

Screening Mosaic F1 Females for Mutations Affecting Zebrafish Heart Induction and Patterning

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ABSTRACT The genetic pathways underlying the induction and anterior-posterior patterning of the heart are poorly understood. The recent emergence of the zebrafish model system now allows a classical genetic approach to such challenging problems in vertebrate development. Two large-scale screens for mutations affecting zebrafish embryonic development have recently been completed; among the hundreds of mutations identified were several that affect specific aspects of cardiac morphogenesis, differentiation, and function. However, very few mutations affecting induction and/or anterior-posterior patterning of the heart were identified. We hypothesize that a directed approach utilizing molecular markers to examine these particular steps of heart development will uncover additional such mutations. To test this hypothesis, we are conducting two parallel screens for mutations that affect either the induction or the anterior-posterior patterning of the zebrafish heart. As an indicator of cardiac induction, we examine expression of *nkx2.5*, the earliest known marker of precardiac mesoderm; to assess anterior-posterior patterning, we distinguish ventricle from atrium with antibodies that recognize different myosin heavy chain isoforms. In order to expedite the examination of a large number of mutations, we are screening the haploid progeny of mosaic F1 females. In these ongoing screens, we have identified four mutations that affect *nkx2.5* expression as well as 21 that disrupt either ventricular or atrial development and thus far have recovered several of these mutations, demonstrating the value of our approach. Future analysis of these and other cardiac mutations will provide further insight into the processes of induction and anterior-posterior patterning of the heart. Dev. Genet. 22:288-299, 1998.

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INTRODUCTION

Formation of the vertebrate heart requires the integration of inductive, patterning, and morphogenetic events (DeHaan, 1965; Fishman and Chien, 1997). Descriptive and fate-mapping studies in various model

systems have delineated the key events in vertebrate heart development. In all vertebrates, the cells that give rise to the myocardium (muscular layer of the heart) are among the first to gastrulate. Subsequent to gastrulating, these myocardial progenitors are found in bilateral regions of the anterior lateral plate mesoderm in close apposition to the anterior endoderm. These bilateral populations of myocardial progenitors later move towards the midline and fuse to form the definitive heart tube, enclosing the endocardial progenitors in the process. As development proceeds, contractile proteins appear within the myocardial precursors; the organization of these proteins into nascent myofibrils precedes the initiation of contractions. The anterior-posterior (A-P) patterning of the definitive heart tube becomes apparent via the visibly distinct formation of the ventricular and atrial chambers at its anterior and posterior ends, respectively. Subsequently, the heart loops in a rightward direction, initiating the division of the heart into left and right sides in higher vertebrates.

Despite this detailed descriptive knowledge, relatively little is known about the molecular mechanisms that guide vertebrate heart development. We are particularly interested in understanding how the early events of induction and anterior-posterior patterning of the heart are accomplished. Neither the identity nor the origin of the signals that direct these processes is known. Similarly, the differentiation pathways that lie downstream of these signals are for the most part uncharacterized. Although a great deal certainly remains to be discovered, a few potentially important

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players in cardiac induction and patterning have been identified.

The first molecular evidence of cardiac induction is the expression in the precardiac mesoderm of the homeobox gene *nkx2.5*, a homologue of the *Drosophila* gene *tinman* (Komuro and Izumo, 1993; Lee *et al.*, 1996; Lints *et al.*, 1993; Schultheiss *et al.*, 1995; Tonissen *et al.*, 1994). *Drosophila tinman* mutants lack the dorsal vessel (the heart equivalent in insects) (Azpiazu and Frasch, 1993; Bodmer, 1993), while the hearts of *Nkx2-5* mutant mice fail to undergo looping morphogenesis and exhibit some cardiac gene expression defects. These data indicate that *Nkx2-5* is critical for normal heart development (Lyons *et al.*, 1995), but the broadly normal pattern of cardiac gene expression in these mutant mice suggests that *Nkx2-5* by itself does not play a role equivalent to that of *tinman* in the fly.

How *nkx2.5* expression is induced in the precardiac mesoderm is not clear. Substantial evidence implicates the endoderm as a source of heart-inducing signals (Nascone and Mercola, 1996), and in avian embryos BMP-2 expressed in the anterior endoderm may induce or maintain expression of *nkx2.5* in the precardiac mesoderm (Schultheiss *et al.*, 1997). The dorsal organizer may also participate in cardiac induction (Sater and Jacobson, 1990), either directly or via influences on the endoderm. Clearly many genes involved in the production and reception of heart-inducing signals await discovery.

Anterior-posterior patterning of the heart is manifest in the substantial histological and physiological differences between the anterior ventricle and the posterior atrium. Although these distinctions do not become apparent until relatively late in cardiac development, allocation of cells to the ventricle or the atrium may occur much earlier. Indications of an A-P pattern are apparent even before the anterior (ventricular) and posterior (atrial) chambers are morphologically obvious. For instance, the expression of chamber-specific myosin isoforms becomes restricted within the heart tube prior to chamber demarcation and valve formation (Bisaha and Bader, 1991; Kubalak *et al.*, 1994; O'Brien *et al.*, 1993; Stainier and Fishman, 1992; Yutzey *et al.*, 1994). Cell fate analyses in chick and zebrafish further suggest that separate groups of cells in the pre-gastrula embryo may give rise to each cardiac chamber (Gonzalez-Sanchez and Bader, 1984; Yutzey *et al.*, 1995; Stainier *et al.*, 1993). Additionally, the A-P location of cells in the chick heart tube corresponds to their relative position during ingression through the primitive streak (DeHaan, 1965).

Neither the molecular players that establish cardiac A-P patterning, nor those that execute the chamber-specific differentiation pathways, are known. Retinoid signalling pathways may influence the early definition of the cardiac A-P axis; for example, administration of low doses of retinoic acid to vertebrate embryos during gastrulation can transform anterior (ventricular) por-

tions of the heart tube into posterior (atrial) tissue (Stainier and Fishman, 1992; Yutzey *et al.*, 1995). However, the importance of endogenous retinoid signalling for heart patterning remains uncertain.

Attempts to understand cardiac induction and A-P patterning using genetic means have been limited by a requirement for prior knowledge of potentially important genes, either by virtue of expression pattern, homology to other genes, or relevant biochemical activity. The advent of the zebrafish model system, however, permits a classical genetic approach to issues in vertebrate development (Kimmel, 1989; Driever *et al.*, 1994). The power of this approach has been well demonstrated in studies of invertebrates such as *Drosophila* and *C. elegans* (St. Johnston and Nusslein-Volhard, 1992; Horvitz, 1988). Similar efforts in zebrafish should eventually provide a complete description of the genes controlling vertebrate development.

A number of characteristics make the zebrafish suitable for genetic analysis (Kimmel, 1989). Adults are small (3-4 cm long), easy and fairly inexpensive to raise and maintain, and reach sexual maturity in 2-3 months. As many as several hundred progeny can be obtained from a single mating pair at weekly intervals. External fertilization and rapid development permit continual observation, which is aided by the zebrafish embryo's transparency. Furthermore, numerous methods exist for creating different types of mutations, such as deletions (Walker and Streisinger, 1983), point mutations (Mullins and Nusslein-Volhard, 1993; Solnica-Krezel *et al.*, 1994), and insertions (Gaiano *et al.*, 1996).

The embryonic zebrafish heart is particularly amenable to such genetic studies (Stainier and Fishman, 1994). The heart's ventral location, combined with the transparency of the embryo, allows detailed *in vivo* observation. Additionally, the heart develops rapidly, beginning to beat at around 22 hours post-fertilization (hpf), with morphological chamber demarcation and looping completed soon thereafter (36 hpf). Most importantly, the zebrafish heart forms in a manner similar to the hearts of other vertebrates, indicating that information gained from studies of the zebrafish will prove broadly relevant.

