REVIEW

Zebrafish mutations and functional analysis of the vertebrate genome

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As the first draft of the human genome nears completion, we are faced with the challenge of defining the functions of the 70,000–100,000 genes contained within the sequence. A powerful combination of large-scale forward genetics and elegant cellular analysis ensures that the zebrafish will have an important role in the functional analysis of the vertebrate genome. Genetic screens have identified mutations that define the functions of hundreds of essential genes—a number that will increase considerably as ongoing screens search for previously unexplored mutant phenotypes (Driever et al. 1996; Haffter et al. 1996). The functions of mutated genes can be examined at the cellular level by sophisticated phenotypic analysis, such as lineage tracing and cellular transplantation, of mutant embryos (Kimmel 1989). Thus, the facile genetics of zebrafish allows the rapid isolation of mutations, and phenotypic analysis can reveal gene functions that are difficult to uncover in other systems. Over the past several years strategies to clone mutated genes have been developed, and ongoing efforts to improve the genomics infrastructure for the zebrafish will accelerate the molecular analysis of zebrafish mutations. Here we review some recent advances in genomic resources that will augment the cloning of mutated zebrafish genes by positional cloning, the candidate gene approach, and insertional mutagenesis.

Positional cloning

Positional cloning is the identification of a mutated gene based on its chromosomal map position. This involves high-resolution genetic mapping of the mutation, assembly of a set of contiguous genomic clones spanning the region of the mutated gene, cataloging the genes in the region, and winnowing this list of genes until the culprit is identified. This can be an arduous process, but positional cloning is an unbiased approach that can be applied to any gene whose inheritance can be traced, without prior biochemical knowledge of the gene or the process in which it acts.

The first step in a positional cloning project is the identification of a DNA sequence tightly linked to the mutation of interest. Dense genetic maps accelerate this process, as the likelihood that a mutation will lie very near a genetic marker increases as more markers are mapped. Prior to 1994, there was no genetic map for the zebrafish, but efforts since then have produced several useful maps (Postlethwait et al. 1994, 1998; Johnson et al. 1996; Knapik et al. 1996, 1998; Gates et al. 1999; Shimoda et al. 1999). The current state of the art is a map comprised of 2600 simple-sequence length polymorphisms (SSLPs) (Shimoda et al. 1999). SSLPs are tracts of short repeated sequences, often CA dinucleotides, in which the number of repeats varies among different strains of fish. The abundance of SSLPs in the genome and the ease of their detection in simple gel-based assays make them the markers of choice for mapping studies in organisms with complex genomes. The zebrafish genome contains $\sim 1.7 \times 10^9$ bp (Hinegardner and Rosen 1972), so the average distance between SSLPs is currently ~ 650 kb. At this density, a substantial fraction of mutations is within a few hundred kilobases of a SSLP, well within striking distance of a positional cloning project. For loci that are not near a mapped SSLP, targeted screens can identify new amplified fragment length polymorphism (AFLP) or random amplified polymorphic DNA (RAPD) markers in the region of the mutation (for review, see Talbot and Schier 1999). An ongoing effort to double the number of mapped SSLPs (<http://zebrafish-mgh.harvard.edu/mapping/>) will diminish the need for these tailored screens and bring the density of genetically mapped SSLPs to about the same level as for mouse and human (400–500 kb average intermarker distance).

The second phase of a positional cloning project is to isolate a series of overlapping genomic clones that connects markers on either side of the mutation, ensuring that the gene lies within the cloned interval (the critical interval). Genomic libraries suitable for this purpose are now available in bacterial artificial chromosome (BAC), P1-derived artificial chromosome (PAC), and yeast artificial chromosome (YAC) vectors (Amemiya and Zon 1999; Amemiya et al. 1999; Zhong et al. 1999). Clones from these libraries contain fragments of ~ 100 kb (BAC and PAC) or 470 kb (YAC), so that, in favorable cases, only a few clones may be needed to span the genetically

