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High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs

(transgenics)

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ABSTRACT **With the goal of developing techniques for DNA insertional mutagenesis in zebrafish, we established procedures for rapidly obtaining and injecting large numbers of fertilized eggs. Using either of two plasmid constructs, we injected uncut DNA into fertilized eggs at the one- or two-cell stage. Fish hatched from injected eggs were raised to sexual maturity, and the frequency of transgenic founder fish was determined by pair-mating the fish and testing DNA extracted from pools of their 16-hr-old offspring by the polymerase chain reaction (PCR) and then Southern analysis. Eggs injected with one of two different plasmids yielded no transgenic fish, but 7–25% (19 of 115 overall) of the eggs injected with the other plasmid transmitted the injected sequences to their offspring (F_1). Of seven lines studied further, all were able to pass the foreign DNA sequences to the next (F_2) generation. Inheritance in the F_2 generation was Mendelian in the five lines tested. PCR and Southern analysis indicated that the plasmid sequences were present in multiple copies, probably tandemly arranged. Two founder fish carried more than one independent integration of the plasmid sequences. The line studied in more detail was a mosaic carrying two independently segregating copies of the transgene in one germ cell and a third copy in another germ-line precursor cell. The ability to obtain and inject large numbers of zebrafish eggs combined with a high frequency of germ-line integration may be steps toward the goal of being able to perform insertional mutagenesis with this organism.**

Following the pioneering work of Streisinger and his colleagues (1–4), a number of laboratories are working to develop the zebrafish as a vertebrate system for genetic analysis. The organism is particularly attractive for this purpose because females can produce a large number of eggs, early development is rapid (gastrulation is at 5 hr; somites form between 10–20 hr), the embryo is transparent throughout the early developmental processes, and the early stages of development can occur in haploid embryos (5–8). These attributes, combined with the fact that the generation time is only 60–70 days and that one can raise large numbers of fish in a relatively small space, suggest that it might be possible to conduct genetic screens of mutagenized populations of zebrafish and thus to identify novel genes that regulate developmental processes in vertebrates (6–9).

While conditions have been partially established for performing γ-ray or chemical mutagenesis with zebrafish (10–12), at present these approaches do not lend themselves to subsequent cloning of the mutated genes. For this reason it would be desirable to be able to mutagenize fish by DNA insertion, thus providing a hybridization tag for the mutated genes. Since work from Westerfield's laboratory (13, 14) has already shown that it is possible to obtain transgenic zebrafish by injecting plasmid DNA into fertilized fish eggs, we began by attempting to repeat and extend their studies. Here we report success in rapidly obtaining large numbers of eggs for injection and also report a high frequency of germ-line transmission of an injected plasmid DNA sequence.

MATERIALS AND METHODS

Fish Maintenance and Egg Collections. Fish were raised and maintained on a light/dark cycle essentially as described in ref. 6. Injection experiments 1, 2, 4, and 5 were begun at the Max Planck Institute in Tubingen, F.R.G. Founder fish identified in initial screens were taken to Cambridge, MA, where F_1 and F_2 fish were generated, raised, and tested. Experiment 3 was performed in Cambridge. In Tubingen fish were maintained on charcoal-filtered tap water; in Cambridge they were maintained on reverse osmosis-purified water to which Instant Ocean (Aquarium Systems, Mentor, OH) was added (50 mg/liter). All fish used were bred in the laboratory from stocks obtained originally from pet stores in Bombay and Tubingen.

Eggs for injection usually were obtained by placing about 10–25 6- to 12-month-old fish, in a female/male ratio of 2:1, in a 15-gallon (0.056 m^3) tank at least 1 day before eggs were needed. Mating occurred in the morning shortly after the lights came on. Eggs were usually collected by placing a nuptial chamber in the tank for 10 min. This device is a plastic box, approximately 7.6 × 15.2 cm, with a slightly shallower and snugly fitting inner box whose bottom has been removed and replaced with a stainless steel mesh to allow eggs to drop through and be caught in the outer box. On some days the fish did not lay any eggs; however, 85% of the time (recorded on 21 days during one 35-day period) about 100–1000 eggs were obtained in 10 min. The average was ≈450 eggs in 10 min.

