Reverse genetics in zebrafish

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Lekven, Arne C., Kathryn Ann Helde, Christopher J. Thorpe, Rebecca Rooke, and Randall T. Moon. Reverse genetics in zebrafish. Physiol Genomics 2: 37–48, 2000.—The zebrafish has become a popular model system for the study of vertebrate developmental biology because of its numerous strengths as a molecular genetic and embryological system. To determine the requirement for specific genes during embryogenesis, it is necessary to generate organisms carrying loss-of-function mutations. This can be accomplished in zebrafish through a reverse genetic approach. This review discusses the current techniques for generating mutations in known genes in zebrafish. These techniques include the generation of chromosomal deletions and the subsequent identification of complementation groups within deletions through non-complementation assays. In addition, this review will discuss methods currently being evaluated that may improve the methods for finding mutations in a known sequence, including screening for randomly induced small deletions within genes and screening for randomly induced point mutations within specific genes.

Zebrafish as a Molecular Genetic Model System for Study of Vertebrate Embryonic Development

The zebrafish (Danio rerio) has become a popular model system for the study of vertebrate developmental biology because of its numerous strengths as a molecular genetic and embryological system. Techniques for manipulation of single cells and tissues have been very successful in the analysis of early embryonic development in zebrafish (for example, see Refs. 20 and 28). Moreover, the use of zebrafish for genetic analyses of development and physiology is well established. Specifically, in addition to two large-scale screens that identified more than 600 genes required during early embryogenesis based on the analysis of diploid phenotypes (13, 18), a number of other small- and large-scale screens are identifying numerous other loci required for different physiological processes. Zebrafish also are amenable to some genetic tricks that increase their utility; for example, the ability to generate haploid embryos facilitates genetic screens by eliminating a generation or more from crossing schemes (44). Such genetic screens, based on analysis of gynogenetic haploid phenotypes (e.g., Ref. 1) or gynogenetic diploid phenotypes (16), have already proven quite successful in identifying genes required during embryogenesis. Thus the zebrafish is a desirable system in which to carry out classic genetic screens to identify loci required during vertebrate embryonic development and to analyze the resulting phenotypes with powerful embryological techniques.

Not all investigators approach biological questions from a classic genetic direction, however. For them the zebrafish is also a potent model system for the analysis of vertebrate development and physiology from a molecular standpoint. In addition to the ability to assess gene function through overexpression from injected RNAs or DNAs in embryos, zebrafish transgenic technologies are being developed at an increasing rate. For example, transgenic lines expressing reporter genes under the control of tissue-specific promoters have been generated (19, 27). In addition, fish embryos are amenable to conditionally inducible gene expression (e.g., see Refs. 11 and 38). Furthermore, the GAL4-UAS system for driving the overexpression of transgenes, which has been an extremely useful analytical tool in Drosophila, has now been demonstrated to work effectively in zebrafish (36). The ability to control the spatial and temporal expression of gene constructs or reporter constructs in vivo is a powerful tool in the analysis of gene function, as evidenced by the concerted efforts...
that have been undertaken in other systems. The rapid development of these techniques, in addition to transposable elements (22, 26), highlights the significant battery of genetic tools available in zebrafish.

Despite all of the strengths mentioned, one technology lacking in the zebrafish system, indeed in any vertebrate system other than the mouse, is the ability to target mutations to specific genes through homologous recombination. The utility of this technology is apparent to anyone who has tried to determine a gene’s function after cloning based on homology.

To fully determine the function of a gene in vivo requires a combined analysis of misexpression and loss-of-function effects. In systems lacking gene knockouts through homologous recombination, how is it possible to analyze loss-of-function effects of a gene for which no mutant exists?

To add complexity to the problem of in vivo loss-of-function analysis, vertebrate genomes often contain multiple members of a gene family that have overlapping expression domains. This genetic redundancy may make the recovery of null mutations through a classic genetic approach difficult, as mutant phenotypes for each individual gene may be too subtle to detect. For example, the myogenic transcription factors myoD and myf-5 had been initially identified by their ability to make fibroblasts differentiate as muscle in vitro (6, 10), but the primary analysis of mouse knockouts of either locus showed that muscle development in neonates was essentially normal (7, 34). However, subsequent analyses by Kablar et al. (25) showed that the development of distinct subsets of skeletal muscle populations is delayed in these mutants. Embryos mutant for both myoD and myf-5 show no skeletal muscle development (35). This example highlights several points: 1) because of the subtle phenotypes, a mutant in myoD might not be found during a classic type of genetic screen, but, as also illustrated by Kablar et al. (25), this would depend on the sensitivity with which one assays for phenotypes; 2) genes of the same family that overlap in their expression can compensate for each other to differing extents. Thus it is by directly obtaining mutants in each family member individually and then by studying the compound mutants that one uncovers all the roles of a particular gene family, as a compound mutant may have a more severe phenotype than the additive phenotype of the single mutants.