Two recent large-scale screens, one performed in Boston (Driever *et al.*, 1996) and the other in Tübingen (Haffter *et al.*, 1996), have generated a collection of nearly 2000 mutations that identify at least 400 genes critical for zebrafish development (Table 1). Both groups used a traditional two-generation breeding scheme to uncover recessive mutations in diploid F3 progeny (Figure 1A). Visual inspection at several timepoints following fertilization (6-12 hpf, and approximately 1, 2, and 5 days post-fertilization) revealed morphological abnormalities. In general, mutations that produced specific developmental defects were propagated for further analysis.

In these screens, mutations affecting the heart were identified in embryos at 30-36 hpf (Chen *et al.*, 1996b;

TABLE 1. Mutations Affecting Cardiac Development Identified in the Large-Scale Zebrafish Screens*

Boston		Tübingen	
Mutagenized genomes screened	2337	Mutagenized genomes screened	3857
Total loci identified for which complementation testing done	220	Total loci identified for which complementation testing done	372
Phenotypic classes		Phenotypic classes	
Ventricle defect (<i>pandora</i>)	1	Ventricle defect (<i>lonely atrium</i>)	1
Cardia bifida (e.g., <i>miles apart</i>)	2	Cardia bifida (e.g., <i>casanova</i>)	4
Large heart (e.g., <i>heart of glass</i>)	3	Large heart (<i>santa</i>)	1
No endocardium (<i>cloche</i>)	1	Chamber position (<i>overlooped</i>)	1
Small heart (<i>heart and soul</i>)	1	Thin matrix (e.g., <i>scotch tape</i>)	2
No valve (e.g., <i>jeekyll</i>)	2		

*Summary of the results of the recently completed Boston and Tübingen zebrafish screens. The total number of mutagenized genomes screened and the total number of identified loci for which complementation testing has been done are given. The 19 genes that were found to affect cardiac development are grouped by phenotypic class, and an example of each is given in parentheses. In some cases, allelism between mutations found in the two independent screens has yet to be tested. In addition to those listed here, many mutants displayed abnormal cardiac function, and some of these phenotypes may be due to defects in myocardial differentiation.

Stainier *et al.*, 1996); their diverse yet specific phenotypes demonstrate that single gene mutations can perturb discrete events in cardiac development (Table 1). For example, *cloche* mutant embryos lack the endocardial lining of the heart (Stainier *et al.*, 1995). Further studies of *cloche* demonstrate that this gene affects the development of all endothelial cells and acts upstream of *flk-1*, the earliest characterized marker of endothelial precursors (Liao *et al.*, 1997). These screens also identified mutations that cause cardia bifida (bilateral hearts), affect heart size, eliminate the cardiac valves, or disrupt the cardiac matrix. Many additional mutants display abnormal cardiac function; some of these phenotypes may be due to defects in myocardial differentiation.

In contrast, none of these mutations appears to disrupt cardiac induction severely (i.e. the mutant entirely lacks myocardial tissue), and only two mutations, *pandora* and *lonely atrium*, specifically affect the A-P patterning of the heart (Table 1) (Stainier *et al.*, 1996; Chen *et al.*, 1996b). In both of these mutants, the heart appears entirely atrial; the fate of the anterior (ventricular) tissue in these mutants is not known. That no mutants entirely lacking a heart were identified may be due to functional overlap of genes controlling cardiac induction or perhaps because such mutants die prior to screening (i.e. 30-36 hpf). The underrepresentation of these phenotypic classes may also be due to how the screens were conducted. Both groups agree that it is difficult to estimate the degree of saturation that their screens achieved and suggest that many loci remain to be discovered (Driever *et al.*, 1996; Haffter *et al.*, 1996). This possibility may be particularly true in regards to heart development. More than one allele was identified for only 6 of the 17 genes that affect cardiac development (24 mutations total), and for only two genes, *miles apart* and *santa*, did each group find an allele. Also, while mutations causing some phenotypes were detected in both screens (e.g. large heart), other phenotypes were seen only in one or the other screen

(e.g. no valves and thin matrix) (Table 1). Finally, screening with morphological criteria may have failed to detect certain cardiac phenotypes -- for example, those that resulted in early lethality or those in which the heart had only subtle abnormalities.

Defining the genetic pathways that control induction and A-P patterning of the heart will require additional relevant mutations. We hypothesize that by adjusting both the method and the criteria of screening, we will detect such mutations. Here we describe the progress of two screens directed at identifying mutations affecting induction and/or A-P patterning of the heart.

MATERIALS AND METHODS

Mutagenesis

Twenty-four wild-type males (AB background) were mutagenized essentially as described (Riley and Grunwald, 1995). Briefly, a 1 gram isopac of N-nitroso-N-ethylurea (ENU) (#N3385, Sigma, St. Louis, MO) was dissolved in 5 mM MES (pH 6.1) to a concentration of 50 mM. Three to five males were placed in disposable buckets containing 500 ml system water buffered with 3 mM MES (pH 6.1). Either 8 or 10 ml of 50 mM ENU was then added, yielding final ENU concentrations of 0.8 and 1.0 mM ENU respectively. Following a one-hour incubation at room temperature (21-23°C) in a closed fume-hood, the fish were removed from the ENU and allowed to recover in fresh system water for 2.5 hours at 25°C, then returned to the aquarium system. The ENU-treated males were crossed to wild-type females (of AB or Hong Kong backgrounds) that evening and every other evening subsequently for a total of four outcrosses, and the resulting F1 progeny were raised using tetrahymena (Gerson and Stainier, 1995).

Generating haploid embryos

When the F1 females neared sexual maturity, they were separated into tanks containing 20-30 females

and 4-6 males. This ratio of females to males facilitated optimal growth of the females, while maintaining their fecundity. Ten days prior to screening, F1 females were crossed to wild-type males; if the cross yielded embryos, the female was assigned an identification number (e.g. F1 female #86) and transferred to her own 1-liter tank to ensure optimal feeding. These advance crosses served a dual purpose: first, only those females known to be

fertile were screened; second, should a female die after screening, an interesting mutation could still potentially be recovered from the advance outcross progeny.

Eggs were extruded from females as described (Westerfield, 1993). Since the individual identity of females had to be maintained, several females could not be incubated with a single male during the night prior to squeezing. Instead, a male was placed below, and the female above, the mesh of a standard breeding trap; this enabled appropriate conditioning to occur without permitting the female to lay her eggs. Sperm was collected by dissecting testes from adult males and then homogenizing each pair in 200 ml of I-buffer (Westerfield, 1993). The sperm was inactivated by ultraviolet irradiation in a UV Stratalinker 2400 (Stratagene, La Jolla, California) for 18 seconds and then diluted with an additional 200 ml of I-buffer. The sperm from one male was used to fertilize five clutches of eggs.

The eggs were examined at the mid-blastula stage and the unfertilized and obviously abnormal ones were discarded. The developing embryos were divided into two groups: 40% were raised at room temperature and fixed at approximately the 8-somite stage of development for wholemount in situ hybridization (see below); the remainder were kept at 28.5°C and analysed by immunofluorescence at approximately 30 hpf (see below). In cases where a large number of haploids were obtained, we limited our screening to 100 embryos per clutch for in situ hybridization and 70 per clutch for immunofluorescence. In a few cases, small clutches were analyzed at only one timepoint with only one set of molecular markers.