Dechorionation by Pronase and Microinjection. Procedures were essentially as described by Stuart *et al.* (13, 14). Self-digested Pronase at 0.5 mg/ml was used to remove chorions. The reaction was stopped by removing as much of the Pronase as possible and then pouring the eggs into water and decanting three or four times with several hundred milliliters each time. DNA to be injected was mixed with fluorescein dextran (12%) and phenol red (2%) and injected at 50 μg/ml in 0.1 M KCl (experiments 1–3). In experiments 4 and 5, the fluorescein dextran–phenol red solution was diluted 1:20, and DNA concentration was 25–35 μg/ml, since this condition was found to result in greater transient gene expression from injected plasmids. For injection, dechorionated eggs were pipetted onto an agarose ramp formed by resting a wide glass slide in the lid of a 60-mm Petri dish containing about 15 ml of molten 1% agarose. Removing the slide left a ramp terminating in a groove. Eggs rolled down the ramp and lined up in the groove where the majority could be injected into the cytoplasm above the yolk at the one- or

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Abbreviations: RSV, Rous sarcoma virus; LTR, long terminal repeat; SV40, simian virus 40.

two-cell stage. Injections were performed under a dissecting microscope.

Detecting Transgenic Fish by the Polymerase Chain Reaction (PCR) and Southern Blotting. DNA was extracted from pools of 50 to several hundred embryos (obtained from mating a single pair of fish) at 16 hr of development by lysing in 10 mM Tris, pH 8.0/10 mM EDTA/100 mM NaCl/0.4% SDS/0.5 mg of Pronase per ml (TE buffer) at 42°C for 12 hr. After extraction with phenol and precipitation with ethanol, the DNA was resuspended in TE buffer. Approximately 1 μg of DNA was used in a PCR reaction modified from that of Zimmer and Gruss (15): 67 mM Tris (pH 8.8) containing 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 170 μg of bovine serum albumin, 2 mM $MgCl_2$, 0.2 μM each PCR primer, 800 μM each dNTP, and 2.5 units of *Taq* DNA polymerase (Pharmacia). The reaction was carried out at 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min for 35 cycles with a 1-min initial 94°C denaturation step, and a 10-min final 72°C elongation step. The primers within the *lacZ* gene used in the initial screening (PC25 and PC26 in Fig. 1) were kindly provided by David Stott (who originally described them as BG2C and BG3C). These primers generated a PCR product of 196 base pairs (bp). The second pair of primers (PC28 and PC26 in Fig. 1), with the 5′ primer in the Rous sarcoma virus (RSV) long terminal repeat (LTR), generated a product of ≈1.2 kb. For Southern analysis, ≈10 μg of DNA was digested with the appropriate restriction enzymes (New England Biolabs) for 16 hr and was loaded on a 0.7% agarose gel, electrophoresed, and transferred to nitrocellulose (Schleicher & Schuell) according to standard procedures (16). Hybridizations were carried out at 42°C in a 50% formamide solution for 16 hr in the presence of 1×10^6 cpm/ml of probe labeled with [^{32}P]dATP by the random priming method (17). The probes were derived from the *lacZ* region or the RSV LTR region of pRSV-βGal. The former was generated by using the primers PC17 and PC18 (Fig. 1). The blots were washed in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 0.05% $Na_4P_2O_7$ and 0.1% *N*-lauroylsarcosine at 37°C for 1 hr followed by higher stringency washes in 0.1 × SSC at 42°C for 1 hr and at 55°C for 30 min. Exposure to film was carried out at −80°C with enhancing screen for 24–72 hours.

RESULTS

DNA Injections and Survival Rates of Injected Eggs. We wished to inject the cytoplasm of fertilized eggs at the one-cell stage. This stage lasts about 40 min at 28°C. We obtained large groups of almost synchronously fertilized eggs by natural matings as described. In some experiments, some of the eggs were placed at 20.5°C for 20–30 min to delay the time to first cleavage. By using these methods, one person was able to inject up to about 300 eggs in about 1.5 hr while the majority were still at the 1-cell stage.

Dechorionated eggs were injected with either of two plasmids. One, pCH110 (commercially available from Pharmacia), contains the *lacZ* gene under the control of the simian virus 40 (SV40) enhancer–promoter; the other, pRSV-βGal (18), contains the *lacZ* gene under the control of the RSV LTR. To ensure that only successfully injected eggs were raised, DNA was mixed with fluorescein–dextran and the following morning injected embryos were screened microscopically. Only fluorescent embryos were raised as potential transgenics.