Finally, the completion of sequencing phases of the different planned and ongoing genome projects will certainly reveal a plethora of genes for which there are no existing mutations. To assign a function to any region of the genome, an undertaking referred to as functional genomics, it will be advantageous to have a high-throughput method for generating mutations at molecularly identified loci in any region of the genome. One strategy employed with mice for functional genomic analyses involves generating large chromosomal deletions and then defining functional units within the deletions (24). This is an advantageous approach, because the deletions generated are useful for a number of purposes in functional genomics, including the ordering of loci in a defined genetic interval, determining the strength of mutant alleles in trans to the deletion, and quickly testing large regions of the genome for specific genetic activity.

This review will discuss the current methods in use to generate mutations in known genes in the zebrafish, which primarily involve high-throughput screening for complementation groups within specified genomic intervals delineated by chromosomal deletions. Also, this review will discuss methods currently being evaluated that may improve the methods for finding mutations in a known sequence, including screening for randomly induced small deletions within genes and screening for randomly induced point mutations within specific genes.

**Screening for Complementation Groups Within Defined Genetic Intervals Delineated by Chromosomal Deletions**

**Generation of chromosomal deletions.** The first step in screening for complementation groups within a chromosomal deletion is the generation of the deletion itself. Studies from the early 1980s showed that gamma rays are an efficient mutagen in zebrafish at different developmental stages (8, 43). However, although the high-specific locus mutation rate and high rate of induction of lethals suggested that these might be large deletions, the nature of the lesions induced was not determined. In a clever use of the ability to produce gynogenetic haploid embryos, Fritz et al. (14) developed a protocol using multiplex PCR to screen haploid embryos from mutagenized female fish for the presence of deletions at specific loci (Fig. 1A). Their work was a part of an ongoing genetic screen at the University of Oregon in which sperm irradiated with gamma rays was used to fertilize wild-type eggs to produce the parental generation. Gynogenetic haploid embryos are produced from the parental females, and DNA from these embryos serves as a template for multiplex PCR using primers for any desired locus. Deletion carriers are identified by the failure to amplify a locus from some fraction of the haploid embryos.

This scheme of coupling efficient mutagenesis with high-throughput molecular screening represents a very efficient way to screen for deletions anywhere in the genome. Fritz et al. (14) identified from this screen an average of one mutation for every 230 loci screened, or a specific locus frequency of ~0.5%. Considering the ease of screening and the modest requirements for fish tank space, this screen represents a successful and efficient mutagenesis and screening protocol that can be implemented by even a single researcher with a modestly sized zebrafish facility. In fact, this method is currently in use as part of the zebrafish genome initiative to generate a deletion panel encompassing the entire genome (M. Halpern and A. Fritz, Principal Investigators. Zebrafish Deletion Project. [Online], Carnegie Institution of Washington. http://www.eiwemb.edu/zdp.html).