In situ hybridization

Wholemount in situ hybridizations were performed as described (Schier *et al.*, 1997), using probes for zebrafish *nkx2.5* (Lee *et al.*, 1996) and *gata-1* (Detrich *et al.*, 1995); hybridizations were at 70°C in buffer containing 50% formamide and 5 µg/ml tRNA. Photo-

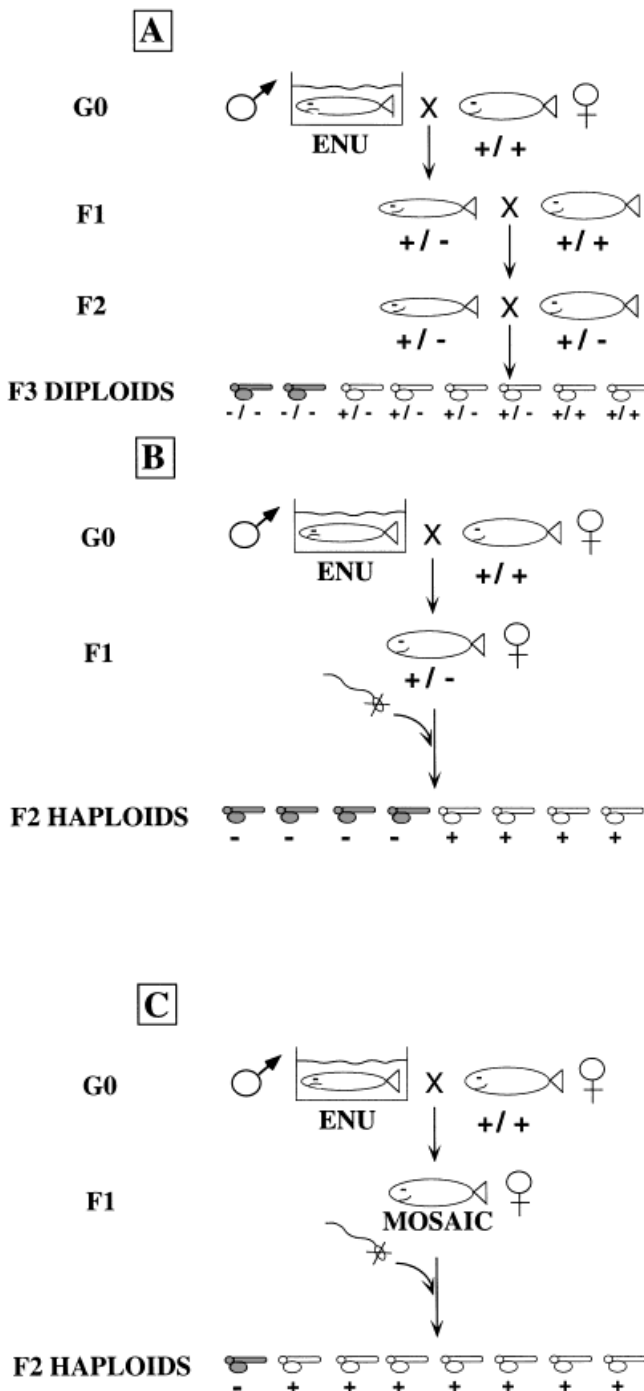


Fig. 1. Three approaches to screening for mutations in zebrafish. Panels (A), (B), and (C) show three alternative ways to screen for mutations affecting embryonic development in zebrafish. In a traditional two-generation breeding scheme (A), males are mutagenized and outcrossed to wild-type females in order to generate heterozygous F1 founders. Individual F2 families are raised from these founders, and sibling intercrosses performed to reveal recessive mutations in one quarter of the F3 generation (shaded embryos). By using UV-inactivated sperm to produce gynogenetic haploid embryos from F1 females (B), one can observe the effects of mutations without requiring an additional generation to breed them to homozygosity. In this case, one half of the haploid F2 embryos will be mutant (shaded embryos). If the F1 females are instead mosaic (C), a variable proportion of the F2 haploid embryos will be mutant -- for example, one of eight embryos (shaded). The precise number of mutant embryos depends upon the proportion of the F1 germline cells heterozygous for the mutation. Mosaic F1 fish, produced by crossing mutagenized males directly after mutagenesis, are able to carry a much greater mutational load than non-mosaic F1 fish.

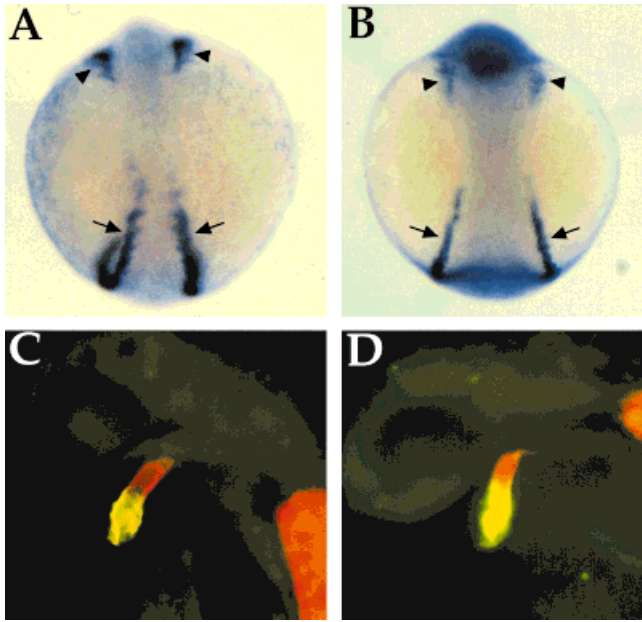


Fig. 2. Molecular markers that monitor cardiac induction and A-P patterning are expressed similarly in diploid and haploid embryos. In (A) and (B), embryos were examined by wholemout *in situ* hybridization using riboprobes directed against *nkx2.5* and *gata-1*. Expression of both *nkx2.5* (arrowheads) and *gata-1* (arrows) in similarly staged diploid ((A), 10-somite stage) and haploid ((B), 8-somite stage) embryos is essentially identical. Embryos are viewed dorsally, with anterior to the top. In (C) and (D), embryos were stained with S46 and MF20 monoclonal antibodies at 30 hpf. In both the diploid embryo (C) and the haploid embryo (D), red fluorescence indicates MF20 (TRITC) staining of ventricular and skeletal muscle, and yellow fluorescence indicates the overlap of S46 (FITC) and MF20 (TRITC) staining of atrial muscle. Skeletal muscle (red) is visible in the lower right of (C) and the upper right of (D). Embryos are viewed laterally, with the head to the left.

graphs were taken using a Leica MZ12 stereomicroscope and Kodak Ektachrome 160T film, and were processed using Adobe Photoshop 3.0.

Immunofluorescence

Embryos were prepared for wholemout immunofluorescence as described previously (Stainier and Gilbert, 1990). The secondary reagents goat anti-mouse IgG1-FITC (fluorescein isothiocyanate) and goat anti-mouse IgG2b-TRITC (tetramethylrhodamine isothiocyanate) (Southern Biotechnology Associates, Birmingham, Alabama) recognize the monoclonal antibodies S46 (generous gift of Dr. Frank Stockdale, Stanford University) and MF20 (Bader *et al.*, 1982) respectively. Photographs were taken using a Zeiss Axioplan microscope and Fujichrome 1600 ASA film, and images were processed using Adobe Photoshop 3.0.