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defined interval containing the mutation. High-resolution genetic mapping can determine which clone or clones contain the mutated gene, because mapping crosses comprised of thousands of individuals can be screened to identify rare recombinants with crossovers lying very near the mutation. In the cloning of *one-eyed pinhead* (*oep*), for example, mapping crosses containing 3122 individuals localized the mutation to a region of ~100 kb (Zhang et al. 1998). Similarly, high-resolution mapping narrowed the critical intervals to 120–150 kb in the analysis of *sauternes* (*sau*), *weissherbst* (*weh*), and *gridlock* (*grl*) (Brownlie et al. 1998; Donovan et al. 2000; Zhong et al. 2000). Thus, mapping studies can limit the critical interval to a region defined by one or two genomic clones, which greatly simplifies the search for the mutated gene.

Genes in the critical interval may be sought by a number of approaches ranging from screening cDNA libraries with genomic clones to sequencing of BAC and PAC clones. Microinjection of genomic clones can partially rescue zebrafish mutant phenotypes (Yan et al. 1998), so phenotypic rescue could provide another assay to identify genomic clones containing the gene of interest. Similarly, injection of cloned RNAs can provide a rapid screen to determine if any genes from the critical interval are able to rescue the mutant phenotype.

Four genes have been identified by positional cloning in zebrafish. The first of these, *oep*, encodes an extracellular, membrane-associated protein in the EGF-CFC family (Zhang et al. 1998). Before the identification of *oep*, EGF-CFC genes were known in frog, mouse, and human, but *oep* was the first EGF-CFC gene for which a mutation was available. Interestingly, there are no known invertebrate EGF-CFC proteins, and it is possible that these factors are vertebrate-specific developmental regulators. Little is known about the biochemical activities of the EGF-CFC proteins, but recent data suggest that *Oep* is a cofactor for signals in the Nodal subclass of the TGF- β superfamily (Gritsman et al. 1999). The analysis of *oep* illustrates the potential of zebrafish genetics to define novel gene functions.

The three other genes, *sau*, *weh*, and *grl*, represent models of human disease. *sau* mutants have anemia associated with reduced hemoglobin levels, and the *sau* gene encodes Δ -aminolevulinic synthase (Alas2), an enzyme in the heme biosynthetic pathway (Brownlie et al. 1998). In humans, congenital sideroblastic anemia is caused by mutations in *ALAS2*, and the *sau* mutant is the first animal model of this disease. A similar anemia phenotype is characteristic of *weh* mutants, and positional cloning revealed that *weh* encodes a novel iron transporter that is conserved among vertebrates (Donovan et al. 2000). *grl* mutants have a cardiovascular patterning defect that resembles the human malformation coarctation of the aorta (Weinstein et al. 1995). The *grl* gene encodes a novel Hairy/Enhancer of split homolog, and this discovery identifies a new regulator of cardiovascular development in the vertebrate embryo (Zhong et al. 2000).

The zebrafish gene map and the candidate gene approach

In the candidate gene approach, one examines genes with some property expected of the mutated locus to determine if any is disrupted in the mutants. Candidate genes have been selected by expression pattern, knowledge of mutants with similar phenotypes in other organisms, and inferences about the biochemical pathway disrupted by the mutation (see references in Table 1). Mapping studies provide an efficient test of candidate genes, as genes not linked to the mutation are excluded rapidly. Phenotypic rescue assays are also valuable in testing candidate genes. The candidate gene approach has been quite successful, netting 23 loci as of this writing (Table 1).

Recent advances in genomic resources for the zebrafish will accelerate the identification of candidate genes for mutations. Sequencing projects have generated >50,000 expressed sequence tags (ESTs) that define $\geq 11,500$ zebrafish transcripts (S. Johnson, pers. comm.; <http://zfish.wustl.edu/>). Thus, cDNA sequencing projects are rapidly identifying genes that may be considered as candidates for zebrafish mutations. Efforts to map zebrafish genes and ESTs facilitate the testing of candidate genes, and, as the number of mapped transcripts increases, candidate genes may be identified increasingly by map position rather than by more subjective criteria. Published genetic linkage maps localize ~900 cloned genes and ESTs (Postlethwait et al. 1998; Gates et al. 1999; Kelly et al. 2000), and ~250 additional transcripts have been mapped in ongoing projects as of March 2000 (<http://zebrafish.stanford.edu>). Genetic linkage maps now cover essentially the entire genome (Knapik et al. 1998; Postlethwait et al. 1998; Gates et al. 1999; Shimoda et al. 1999), so any gene (or other DNA sequence) that contains a polymorphism can be mapped by this method. Although genetic mapping is robust and reliable, the effort required to screen genes for polymorphisms limits the application of meiotic mapping in very large-scale projects.