The percentage of eggs surviving to sexual maturity varied greatly. Losses were due to incomplete fertilization, rupture of fragile dechorionated eggs, damage caused by microinjection, or loss of embryos during raising. Average survivals for these steps, recorded in six or more experiments, were: successful fertilization, 81%, surviving dechorionation, 65%, successful injection, about 50%, surviving to sexual maturity, about 85%. These rates lead to a calculated average overall survival of 23%.

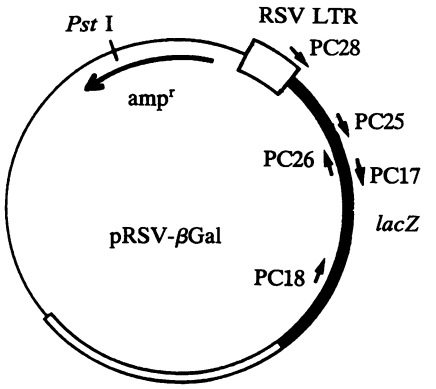

FIG. 1. Map of pRSV-βGal (18). The *lacZ* gene is shown as a solid thick line, and the RSV LTR, as a large open box. The relative locations of PCR primers used to identify transgenic fish and to generate probes for Southern hybridization are indicated. amp, Ampicillin resistance gene.

High-Frequency Germ-Line Transmission of Injected Plasmid DNA Sequences. Injected eggs were raised to sexual maturity and then pair-mated to each other or to uninjected fish. Eggs from each mating were harvested and incubated at 28°C for about 16 hr, and then DNA was prepared from the embryos. Plasmid sequences were detected by PCR with two primers located within the *lacZ* gene (PC25 and PC26 in Fig. 1). The assay was sensitive enough to detect a single copy of the plasmid DNA if it were present in 1 egg of 20. There were no germ-line transgenic fish among those injected with pCH110 (Table 1). However, transgenics were identified among fish injected with the pRSV-βGal construct. To confirm the initial results, all DNA samples were retested by PCR with different 5′ primers: a primer designated PC28, which initiates in the RSV LTR (see Fig. 1), was used to test samples from fish injected with pRSV-βGal; and one that initiates in the SV40 enhancer–promoter was used for fish injected with pCH110. The results of one such PCR test are shown in Fig. 2. In the cases where embryos were positive for the RSV-βGal sequences and both parental fish had been injected, each fish was then remated to an uninjected control fish and their offspring pool was tested to determine which fish of the original pair (or both) was transgenic. Eggs from additional matings were also tested to confirm consistent germ-line transmission of the injected plasmid DNA sequences in putative transgenics: 15 of 15 tested remained

Table 1. Frequency of transgenic founder zebrafish

Experiment	DNA injected	Injected fish tested, no.	Positive (founders)*	
			No.	%
1	RSV-*lacZ*	32	6	19
2	RSV-*lacZ*	43	3	7
3	RSV-*lacZ*	40	10	25
4	SV40-*lacZ*	54	0	0
5†	SV40-*lacZ*	19	0	0

In some experiments, fish hatched from eggs injected on different days were pooled as young adults. Fish in experiments were derived from two independent injections (experiment 1), one injection (experiment 2), four injections (experiment 3), four injections (experiment 4), and one injection (experiment 5).

*Number that transmit plasmid sequences to their offspring, detected first by PCR analysis and confirmed for most fish by Southern analysis (see text).

†Linearized plasmid was used in this experiment. All others were uncut.

