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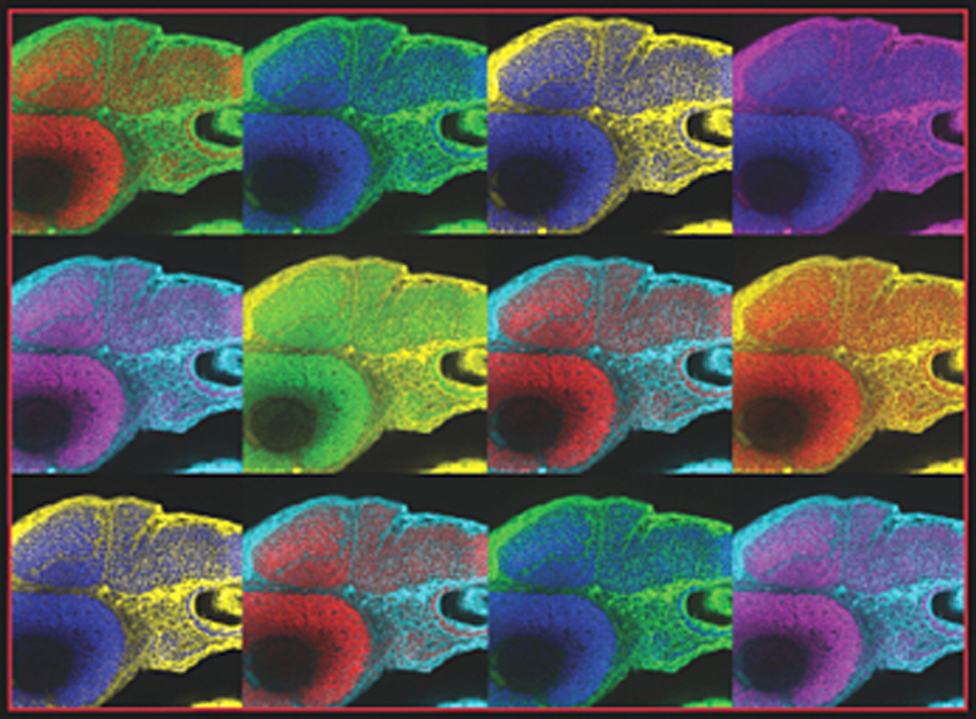

Edited by

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Methods in Cell Biology

VOLUME 77

The Zebrafish: Genetics, Genomics, and Informatics

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PREFACE

Monte, Len, and I welcome you to two new volumes of *Methods in Cell Biology* devoted to *The Zebrafish: Cellular and Developmental Biology* and *Genetics, Genomics, and Informatics*. In the five years since publication of the first pair of volumes, *The Zebrafish: Biology* (Vol. 59) and *The Zebrafish: Genetics and Genomics* (Vol. 60), revolutionary advances in techniques have greatly increased the versatility of this system. At the Fifth Conference on *Zebrafish Development and Genetics*, held at the University of Wisconsin in 2003, it was clear that many new and compelling methods were maturing and justified the creation of the present volumes. The zebrafish community responded enthusiastically to our request for contributions, and we thank them for their tremendous efforts.

The new volumes present the post-2000 advances in molecular, cellular, and embryological techniques (Vol. 76) and in genetic, genomic, and bioinformatic methods (Vol. 77) for the zebrafish, *Danio rerio*. The latter volume also contains a section devoted to critical infrastructure issues. Overlap with the prior volumes has been minimized intentionally.

The first volume, *Cellular and Developmental Biology*, is divided into three sections: Cell Biology, Developmental and Neural Biology, and Disease Models. The first section focuses on microscopy and cell culture methodologies. New microscopic modalities and fluorescent reporters are described, the cell cycle and lipid metabolism in embryos are discussed, apoptosis assays are outlined, and the isolation and culture of stem cells are presented. The second section covers development of the nervous system, techniques for analysis of behavior and for screening for behavioral mutants, and methods applicable to the study of major organ systems. The volume concludes with a section on use of the zebrafish as a model for several diseases.