Recently developed radiation hybrid (RH) maps of the zebrafish genome provide an alternative mapping strategy that does not necessitate the identification of polymorphisms (Geisler et al. 1999; Hukriede et al. 1999). This method orders markers based on their coretenation in panels of rodent-zebrafish hybrid cell lines. The distance separating two markers is inversely related to the frequency at which they are present together in the same RH lines. Because only the presence or absence of a sequence in the hybrid lines needs to be determined to assign a map location, RH mapping uses very straightforward agarose gel assays that are amenable to automation and high throughput. Although RH maps tend to have more gaps than meiotic maps, two independent sets of zebrafish-rodent radiation hybrid lines are available, and the two maps together cover >90% of the zebrafish genome. Approximately 500 genes and ESTs have been localized in at least one of the two published RH maps, and ~250 of these were not previously assigned in mei-

Table 1. Mutated genes identified by positional cloning and the candidate approach

Strategy	Mutation	Gene product	Reference
Positional cloning	<i>one-eyed pinhead</i>	EGF-CFC domain	Zhang et al. (1998)
	<i>sauternes</i>	aminolevulinate synthase	Brownlie et al. (1998)
	<i>weissherbst</i>	Ferroportin1	Donovan et al. (2000)
Candidate gene	<i>gridlock</i>	Hairy/Enhancer of split homolog	Zhong et al. (2000)
	<i>no tail</i>	Brachyury homolog, T-box	Schulte-Merker et al. (1994)
	<i>floating head</i>	Xnot homolog, homeodomain	Talbot et al. (1995)
	<i>no isthmus</i>	Pax2.1	Brand et al. (1996)
	<i>dino/chordino</i>	Chordin	Schulte-Merker et al. (1997)
	<i>swirl</i>	Bmp2b	Kishimoto et al. (1997); Nguyen et al. (1998)
	<i>pipetail</i>	Wnt5	Rauch et al. (1997)
	<i>nic1</i>	acetylcholine receptor α -subunit	Sepich et al. (1998)
	<i>valentino</i>	Kreisler homolog, bZip	Moens et al. (1998)
	<i>acerebellar</i>	FGF8	Reifers et al. (1998)
	<i>sonic you</i>	Sonic hedgehog	Schauerte et al. (1998)
	<i>cyclops</i>	Nodal homolog; TGF- β	Rebagliati et al. (1998a) Sampath et al. (1998)
	<i>squint</i>	Nodal homolog; TGF- β	Feldman et al. (1998)
	<i>spadetail</i>	VegT homolog; T-box	Griffin et al. (1998)
	<i>yquem</i>	uroporphyrinogen decarboxylase	Wang et al. (1998)
	<i>you-too</i>	Gli2	Karlstrom et al. (1999)
	<i>bozozok</i>	homeodomain	Fekany et al. (1999)
	<i>somitabun</i>	Smad5	Hild et al. (1999)
	<i>minifin</i>	Tolloid	Connors et al. (1999)
	<i>sparse</i>	c-Kit	Parichy et al. (1999)
	<i>nacre</i>	microphthalmia-related protein	Lister et al. (1999)
	<i>faust</i>	Gata5	Reiter et al. (1999)
	<i>soulless</i>	Phox2a	Guo et al. (1999)
	<i>snailhouse</i>	Bmp7	Dick et al. (2000); Schmid et al. (2000)

otic maps (Geisler et al. 1999; Hukriede et al. 1999). Moreover, ~2500 additional genes and ESTs have been localized in ongoing RH projects, and these initiatives aim to map a total of 15,000 transcripts over the next 2 years (<http://zfsh.wustl.edu/>; <http://zfRHmaps.tch.harvard.edu/ZonRHmapper/>; <http://wwwmap.tuebingen.mpg.de/>; <http://dir.nichd.nih.gov/lmg/lmgdevb.htm>).