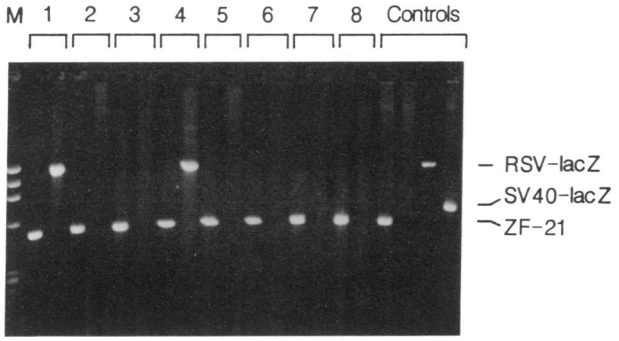

FIG. 2. Identification of germ-line transgenic fish by PCR. Genomic DNA was extracted from a pool of embryos from a single mating and was used in two PCR reactions that were run in adjacent lanes on a 2% agarose gel: samples 1–4 test genomic DNAs from embryo pools of four fish injected with pRSV-βGal, and samples 5–8 test pools from four pCH110-injected animals. The first lane in each pair is a positive control reaction containing primers from the zebrafish homeobox gene, *ZF-21* (19), generating a 500-bp product; the second contains primers specific to the injected plasmids. The control lanes contain DNA from uninjected fish. The first control lane contains *ZF21* primers, and the second, third, and fourth lanes contain both sets of primers directed to the injected plasmids. The third and fourth control lanes also contain 100 fg of the appropriate plasmid. Primers for fish injected with pRSV-βGal generate a 1.2-kb product (third control lane), and the primers directed to pCH110 generate a 0.6-kb product (fourth control lane). Samples 1 and 4 demonstrate PCR amplification of the RSV-lacZ sequences and consequent germ-line transmission in these fish. Lane M: *Hae* III-digested OX174 size markers.

positive. Finally, DNA from pooled (F_1) progeny of PCR-positive transgenics was analyzed by Southern blotting, first using an enzyme (*Pst* I) that cuts once in the plasmid sequence: 16 of 16 analyzed by this method were positive (Fig. 3). These studies showed that 7–25% of the injected eggs in different experiments gave rise to fish that transmitted the injected sequences to their offspring (Table 1).

State of the DNA in Transgenic Fish. Consistent with the results of Stuart *et al.* (13), all of the founder (F_0) fish we tested (11 of 11) were found to be mosaic. The percentage of their F_1 progeny that received the transgene ranged from 6% to 24% for different founders (Table 2). A deviance from 50% transmission could indicate that the foreign DNA was not integrated in the genome of the founder or, alternatively, it could be due to mosaicism in the germ line of the F_0 fish. The latter possibility is supported by the result that in the five lines tested, although <50% of F_1 fish inherited the foreign DNA sequences, F_1 fish that did inherit the injected plasmid sequence transmitted it to ≈50% of their (F_2) offspring, the result expected if the DNA were integrated (Table 2). Further evidence for mosaicism in the F_0 fish is obtained from Southern analysis. Bands representing plasmid sequences are less intense in DNA from pools of embryos obtained from a founder fish compared with pools obtained from its F_1 or F_2 generations (not shown).

Table 2. Mosaicism of founder fish and Mendelian inheritance of plasmid sequences in the F_2 generation

F_0 line	F_1 positive fish*			F_2 positive fish†	
	Line‡	No.	%	No.	%
F2		12/50	24	20/43	46
F9		12/81	15		
F17		7/126	6	23/49	47
M14		14/59	24	19/38	50
M9		33/116	34		
	M9 2/4	7	6§	23/46	50
	M9 1	10	15§	25/46	54
	M9 1/3	7			
	M9 3	8	13§		
	ND	1			
		33	34		

*Two- to three-month-old F_1 fish were pair-mated, and DNA from pools of their eggs was analyzed by PCR and, in the case of M9, by Southern blotting to identify fish transmitting pRSV-βGal sequences.

†DNA from individual two- to seven-week-old F_2 fish was analyzed by PCR for the presence of pRSV-βGal sequences.

‡The 33 M9 F_1 transgenic fish inherit band 1, band 3, bands 1 and 3, or bands 2 and 4 of Fig. 4*B*. ND, not determined.

§Percent of 116 total M9 F_1 fish.

From the intensity of the PCR products (Fig. 2), it appeared that transgenic fish frequently harbored the foreign DNA sequences in multiple copies. Southern analysis was consistent with this result and also suggested that the copies are often in tandem arrays: digestion of the DNA of many of the transgenic fish with an enzyme that cuts once in the plasmid sequence yielded a unit length [13.5 kilobases (kb)] band (Fig. 3). Southern analysis also revealed, however, that some fish did not have unit length bands after digestion with *Pst* I. For example, using the *lacZ* region probe, all fish of the M14 line (Fig. 3, lane 4) have four intensely hybridizing bands and several fainter bands, none of which is of unit length. The intensity of the darker bands in this and other such lines indicates that multiple copies of plasmid sequences are present and thus the unit length bands are not likely to be junction fragments. It seems likely that in these lines amplification occurred subsequent to rearrangements of the plasmid sequences.

