

Insertional mutagenesis in zebrafish rapidly identifies genes essential for early vertebrate development

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To rapidly identify genes required for early vertebrate development, we are carrying out a large-scale, insertional mutagenesis screen in zebrafish, using mouse retroviral vectors as the mutagen. We will obtain mutations in 450 to 500 different genes—roughly 20% of the genes that can be mutated to produce a visible embryonic phenotype in this species—and will clone the majority of the mutated alleles. So far, we have isolated more than 500 insertional mutants. Here we describe the first 75 insertional mutants for which the disrupted genes have been identified. In agreement with chemical mutagenesis screens, approximately one-third of the mutants have developmental defects that affect primarily one or a small number of organs, body shape or swimming behavior; the rest of the mutants show more widespread or pleiotropic abnormalities. Many of the genes we identified have not been previously assigned a biological role *in vivo*. Roughly 20% of the mutants result from lesions in genes for which the biochemical and cellular function of the proteins they encode cannot be deduced with confidence, if at all, from their predicted amino-acid sequences. All of the genes have either orthologs or clearly related genes in human. These results provide an unbiased view of the genetic construction kit for a vertebrate embryo, reveal the diversity of genes required for vertebrate development and suggest that hundreds of genes of unknown biochemical function essential for vertebrate development have yet to be identified.

Introduction

The development of a multicellular organism requires the integration of diverse biological processes, including growth, differentiation and cellular physiology, as well as cell-type specification and patterning. Analysis of the genetic basis of development in vertebrate animals is following the approach taken in *Drosophila melanogaster* and *Caenorhabditis elegans* of using forward genetic screens to identify the genes necessary for embryonic development. Such genetic screens in zebrafish have shown that it is possible to obtain mutations that are involved in all of the aforementioned processes^{1,2}. In addition, the isolation of mutants with defects primarily in the development of one or a few embryonic organs, such as kidney or heart, reveals that the formation of functional organs in zebrafish can be analyzed using a forward genetic approach^{1,2}.

It has been estimated from large-scale chemical mutagenesis screens in zebrafish that roughly 800 genes can be mutated to yield relatively specific or localized defects during development

that can be identified by a visual screen of embryos up to five days post-fertilization². These genes may be involved in the range of processes needed for the development of specific organs and structures. Approximately 1,600 additional genes can be mutated to yield less specific phenotypes or recurring syndromes; these may be genes whose products are required in many cell types. Identification of these 2,400 genes would contribute significantly to understanding vertebrate development. However, the cloning of chemically mutated genes requires either arduous positional cloning or a candidate gene approach. Since the initial description, five and a half years ago, of hundreds of mutants, representing about half or more of the genes that yield specific types of defects, the genes underlying only about 50 mutants have been reported. Most were cloned by the candidate-gene approach and may thus represent only a fraction of the types of genes important for specific developmental processes in zebrafish. Many of these cloned genes encode transcription factors, ligands and their receptors, and many were previously identified as important developmental genes in other species.

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We designed a method of insertional mutagenesis for zebrafish using a Moloney murine leukemia–based retroviral vector as a mutagen^{3,4}. We find that most retrovirally mutated genes can be tentatively identified in as little as two weeks. The ease of gene cloning has allowed us to take a relatively unbiased approach to identifying the genes required for early development as we do not have to select a small number of mutants for positional cloning and are not biased towards known genes, as is the case with a candidate-gene approach. Notably, the types of genes we have identified encode a wide range of proteins, including many without known biological or biochemical functions.

Results

Identification of retrovirus-induced mutations

We use previously described^{3,4} methods for inducing insertions in the fish germ line using high-titer pseudotyped mouse retroviruses, for identifying embryonic mutations and for cloning the mutated genes. After identifying a proviral insert potentially linked to the mutant phenotype, we clone DNA flanking this insert using inverse PCR. In slightly less than 50% of the cases, sequencing this junction fragment yields a candidate gene by homology search against the National Center for Biotechnology Information (NCBI) database. In the remaining cases, we obtain and sequence additional genomic DNA adjacent to one or both sides of the original junction fragment using small chromosomal walks. In about two-thirds of all cases, this two-step procedure yields a candidate gene. We then use RT–PCR and RACE to obtain the rest of the cDNA. The position of the insertion within the gene for each mutant is shown in Web Fig. A online.

