MINIREVIEW

From screens to genes: prospects for insertional mutagenesis in zebrafish

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The systematic, and now classic, genetic screens for mutations affecting the development of Drosophila and Caenorhabditis elegans have laid the foundation for the study of developmental mechanisms in these invertebrates [Nüsslein-Volhard and Wieschaus 1980; Horvitz and Sternberg 1991]. The subsequent molecular isolation and characterization of the affected genes has allowed a thorough understanding of the mechanisms underlying development. A similar genetic approach has recently been taken in a vertebrate. Large-scale genetic screens in zebrafish (Danio rerio) have identified >400 genes essential for the early development of this vertebrate [Driever et al. 1996; Haffter et al. 1996]. Phenotypic analysis of zebrafish mutants has already provided interesting insights into developmental mechanisms, but only a handful have been cloned. Because the mutagen ethylnitrosourea (ENU) predominantly causes point mutations, cloning of the mutations is rather laborious at this stage, and depends on positional cloning and candidate gene approaches [for review, see Collins 1995]. The recent development of pseudotyped retroviruses as insertional mutants in zebrafish provides a novel tool to identify and efficiently clone zebrafish mutations. As described by Hopkins and co-workers [Gaiano et al. 1996b; Allende et al., this issue], retroviral vectors insert into the zebrafish genome and can disrupt essential genes. The integrated DNA can then be used to isolate affected genes rapidly. We discuss the strategies and results of recent chemical and insertional genetic screens and assess different approaches to clone essential zebrafish genes.

Chemical mutagenesis with ENU efficiently induces mutations in essential genes

Two large-scale chemical mutagenesis screens were recently performed, using a standard three-generation inbreeding scheme, to identify embryonic and larval mutants as homzygous diploid animals [Driever et al. 1996; Haffter et al. 1996]. Germ-line mutations were induced by ENU in males [G0; see Fig. 1A], transmitted to their F1 offspring, expanded by breeding to produce F2 families, and bred to homozygosity in F3 embryos by interbreeding F2 fish. The two screens led to the identification of >6000 mutants, two-thirds of which showed general defects [growth retardation or degeneration] and were discarded. Complementation analysis of the remaining mutants indicated that the two screens identified >400 genetic loci involved in various aspects of development. Based on the average allele frequency, estimates indicate that >50% of genes that could be identified by such screens remain to be isolated. Nevertheless, the screens were efficient in inducing and identifying mutants: 1.3-1.4 mutants were identified in the progeny of each F2 family. These numbers are similar to the efficiency by which embryonic lethal mutations were identified in the Drosophila chemical mutagenesis screens. The major limitation of zebrafish compared to Drosophila is, therefore, not the rate of chemical mutagenesis but the limited number of fish that can be raised, housed, and screened.

Insertional mutagenesis with pseudotyped retroviruses disrupts essential genes

Insertional mutants provide an extremely useful tool to rapidly clone disrupted genes. This approach has been very successful in C. elegans and Drosophila, using transposable elements. For instance, P element-mediated mutagenesis in Drosophila has allowed the cloning of many of the genes originally identified in chemical mutagenesis screens, and has also led to the identification and cloning of many more genes in recent years [for review, see Spradling et al. 1995]. Experiments in the mouse introducing transgenes by pronuclear injections, gene trapping, or retroviral integration have also highlighted the power of insertional mutagenesis in vertebrates [for review, see Jaenisch 1988; Gossler and Zachgo 1993].

The retroviral approach has now been applied successfully to disrupt and rapidly clone essential genes in zebrafish [Allende et al. 1996; Gaiano et al. 1996b]. Burns et al. (1993) initially developed a pseudotyped retroviral vector that contains a genome based on the Moloney murine leukemia virus (MoMLV) and the envelope glycoprotein of the vesicular stomatitis virus (VSV). The pseudotyped retrovirus was used to infect zebrafish cell lines [Burns et al. 1993] and embryos [Lin et al. 1994a], and proviral insertions were initially transmitted at a low frequency to subsequent generations. More recently, higher-titer virus stocks [2 x 10^6 cfu/ml] have been produced, and the efficiency of insertion and germ-line transmission has been improved 100-fold [Gaiano et al. 1996a]. In a pilot screen, ~1/70 of insertions have now

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been found to produce embryonic or larval phenotypes (Allende et al. 1996; Gaiano et al. 1996b).