Recovery of mutations

If a potentially interesting phenotype was seen in at least 5% of the embryos in a clutch, the progeny from the initial outcross were raised, and the female was crossed to wild-type males at weekly intervals to ensure

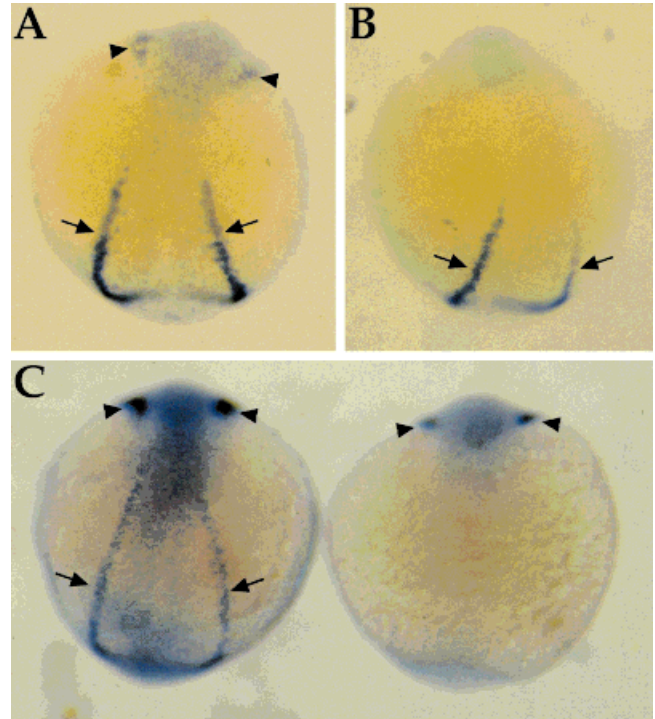


Fig. 3.

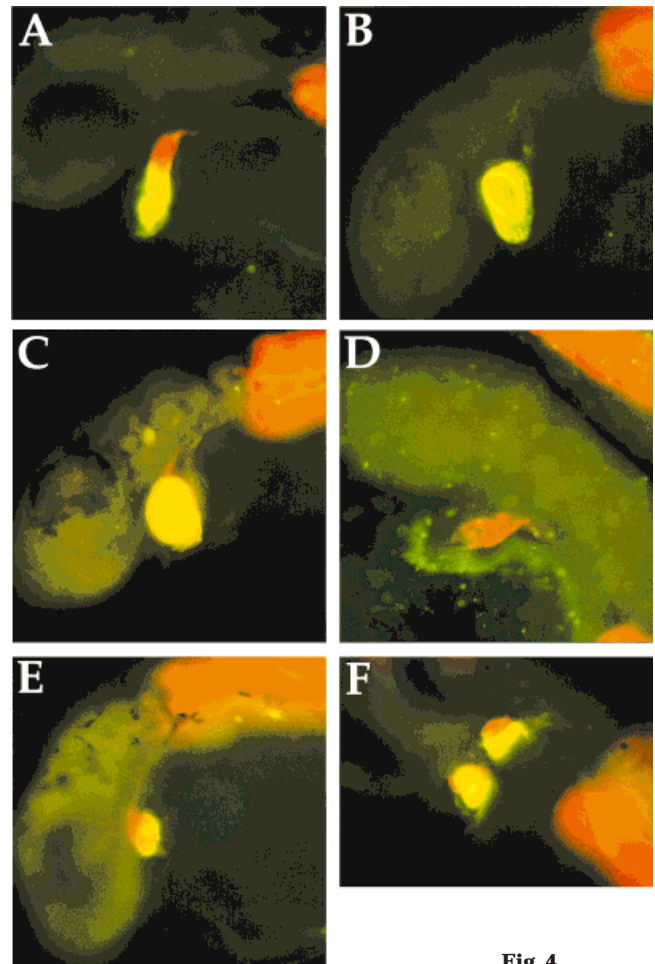


Fig. 4.

that sufficient F2 progeny existed to permit recovery of the mutation. In order to confirm the presence of an interesting mutation in the germline of an F1 female, the female was squeezed again at least six weeks later. To determine whether the mutation could be acting in a dominant fashion, F1 females were further crossed to wild-type males and the diploid progeny examined as above.

In order to recover mutations in the F2 generation, we squeezed F2 females as described above. Unlike the mosaic F1 generation, the F2 fish are heterozygous for the induced mutations; the phenotype of interest should therefore appear in half of their haploid progeny. Identified F2 heterozygotes are outcrossed to wild-type males; intercrossing the F3 generation will allow the characterization of the phenotypes of diploid mutants, although further outcrosses may be necessary to ensure that these lines carry only a single embryonic lethal mutation.

RESULTS

Examining the haploid progeny of mosaic F1 females expedites the screening process

In order to expedite screening a large number of mutations, we took advantage of the ability of zebrafish

embryos to develop as gynogenetic haploids (Kimmel, 1989). Screening haploid embryos allows rapid assessment of whether interesting mutations, either dominant or recessive, are present in the mother's germline. This strategy minimizes time and tank space requirements in comparison to a traditional two-generation breeding scheme (Figure 1). Despite the convenience of using haploid embryos, this approach is not suitable for all analyses. Haploid embryos have a limited lifespan, usually no more than three days, and exhibit a number of developmental abnormalities. These haploid-specific defects do not, however, obscure observation of much of embryonic development; previous haploid screens have identified several important zebrafish mutations (e.g. *no tail* (Halpern *et al.*, 1993)). Furthermore, since the processes we seek to study occur normally in haploids (see below), we chose to analyze haploid embryos in our screen.

There are various approaches to generating chemical mutations in zebrafish (Figure 1). Standard ENU mutagenesis and breeding yields a non-mosaic F1 generation that carries an average of one embryonic lethal mutation per mutagenized genome. Mutations can then be identified either in diploid F3 embryos (Figure 1A), or in haploid F2 embryos (Figure 1B). Another strategy uses an alternative protocol in which males are outcrossed immediately following mutagenesis for a maximal period of two weeks (Riley and Grunwald, 1995) (Figure 1C). This protocol ensures that the paternal gametes contributing to the F1 generation were post-meiotic at the time of mutagenesis; the induced mutations are therefore not fixed into the genome until at least the first round of zygotic DNA replication (mutations are often not fixed until the second or third round), and as a result the F1 progeny are genetically mosaic. Mosaic fish are able to carry a mutational load estimated to be as much as ten-fold greater than that of non-mosaic fish, presumably because homozygous wild-type cells within all tissues compensate for less robust heterozygous cells (Riley and Grunwald, 1995). Theoretically, this protocol allows the observation of approximately ten embryonic lethal mutations in each clutch of F2 haploid embryos. The presence of multiple mutations in each haploid embryo and the unpredictable representation of any particular mutation within the germline may make detection of relevant phenotypes difficult and may also complicate efforts to recover mutations of interest. We nonetheless undertook such a mosaic approach (Figure 1C) because of the substantially increased efficiency that it provides and the presumed low frequency of mutational events of interest to us.

Using molecular markers to monitor cardiac induction and A-P patterning

In order to detect mutations that specifically affect cardiac induction and A-P patterning, we selected molecular markers that allow us to monitor these processes. In each case, it was important to confirm that

Fig. 3. Wholemout in situ hybridization analysis demonstrates mutants lacking either *nkx2.5* or *gata-1* expression. Haploid embryos were examined by wholemout in situ hybridization using riboprobes directed against *nkx2.5* and *gata-1* at approximately the 8-somite stage. Arrowheads indicate *nkx2.5* expression, and arrows indicate *gata-1* expression. All embryos are viewed dorsally, with anterior to the top. Panels (A) and (B) show sibling haploid embryos; the embryo in (B) lacks expression of *nkx2.5* (arrowheads) but displays normal *gata-1* staining (arrows). Panel (C) shows sibling haploid embryos from a different clutch; while *nkx2.5* staining (arrowheads) is present in both, the embryo on the right does not express *gata-1* (arrows).