To confirm that the correct junction fragment (and gene) have been cloned, we carry out linkage analysis using primers designed to amplify different-sized products from chromosomes with or without the putative mutagenic insert in a PCR-based assay. We have shown that mutant embryos are invariably homozygous with respect to the putative mutagenic insertion, whereas their wildtype siblings are heterozygous or non-transgenic with respect to the insert. We genotype a minimum of 24 mutant and 24 wildtype embryos in most cases (see Web Fig. A online). We consider that if no recombinants are seen, the insert should lie no further than 3 cM (2 Mb) from the mutation responsible for the mutant phenotype. Thus, if no other insertion showing linkage to the phenotype is present, we consider the insert with tight linkage to be the probable cause of the mutation. A caveat is that during the course of the screen, we have identified mutants that are clearly not linked to a detectable proviral insert and probably result from spontaneous mutations. Thus, it is possible that a proviral insert could be linked to a mutation, but not be its cause. We estimate that this situation could escape detection in at most 1% of our mutants.

To obtain further evidence that the correct gene has been identified, we use RT–PCR or *in situ* hybridization to determine whether expression of the gene is disrupted by the appropriate insertion. In all cases reported here that were analyzed in this way

(37 cases), gene expression was reduced or ablated, or the transcript was altered, as a result of the insertion shown to be linked to the mutant phenotype (see Web Fig. A online).

In this screen, we kept all embryos with mutant phenotypes visible in a dissecting microscope by five days post-fertilization. As seen in chemical mutagenesis screens, about one-third of the insertional mutants show highly specific developmental defects involving one or a few organ systems, whereas the majority have one of several more general, frequently recurring syndromes⁵. Similar to most embryonic mutants isolated in zebrafish by any method of screening, almost all of our mutants are recessive-lethals, and homozygous mutant embryos die between three and ten days of age.

Classification of mutant phenotypes

The first 75 embryonic-lethal mutants in this screen for which the disrupted gene has been identified are listed in Fig. 1 (for an expanded version, see Web Fig. A online). Mutants are grouped according to their phenotypic defects, following a previously described classification system^{1,2}. Most designations were made