To obtain transgenic fish, blastula embryos were injected with ~20,000 infectious particles, some of which infect germ cells (see Fig. 1). Retroviral insertions were transmitted to F1 offspring by breeding G0 founder fish and Southern blot analysis was performed to determine which F1 animals carried retroviral insertions. About 30% of F1 embryos derived from a given G0 fish were found to be transgenic, and, on average, 11 different insertions were present in the mosaic germine of each G0 founder fish (Gaiano et al. 1996a). F2 families were raised and F2 fish heterozygous for the same insertion were intercrossed. The resulting F3 progeny were screened for mutant phenotypes. In a small-scale screen, Gaiano et al. (1996b) analyzed 217 retroviral insertions and found 3% of F1 animals carrying three or more insertions. By breeding F1 fish with several insertions, F2 families could be raised that contain six or more different insertions and these animals can be identified by Southern analysis of ~20 F1 animals. At this rate, insertional mutagenesis would identify ~0.1 essential genes in each F2 family, ~15-fold less than chemical mutagenesis screens. At this rate for insertional mutagenesis, it is unlikely that all mutations identified in the chemical screens will be isolated. However, variations on the breeding and screening schemes, and potential advances in retroviral vectors could make insertional mutagenesis more widely applicable.

Some benefit could be derived by increasing the number of retroviral insertions in each F2 family [Fig. 1A]. When two founders are crossed to each other, ~15% of F1 animals harbor three or more insertions. By breeding F1 fish with several insertions, F2 families could be raised that contain six or more different insertions and these animals can be identified by Southern analysis of ~20 F1 animals. At this rate, insertional mutagenesis would identify ~0.1 essential genes in each F2 family, ~15-fold less than chemical mutagenesis screens.

Another possible improvement of the screening protocol is to use F1 fish directly for identifying mutations, instead of raising large numbers of F2 families [Fig. 1B]. A given insertion is found on average in ~4% of F1 fish derived from a G0 founder (Gaiano et al. 1996a; A. Amsterdam, pers. comm.). Therefore, Southern blot analysis of ~100 fish from an F1 family should identify transgenic progeny. This strategy could allow the identification of

Insertional mutagenesis and large-scale screening

The advantage of using an insertional mutagen lies in the rapid cloning of the disrupted genes. However, the present insertional mutagenesis protocol is >20-fold less efficient in inducing lethal mutations as compared to chemical mutagenesis [Allende et al. 1996; Gaiano et al. 1996b]. Using F2 families that carry two insertions, one in about 35 F2 families would show an embryonic or larval phenotype, compared to virtually every F2 family in chemical mutagenesis screens. At this rate for insertional mutagenesis, it is unlikely that all mutations identified in the chemical screens will be isolated. However, variations on the breeding and screening schemes, and potential advances in retroviral vectors could make insertional mutagenesis more widely applicable.
0.1 mutant phenotypes in the F2 progeny of each F1 family. Although still a formidable screen, eliminating the F2 generation would dramatically reduce fish raising and maintenance required in the F3 protocol.

A third variation to the protocol would exploit the ability to produce gynogenetic haploid and diploid embryos (Kimmel 1989), thus obviating the need for Southern blot analysis of F1 fish [Fig. 1C]. Chemical mutagenesis screens of gynogenetic embryos have identified mutations affecting patterning and morphogenesis, although subtle or late-appearing phenotypes can be difficult to detect because of epigenetic defects, particularly in haploid embryos. An insertional screen of gynogenetic embryos from F1 females could be quite efficient even in the absence of a prescreen for insertions using Southern blot analysis, as more than half of F1 fish are transgenic.

In addition to these improvements in breeding and screening schemes, two major technical developments could make insertional mutagenesis in zebrafish more powerful. First, if the frequency of retroviral insertions continues to improve as it has in the last two years, the number of insertions per G0 germ cell could rise and a higher proportion of F1 offspring might carry the same insertion. This might be achieved by injecting more virus, at earlier stages of development, at different temperatures, or at multiple time points.

Another major technical improvement could come from the construction of retroviral promoter or gene trap vectors that allow for the preselection of fish carrying insertions interrupting transcribed genes, a strategy that has been successfully used in mouse (for review, see Gossler and Zachgo 1993). Expression of lacZ and green fluorescent protein (GFP) can be monitored in vivo in zebrafish [Lin et al. 1994b; Amsterdam et al. 1995], facilitating the identification of animals carrying a particular transgene. The frequency of identifying mutant phenotypes per insertion would likely decrease using gene or promoter trap vectors, because only insertions in the correct orientation and location in a gene would be expressed. However, this disadvantage would be offset by the more efficient identification of insertions in essential genes, and the raising and screening of a much smaller number of F1 fish. In the mouse, retroviral gene trap insertions have a fivefold higher frequency [about 1/4] of causing phenotypes as compared to “random” retroviral insertions [for review, see Jaenisch 1988; Gossler and Zachgo 1993].