One interesting finding in this study was that a large number of the mutations recovered were translocations. Why would translocations be recovered with this
A: outline of the gynogenetic based screen of Fritz et al. (14). Briefly, sperm is squeezed from males, then irradiated with gamma rays. Sperm is used to fertilize wild-type eggs, and resultant embryos are raised to adulthood. Haploid embryos from each adult female are tested for presence of multiple PCR products. Female fish showing a loss of a specific PCR product in their embryos are outcrossed as in D to recover the mutation. B: outline of androgenetic-based screen. Wild-type embryos, 1–3 h old, are irradiated with gamma rays. Surviving embryos are raised to adulthood; 80–90% of these adult fish are male. Haploid embryos from each adult male are tested for presence of multiple PCR products, and fish identified as carriers of deletions are outcrossed for recovery of the mutation (D or E). C: outline of screen for 4,5,8-trimethylpsoralen (TMP)-induced deletions. Sperm squeezed from wild-type males is incubated with TMP and irradiated with ultraviolet light (UV). Treated sperm is used to fertilize wild-type eggs, and resulting embryos are raised to adulthood. Haploid embryos from adult males and females are tested for presence of multiple PCR products, and fish identified as carriers of deletions are outcrossed to recover the mutation. D: scheme for recovery of translocations or deficiencies. Identified fish harboring deletions are outcrossed to wild-type fish. When progeny reach adulthood, haploid embryos are produced from individual fish and tested for presence of mutation. F1 individuals are heterozygous for the deficiency (m). E: scheme for simplified recovery of deficiencies. Fish heterozygous for the deficiency (Df) and an allele of a polymorphic PCR-amplifiable locus removed by the deficiency (ssa1) are crossed to fish homozygous for a second allele of the polymorphic locus (ssa2). Progeny will either be heterozygous for the ssa locus and amplify both ssa1 and ssa2 or will be deficiency carriers and only amplify ssa2. At 4 wk of age, the fish have large enough fins to clip for DNA extraction without harming the fish. This fin DNA can be used for genotyping by the PCR method mentioned. A description of the methods follows here. B: AB strain blastula stage embryos (1–3 h after fertilization) are irradiated with 250–350 rads gamma rays, according to Ref. 43. Surviving embryos (the G0 population) were raised to adulthood; 80–90% of them were male. Sperm squeezed from the adult male fish was used to produce androgenetic haploids (42). Haploid embryos were raised for 10 h, at which point they were transferred to 4% paraformaldehyde-PBS at 4°C until processed. Since G0 fish are mosaic for gamma ray-induced mutations, up to 24 embryos were tested from each individual male fish. Individual embryos were dechorionated and transferred into 0.2-ml PCR tubes containing 50 µl embryo DNA prep medium (EDPM) (45). Samples were incubated at 55°C for 1 h, then 99°C for 15 min. Ten-microliter aliquots of each DNA sample were used in two multiplex PCR reactions containing primers to several known wnt and wnt pathway genes (GenBank). Deletions are revealed by the lack of amplification of one of the PCR products. C: TMP was dissolved in 100% ethanol, diluted in DMSO for 100× stock solutions, and stored at −20°C. Mature sperm was collected in Hanks’ medium (45) and incubated in the dark on ice with 1% DMSO with TMP for final concentrations ranging between 1 ng/ml and 5 pg/ml TMP. After a 10- to 15-min incubation, the mixture was exposed for 6 min to long-wave UV (366 nm) with a 115-V lamp (UVP, Upland, CA) at a distance of 3.175 cm, with shaking every 30 s. The mutagenized sperm was then used to fertilize wild-type eggs. The surviving fish (G0) are screened by multiplex PCR amplification from eight haploid embryos per fish, as described above.
screening protocol? Because of the segregation properties of reciprocal translocations during meiosis, 1/4 of the meiotic products from a carrier of the translocation will be missing any locus distal to either translocation partner breakpoint (other meiotic products will have duplications for either translocation partner). A smaller proportion of the recovered mutations were simple deletions, i.e., they segregate to 50% of the germ cells of a carrier, but, interestingly, some of the translocations were also found to be able to resolve into simple deletions in subsequent generations. The sizes of these mutations varied but on average were larger than 10 cM, as judged by genetic markers from Postlethwait et al. (31). Thus this method of combining random mutagenesis with molecular detection of mutations is an effective way in which to generate deletions encompassing theoretically any point in the genome.

The drawback of depending on the meiotic properties of translocations to create deletions is that germ cells carrying the deletion only represent 25% of the total germ cells from a carrier. Since a mating between two zebrafish can produce hundreds of embryos, however, in most applications the lower proportion of mutant progeny from a cross will have no impact on the results. For example, one can use a translocation stock to determine the strength of a point mutation in trans to a deletion. On the other hand, an application where relying on a deletion derived from a translocation segregation can make things more laborious is when screening for complementation groups within the deleted region. A typical complementation screen is done by crossing a fish carrying randomly induced point mutations to a fish that is a carrier for the deletion/translocation. Since only 25% of the gametes from a fish carrying a translocation will have the deleted portion of a chromosome and the mutagenized fish can have maximally only 50% mutant gametes, there will be only a maximum of 12.5% of the embryos from such a cross potentially exhibiting a mutant phenotype. When the fish carrying new mutations has been produced in such a way that it is mosaic (see below), then this number could be much lower. Such low numbers can make the detection of phenotypes difficult. In addition, in the case of a gene of interest that lies close to its centromere, a reciprocal translocation-derived deletion will necessarily involve a large number of other genes that may also have early required functions. Removal of other linked loci by the deletion may thus obscure a phenotype associated with the gene of interest. Because of these facts, and to find methods to complement existing deletion screens, we have evaluated two other methods of mutagenesis for their ability to induce deletions in zebrafish: gamma irradiation of blastula stage embryos, and treatment of sperm with the chemical mutagen 4,5’-8-trimethylpsoralen (TMP). Our results show that blastula mutagenesis with gamma rays is an efficient way to generate simple deletions, both terminal and interstitial, and mutagenesis of sperm with TMP is an efficient approach in zebrafish that creates both translocations and deletions (below).