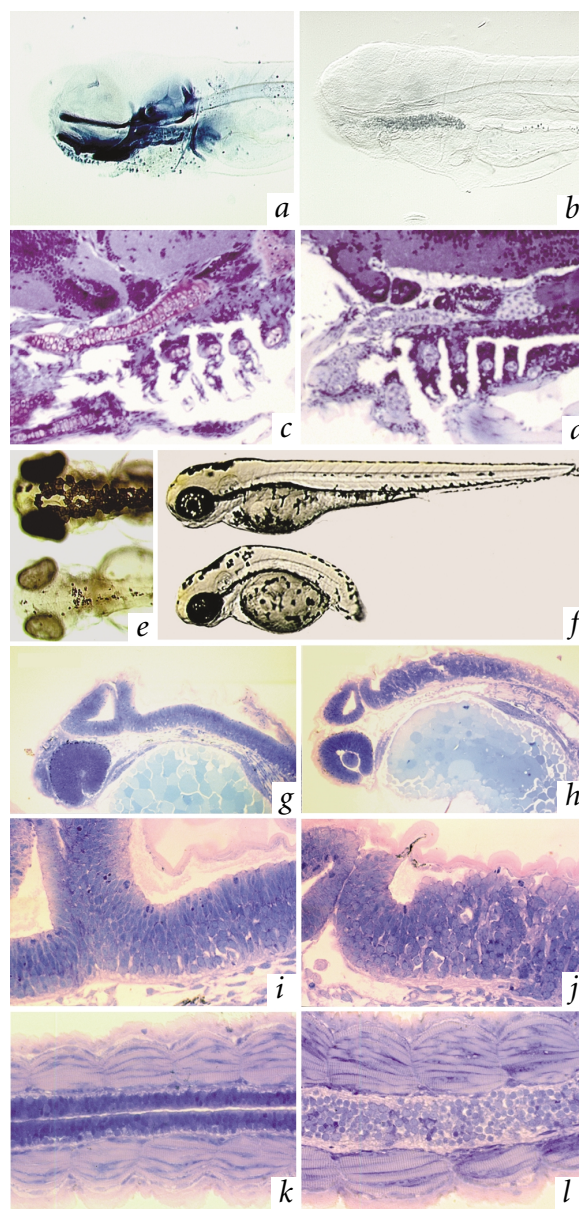


Fig. 2 Examples of mutant phenotypes isolated by insertional mutagenesis. **a–d**, hi954 mutant embryo showed cartilage defects, in that it did not stain with Alcian blue, as seen in these lateral views of wildtype (**a**) and hi954 (**b**) embryos at 5 d. This lack of staining did not correspond entirely to a loss of either jaw/arch structures or cartilage cells, as these features were present in sagittal sections of mutant embryos (compare wild type (**c**) with hi954 (**d**), cartilage cells stained in pink with Fuscian). **e**, A mutation affecting pigment is represented by hi923. These mutants (bottom) had melanophores, yet they appeared much smaller than those in wildtype embryos (above). **f**, In hi2092 mutants (bottom), the posterior portion of the body was significantly reduced, as shown in these day-3 embryos. **g–l**, 33h hi904 mutant embryo had a severe morphological defect in the brain and CNS (wildtype in **g, i, k**; hi904 in **h, j, l**). Higher magnifications of sagittal sections showed a high degree of disorganization of neuronal cells in the brain (compare **i** and **j**) and neural tube (compare **k** and **l**).

Table 1 • Types of genes bearing insertional and chemically induced mutants

Protein category	Phenotype:	Type of mutant		
		Insertional Specific	General	Chemical Specific ^a
DNA replication and cell cycle		1	6	0
Chromatin/transcription associated factors		0	7	0
Transcription factors		6	0	21
RNA processing		0	6	0
Helicases		1	2	0
Translation/post-translation		2	10	0
Protein folding		0	4	0
Kinases		1	1	0
Organelle		7	2	0
Receptors and ligands		3	0	22
Metabolic enzymes		2	3	3
Other		2	2	4
Unknown function		7	9	0
Total		32	52	50

^aAll of the chemical mutants for which the mutated gene has been cloned have specific developmental phenotypes. The numbers indicate the total mutants for each category. The numbers for the chemical mutants include all those published or reported as of 1 October 2001.

solely on the basis of examination by low-power microscopy. In some cases, mutant embryos were sectioned, stained with Alcian blue to visualize cartilage or stained with acridine orange to identify those mutants with apoptotic tissues. As considerable study is needed to accurately describe the phenotype of any mutant, the classifications given here must be considered preliminary. In some cases, further study may identify additional defects. For example, a mutation in *vhnf1* was initially found to result in a kidney mutant. However, further analysis, including study of additional alleles, showed that the gene is required for development of kidney, pancreas, liver and hindbrain⁶.

As in large-scale chemical mutagenesis screens, insertional mutants with unique, relatively specific developmental defects include those in which phenotypes are observed primarily in the brain, eyes, jaw, arches or cartilages, midline, ear, fins, liver, gut, kidney, muscle, pigment, body shape, motility or touch insensitivity in the absence of any visible structural abnormality, and motility or altered touch sensitivity in combination with some degree of visible morphological defects. Mutants classified as having more general developmental defects include those with extensive cell death in the central nervous system (CNS), those with a small head and eyes (some of which initially showed limited cell death in the head, some of which did not), embryos that show retardation, mutants that have several defects (meaning that many organs and structures are visibly affected while others seem grossly normal) and mutants in which the only apparent phenotype is failure to inflate the swim bladder (although this last trait is seen in almost all embryonic mutations of zebrafish, regardless of what other defects are also present). The large phenotypic class of mutants with these common and general defects was discarded in chemical mutagenesis screens^{1,2}. As our method of mutagenesis allows rapid identification of mutated genes, we kept these mutants to generate a more complete picture of the genes required for the development of the five-day-old embryo.

Although the number of mutants described here is small and the characterization preliminary, the range of phenotypes we observed seems indistinguishable from the spectrum of phenotypes induced by ENU mutagenesis^{1,2,5}. Of the 75 mutants, 6 harbor insertional alleles of chemically induced or radiation-induced mutations for which the mutated gene has been reported (Fig. 1); others may prove to be allelic when complementation studies are carried out.

Pictures of four insertional mutants with relatively specific developmental defects are shown in Fig. 2. Mutations in a variety of genes produce phenotypes involving cartilage. For example, hi954, a mutant in which the cartilage does not stain with Alcian blue, but in which cartilage cells can be seen in sections (Fig. 2*b,d* versus Fig. 2*a,c*), results from a disruption of the gene encoding UDP-glucuronic acid decarboxylase. The ENU-induced mutant *jekyll* shows a very similar Alcian blue staining pattern, although it has in addition a cardiac valve defect not seen in hi954 (refs 7,8). The gene disrupted in *jekyll* was recently found to encode UDP-glucose dehydrogenase⁹.