The second volume, *Genetics, Genomics, and Informatics*, contains five sections: Forward and Reverse Genetics, The Zebrafish Genome and Mapping Technologies, Transgenesis, Informatics and Comparative Genomics, and Infrastructure. In the first, forward-genetic (insertional mutagenesis, maternal-effects screening), reverse-genetic (antisense morpholino oligonucleotide and peptide nucleic acid gene knockdown strategies, photoactivation of caged mRNAs), and hybrid (target-selected screening for ENU-induced point mutations) technologies are described. Genetic applications of transposon-mediated transgenesis of zebrafish are presented, and the status of the genetics and genomics of *Medaka*, the honorary zebrafish, is updated. Section 2 covers the zebrafish genome project, the cytogenetics of zebrafish chromosomes, several methods for mapping zebrafish genes and mutations, and the recovery of mutated genes via positional cloning.

The third section presents multiple methods for transgenesis in zebrafish and describes the application of nuclear transfer for cloning of zebrafish. Section 4 describes bioinformatic analysis of the zebrafish genome and of microarray data, and emphasizes the importance of comparative analysis of genomes in gene discovery and in the elucidation of gene regulatory elements. The final section provides important, but difficult to find, information on small- and large-scale infrastructure available to the zebrafish biologist.

The attentive reader will have noticed that this Preface was drafted by the first editor, Bill Detrich, while he (I) was at sea leading the sub-Antarctic ICEFISH Cruise (International Collaborative Expedition to collect and study Fish Indigenous to Sub-antarctic Habitats; visit www.icefish.neu.edu). Wearing my second biological hat, I study the adaptational biology of Antarctic fish and use them as a system for comparative discovery of erythropoietic genes. Antarctic fish embryos generally hatch after six months of development, and they reach sexual maturity only after several years. Imagine attempting genetic studies on these organisms! My point is that the zebrafish system and its many advantages greatly inform my research on Antarctic fish, while at the same time I can move genes discovered by study of the naturally evolved, but very unusual, phenotypes of Antarctic fish into the zebrafish for functional analysis. We the editors emphasize that comparative strategies applied to multiple organisms, including the diverse fish taxa, are destined to play an increasing role in our understanding of vertebrate development.

We wish to express our gratitude to the series editors, Leslie Wilson and Paul Matsudaira, and the staff of Elsevier/Academic Press, especially Kristi Savino, for their diligent help, great patience, and strong encouragement as we developed these volumes.

H. William Detrich, III
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This volume is dedicated to Jose Campos-Ortega and Nigel Holder, departed colleagues whose wisdom and friendship will be missed by the zebra fish community

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PART I

Forward and Reverse Genetics

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CHAPTER 1

Retroviral-Mediated Insertional Mutagenesis in Zebrafish

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I. Introduction

Large-scale chemical mutagenesis screens have resulted in the isolation of thousands of mutations in hundreds of genes that affect zebrafish embryonic development (Driever *et al*., 1996; Haffter *et al*., 1996). These screens have used an alkylating agent, ethyl nitrosourea (ENU), to induce mutations, primarily by causing base pair substitutions. Approximately 100 of the genes disrupted by these mutations have been isolated till March 2004, primarily through a candidate gene approach and less frequently by pure positional cloning (Postlethwait and Talbot, 1997), and many other chapters in this volume are devoted to describing this. However, positional cloning remains arduous.

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Insertional mutagenesis is an alternative to chemical mutagenesis in which exogenous DNA is used as the mutagen. Although insertional mutagenesis is usually less efficient than ENU, insertions serve as a molecular tag to aid in the isolation of the mutated genes. Several methods can be employed to insert DNA into the zebrafish genome, including DNA microinjection (Culp *et al*., 1991; Stuart *et al*., 1988), or microinjection of DNA aided by retroviral integrases (Ivics *et al*., 1993) or a transposable element's transposase (Davidson *et al*., 2003; Kawakami *et al*., 2000; Raz *et al*., 1997); reviews and updates on these methods can be found in other chapters in this volume. However, to date, by far the most efficient way to make a large number of insertions in the zebrafish genome is to use a pseudotyped retrovirus.