**An Androgenetic Screen for Gamma Ray-Induced Deletions**

In addition to sperm mutagenesis, gamma irradiation of blastula stage embryos has also been shown to be an efficient way of inducing mutations that have the characteristics indicative of large deletions (43). Anecdotal evidence also suggested that the mutations generated by blastula mutagenesis might be more genetically favorable (i.e., simple deletions or inversions which segregate to 50% of a carrier’s germ cells) than those induced by sperm mutagenesis (translocations and complex rearrangements; C. Walker, personal communication). Because of these data, we chose to evaluate this method as an alternative to sperm irradiation for the production of chromosomal deletions. Interestingly, gamma irradiation of blastula stage embryos results in predominantly male development (Ref. 43 and unpublished observations), necessitating the ability to produce androgenetic haploids to be able to perform a screen in the same manner as Fritz et al. (14). Two methods have been developed to produce androgenetic haploids: one is based on X-ray irradiation of eggs (9), and the other is based on ultraviolet (UV) irradiation to eliminate the maternal genome (42). As a matter of practice, only the UV method for generating androgenetic haploids (42) is feasible for this screen because of the requirement for rapid production of large numbers of treated eggs.

A schematic diagram of our blastula-irradiation screening protocol is shown in Fig. 1B, and Fig. 2 summarizes the results of this screen. Approximately 200 G0 fish were tested during our blastula-irradiation screen at 17 loci each, giving a total of ~3,400 loci tested. Thirty-four different mutations were detected during the screen, giving an average specific locus frequency of ~1%, in agreement with previously published results (43). The 34 mutations were carried by 30 fish; there were instances of fish carrying multiple deletions at unlinked loci. Since early blastula stage embryos (64–256 cell stages) were mutagenized, and embryos at these stages contain 4–5 germ cell precursors (43, 47), the resulting adult fish are likely to be mosaic for newly induced mutations in both their soma and germ lines. Our results are consistent with this, and we saw a range of 4–50% mutant germ cells from any particular fish. Of the 34 different mutations, 18 were represented in their respective germ lines at greater than 10%.

Once fish carrying a desired mutation are identified, they are outcrossed to wild-type females, then the

<table>
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<th>γ-ray sperm (B)</th>
<th>γ-ray embryo (B)</th>
<th>TMP sperm (C)</th>
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<td>34</td>
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<td>4</td>
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<tr>
<td>ave. frequency/locus</td>
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<td>1/485</td>
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Fig. 2. Comparison of the specific locus frequency of deletions found in 3 separate screens.
outcross progeny are tested in the same manner as the G0 parents. During our screen, we attempted to recover 16 of the 34 mutations; these were selected due to their presence in >10% of their founders' gametes (Fig. 2). We were successful in recovering 7 mutations of the 16. A potential cause of not being able to recover all 16 putative mutations is the decreased viability of fish heterozygous for the mutations compared with their wild-type siblings. In the recovered mutant stocks, we have noticed a decreased viability of heterozygotes during larval life (weeks 2–4 after fertilization). The decreased viability of heterozygotes may be due to either genetic background or haploinsufficiency associated with the deletion, leading to a substantial delay of growth compared with their wild-type siblings under crowded conditions. From our blastula irradiation screen, then, the practical specific locus frequency, considering the recovery rate, is ~0.4%. This number is comparable to that observed by Fritz et al. (14).

A significant difference between mutagenesis with gamma rays of blastula embryos vs. sperm that was revealed by our screen is in the proportion of recovered mutations that are apparent deficiencies (Fig. 3). Gamma irradiation of sperm results in primarily translocations being induced; 7 of 12 mutations recovered and mapped by Fritz et al. (14) were translocations. The other mutations included 2 deficiencies and 3 complex rearrangements. Of the 7 mutations recovered in our blastula irradiation screen, all 7 exhibited characteristics diagnostic of deficiencies, i.e., 2 neighboring loci deleted and 50% of gametes from a heterozygous fish showing the mutation (Fig. 3). We have attempted to analyze the sizes of five of the deletions using markers from three genetic maps (15, 31, 37). In general, the results show that in addition to terminal deletions, interstitial deletions have also been recovered, and the sizes of the deletions vary from <10 cM to >30 cM.

In conclusion, mutagenesis of blastula stage zebrafish embryos with gamma rays efficiently induces multilocus deletions and has several advantages that will complement the deficiency screens that utilize sperm mutagenesis, namely, that simple deletions are recovered, and they represent varying sizes of both interstitial and terminal deletions. Deletions are useful for a number of purposes, including genetic mapping and allelic analysis. Furthermore, and more pertinent to this review, deletions can be used effectively to scan a limited region of the genome for complementation groups (see below).