Most mutants with pigmentation abnormalities that we have isolated so far have disruptions in genes encoding proteins associated with cytoplasmic organelles. The mutants hi577a, hi923 (Fig. 2*e*) and hi1207, as well as the previously reported⁴ hi112, have reduced pigmentation in both the body and eyes and all are the result of mutations in different subunits of vacuolar ATP synthase. The gene disrupted in another mutant with a pigmentation abnormality, hi1463, encodes a protein similar to lysosomal membrane protein II (LIMP II). Pigmentation mutant 2499a results from a lesion in a gene encoding a vacuolar protein sorting protein that is homologous to *D. melanogaster* deep orange. A number of mutations affecting eye color in *D. melanogaster* and coat color in mouse involve genes that encode membrane components of vesicles involved in pigmentation^{10,11}. As many of these proteins are also expressed in lysosomes^{12,13}, it is not unexpected that strong alleles of these mutations would result in lethality.

A mutant (hi2092) with an insertion in the well known developmental gene *caudal* has a shortened trunk and tail and no yolk extension (Fig. 2*f*). The mutant hi904 has severe disorganization of the brain and CNS (compare Fig. 2*g* with Fig. 2*h*); in higher-magnification sections, the cells appear more loosely organized than those of wildtype embryos (Fig. 2*i-l*). The gene mutated in hi904 has some recognizable motifs but no known biochemical function and has orthologs in fly and human.

Genes required for early vertebrate development

The types of genes mutated in the two broad phenotypic classes of mutants, those with relatively specific and those with widespread defects, including insertional mutants previously reported from our pilot screen^{3,4,14,15}, are shown in Table 1. It has been suspected that because zebrafish embryos receive maternally encoded gene products, development can proceed to multicellular stages even if the embryo harbors a mutation in a cell-essential gene¹⁶. It has been thought that many mutants with so-called nonspecific developmental defects may result from mutations in cell-essential genes¹. By contrast, mutants with more specific developmental phenotypes might result from lesions in genes required specifically for normal development and function of multicellular structures, or possibly from lesions in housekeeping genes with tissue-specific expression or with more than one biochemical function. Although the data are limited, our findings suggest that genes required for protein synthesis, RNA processing, DNA replication and chromatin assembly



often give rise to nonspecific developmental phenotypes when mutated. As expected, genes encoding transcription factors, receptors and ligands are well represented among mutants with specific developmental phenotypes; however, so are many genes encoding organelle proteins, a diverse array of other types of genes and many genes of unknown biochemical function.

One of the largest categories of genes responsible for both phenotypic classes of mutants is that of genes for which a biochemical function cannot be predicted. These account for 20% of the mutants we have identified. The proteins that these genes are predicted to encode differ in their degree of novelty. One protein (hi459) and its closest human homolog resemble a protein of known function in mammals (ADP-ribosylation factor-related), but the similarity is not sufficient to conclude that they would carry out the same biochemical or cellular function (see Web Fig. B online). Some proteins share recognizable motifs with proteins of known biochemical or cellular functions (hi904, zinc finger; hi447 and hi821a, WD40 repeats). The eleven others do not have any identifiable protein motifs (searched against Conserved Domain Database at NCBI). For all of the genes, there is either a clearly identifiable human ortholog, or at least a human gene with some similarity that can be identified (using protein-protein BLAST (blastp) or translated db (tblastn) searches against the nr and human genome databases, $P < 10^{-29}$). Some do not yet have recognizable orthologs in *D. melanogaster* or in yeast (hi572 and hi1262) and may thus be encoded by genes specific to or conserved only in vertebrate development. Others (hi428, 904, 1548, 591) do not have recognizable orthologs in yeast and could thus be specific to animals. However, divergence in amino-acid sequence could make it difficult to identify the orthologs of some genes in these species. Some of the genes of unknown biochemical function are highly conserved from yeast to mammals (see Web Fig. C online).

Discussion

Large-scale genetic screens that used ENU as a mutagen in zebrafish provided the first broad view of the types of embryonic and early larval mutants that can be obtained in this species, and allowed rough estimates of the number of genes required for early development^{1,2}. However, the difficulty of cloning the mutated genes precluded obtaining an equally revealing picture of the genes required for early developmental processes in zebrafish. The results presented here provide the first large-scale, unbiased view of the genes required for the development of a vertebrate embryo. They reveal an array of genes, including genes probably involved in cellular biological processes and physiology. Although these functions have been thought of as distinct from development, they have recently been shown, in some cases, to be inseparable even from specific developmental processes¹⁷. Table 1 shows a comparison of the types of genes cloned in our lab with those cloned and reported by all other zebrafish labs. Other labs have used primarily candidate-gene cloning, and less frequently purely positional cloning, of chemical or radiation-induced mutants with specific developmental phenotypes. The bias towards genes encoding transcription factors, receptors or ligands presumably results from the bias imposed by selecting candidate genes in advance, and from a bias in choosing mutants with particular types of developmental defects for the laborious process of candidate or positional cloning.