Conservation of synteny among vertebrates

In addition to providing many candidates for mutations, gene mapping projects allow comparisons among the genomes of zebrafish and other vertebrates. Analysis of zebrafish genes and their mammalian counterparts has identified >40 groups of genes that are syntenic (i.e., on the same chromosome) in both zebrafish and human (Postlethwait and Talbot 1997; Amores et al. 1998; Postlethwait et al. 1998; Gates et al. 1999; O'Brien et al. 1999). Some of these groups are fairly large, containing as many as nine orthologous genes that are syntenic in both species, but current comparative maps contain many gaps, or regions where corresponding zebrafish and human segments have not yet been identified. Analysis of the many zebrafish genes that have been mapped in the last year will define new regions of conserved synteny and will provide important information on the extent to which gene order has been preserved in segments of con-

served synteny. Despite the current gaps in the zebrafish-human comparative map, conservation of synteny proved valuable in defining a candidate gene for the *you-too* (*yot*) mutation (Karlstrom et al. 1999). The *yot* locus mapped to zebrafish linkage group (LG) 9, which contains genes with orthologs on human chromosome 2. Inspection of human genes on chromosome 2, together with an inference that *yot* mutations might disrupt a Hedgehog signaling pathway, identified *gli2* as a candidate for *yot*. In another example, conservation of synteny between zebrafish LG 11 and human chromosome 20, combined with the possibility that the *snailhouse* (*snh*) mutation disrupts a bone morphogenetic protein (BMP) signaling pathway, identified the *bmp7* gene as a candidate for *snh* (Schmid et al. 2000). Thus comparative analysis allows dense mammalian gene maps to be used in defining candidates for zebrafish mutations. Conversely, comparative mapping may also help determine which zebrafish mutations correspond to human genes of unknown function.

Insertional mutagenesis

A third approach in which mutated zebrafish genes can be cloned is from insertional mutants. In contrast to the more commonly used chemical mutagen ethylnitrosurea (ENU), which usually induces single-base changes in

DNA, insertional mutagens deposit an exogenous DNA tag within the gene that they mutate. Using inverse PCR, one can readily clone genomic DNA that flanks either side of a mutagenic insert and sequence it. By searching the sequence for homologies to sequences in the databases, either nucleotides or implied amino acid sequences, one can often identify the disrupted gene.

The only insertional mutagen that is efficient enough at present to allow the isolation of a large number of mutants in zebrafish is pseudotyped mouse retroviral vectors (Gaiano et al. 1996; Amsterdam et al. 1999). Retroviruses are effective mutagens because they integrate with precision and are known to insert at many sites in host genomes. Currently a large insertional mutagenesis screen under way in the Hopkins laboratory aims to isolate ~600–1200 embryonic lethal mutations. This number should represent ~20%–40% of the embryonic lethal loci that can be obtained by morphological screens (Haffter et al. 1996). The screen is 10% complete and is on track to be finished in 1.5 years (6/01). To date, ~150 mutants have been isolated, of which 103 have been provisionally confirmed by showing linkage of a specific proviral insert to the mutant phenotype. Of these 103 mutants, candidate mutated genes have been cloned for 40, and junction fragments flanking mutagenic inserts have been cloned for an additional 30. Of these 70 cloned junction fragments and genes, 69 were cloned within this single laboratory. Among the cloned genes, one was known previously to be the cause of a form of human diabetes.

Currently the ease of cloning any particular mutated gene depends on the distance between the insert that is genetically linked to the mutation and an identifiable exon. Of the genes cloned to date, in about half the cases exons matching cDNA or genomic sequences in the data base were found within the first fragment cloned by inverse PCR (A. Amsterdam and N. Hopkins, unpubl.). These fragments ranged in size from 80 bp to 5 kb and averaged ~1.5 kb. In those cases where a gene is not found within the first cloned junction fragment, additional genomic DNA flanking these sequences can be cloned and sequenced rapidly, again using inverse PCR or linker-mediated PCR. At present, a single scientist is capable of cloning junction fragments for three to six insertional mutants per week, of which about half, when sequenced, allow immediate identification of the candidate mutated gene.