A Screen for TMP-Induced Deletions

Because the lesions caused by gamma rays are predominantly on the order of tens of centimorgans, and thus are likely to involve large numbers of genes, it would be advantageous to find a mutagenesis protocol for zebrafish that would result in deletions of a smaller size, ideally even deletions within only one gene. We chose to investigate the mutagen TMP. TMP is a planar three-ringed molecule that intercalates between base pairs in DNA. When activated by long-wave UV light, either or both of the end rings can covalently attach to adjacent thymine bases, forming either a monoadduct or an interstrand crosslink, both of which are mutagenic (reviewed in Ref. 21). Mutagenesis with the chemical TMP has been shown in Caenorhabditis elegans to create deletions ranging in size from 100 bp to 15 kbp (46) and thus would be an attractive candidate for use on zebrafish to create smaller size deletions.

We recently performed a small-scale screen using TMP to investigate its ability to create deletions in zebrafish. The mutagenesis protocol we developed (see Fig. 1C) is based on Ref. 46 and is similar to one recently published for zebrafish (4). A typical dose-response curve showing embryonic survival following treatment of sperm with TMP and long-wave UV light is shown in Fig. 4A.

The final mutagenesis rate is greatly affected by variability in either the concentration of TMP or the amount of long-wave UV exposure, which is required for TMP to covalently alter DNA. Therefore, it is anticipated that individual researchers will have to determine empirically the optimal dose of TMP for their source of long-wave UV light. Of practical note, ~1/4 of mutagenized fish are female; although this is not a severe skew in the sex ratio, it is important to be able to screen male fish for mutations.

As a prelude to screening for deletions induced by TMP, we tested the efficiency of TMP as a mutagen by two morphological assays. First, we found that TMP efficiently induces new alleles of the recessive viable embryonic pigment mutation golden. The results are shown in Fig. 4B. The second assessment of TMP as a mutagen involved inducing and identifying new mutations that can be recovered in the next generation. In a small morphology-based screen for embryonic lethal syndromes in genotypic haploid embryos, one-third of the female fish carried a unique patterning defect (described in Fig. 4C). Since fewer patterning defects can be identified in haploid embryos than in diploid embryos, this method presents a low estimate of the number of embryonic lethal mutations carried by the mutagenized fish.

To determine whether TMP induces deletions, we analyzed the mutagenized fish described above with a multiplex PCR-based screen (as described above for the

<table>
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<td>terminal</td>
<td>&lt;37&lt;sup&gt;b&lt;/sup&gt;</td>
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Fig. 3. Comparison of mutations recovered from blastula irradiation and TMP sperm mutagenesis screens. <sup>a</sup>Reference 15. <sup>b</sup>Reference 37.
gamma ray-mutagenized fish). The germ lines of 90 G₀ fish were screened at 18 loci each, for a total of 1,620 fish loci screened (Fig. 2). Eight mutations were identified, corresponding to a specific locus frequency of 0.5%. This is a high specific locus frequency, close to that found with gamma rays. Four mutation lines were recovered, three of which segregate in the F₁ generation as balanced translocations and one which segregates as a simple deletion (Fig. 3). Using markers from two meiotic maps of the zebrafish genome, we determined the size of this deletion to be between 27.2 and 37.3 cM.

The results obtained in this pilot screen indicate that TMP mutagenesis of mature sperm leads to an efficient induction of translocations and large deletions, similar to the range of mutations induced by gamma ray treatment of mature sperm. This method may be useful to researchers who want to create deletions but do not have access to a gamma ray source.

Although it is encouraging that TMP is an efficient mutagen in zebrafish, we would still like to find a mechanism for creating single-gene null mutations. As described above, gamma ray mutagenesis of mature sperm efficiently induces translocations, whereas mutagenesis of blastula stage embryos induces primarily, if not entirely, deletions. Since the stage of mutagenesis with gamma rays effects the type of lesion created, we are currently investigating the effect of TMP mutagenesis on blastula stage embryos in hopes that the molecular lesions created will be smaller deletions. In addition, it may be important to investigate the effect of different doses of TMP on the type of molecular lesion formed.

**Using Deletions to Look for Noncomplementing Point Mutations**

Deletions generated in the types of screens described so far will most likely remove multiple loci. Thus when analyzing the phenotype of embryos homozygous for such a deletion, it may be difficult to determine whether aspects of the phenotype are due to the removal of any one specific gene within the deletion. One can begin to address this limitation by generating embryos transheterozygous for overlapping deletions, thus effectively removing a smaller portion of the genome. Alternatively, one can use the deletions to screen for point mutations with complementation testing; this has the important advantage of possibly recovering several different alleles of varying strength, which can be useful in structure-function analyses.

To identify complementation groups within a deletion, one crosses fish carrying random point mutations to fish heterozygous for the deletion (Fig. 5). If the fish carrying the point mutation are heterozygous for a mutation that lies within the deletion interval, then 25% of the resulting embryos from such a cross should show some mutant phenotype, if the gene in question is required for normal development. Clearly, the more sensitively one can assay the progeny from test crosses for defects, the more new mutations will be observed and recovered.