Fig. 4. Immunostaining with the S46 and MF20 antibodies reveals four categories of mutant phenotypes. Haploid embryos were stained with S46 and MF20 monoclonal antibodies at 30 hpf. Red fluorescence indicates MF20 (TRITC) staining of ventricular and skeletal muscle, and yellow fluorescence indicates the overlap of S46 (FITC) and MF20 (TRITC) staining of atrial muscle. Panels (A) - (E) show lateral views, with the head to the left. In (A), the ventricle (red) and atrium (yellow) of a wild-type haploid embryo are visible, as well as some skeletal muscle (red, upper right) in the trunk. The embryos in (B) and (C) exhibit defects in ventricle development. In (B), the single chamber (yellow) of a mutant haploid embryo is visible, in addition to some skeletal muscle (red, upper right). In (C), the single major chamber (yellow) of a mutant haploid embryo is attached to a short stalk of S46-MF20⁺ tissue (red) at its anterior end; some skeletal muscle (red, upper right) is also visible. The mutant haploid embryo shown in (D) has a defect in atrium development. Its single cardiac chamber (red) is visible; some skeletal muscle of this embryo (red, lower right) and some skeletal muscle of a neighboring embryo (red, upper right) are also shown. The high level of green background in (D) is due to deliberate photographic overexposure to show the lack of genuine S46 staining. (E) shows a mutant haploid embryo with a small heart that has both a ventricle (red) and an atrium (yellow). Some skeletal muscle (red, upper right) is also visible. (F) shows a ventral view of a mutant haploid embryo, with its head in the upper left. This embryo exhibits cardia bifida; two bilateral hearts, each with a ventricle (red) and an atrium (yellow), are visible. The view of one of these ventricles is partially obscured. Some skeletal muscle (red, lower right) of this embryo is also visible.

TABLE 2. New Mutations Identified by Screening With Molecular Markers*

<i>nkx-2.5/gata-1</i>		S46/MF20	
F2 haploid clutches screened	311	F2 haploid clutches screened	339
Embryos screened per clutch (average)	30	Embryos screened per clutch (average)	32
Phenotypic classes		Phenotypic classes	
No <i>nkx2.5</i> expression	4	Ventricle defect	20
No <i>gata-1</i> expression	3	Atrium defect	1
		Small heart	4
		Cardia bifida	10

*Summary of the results of our two parallel screens. The total number of clutches screened with each set of molecular markers and the average number of embryos in each clutch are indicated, along with the number of mutations in each phenotypic class.

haploid embryos express these markers in a manner similar to diploids.

The homeobox gene *nkx2.5* is the earliest marker of precardiac mesoderm in all vertebrates (Komuro and Izumo, 1993; Lints *et al.*, 1993; Schultheiss *et al.*, 1995; Tonissen *et al.*, 1994). In zebrafish, *nkx2.5* expression in the precardiac mesoderm initiates soon after the onset of somitogenesis, indicating that cardiac induction has occurred by this time (Chen and Fishman, 1996a; Lee *et al.*, 1996); mutations affecting *nkx2.5* expression may therefore identify genes important for the induction or early differentiation of the precardiac mesoderm. Embryos were examined by wholemount in situ hybridization with a probe for *nkx2.5* at approximately the 8-somite stage (arrowheads in figure 2, A and B). Analysis at this stage, safely after the onset of *nkx2.5* expression, ensures that developmental asynchrony within a clutch does not confound the identification of mutant embryos. Additionally, a probe for the hematopoietic transcription factor gene *gata-1* (Detrich *et al.*, 1995) is used simultaneously, both as an internal control for staining quality as well as to broaden the range of detectable phenotypes (arrows in Figure 2, A and B). Importantly, haploid and diploid embryos express *nkx2.5* and *gata-1* similarly (compare Figure 2, A and B).

In order to detect mutations affecting cardiac A-P patterning, we chose to examine the expression of chamber-specific myosin heavy chain (MHC) isoforms, early indicators of anterior (ventricular) and posterior (atrial) cell identities (Stainier and Fishman, 1992). The monoclonal antibody S46 recognizes an atrial-specific MHC isoform (generous gift of Dr. Frank Stockdale, Stanford University), while the monoclonal antibody MF20 recognizes both atrial and ventricular MHCs (Bader *et al.*, 1982). These antibodies therefore distinguish the atrial (S46⁺MF20⁺) from the ventricular myocardium (S46⁻MF20⁺), allowing visualization of A-P pattern in the zebrafish heart tube prior to the morphological demarcation of the chambers. We visualize S46 with green fluorescence and MF20 with red

fluorescence; thus, the anterior (ventricular) end of the heart tube appears red and the posterior (atrial) end of the heart tube appears yellow (overlap of green and red fluorescence) (Figure 2, C and D). The somites also appear red, since MF20 additionally recognizes skeletal MHC, thereby providing a control for effective immunostaining. Most relevant for our purposes, the expression patterns of these MHCs are identical in haploid and diploid embryos at 30 hpf (compare Figure 2, C and D).

In order to screen at the appropriate time points for mutations that affect cardiac induction or A-P patterning, we divided each clutch of haploid embryos. One group of embryos was analyzed for *nkx2.5* and *gata-1* expression at approximately the 8-somite stage, while the remaining embryos were stained with S46 and MF20 at approximately 30 hpf (Table 2). In a few cases, small clutches were analyzed at only one timepoint with only one set of molecular markers. To date, we have detected 42 interesting mutations in a total of 340 clutches examined. The phenotypes observed fall into several distinct categories.

Mutations eliminating *nkx2.5* expression

In four cases we detected embryos lacking *nkx2.5* expression (Table 2). In each, the mutant embryos appear morphologically normal, with a recognizable head, trunk, and tail, and express *gata-1* in an essentially normal pattern (Figure 3, compare A and B). No previously identified zebrafish cardiac mutants are known to lack *nkx2.5* expression, suggesting that these mutations define novel loci.

Additionally, three mutations were found that eliminate *gata-1* expression (Table 2); *nkx2.5* expression in these embryos is unaffected (Figure 3C). Mutations in several previously identified loci (e.g. *moonshine* (Ransom *et al.*, 1996), *spadetail* (Detrich *et al.*, 1995) and *cloche* (Stainier *et al.*, 1995)) similarly cause a severe reduction or elimination of *gata-1* expression. These mutants may therefore represent new alleles of these genes or other genes required for early *gata-1* expression.

Mutations affecting cardiac chamber formation

Analyzing the expression of MHC isoforms revealed 21 mutations affecting the A-P pattern of the definitive heart tube (Table 2). Twenty of these mutations affect the development of the ventricle. In these mutant embryos, only one cardiac chamber is visible (Figure 4A, B, and C). The S46 and MF20 antibodies both stain this chamber, identifying it as an atrium. In seven of 20 cases, this rounded S46⁺MF20⁺ (yellow) chamber appears completely isolated, with no anterior S46⁺MF20⁺ (red) tissue (Figure 4B). In the other 13, a small stalk of S46⁺MF20⁺ (red) tissue emerges from its anterior end (Figure 4C); it is unclear whether this S46⁺MF20⁺ tissue is a ventricular rudiment or part of the outflow tract. These two types of ventricle defects resemble the

phenotypes of the previously identified mutations *lonely atrium* (Chen *et al.*, 1996b) and *pandora* (Stainier *et al.*, 1996), respectively. Complementation testing will resolve whether these new mutations represent novel genes controlling differentiation of the anterior portion of the heart tube.

A single mutation affects the development of the posterior heart tube (Table 2, Figure 4D). In this case the sole visible chamber expresses only the MF20 antigen and not the S46 antigen, suggesting that it is a ventricle; the morphology of this chamber also resembles a normal ventricle. Although no S46 staining is visible in the mutant embryos, simultaneously stained siblings had normal S46 reactivity (data not shown). This phenotype is novel; no such defect in atrial differentiation has been described to date.