Even the most efficient retroviral insertional mutagen has an overall frequency of mutagenesis that is probably severalfold lower than that of ENU. The viruses for which the most extensive data are available have a roughly ninefold lower frequency of mutagenesis (Amsterdam et al. 1999). Furthermore, inducing mutations with retroviral vectors is extremely laborious as compared with ENU mutagenesis. However, significant advances in the efficiency of retroviral insertional mutagenesis have been made recently, and as a result, it is now possible to generate a million proviral insertions in the fish germ line in ~2 months (W. Chen, S. Burgess, and N.H., unpubl.). Only ~20% of the inserts that are gener-

ated can be screened efficiently and the protocol for breeding the insertions to homozygosity involves determining the proviral copy number in a large number of individual F1 fish, a laborious step. Nonetheless, the ability to easily clone almost every mutant obtained must be weighed against these disadvantages.

For now it seems clear that it is the power of the two methods together, chemical and insertional mutagenesis, that will advance the zebrafish field most rapidly in the near future. The highly efficient production of mutants by ENU has already provided an overall picture of the range of embryonic phenotypes that can be obtained, and yielded molecular entry points to a number of developmental pathways. Insertional mutagenesis is now fulfilling its promise of identifying a large number of genes essential for the normal development of the zebrafish embryo.

Although still unproven as mutagens, several transposable elements show promise as possible future insertional elements for the zebrafish. These include *mariner* (Fadool et al. 1998), *Tc3* (Raz et al. 1998), and *sleeping beauty*, an engineered *Tc-1* like element being developed by Hackett and his colleagues (Ivics et al. 1997).

Genome duplication in the evolution of teleosts

Comparative analyses suggest that a genome duplication occurred early in the evolution of the lineage leading to zebrafish. Numerous studies have revealed that some gene families in zebrafish have more members than the corresponding families in mammals (see Wittbrodt et al. 1998; Force et al. 1999; and references therein). For example, there are four *engrailed* (*eng*) genes and seven *hox* complexes in zebrafish, whereas mammals have only two known *eng* genes and four known *hox* complexes (Ekker et al. 1992; Amores et al. 1998; Force et al. 1999). The seven zebrafish *hox* clusters reside on different linkage groups, LG 3, 9, 11, 12, 16, 19, 23, as do the four *eng* genes (LG 1, 7, 9, 19), indicating that these extra genes are not the result of tandem duplications. (It is of course possible that tandem duplications have occurred elsewhere, accounting for some extra genes.) Mapping studies instead suggest that *hox*-containing chromosomes (or large chromosomal segments) were duplicated in the lineage leading to zebrafish, as members of other expanded gene families reside on the same linkage groups as some of the *hox* clusters (Amores et al. 1998). Similarly, mapping studies have identified other pairs of chromosomal segments that contain duplicate genes (Postlethwait et al. 1998; Gates et al. 1999). The finding of large, duplicated chromosomal segments suggests that a genome duplication occurred in the lineage leading to zebrafish after its divergence from the tetrapod lineage (Amores et al. 1998; Postlethwait et al. 1998; Gates et al. 1999). Although the time of this duplication is not certain, the available evidence suggests that it occurred early in the evolution of the teleost lineage. Several teleosts, including medaka and pufferfish, have expanded gene families (Wittbrodt et al. 1998; Naruse et al. 2000; Smith et al. 2000), and phylogenetic analyses suggest that duplicate

genes existed in the last common ancestor of these species (Amores et al. 1998; Gates et al. 1999).