Some of the genes we have identified are important in growth control, are involved in human diseases or both. For example, *pescadillo* (hi2), a BRCT motif-containing gene identified in our pilot screen and required for the normal size of some but not all embryonic organs¹⁵, was recently identified as a gene whose expression is elevated in p53-deficient tumor cell lines and is

thought to be involved in cell-cycle check points^{18,19}. The transcription factor gene *vhnf1*, mutated in the kidney and pancreas mutant hi548, underlies a genetic form of human diabetes, maturity onset diabetes of the young type V, in which individuals have kidney abnormalities in addition to diabetes^{20,21}. It seems likely that other genes we identify in our screen will also be involved in human diseases, possibly including diseases for which the genetic basis has not yet been determined.

A concern in using insertional mutagens is whether they are biased in their insertion sites and whether they can induce mutations in all genes. That retroviruses have some bias in integration sites in zebrafish is suggested by the fact that when more than one insertional allele of a gene is recovered, it is often the case that the insertions lie close together. So far, we have recovered 320 insertional mutants for which we have cloned genomic DNA flanking the mutagenic insert; these represent 254 loci: 205 loci (81%) are represented by a single allele, 37 loci (15%) by 2 alleles, 10 loci (4%) by 3 alleles, and 1 locus each by 4 and 7 alleles. Because the single allele class is so high and the data do not fit a Poisson distribution, it is impossible to estimate the total number of genes mutable by retroviral insertion, except that it must be at least several-fold higher than the number recovered so far. The allele distribution in large-scale ENU mutagenesis screens also did not fit a Poisson distribution^{1,2}, indicating that all genes are not mutated with equal frequency with either mutagen, and that retroviruses may be no more biased in the genes they mutate than are chemical mutagens.

Upon the completion of our current screen, we will have isolated insertional mutations in around 450–500 genes, roughly 20% of the 2,400 genes estimated to be required for the development of a zebrafish embryo. This will include the identification of approximately 100 genes of unknown biochemical function to which we will be able to assign a biological role. As we will clone all of these mutated genes without selecting for certain types of phenotypes, we should be able to estimate the number of different types of gene products—transcription factors, signaling molecules and vesicle trafficking proteins and so on—that are essential for embryonic development or for the development of any given embryonic structure. Finally, this large collection of mutants will be a valuable community resource, supplementing the large number of chemical mutants, with the benefit that the mutated gene will have already been identified.

Methods

Mutagenesis. We carried out the preparation of virus, injection of embryos and breeding and screening scheme as previously described⁴. Mutant lines will be made available upon request.

Genotyping embryos. For linkage analysis, we sorted embryos from heterozygous parents into phenotypically wildtype and mutant groups. In most cases, we genotyped 24 embryos of each group by PCR. We used a pair of genomic primers flanking the responsible mutagenic viral insertion and a viral-specific primer in a single reaction. A viral insertion leads to amplification between the viral-specific primer and one of the genomic primers, while disrupting amplification between the genomic pair. Thus, wildtype and mutated DNA yield distinctive PCR products. In a few cases, we determined genotypes by Southern blot using genomic sequence flanking the insertion as a probe, as previously described³.

cDNA cloning. We identified putative exons by comparing the sequences of the genomic DNA adjacent to the mutagenic insert with the public databases, using BLAST²². We used either RT-PCR or RACE to obtain the rest of the cDNA. If homology was found to a zebrafish expressed sequence tag for which both 5' and 3' sequence was available, we used RT-PCR; otherwise, we carried out RACE using the SMART RACE kit (Clontech). The source of RNA for either approach was a pool of embryos at 1 d, 2 d, 3 d, 4 d and 5 d post-fertilization.

Alcian blue staining. We carried out cartilage-staining procedures as previously described²³, except that HCl was used in place of acetic acid and the pH of the final solution was 1.0.

Accession numbers. Accession numbers for mutated genes can be found in Web Fig. A online.

Note: Supplementary information is available on the Nature Genetics website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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