In addition to detecting mutations that alter the A-P patterning of the heart tube, screening with these antibodies also revealed mutations affecting other aspects of heart morphogenesis. We have identified four mutations that reduce the overall size of the heart, without eliminating either chamber (Table 2; Figure 4E). One previously identified mutation, *heart and soul* (Stainier *et al.*, 1996), produces a similarly small heart. We have also detected ten cases in which the embryos show bilateral heart tubes (cardia bifida) with proper A-P orientation (Table 2; Figure 4F). These ten mutations may be alleles of known zebrafish genes which cause this phenotype (see below) (Chen *et al.*, 1996b; Stainier *et al.*, 1996) (Table 1), or identify new genes involved in heart tube fusion.

Altogether, by screening with molecular markers that monitor cardiac induction and A-P patterning, we have identified many new mutations affecting these and other steps in heart development. While some of these mutations join members of previously described phenotypic classes, other mutations establish new classes and likely represent novel loci. These results demonstrate the potential for a small-scale focused screen to yield a large collection of relevant mutations.

Germline clone sizes provide evidence for genetic mosaicism

Since the F1 females examined were genetically mosaic, each mutation's representation within a clutch (the 'germline clone size') was unique. For instance, 26% (13 of 50) of the haploid embryos from F1 female #99 had the atrial defect shown in Figure 4D, while only 10% (7 of 72) of the progeny of F1 female #81 had small hearts (Figure 4E). The apparent germline clone size of the cardiac mutations ranged widely, from less than 5% to 53%; a similar range was reported by Riley and Grunwald (1995). Eight clutches in which more than one interesting phenotype was observed provide further evidence of mosaicism. For example, in addition to the 26% of embryos exhibiting an atrial defect, 8% (4 of 50) of the haploid embryos from F1 female #99 exhibited cardia bifida. Germline clone sizes were gen-

erally consistent when F1 females were squeezed a second time: for example, 28% (9 of 32) of the second clutch of haploid embryos from female #99 had the same atrial defect, compared to 26% initially; and 8% (2 of 24) of female #81's second clutch had small hearts, compared to 10% initially. Thus, although germline clone sizes vary for different mutations, they do not seem to vary greatly over time within the germline of an individual mosaic F1 female. In a few cases, we did observe substantial variations, typically when the total number of embryos inspected was small (i.e. less than 20).

We retained all F1 females from which at least 5% of the haploid embryos exhibited an interesting phenotype. Although somewhat arbitrary, we selected this threshold for two reasons. First, we did not want to be misled by the appearance of haploid-specific abnormalities that resemble interesting mutant phenotypes. Haploid embryos from wild-type females of similar genetic background to our F1 females exhibit cardiac defects at a low frequency; approximately 2% of these haploids have ventricle defects, while another 2% show cardia bifida (data not shown). Secondly, the number of F2 fish heterozygous for any particular mutation should be directly proportional to the number of haploid embryos displaying the mutant phenotype. If such a mutation constitutes less than 5% of an F1 female's germline, then at most one in 20 of the F2 generation will be heterozygous, making the recovery of such mutations rather tedious and somewhat unpredictable. As a result of this 5% cut-off, we have undoubtedly discarded relevant mutations.

Mutations identified in haploid embryos can be recovered in the F2 generation

Recovery of mutations from F1 screens is further complicated by the possibility that mutations detected in haploid embryos may act in a dominant fashion. In order to test this possibility, F1 females are crossed to wild-type males and the diploid progeny examined. Having confirmed that a mutation is indeed recessive, we identify heterozygotes by squeezing F2 females, looking for those from which approximately half of the haploid progeny exhibit the mutant phenotype. We then perform complementation testing in order to determine whether these new mutations represent alleles of previously identified genes. F2 female heterozygotes are further outcrossed to wild-type males to decrease the likelihood that a single F3 individual will carry multiple mutations. Intercrossing the resulting F3 generation allows the characterization of each mutation in diploid embryos.

As our screens are still in progress, recovery efforts are ongoing. Thus far, none of the mutations described here appears to act in a dominant fashion, although as expected we have observed some dominant syndromes (data not shown). For example, a distinct head defect appeared in approximately 4% of both the haploid and

diploid progeny of F1 female #477. Of the 42 mutations described in this paper, we have begun efforts to recover 28, and have already successfully recovered five: two that cause ventricle defects, the one causing an atrial defect, one affecting heart size (S. Horne, pers. comm.), and one causing cardia bifida (E. Kupperman and J. Reiter, pers. comm.). The germline clone sizes of these mutations varied from 12% to 27%, and in each case we have identified F2 heterozygotes at least as frequently as predicted. In cases with smaller germline clone sizes, recovery has proved more challenging; for instance, in the case of F1 female #17, where a ventricle defect appeared in 5% of the haploid progeny, none of 22 F2 females screened revealed a similar defect. Complementation testing of the recovered mutations is underway; this work has demonstrated that the recovered cardia bifida mutation represents a new allele of the *casanova* gene (J. Reiter, pers. comm.; Chen et al., 1996b).

DISCUSSION

Using molecular markers to examine the haploid progeny of mosaic F1 females we have identified numerous mutations that specifically disrupt cardiac induction and A-P patterning in zebrafish. Additionally, we have uncovered mutations that block fusion of the bilateral cardiac primordia, others that cause an abnormally small heart, and still others that prevent early expression of the hematopoietic gene *gata-1*. The use of mosaic F1 females has made our screen particularly efficient, enabling us to identify a total of 42 interesting mutations in 340 clutches examined. Our results further demonstrate that such mutations can be recovered in the F2 generation, permitting their future study. We conclude that screening the haploid progeny of mosaic F1 females with appropriate molecular markers is an efficient way to identify mutants with defined phenotypes.

For the majority of the mutations described above, the process of identifying F2 heterozygotes is underway. Because the F1 females are mosaic, it is difficult to estimate the total number of embryonic lethal mutations that we have examined; assuming a maximum of ten embryonic lethal mutations per F1 female (Riley and Grunwald, 1995), we have inspected at most 3400 mutations. The degree of genome saturation this number represents is not known, but this effort compares reasonably to previous zebrafish screens (Driever et al., 1996; Haffter et al., 1996) (Table 1). Complementation testing of our mutations will of course be most informative in this regard (see below).

Mutations affecting cardiac induction

The mutations lacking *nkx2.5* expression provide unique entry-points to the study of cardiac induction; no previously identified zebrafish heart mutations are known to eliminate *nkx2.5* expression. Because we examined *nkx2.5* expression at only a single time point

(i.e. the 8-somite stage), it is not yet clear whether these mutations entirely prevent, or perhaps only delay, *nkx2.5* expression. Molecular and morphological examination of mutants at later stages will be required to resolve this issue. In either case, the genes affected by these mutations could function in the production of heart-inducing signals, the reception of such signals by the future precardiac mesoderm, or the response of the mesoderm to these signals. Defects in cardiac induction could also result from improper dorsal-ventral patterning of the mesoderm (Jacobson and Sater, 1988), although the normal *gata-1* expression seen in these mutants suggests that their mesodermal patterning is largely intact.

Given that no mutation that completely eliminates the heart is known, it seems likely that no single gene plays a role in vertebrate heart development equivalent to that of *tinman* in *Drosophila* (Lyons et al., 1995). It is intriguing that in three of the four cases in which *nkx2.5* expression was affected, embryos with various abnormal heart phenotypes were also detected at 30 hpf (data not shown). This may indicate an important role for *nkx2.5* in zebrafish heart development, as previously suggested by the overexpression studies of Chen and Fishman (1996a). Whether these cases do in fact represent early and late manifestations of a single mutation, or instead the effects of two different mutations, remains to be determined.