Retroviruses have an RNA genome and, on infection of a cell, reverse transcribe their genome to a DNA molecule, the provirus. The provirus integrates into a host cell chromosome, where it remains stable and is thus inherited by all the descendants of that cell. Replication-defective retroviral vectors, unlike nondefective retroviruses, are infectious agents that can integrate into host DNA, but whose genetic material lacks the coding sequences for the proteins required to make progeny virions. Retroviral vectors are made in split-genome packaging cells, in which the genome of the retroviral vector is expressed from one integrated set of viral sequences, whereas the retroviral genes required for packaging, infection, reverse transcription, and integration are expressed from another locus. The most widely used retroviral vectors have been derived from a murine retrovirus, the Moloney murine leukemia virus (MoMLV), resulting in replication-defective viruses that can be produced at very high titers. Initially, these retroviruses were only capable of infecting mammalian cells, but their host range can be expanded as described later.

Retroviruses have a host range, or tropism, which is frequently determined by their envelope protein, which recognizes and binds to some specific component, usually a protein, on the surface of the cell to be infected. Cell types that have an appropriate receptor can be infected by the retrovirus, whereas those that do not are refractory to infection. The host range of a virus can be changed by pseudotyping, a process in which virions acquire the genome and core proteins of one virus but the envelope protein of another. One way to enable this situation in split-genome packaging cells is to simply substitute the gene encoding the alternative envelope protein for the usual one. Although there is some specificity as to which envelope proteins can be pseudotyped with which viral genomes, one such combination that is particularly useful allows the MoMLV viral genomes and core proteins to be pseudotyped with the envelope glycoprotein (G-protein) of the vesicular stomatitis virus (VSV; Weiss *et al*., 1974). VSV is a rhabdovirus that is apparently pantropic; it can infect cells of species as diverse as insects and mammals (Wagner, 1972). MoMLV vectors pseudotyped with VSV-G possess two qualities essential for their use in high-frequency germline transgenesis in zebrafish: the extended host range allows for the infection of

fish cells, and the VSV-G-pseudotyped virions are unusually stable, allowing viruses to be concentrated 1000-fold by centrifugation (Burns *et al*., 1993).

When pseudotyped retroviral vectors are injected into zebrafish blastulae, many of the cells become independently infected, producing a mosaic organism in which different cells harbor proviral insertions at different chromosomal sites. When cells destined to give rise to the germ-line are infected, some proportion of the progeny of the injected fish will contain one or more insertions (Lin *et al*., 1994). When a sufficiently high-titer virus is used, one can infect a very high proportion of the germline of injected fish (Gaiano *et al*., 1996a). With very high-titer virus, on average, about 25–30 independent insertions can be inherited from a single founder, though any given insertion will only be present in about 3–20% of the offspring (Chen *et al*., 2002; A. Amsterdam, unpublished data). However, the progeny are nonmosaic for the insertions and transmits them in a Mendelian fashion to 50% of then progeny. Furthermore, because more than one virus can infect a single cell, some germ cells contain multiple insertions, and thus offspring can be born with as many as 10–15 independently segregating insertions (Amsterdam *et al*., 1999; Chen *et al*., 2002; Gaiano *et al*., 1996a). This remarkable transgenesis rate has made it possible to conduct an insertional mutagenesis screen, which has allowed isolation of hundreds of insertional mutants and rapid cloning of the mutated genes (Amsterdam *et al*., 1999, 2004a; Golling *et al*., 2002).

II. Mutagenesis

To establish the frequency of mutagenesis with retroviral vectors in the zebrafish, we carried out a pilot screen in which we inbred more than 500 individual proviral insertions, one at a time, and screened for recessive phenotypes that could be visually scored in the first 5 days of embryonic development. We found six recessive embryonic lethal mutations, a frequency of about one mutation per 80–100 insertions (Allende *et al*., 1996; Becker *et al*., 1998; Gaiano *et al*., 1996b; Young *et al*., 2002). We also found one viable dominant insertional mutation (Kawakami *et al*., 2000). Although this rate was too inefficient to conduct a large-scale screen by breeding one insertion at a time, by using the ability of founders to transmit multiple insertions to individual F1 progeny an average of about 12 inserts can be screened per family, allowing the recovery of about one insertional mutation per seven families screened (Amsterdam *et al*., 1999; Amsterdam, unpublished data). This is only 7- to 10-fold lower than the frequency observed in analogously performed (three-generation diploid) ENU screens (Driever *et al*., 1996; Haffter *et al*., 1996; Mullins *et al*., 1994; Solnica-Krezel *et al*., 1994). The strategy to produce, select, and breed the fish for such an insertional mutagenesis screen is outlined in this section.