Important questions are whether retroviruses integrate randomly into the genome, and whether all genes can be disrupted with this method. Examination of 42 proviral insertions revealed no obvious integration site bias (see Allende et al. 1996). However, analysis of P-element integration sites in Drosophila shows that this must be interpreted with caution. Sequence analysis did not reveal integration site biases, but only one-third of the genes identified by chemical mutagenesis can be functionally disrupted by this transposable element (for review, see Spradling et al. 1995). Only large-scale screens will be able to assess whether insertional mutagenesis can identify most essential genes in zebrafish.

Cloning strategies

The advantage of insertional mutagenesis over chemical mutagenesis is the amount of time that can be saved during the cloning of the mutated gene. Using PCR-based approaches, flanking sequences are cloned within a few days. Sequence analysis and comparisons then often allow the rapid identification of disrupted transcription units. However, even accounting for improvements such as those outlined above, it will be difficult to undertake an insertional screen of the magnitude required to yield as many mutations as have been isolated in large-scale chemical mutagenesis screens already. Thus, ENU-induced mutations will remain an important resource for identifying gene functions. In addition, second-generation chemical mutagenesis screens designed to identify relatively rare mutations that cause very specific phenotypes are underway in several laboratories (e.g., Henion et al. 1996). These screens would not be possible with the reduced efficiency of insertional mutagenesis. Thus other approaches are required to isolate mutant loci for which insertional alleles are not available.

The progress in cloning efforts in humans and mice is instructive in this regard, because the isolation of human genetic disease loci, for example, involves many of the same challenges as isolating genes defined in chemical mutagenesis screens in zebrafish. Positional cloning is one strategy that has been used successfully. In this approach, one uses a DNA probe near the gene of interest, usually identified in genetic mapping studies, to isolate by chromosomal walking clones that encompass the mutation. The next step is to identify transcription units within the chromosomal walk and then to determine which gene corresponds to the mutant locus.

The ability to identify markers tightly linked to mutations makes positional cloning feasible in zebrafish (Postlethwait et al. 1994). The average interval between zebrafish markers is currently ~1500 kb [Postlethwait et al. 1994; Knapik et al. 1996], so the average interval between a mapped mutation and its closest marker is ~750 kb. This distance can be narrowed substantially with bulked segregant analysis, which allows the directed screening of thousands of PCR-based markers to identify those that are very close to the mutation of interest [Postlethwait et al. 1994 and references therein]. In addition, the ability to identify rare recombinants in large mapping crosses (1000 or more individuals) scored by PCR can resolve markers and mutations spaced as closely as 0.1 cM, which represents a physical distance of 60 kb on average in zebrafish [Postlethwait et al. 1994]. Mapping at this resolution allows the progress of a walk toward a mutation to be monitored closely and narrows the search for the gene after the walk is completed.

The candidate gene approach is the second major strategy used to isolate mutant loci in mouse and human, and a few genes in zebrafish have been cloned using this approach (Schulte-Merker et al. 1994; Talbot et al. 1995). In this approach, one identifies genes that have already been cloned and found to have some property (e.g., expression pattern) expected of the mutant locus. The genetic map provides an efficient test of candidate genes by allowing assessment of linkage between a mutation and
a candidate gene. Unlinked loci are excluded, and further experiments determine whether any of the linked candidates are the gene of interest.

With the establishment of a dense transcript map, the candidate gene approach becomes extremely powerful [for review, see Collins 1995]. This is best illustrated in humans, in which >16,000 transcribed sequences, representing about one-fifth of human genes, have been mapped [Schuler et al. 1996]. Over the next few years most transcription units in the human genome will be mapped and sequenced. Thus the goal of genetic analysis will not be to isolate new genes, but rather to identify new gene functions. Candidate genes will be routinely identified as transcribed sequences that map to the same interval as a mutation (the “positional candidate” approach). The zebrafish genetic map and radiation hybrids [C. Kwok, R.M. Korn, M.E. Davis, D.W. Burt, R. Critcher, B.H. Paw, L.I. Zor, P.N. Goodfellow, and K. Schmitt, pers. comm] provide the framework to construct a dense zebrafish transcript map. As more genes are mapped, many chemically induced mutations will be cloned by the positional candidate approach.

Conclusions

As in all other genetic organisms, several combined approaches will lead to gene isolation in zebrafish, including positional cloning, candidate gene approaches, and insertional mutagenesis. Genes disrupted with retroviral transgenes can be rapidly cloned, and this is an important advantage over mutations induced by chemical mutagens. Whereas cloning, but not generation, of mutations is rapid with insertional mutagens, the opposite is true for chemical mutagens. Further development of zebrafish genome resources and improvements in the efficiency of insertional mutagenesis should soon lead to the molecular isolation of many of the genes involved in zebrafish development.

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