Mutations affecting cardiac A-P patterning

The large number of mutations affecting cardiac A-P patterning provides a valuable resource for the analysis of this poorly understood process. Many distinct defects could cause the abnormal ventricle development observed in 20 of our mutants. These mutants may fail to specify anterior cell identities, due to the disruption of a critical signal, receptor, or differentiation gene. Alternatively, these mutations may cause a homeotic transformation of myocardial cell fate from anterior to posterior, or may interfere with the maintenance or maturation of cells initially specified as ventricular myocytes. A number of these possibilities are probably represented among the large group of mutations producing ventricle defects.

In light of the large number of mutations impacting the ventricle, it is noteworthy that we detected only a single mutation which affects atrial development. The possible deficiencies in this case resemble the scenarios described for the ventricle defects; a normal atrium may not form as the result of problems in atrial cell specification, maintenance, or maturation. The relative ease of disrupting ventricular development, in comparison to atrial development, is striking, and suggests that the process of cardiac A-P patterning may be primarily a matter of inducing anterior (ventricular) cell fates.

Mutations affecting heart size, heart tube fusion, and hematopoiesis

Although not the principal focus of our screens, the mutants displaying a small heart are a further measure of its success. Developing hearts may not achieve normal size for a number of reasons: initial induction of cardiac tissue may be faulty; growth of the cardiomyocytes may be stunted; aberrant cell death may eliminate myocyte precursors; or the morphogenetic processes that form the heart tube may go awry. Screening one clutch of embryos known to contain small heart mutants at 30 hpf revealed smaller than normal patches of *nkx2.5* expression at the 8-somite stage (data not shown), suggesting that in this particular case there may be a problem in early cardiac development. Further study will reveal which of the above scenarios apply in the other mutations causing small hearts.

Additionally, our efforts have added to the already substantial collection of cardia bifida mutants. The large number of mutations in this phenotypic class suggests that the requirements for successful fusion of the primitive myocardial tubes are complex. These requirements, both intrinsic and extrinsic to the myocardial cells, likely include adhesion, migration, and the temporal coordination of these processes. More detailed characterization will reveal whether these or other aspects of cell behavior are abnormal in any of the cardia bifida mutants.

We have also identified three mutants that at an early stage do not express the hematopoietic transcription factor gene *gata-1*. This lack of *gata-1* expression may result from a failure to induce blood progenitors or to specify ventral mesoderm properly. Alternatively, the *gata-1* deficiency in *cloche* mutants has been proposed to result from either a defect in a common hemangioblast progenitor of blood and endothelial tissues or from a failure by incompletely differentiated endothelial cells to induce hematopoietic development (Stainier *et al.*, 1995). A similar scenario may underlie the lack of *gata-1* expression in these new mutants; it will therefore be interesting to examine their endothelial cells as well.

Molecular, cellular, and genetic characterization of mutants

As a first step towards characterizing these new mutants, complementation testing will resolve which mutations represent alleles of previously known genes and which are novel. Such analysis has demonstrated that one of the cardia bifida mutations is in fact a new allele of the *casanova* gene (Chen *et al.*, 1996b), while the recovered small heart mutation does not represent an allele of *heart and soul* (S. Horne, pers. comm.). Additionally, determining the number of alleles identified per gene should provide an indication of the degree of saturation achieved in our screens. Examining both heart morphology and the expression of additional

markers of cardiac differentiation should demonstrate the spectrum of cardiac defects in each mutant. Furthermore, general inspection of embryonic morphology will indicate the degree of pleiotropy for each mutation.

This morphological and molecular characterization will facilitate epistasis analysis, allowing these genes to be ordered into developmental pathways by examining double-mutant combinations. Performing cell transplantations in order to test the cell-autonomy of each affected gene's action will also be critical, indicating which genes function intrinsically within the heart and which act extrinsically, i.e. in tissues that influence the heart. Ultimately, cloning of the affected genes may demonstrate the importance of known molecules in heart development or better yet will reveal novel genes and pathways.

A combination of screening strategies with general utility

Previous work by other groups established that the individual components of our approach facilitate genetic screens in zebrafish. The mosaic approach has been used to isolate alleles of previously known genes and also novel loci in diploid screens (Riley and Grunwald, 1995; Riley and Grunwald, 1996); haploid screens have identified several mutations, for example *no tail* (Halpern *et al.*, 1993; Fritz *et al.*, 1996); and marker-based screens have succeeded in identifying mutations affecting various aspects of neural crest and brain development (Henion *et al.*, 1996; Moens *et al.*, 1996). We have combined these strategies--creating a mosaic F1 generation, analyzing haploid embryos, and using molecular markers--to perform our screens. A similar strategy is also being employed in Eugene, Oregon to screen for mutations affecting mesodermal and brain patterning (C. Kimmel, pers. comm.). We believe this approach significantly enhances the efficiency of screening, albeit at the cost of increased difficulty in recovering mutations. However, our success in recovering several mutations suggests that it is feasible. In fact, we have successfully recovered two distinct mutations from the progeny of a single F1 female, highlighting the efficiency and reliability of our approach.

One concern with our approach is the possibility that detecting a severe phenotype in haploid embryos does not predict the severity of the phenotype in homozygous mutant diploids. It is notable that our two most commonly detected phenotypes, ventricle defects and cardia bifida, can be observed at a low frequency (2%) in wild-type haploid embryos. Haploid embryos may therefore represent a sensitized background, at least for certain cardiac phenotypes. On the other hand, the phenotypes of several previously identified cardiac mutants appear quite similar in haploids and diploids (W.-Y. Liao, J. Reiter, E. Kupperman, S. Horne, D. Y., J. A., and D.Y.R.S., unpublished data), indicating that haploid phenotypes can mimic diploid phenotypes. Whether the mutations we have identified will affect

haploids more severely than diploids remains to be determined.

In conclusion, the success of our screens provides evidence for the usefulness of a novel combination of approaches to screening for mutations in zebrafish. This strategy has enabled us to examine rapidly a large number of embryonic lethal mutations for their effects on cardiac induction and A-P patterning and has yielded numerous mutants exhibiting novel as well as previously observed phenotypes. Already, this collection of mutants exceeds the number of published mouse mutations with phenotypes relevant to cardiac induction and A-P patterning and will extend the unique potential of the zebrafish system to studying these processes. We expect that future screens utilizing mosaic F1 females and molecular criteria will aid studies of other aspects of heart development, and of vertebrate development more generally.