Interestingly, two founder fish had multiple integration events. One such fish was studied further. Fig. 4 *A* and *B* compares the Southern analysis of DNA from an offspring pool of M9, the founder fish, to that of offspring pools of individual F_1 fish. DNA from the pool of embryos of the founder fish contains four bands that hybridize to a probe derived from the *lacZ* region of the injected pRSV-βGal

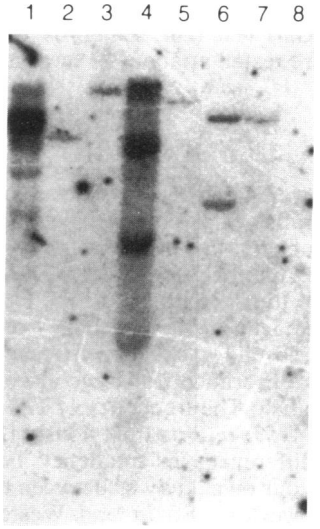

FIG. 3. Confirmation of transgenic fish by Southern analysis. *Pst* I digests of genomic DNA were subjected to Southern analysis by hybridizing with a probe from the *lacZ* gene (generated by using primers PC17 and PC18 in Fig. 1). Comparisons of hybridizing band patterns between the offspring pools of F_1 transgenic fish (lanes 1–6) and 30 pg of the pRSV-βGal plasmid (lane 7), equivalent to ≈1.34 copies per diploid genome. Genomic zebrafish DNA in lanes: 1, F2-45; 2, F9-45; 3, F17-114; 4, M14-5; 5, M9-51; 6, M9-37; 8, offspring pool from a nontransgenic F_1 fish derived from transgenic fish F17.

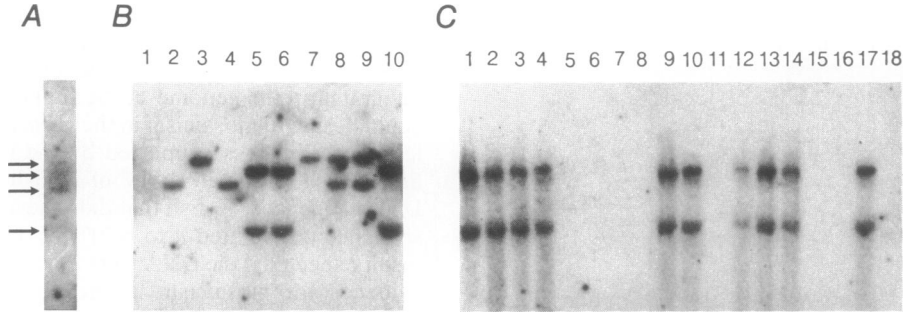

FIG. 4. Identification of a transgenic fish carrying multiple insertions and Mendelian inheritance in the F_2 generation. Southern analysis was performed as in Fig. 3. (*A*) Plasmid sequences transmitted by founder fish M9. (*B*) Sequences inherited by individual F_1 fish derived from M9. (*C*) Sequences present in 18 randomly selected F_2 fish derived from an F_1 fish of the type shown in lanes 5, 6, and 10 of *B*. For *A* and *B*, DNA was derived from offspring pools of M9 or individual PCR-positive F_1 fish, respectively; while in *C*, DNA was derived from individual 7-week-old fish. Lanes in *B*: 1, negative sibling of F1 transgenics; 2–10, F_1 transgenic fish that inherit band 1, band 3, bands 1 and 3, or bands 2 and 4.

plasmid (Fig. 4*A*). In contrast, when individual F_1 fish were raised and mated and DNA from pools of their progeny was analyzed, it is apparent that individual F_1 fish transmit only one or two of the four bands that were transmitted by the founder (Fig. 4*B*). One pair of bands that represents a single integration event, or conceivably two closely linked integrations, (designated 2/4 and represented by the two bands, 2 and 4, in Southern blots of *Pst* I-digested DNA) is never seen in the same F_1 fish as two other integrations (represented by bands 1 and 3), suggesting that the 2/4 integration(s) occurred in a different germ-line precursor cell than the integrations represented by bands 1 and 3. The latter occurred in the same germ-line precursor but are shown to segregate independently to the F_1 generation, indicating no genetic linkage between these two integration events (Fig. 4*B*). As shown in Fig. 4*C* and Table 2, Southern analysis of DNA prepared from individual F_2 progeny of an F_1 fish that inherited bands 2/4 shows Mendelian inheritance of the integration event represented by these two bands.