A. Making Founder Fish That Transmit Proviral Inserts at High Frequency to Their F1 Progeny

Founders are produced by injecting late-blastula-stage (512–2000 cells) embryos. Virus must be injected into the space between the cells, and blastula-stage embryos ideally accommodate the injected fluid. At this time, there are four primordial germ cells, and these cells divide two or three times over the course of the infection (Yoon *et al*., 1997). Thus, the injected embryos grow up to be founder fish (F0) with mosaic germ lines. With very good viral stocks, individual founders can contain 25–30 different insertions in their germ lines, with any given insertion present in 3–20% of the gametes (Amsterdam, unpublished data; Chen *et al*., 2002). Individual F1 fish can inherit up to 10 different insertions, and when founders are bred to each other F1 fish can be found with up to 20 different insertions. F1 fish are not mosaic and transmit all their insertions in a Mendelian fashion.

Because the efficiency of the screen relies on the generation of F1 fish with a high number of inserts, it is essential to perform quality control assays on the viral stocks and founder injections before raising and breeding the founders. For every batch of injected embryos, several embryos are sacrificed for DNA preparation at 48 h postinjection and subjected to quantitative Southern analysis or real-time polymerase chain reaction (PCR) to determine the average number of insertions per cell in the entire infected embryo. This number is called the embryo assay value (EAV; Amsterdam *et al*., 1999). In our experience, if the average EAV is above 15 and does not vary much among the individual analyzed embryos, the rest of the founders from that injection session transmit inserts at the rates mentioned previously. Batches with average EAV below 15 transmit somewhat fewer inserts, and usually have greater founder-to-founder variation, and those with average EAV below 5 are quite inconsistent in transmitting multiple inserts to their progeny.

B. Breeding and Screening for Mutations

The breeding scheme for a diploid F3 insertional mutagenesis screen is outlined in Fig. 1. In essence, the goal is to create families with a large number of independent insertions that can be screened simultaneously. This is achieved by selecting and breeding F1 fish that inherit the most inserts from the mosaic founders.

Founder fish can be bred to each other or outcrossed to nontransgenic fish. For reasons that remain unclear, a majority of injected fish grow up to be males; thus, it is most efficient to outcross the best male F0 fish (those from batches with the highest EAV) and inbreed the rest. F1 families of 30 fish are raised, and at 6 weeks of age the fish are fin clipped for DNA preparation and analysis by quantitative Southern or real-time PCR to determine which fish harbor the most insertions. Keeping up to the three top fish per family with at least five inserts strikes a balance between throwing away too many inserts (if fewer fish were kept) and keeping too many 'repeat inserts' (i.e., the same insert inherited by sibling F1 fish). The repeat insert rate is quite low if only three fish are kept, as the average mosaicism