In zebrafish, several protocols have been developed to efficiently induce point mutations in either mature sperm (Fig. 5A; Ref. 33) or spermatogonia (Fig. 5B; Refs. 29 and 40) using the chemical mutagen ethylnitro-
Sperm mutagenesis frequently results in mosaic animals because only a single strand of DNA is altered by the mutagen, and this only becomes fixed after subsequent rounds of DNA replication. Spermatogonial mutagenesis results in fish heterozygous for newly induced mutations, because the mutation becomes fixed following subsequent mitotic divisions during sperm maturation. Which protocol one chooses often depends on the practical consideration of the amount of tank space and staff available. Mutagenesis of mature sperm is reported to result in up to a 10-fold increased specific locus mutation rate over spermatogonial mutagenesis (2% over 0.2%, or 1 fish in 50 carrying a mutation in a specific gene over 1 fish in 500; Refs. 29, 33, 40), possibly because the fish derived from the spermatogenesis regimen are mosaic and can carry a heavier mutational load. However, because the animals are mosaic, a given mutation may only be present in a small fraction (5–10%) of its gametes. Thus, if a mosaic fish carrying a noncomplementing mutation in an essential gene is crossed to a deletion carrier, only a few of the embryos may show a phenotype, making it more difficult to detect candidate point mutants. Because female zebrafish typically lay clutches of more than 200 eggs, it is feasible to collect and score sufficient numbers of embryos to compensate for the lowered percentages of mutants. Also, with the increased specific locus frequency, sperm mutagenesis enables researchers with small to moderately sized fish facilities to carry out this type of screen.

After recovering mutations that lie within a specific genomic interval, it still needs to be demonstrated that a particular phenotype, as defined by the complementation groups, is caused by the lack of a specific gene product. Among the strategies that can be applied to establish definitively the connection between mutation and gene are the following: meiotic mapping to determine whether the mutation is tightly linked to the gene of interest (i.e., within 0.1 cM), sequencing to find an alteration in the mutant gene, and rescuing the

Fig. 5. Outlines of noncomplementation assay procedures. A: to make fish that are mosaic for ethyl-nitrosourea (ENU)-induced mutations (33), adult male fish are incubated in 0.8 mM ENU for 1 h, then immediately outcrossed to wild-type females. Resulting progeny are crossed individually to deficiency carriers. If the mosaic F1 fish is harboring a mutation within the deficiency, then some fraction of the embryos from the test cross (<25%) will show a mutant phenotype. Once a fish carrying a noncomplementing mutation is identified, it is outcrossed to wild-type fish, and the resulting F2 generation (heterozygous for the mutation) is test crossed to the deficiency. B: to produce fish that are heterozygous for ENU-induced mutations (29, 40), adult male fish are subjected to several soaks in water containing ENU. Surviving fish are allowed to rest for 3 wk, at which point any sperm will be derived from spermatogonia at the time of mutagenesis. Mutagenized males are outcrossed to wild-type females, and resulting F1 individuals are crossed to deficiency carriers as in A. Fish harboring mutations in complementation groups within the deficiency will produce 25% mutant embryos in test crosses.
mutant phenotype by microinjecting RNA or DNA corresponding to the wild-type locus. All of these strategies may need to be employed to establish with certainty a gene/mutant connection; however, as the field stands, if one can show by sequence analysis that there is a point mutation that disrupts the coding region of a gene and that segregates with the mutant phenotype, then it can be concluded that the two are causally related.

How well does this approach work in practice? Appel et al. (5) were successful in recovering a point mutation in the deltaA locus, which is deleted by the T(msxb)b220 deficiency generated by Fritz et al. (14). Embryos homozygous for the deficiency show several phenotypic defects, including a neurogenic phenotype. That the neurogenic phenotype is due to the loss of deltaA was suggested by overexpression of dominant negative forms of the gene (5). Out of 270 F1 mosaic fish crossed to T(msxb)b220 carriers, 1 consistently showed the same neurogenic phenotype. Recovery and sequencing of the mutant allele showed a point mutation within one of the EGF repeats of the deltaA protein. Analysis of the mutants revealed that the dlA gene is required for a developmental cell fate choice among floor plate, hypochord, and notochord, as the mutants show an excess of notochord at the expense of floor plate and hypochord (5). Thus, this study demonstrates the feasibility of this approach to reverse genetics in zebrafish. Clearly, recovery of point mutations through noncomplementation assays relies on some aspect of chance; however, by increasing the number of crosses one performs and increasing the sensitivity of how one scores the embryos for mutant phenotypes, for example, by using molecular markers instead of examining gross morphology, the likelihood of recovering one or more alleles of a specific gene is increased.

