m693, m731, m732, m741</i>
Pigmentation darker or dilated melanophores	<i>m266, m368, m417, m761</i>
Iridophores, xanthophores abnormal or absent	<i>m95, m187, m464, m540, m571, m615, m652, m666, m722, m756, m783</i>
Pigmentation pattern	<i>m121, m136, spa<sup>m141</sup>, m179, m204, m280, spa<sup>m442</sup>, spa<sup>m443</sup>, m467, m500, m717</i>

Descriptions of the phenotypes as well as pictures can be retrieved on the Internet/WWW at 'http://zebrafish.mgh.harvard.edu' or 'http://zebrafish.uni-freiburg.de'



since it is the only method presently available with a very low background in non-genetic developmental abnormalities. Furthermore, it provides the least bias against certain types of phenotypes, like those resembling haploid embryos obtained in haploid or early pressure diploid screens (Streisinger et al., 1981; Kimmel et al., 1989; Driever et al., 1994). It also does not bias against genes in certain chromosomal regions, like early pressure half-tetrads, which segregate mutant phenotypes in loci close to the ends of the chromosomes at low rates. However, haploid and early pressure gynogenetic F<sub>2</sub> progeny are much faster, easier and cheaper to generate than F<sub>3</sub> progeny, and therefore will in the future be used extensively for rapid surveys of the zebrafish genome for mutations affecting defined aspects of development.

## (II) Molecular analysis – mapping and cloning

It will be important to link the mutant loci isolated in zebrafish to the repertoire of genes isolated and characterized at a molecular level in other vertebrate systems. This will involve either the isolation of candidate genes, or positional cloning, both of which require a good genetic map for zebrafish. More than one thousand marker (RAPDs and SSLPs) have been placed on the zebrafish genetic map (Postlethwait et al., 1994; Johnson et al., 1996; Knapik et al., 1996), and these laboratories expect to reach a marker density of one marker per centiMorgan (with an average of 590 kbp per cM in zebrafish). The availability of the map has already facilitated the identification of the gene for the *floating head* mutation, the zebrafish homologue of *Xenopus* Xnot (Talbot et al., 1995). The identification of candidate genes will be further accelerated as soon as synteny relationships between the zebrafish and mammalian genomes are characterized by mapping of genes cloned from zebrafish and mammals (Nadeau, 1989; Nadeau et al., 1995; Postlethwait et al., personal communication). Large insert libraries necessary for positional cloning are available (Genome Systems, St Louis, USA). Given the excellent mapping resolution one can obtain in zebrafish (segregation analysis of marker in one thousand progeny by PCR should result in better than 0.1 cM, or 50-100 kbp resolution), and efficient ways to screen genomic regions of this size for sequences expressed in the affected structures of the embryo by in situ hybridization, cloning of mutant genes will hopefully soon be a routine procedure similar to those in invertebrate genetic model systems.

Will these efforts lead to the identification of new genes, or will most of them be homologues of genes previously identified in *Drosophila* and other invertebrates? The analysis of the human genome reveals that thus far about half of all open reading frames have no previously known homologues, and several hundred previously unknown gene families can be predicted (Adams et al., 1995). We hope that zebrafish genetics will significantly contribute to our understanding of vertebrate-specific genetic functions. Many features of vertebrate embryogenesis, such as the neural crest or the notochord, and organogenesis have no obvious counterparts in invertebrates, and a detailed molecular understanding of vertebrate development will have to rely on the combination of forward genetic approaches, molecular characterization and experimental analysis in vertebrate model systems like the zebrafish.

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