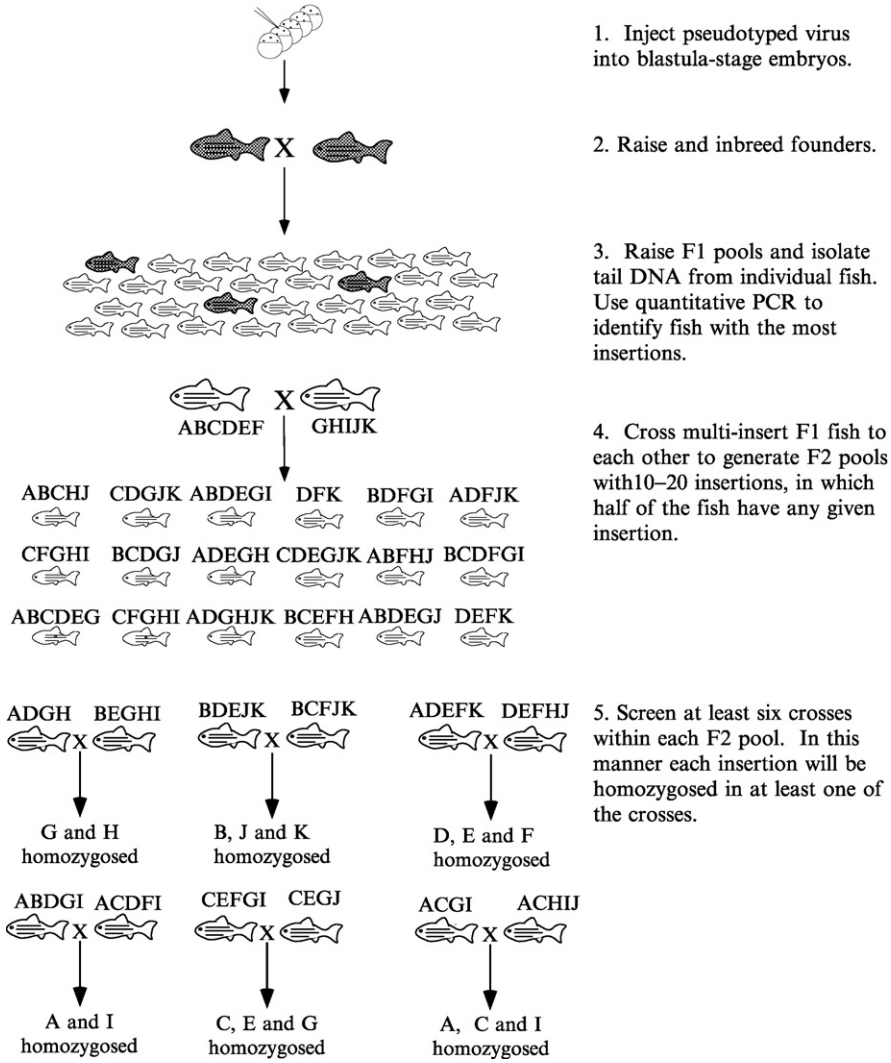

Fig. 1 Insertional mutagenesis breeding scheme.

(i.e., proportion of F1 inheriting a given insert) is about 8%. In our screen, only 3% of the recovered mutations have been caused by reisolating such repeat inserts.

The selected multiinsert F1 fish are pooled together and eventually bred to make F2 families that harbor at least 10 different independently segregating inserts, and in which each insert is present in half the fish. Multiple sibcrosses are then conducted between the F2 fish; because half the fish have any given insert, including one causing a mutation, should be homozygosed in one quarter of the

crosses. On average, six crosses will homozygose 83% of the inserts in the family and ten crosses will screen 95% of them. Every F3 clutch from each F2 family is screened for a phenotype in one quarter of the embryos. In our screen, embryos were scored for any morphological defect visible in a dissecting microscope at 1, 2, and 5 days post fertilization (dpf), as well as for defects in motility and touch response. One aid to screening is that more than 98% of these mutants fail to inflate their swim bladders by 5 dpf; because this is such a highly visible structure, a quick screen for clutches in which one quarter of the embryos fail to inflate their swim bladder often signals the presence of a mutation.

III. Cloning the Mutated Genes

A. Identifying the Mutagenic Insert

The great advantage to using insertional mutagenesis over chemical mutagenesis is that the mutagenic insertion provides a molecular tag that can be used to identify the disrupted gene. However, because the mutagenesis screen described uses multiple insertions to increase the rate of recovery of mutations, the first step after identifying a mutation is to determine which (if any) insertion appears to be responsible for the mutation. DNA is prepared from the tails of the parents of all of the crosses from the F2 family, and, using Southern analysis to distinguish the different insertions, one looks for an insertion that segregates with the phenotype (Fig. 2A). A linked insert (represented by a band of a specific size) will be shared by both parents of every cross that had the phenotype and be in only one or neither of the parents of every cross that lacked the phenotype. In addition, DNA prepared from the mutant embryos is subjected to the same analysis; an unlinked insert would be in only three quarters of the mutant embryos, but a linked insert must be present in all of them (Fig. 2B).