Technologies Being Investigated

A disadvantage of the deletion/point mutation screening method outlined in the previous section is that it is, of necessity, a two-part screen. First, the chromosomal deletion that takes out the gene of interest must be identified, if it does not already exist. Then, the noncomplementation screen is performed to obtain a mutation in only the gene being studied. In practice, this whole procedure requires four to five generations of fish to obtain a mutant for one’s gene of interest.

Another disadvantage of a noncomplementation screen is that, because it is based on analyzing zygotic phenotypes, mutations in genes that are strictly maternally required will not be detected, since embryos homozygous for such a mutation will be phenotypically wild type. Classic genetic screens to identify maternally required genes are very laborious; the required staff and tank space may not be feasible for many zebrafish laboratories.

Taking the previous points together, a procedure by which one could induce single-locus mutations detectable as heterozygotes would save both time and labor for many different applications. To achieve this, we are currently working to adapt two techniques that have proven successful in other systems for use as high-throughput screens in zebrafish. The first involves the detection of point mutations through the use of denaturing gradient gel electrophoresis, and the second is a PCR-based approach for identifying small deletions (2 kb or less) induced by chemical mutagenesis.

Point mutation detection. Many molecular methods have been developed for detecting point mutations in a specific DNA sequence, including forms of heteroduplex analysis and single-strand conformation polymorphism (SSCP) detection (reviewed in Ref. 30), methods using chemical cleavage at mismatches (reviewed in Ref. 39), microarray technologies (17), and sequencing of diploid DNA for heterogeneity (32). The method that we have chosen for finding point mutations carried by mosaic mutagenized zebrafish is heteroduplex analysis, as developed by Bio-Rad with their D-GENE system.

The advantage of heteroduplex analysis is that a heterozygous point mutation can act as a dominant trait, even when the mutant allele is represented in less than half the sample being analyzed. To detect a point mutation in a given stretch of sequence, genomic DNA from individual or pooled embryos is used as template for PCR amplification. The method to identify point mutations by creating and detecting heteroduplexes is shown schematically in Fig. 6A. The locus is amplified using one primer that contains on its 5’ end a “clamp” consisting of 33 G and C bases (GC clamp), which acts as a thermodynamically stable region to prevent complete denaturation during electrophoresis. After amplification of the locus, the samples are boiled to completely denature the DNA, then cooled at a rate that allows any potential heteroduplexes to form. The products are then run on an acrylamide gel that contains a gradient of denaturant parallel to the direction of electrophoresis. The heteroduplexes begin denaturing at a lower denaturant concentration than the homoduplexes, and the partially denatured molecules are retarded in the acrylamide matrix and thus form bands at higher positions in the gel. Thus the presence of a point mutation in 1/2 to 1/10 the amplification product is a dominant trait in this easily read assay.

The advantage of this method for finding mutations in cloned genes is that it precludes the necessity of first generating large deletions and circumvents the possibility that point mutations in the gene will not be found by complementation crosses due to a subtle or absent morphological phenotype. A disadvantage is that a point mutation found by this method may cause no functional change in the protein. Although this method has the potential to detect false positives due to normal allelic variation in populations and to errors early in the PCR amplification, in practice we have not had any false positives. Despite major differences in the techniques, the throughput of this method is not significantly different from that for multiplex PCR screens for loss of sequence. Although it is not possible to simultaneously analyze multiple loci for heteroduplex formation, the loss in throughput is offset by the fact that the traits are dominant and in our system can be visualized when a mutant chromosome represents only 1/8 of the
chromosomes in a DNA sample. Thus one can detect a point mutation if it is represented in one heterozygous embryo of four. To be confident of detecting a mutation that is present in a sufficient proportion of a founder's germ line to be easily recovered, one should test 12 germ cells from that founder. Therefore, one can collect embryos from an intercross of two mutagenized fish and prepare PCR products from three pools of four embryos each. Each pooled DNA sample can be tested at one locus at a time and will occupy one lane on the denaturing gradient gel. Thus 3 lanes will be used to test 12 germ cells from each of the 2 founder fish of that cross (0.67 fish loci screened per lane on the gel). With haploid screening for recessive traits, screening 1 founder fish requires 12 lanes on a gel for up to 10 loci (0.83 fish loci screened/lane on a gel).