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REFERENCES

- Azpiazu N, and Frasch M (1993): *tinman* and *bagpipe*: two homeobox genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev* 7:1325-1340.
- Bader D, Masaki T, and Fischman DA (1982): Immunohistochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. *J Cell Biol* 95:763-770.
- Bisaha JG, and Bader D (1991): Identification and characterization of a ventricular-specific avian myosin heavy chain, VMHC1: expression in differentiating cardiac and skeletal muscle. *Dev Biol* 148:355-364.
- Bodmer R (1993): The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* 118:719-729.
- Chen J-N, and Fishman MC (1996a): Zebrafish *tinman* homolog demarcates the heart field and initiates myocardial differentiation. *Development* 122:3809-3816.
- Chen J-N, Haffter P, Odenthal J, Vogelsang E, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang Y-J, Kane DA, Kelsh RN, Mullins MC, and Nusslein-Volhard C (1996b): Mutations affecting the cardiovascular system and other internal organs in zebrafish. *Development* 123:293-302.
- DeHaan RL (1965): Morphogenesis of the vertebrate heart. In RL DeHaan and H Ursprung (eds.): "Organogenesis." New York: Holt, Rinehart and Winston, pp. 377-419.
- Detrich HW, Kieran MW, Chan FY, Barone LM, Yee K, Rundstadler JA, Pratt S, Ransom D, and Zon LI (1995): Intraembryonic hematopoietic cell migration during vertebrate development. *Proc Natl Acad Sci U S A* 92:10713-10717.
- Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier D, Zwartkruis F, Abdelilah S, Rangini Z, Belak J, and Boggs C (1996): A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* 123:37-46.
- Driever W, Stemple D, Schier A, and Solnica-Krezel L (1994): Zebrafish: genetic tools for studying vertebrate development. *Trends Genet* 10:152-159.
- Fishman MC, and Chien KR (1997): Fashioning the vertebrate heart: earliest embryonic decisions. *Development* 124:2099-2117.
- Fritz A, Rozowski M, Walker C, and Westerfield M (1996): Identification of selected gamma-ray induced deficiencies in zebrafish using multiplex polymerase chain reaction. *Genetics* 144:1735-1745.
- Gaiano N, Amsterdam A, Kawakami K, Allende M, Becker T, and Hopkins N (1996): Insertional mutagenesis and rapid cloning of essential genes in zebrafish. *Nature* 383:829-832.
- Gerson M, and Stainier D (1995): Culturing *Tetrahymena* as an alternative baby food to paramecia. *The Zebrafish Science Monitor* 3:12.
- Gonzalez-Sanchez A, and Bader D (1984): Immunohistochemical analysis of myosin heavy chains in the developing chicken heart. *Dev Biol* 103:151-158.
- Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, and Nusslein-Volhard C (1996): The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* 123:1-36.
- Halpern ME, Ho RK, Walker C, and Kimmel CB (1993): Induction of muscle pioneers and floor plate is distinguished by the zebrafish *no tail* mutation. *Cell* 75:99-111.
- Henion PD, Raible DW, Beattie CE, Stoesser KL, Weston JA, and Eisen JS (1996): Screen for mutations affecting development of zebrafish neural crest. *Dev Genet* 18:11-17.
- Horvitz HR (1988): Genetics of cell lineage. In WB Wood (ed.): "The Nematode *Caenorhabditis Elegans*." Plainview, NY: Cold Spring Harbor Laboratory, pp. 157-190.
- Jacobson AG, and Sater AK (1988): Features of embryonic induction. *Development* 104:341-359.
- Kimmel CB (1989): Genetics and early development of zebrafish. *Trends in Genetics* 5:283-288.
- Komuro I, and Izumo S (1993): *Csx*: A murine homeobox-containing gene specifically expressed in the developing heart. *Proc Natl Acad Sci USA* 90:8145-8149.
- Kubalak SW, Miller-Hance WC, O'Brien TX, Dyson E, and Chien KR (1994): Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis. *J Biol Chem* 269:16961-16970.
- Lee KH, Xu Q, and Breitbart RE (1996): A new *tinman*-related gene, *nkx2.7*, anticipates the expression of *nkx2.5* and *nkx2.3* in zebrafish heart and pharyngeal endoderm. *Dev Biol* 180:722-731.
- Liao W-Y, Bisgrove BW, Sawyer H, Hug B, Bell B, Peters K, Grunwald DJ, and Stainier D (1997): The zebrafish gene *cloche* acts upstream of a *flk-1* homologue to regulate endothelial cell differentiation. *Development* 124:381-389.
- Lints TJ, Parsons LM, Hartley L, Lyons I, and Harvey RP (1993): *Nkx-2.5*: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* 119:969.
- Lyons I, Parsons LM, Hartley L, Li R, Andrews JE, Robb L, and

- Harvey RP (1995): Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeo box gene *Nkx2-5*. *Genes Dev* 9:1654-1666.
- Moens CB, Yan YL, Appel B, Force AG, and Kimmel CB (1996): *valentino*: a zebrafish gene required for normal hindbrain segmentation. *Development* 122:3981-3990.
- Mullins MC, and Nusslein-Volhard C (1993): Mutational approaches to studying embryonic pattern formation in the zebrafish. *Curr Opin Genet Dev* 3:648-654.
- Nascone N, and Mercola M (1996): Endoderm and cardiogenesis: new insights. *Trends Cardiovasc Med* 6:211-216.
- O'Brien TX, Lee KJ, and Chien KR (1993): Positional specification of ventricular myosin light chain 2 expression in the primitive murine heart tube. *Proc Natl Acad Sci U S A* 90:5157-5161.
- Ransom DG, Haffter P, Odenthal J, Brownlie A, Vogelsang E, Kelsh RN, Brand M, van Eeden FJ, Furutani-Seiki M, Granato M, Hammerschmidt M, Heisenberg CP, Jiang YJ, Kane DA, Mullins MC, and Nusslein-Volhard C (1996): Characterization of zebrafish mutants with defects in embryonic hematopoiesis. *Development* 123:311-319.
- Riley BB, and Grunwald DJ (1995): Efficient induction of point mutations allowing recovery of specific locus mutations in zebrafish. *Proc Natl Acad Sci U S A* 92:5997-6001.
- Riley BB, and Grunwald DJ (1996): A mutation in zebrafish affecting a localized cellular function required for normal ear development. *Dev Biol* 179:427-435.
- Sater AK, and Jacobson AG (1990): The role of the dorsal lip in the induction of heart mesoderm in *Xenopus laevis*. *Development* 108:461-470.
- Schier, A. F., Neuhauss, S. C., Helde, K. A., Talbot, W. S., and Driever, W. (1997): The *one-eyed pinhead* gene functions in mesoderm and endoderm formation in zebrafish and interacts with *no tail*. *Development* 124: 327-342.
- Schultheiss TM, Burch JB, and Lassar AB (1997): A role for bone morphogenetic proteins in the induction of cardiac myogenesis. *Genes Dev* 11:451-462.
- Schultheiss TM, Xydas S, and Lassar AB (1995): Induction of avian cardiac myogenesis by anterior endoderm. *Development* 121:4203-4214.
- Solnica-Krezel L, Schier AF, and Driever W (1994): Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* 136:1401-1420.
- St. Johnston D, and Nusslein-Volhard C (1992): The origin of pattern and polarity in the *Drosophila* embryo. *Cell* 68:210-219.
- Stainier DYR, Fouquet B, Chen JN, Warren KS, Weinstein BM, Meiler SE, Mohideen MA, Neuhauss SC, Solnica-Krezel L, Schier AF, Zwartkuis F, Stemple DL, Malicki J, Driever W, and Fishman MC (1996): Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* 123: 285-292.
- Stainier DYR, Lee RK, and Fishman MC (1993): Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. *Development* 119:31-40.
- Stainier DYR, Weinstein BM, Detrich HW, Zon LI, and Fishman MC (1995): *cloche*, an early acting zebrafish gene, is required by both the endothelial and hematopoietic lineages. *Development* 121:3141-3150.
- Stainier DYR, and Fishman MC (1992): Patterning the zebrafish heart tube: acquisition of anteroposterior polarity. *Dev Biol* 153:91-101.
- Stainier DYR, and Fishman MC (1994): The zebrafish as a model system to study cardiovascular development. *Trends Cardiovasc Med* 4:207-212.
- Stainier DYR, and Gilbert W (1990): Pioneer neurons in the mouse trigeminal sensory system. *Proc Nat Acad Sci USA* 87:923-927.
- Tonissen KF, Drysdale TA, Lints TJ, Harvey RP, and Krieg PA (1994): *XNkx-2.5*, a *Xenopus* gene related to *Nkx-2.5* and *tinman*: evidence for a conserved role in cardiac development. *Dev Biol* 162:325-328.
- Walker C, and Streisinger G (1983): Induction of mutations by gamma-rays in pregonial germ cells of zebrafish embryos. *Genetics* 103:125-136.
- Westerfield M (1993): "The Zebrafish Book." Eugene, OR: Univ. of Oregon Press.
- Yutzey K, Gannon M, and Bader D (1995): Diversification of cardiomyogenic cell lineages *in vitro*. *Dev Biol* 170:531-541.
- Yutzey KE, Rhee JT, and Bader D (1994): Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. *Development* 120:871-883.