It is possible that no insert appears linked to the phenotype; in our screen, we found that about one quarter of the mutants recovered were not linked to a detectable insertion. In addition, it is important to note that the identification of an insertion initially linked to the phenotype is not proof that the identified insert is *tightly* linked to the mutation; it is merely a way to either identify the insertion that is a *candidate* for causing the mutation or to conclude that the mutation is not linked to any insert if no insert meets the criteria. This is because recombination rates in the male germline are much lower than in the female germline (Johnson, personal communication; Amsterdam, unpublished data); thus, an insert inherited from an F1 male that is merely on the same chromosome as a noninsertional mutation will often meet the previously mentioned criteria. The mutation and the insert will not have segregated in the F2 generation, and because the mutant F3 embryos must inherit the mutant locus from both parents, even if there is recombination in the female germ line all the mutant embryos will receive the insert from their father. Thus, additional linkage experiments that can distinguish

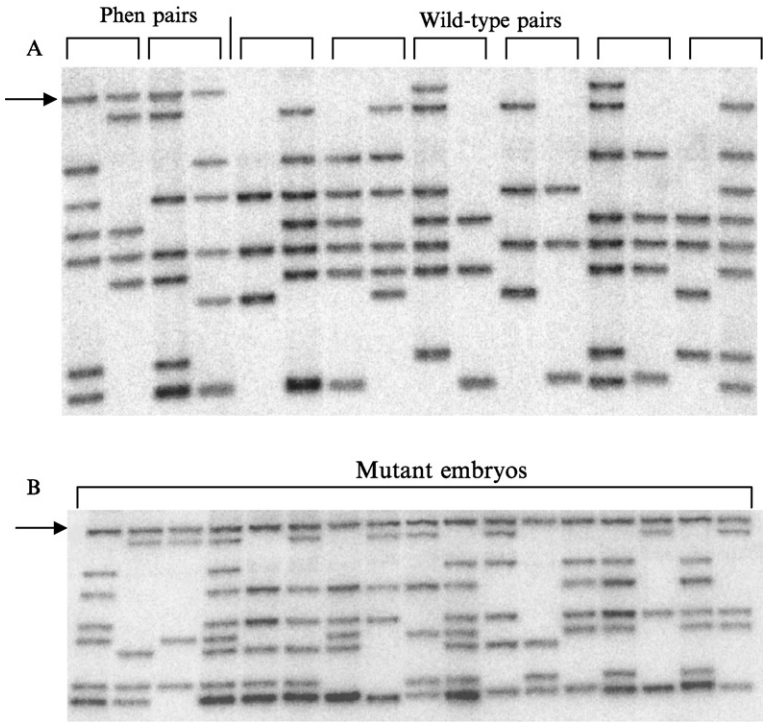

Fig. 2 Identification of the mutagenic insert. (A) Southern analysis of DNA prepared from tail fins of F2 fish: the arrow indicates an insert that is homozygosed in phenotypic pairs but not any of the wild-type pairs. (B) Southern analysis of DNA from individual mutant embryos from the second phenotypic pair in (A) the arrow indicates that the same insert is also present in all the mutant embryos.

heterozygosity from homozygosity for the insert are required, but it is not possible to perform these until genomic DNA flanking the candidate insert is cloned.

Sometimes more than one insert meets the previously mentioned criteria, and thus more than one are candidates to have caused the mutant phenotype. This can be for one of several reasons. First, if more than one insert in the family is on the same chromosome, for the reasons described previously they might fail to segregate from each other. Often this can be resolved by outcrossing a female carrier and repeating the analysis in the next generation, either by further random sib crosses followed by molecular analysis or by using Southern analysis first to identify fish with one or the other insertion and then performing test crosses. Another possibility is that multiple copies of the virus have integrated in tandem, which happens about 3–4% of the time. Usually when this happens, there is a higher-intensity provirus-sized band (if the enzyme used cuts the insert only once) in addition to the true junction fragment band. Finally, there might be too many inserts in the family to accurately distinguish all the inserts (more than 15–20), and