**Nested PCR approach.** In addition to detection of point mutations, the use of PCR to detect randomly induced deletions on the order of 2 kb or less would also be a valuable asset. A technique currently in use by researchers in the *C. elegans* community (23) involves the use of nested pairs of PCR primers to amplify a specific locus from a population of nematodes following mutagenesis with either ethylmethanesulfonate (EMS) or TMP, both of which can induce small (hundreds of base pairs) deletions. Nested primers are designed for one's gene of interest, with the outer set giving a product from 2–3 kb in size and the inner set yielding a
slightly smaller fragment. Animals are mutagenized, and their progeny are grown on many plates in pools of 500 worms/plate. After these worms have begun laying eggs, a fraction of the worms are removed, and their DNA is used as a template for PCR with the nested set of primers. If a deletion has been induced in the gene of interest, then the smaller PCR product derived from the mutant allele is preferentially amplified compared with the full-length wild-type product, allowing for its detection from a pool of DNA containing a large excess of wild-type copies. This approach is becoming increasingly popular with *C. elegans* researchers, particularly with the recent completion of the genome sequencing project, which has provided the community with thousands of genes, both novel and familiar alike, for which there is no corresponding mutant. This has led to the recent formation of the *C. elegans* Gene Knockout Consortium (http://elegans.bcgsc.bc.ca/knockout.shtml), which plans to produce null alleles of every predicted gene in the worm genome using this technique.

We are currently working to adapt this approach for use in zebrafish, using ENU rather than EMS as the mutagen. ENU, like EMS, has been shown to induce small deletions in *C. elegans* ~13% of the time (12). However, in zebrafish, treatment with ENU induces mutations at frequencies an order of magnitude higher than EMS. With the scheme of mutagenizing mature sperm with ENU described above (33), a 2% specific locus frequency has been reported. If 10% of these mutations are deletions detectable by the nested PCR approach, then the frequency of deletions at a given locus should be ~0.2%, which is 2–3 orders of magnitude higher than EMS-induced deletions in *C. elegans*. At this frequency, one would have to screen through ~1,500 fish to have a 95% chance of finding a deletion in a given gene. This is a large, but not insurmountable, number and could prove an efficient means of identifying mutations in a specific gene, particularly if one screens with primer sets for several genes at once. Whether ENU induces deletions at an appreciable rate in zebrafish remains to be determined, however. Thus, in addition to conducting pilot screens using ENU, we are also testing different doses of TMP to determine whether it can induce smaller lesions than those described above.

**Summary**

This article has discussed current methodologies with which to generate and identify mutations in known genes in the zebrafish. The two major genetic screens for embryonic lethal mutations in zebrafish identified more than 600 genes required for embryonic development (13, 18), and already a fair number of them have had their corresponding genes cloned. However, these screens were not saturating, and a large number of mutants were discarded because of a lack of a specific phenotype (13, 18). Thus there is a large number of genes in zebrafish which have no corresponding mutants. In addition, the zebrafish genome project will in the future provide a huge amount of sequence data and a correspondingly large number of genes available for analysis. Determination of the loss-of-function effects of these genes will require a reverse genetic approach, and this approach must be of a high throughput to be applicable to functional genomics studies.

Researchers involved in any genetic model system are faced with the situation of having a larger number of genes than mutants; even those studying mouse genetics are faced with requiring a high-throughput method for identifying mutants, and the generation of knockouts through homologous recombination is not necessarily the most high-throughput method available (24). In this case, screening for point mutants within a small region of the genome delineated by a deficiency as described in this review is an advantageous technique (24).

In the case of the zebrafish, the rapid advancement of insertional mutagenesis (2, 3) and transposable element (22, 26) technologies means that a larger number of techniques are becoming available, in addition to the techniques described in this review, for the possible application to reverse genetics. Moreover, there is considerable effort being put forth by many laboratories to create a powerful zebrafish infrastructure for genomic research. Further information regarding zebrafish genomics can be obtained at several web sites, including the genome deletion project web site (http://www.eiemb.edu/zdp.html), the Washington University Zebrafish Genome Resources Project (S. Johnson; http://zfish.wustl.edu/), the Stanford Zebrafish Genome Project (W. Talbot; http://zebrafish.stanford.edu/), and the Zebrafish Information Network (http://zfish.uoregon.edu/). Add to these resources the wealth of mutants, the large number of elegant embryological techniques, and powerful molecular techniques, and the zebrafish is a choice system for the genetic analysis of vertebrate development and physiology.

**NOTE ADDED IN PROOF**

Two recent publications (A. Wargelius, S. Ellingsen, and A. Fjose. Biochem. Biophys. Res. Commun. 263: 156–161, 1999; and Y. X. Li, M. J. Farrell, R. Liu, N. Mohanty, and M. L. Kirby. Dev. Biol. 217: 394–405, 2000) have described the successful application of double-stranded RNA mediated interference (RNAi) in zebrafish embryos. RNAi has been used in *C. elegans, Drosophila*, mouse, and other systems to specifically block the function of targeted genes. The application of this technique to zebrafish is an exciting new development and promises to be an important tool for determining the function of cloned genes.

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