In many cases, members of duplicate gene pairs have distinct expression patterns (Force et al. 1999; and references therein). It has been proposed that regulatory mutations occurring after duplication result in duplicates having different, complementary subsets of the ancestral expression pattern (Force et al. 1999). This proposal explains the retention of both members of a pair of duplicates, since the function of each gene is required in cell types where its duplicate is not expressed. Five cloned mutations (*swirl*, *sonic you*, *squint*, *cyclops*, and *no isthmus*) correspond to genes with apparent duplicates (see references in Table 1), indicating that duplicate genes can have distinct functions. Two of these genes, *squint* (*sqt*) and *cyclops* (*cyc*), are zebrafish homologs of mouse *Nodal*, which is present in a single copy in the mammalian genome (Erter et al. 1998; Feldman et al. 1998; Rebagliati et al. 1998a,b; Sampath et al. 1998). The *sqt* and *cyc* genes have overlapping but not identical expression patterns, and the genetic interaction between *sqt* and *cyc* demonstrates that these genes also have distinct but overlapping functions. *sqt* single mutants have defects in development of the prechordal plate (Heisenberg and Nüsslein-Volhard 1997), and *cyc* single mutants lack ventral midline cell types in the central nervous system (Hatta et al. 1991), indicating that some functions of each gene cannot be completely satisfied by the action of the other. *sqt;cyc* double mutants have a severe disruption of mesoderm and endoderm (Feldman et al. 1998)—a phenotype not characteristic of either single mutant—so it seems that either *sqt* or *cyc* function is sufficient to support the development of these germ layers. Thus, diverging expression patterns may confer essential functions on many duplicated genes on zebrafish.

Reverse genetic approaches

Phenotype-driven, forward genetic strategies are the forte of the zebrafish, but recent advances have improved the prospects for reverse genetic approaches. One method to obtain a mutation in a cloned gene is to screen for point mutations that fail to complement a deletion that removes the gene of interest. In the first application of this approach in zebrafish, Appel et al. (1999) used a translocation removing a segment of LG 1 to screen for a point mutation in the *deltaA* gene. In a screen of 270 chemically mutagenized F1 fish, four mutations failing to complement the translocation were recovered, and one of these contained a lesion in *deltaA* (Appel et al. 1999). This approach could be applied to genes in other regions of the genome using existing deletions and translocations and by identifying new rearrangements in PCR-based screens for mutations that eliminate a DNA sequence of interest (Fritz et al. 1996; Fisher et al. 1997; Talbot et al. 1998; Imai et al. 2000).

In another approach, recent reports show that double-stranded RNA interference (RNAi) can disrupt the functions of zebrafish genes, although the frequency of specific phenotypes is low and variable and nonspecific ef-

fects may also be obtained (Wargelius et al. 1999; Li et al. 2000). Additional work is needed to assess the specificity and efficacy of RNAi as a tool to inactivate zebrafish genes.

Prospects

Analysis of zebrafish mutations has demonstrated the value of this system for the genetic dissection of vertebrate development and disease. The insights gained over the last few years are only the beginning, as the vast majority of existing mutations have yet to be cloned or even characterized in depth phenotypically. Furthermore, innovative screens for novel phenotypes will expand the reach of zebrafish genetics to new areas of vertebrate biology. Recent progress in zebrafish genomics has accelerated the cloning of mutations—all but six of the genes shown in Table 1 were cloned in the last two years—and ongoing efforts to improve methods and infrastructure will continue these advances. Over the next two years, insertional mutagenesis screens will generate hundreds of mutations, thus increasing the number of cloned genes with essential functions many fold. EST sequencing and mapping projects will expand the candidate approach, and large-scale screens to determine the spatiotemporal expression patterns of ESTs will add an important new dimension to the database of zebrafish genes (e.g., Thisse and Thisse 1999). Ongoing mapping projects will facilitate positional cloning projects, and a clone-based physical map, perhaps constructed using BAC/PAC hybridization or fingerprinting approaches (Marra et al. 1999; Mozo et al. 1999), would be a tremendous additional boost. Ultimately, the sequence of the zebrafish genome will be the most potent tool for the molecular analysis of mutations, and the power of forward genetic approaches ensures that the fruits of the sequence will be worth the effort to obtain it.

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Talbot and Hopkins

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Talbot and Hopkins

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