Because the injected DNA sequences carried the *lacZ* gene and we had shown that this plasmid could direct the expression of β-galactosidase in zebrafish embryos after injection, we tested offspring from transgenic fish for expression of β-galactosidase by the 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) test (20). Embryos from 5 of the 18 founder fish were tested at about 24 hr of age, and all were negative.

DISCUSSION

In these studies we established procedures for obtaining large numbers of zebrafish eggs for microinjection and obtained a high frequency of germ-line transmission of injected DNA. It seems likely that the inherited plasmid DNA sequences are integrated, since they are passed to the F_2 generation with Mendelian ratios. The overall frequency of transgenics obtained with one plasmid was 17% with a rate ranging from 7% to 25% in three experiments. A second construct failed to yield transgenic fish. The frequency of 17% is several times higher than the 4–5% reported previously (14). The reasons for the large differences in frequency between different plasmids within our own study or between these studies and the pioneering work of Stuart and Westerfield and their colleagues remain to be determined; however, a plausible explanation for the difference in our results and those of Stuart *et al.* (13, 14) may be the assay used to detect germ-line transmission.

In the majority of their studies Stuart *et al.* (ref. 14 and M. Westerfield, personal communication) injected a plasmid containing a chloramphenicol acetyltransferase (CAT) gene and determined the frequency of transgenic fish by testing progeny of potential founder fish for CAT gene expression. In contrast, we assayed germ-line transmission by the presence of plasmid DNA sequences in F_1 fish. Although the construct we injected carries a functional *lacZ* gene under the control of an RSV LTR and expresses β-galactosidase in freshly injected embryos, we failed to obtain *lacZ* expression in the F_1 of 5 (of 19) transgenic fish tested so far. Thus, had we determined the frequency of transgenic fish by gene expression, we would have seriously underestimated the frequency, perhaps even failed to detect transgenics at all. It remains to be determined whether the failure of the *lacZ* gene to be expressed in the progeny of our transgenic founder fish is due to rearrangements in the plasmid DNA that rendered the *lacZ* gene or the RSV enhancer–promoter nonfunctional or due to repression after passage through the germ line. Our preliminary Southern analysis has already shown that rearrangement of injected sequences is common, but more extensive analysis would be needed to determine if rearrangements explain the lack of expression. Alternatively, it is possible that expression from the RSV enhancer–promoter or possibly expression of the *lacZ* structural gene itself is repressed after passage through the fish germ line. By analogy, it seems possible that CAT gene expression might have been suppressed in the studies of Stuart *et al.* (14), leading to an apparently lower frequency of germ-line transmission of injected DNA sequences.

As for the difference in frequency of transgenic fish obtained with the two plasmids injected in our own studies, several explanations can be considered. There may be differences in the frequency with which different DNA sequences integrate into fish DNA. Alternatively, differences in the conditions of injection, which varied in the five experiments reported here, may be important. These include the use of low temperature to delay first cleavage in some groups of eggs, the concentration of DNA, and the concentration of fluorescein dextran and phenol red in the injection solution. It is also possible that the DNA preparations differed in some way. Systematic studies to determine the effect of these conditions on the frequency of transgenic fish will be needed to resolve this issue.

Like Stuart *et al.* (13, 14), we found that injected plasmid DNA sequences were inherited in multiple copies. In addition, founder fish were mosaic in all (11 of 11) cases studied, indicating that injected DNA does not usually integrate immediately. In mice, transgenes are frequently inherited as tandem arrays and mosaicism is not uncommon (21–29). A novel finding was the two fish in which multiple integrations had occurred, where the one fish studied further contained integrations in two different germ-line precursor cells. We detected multiple integrations in two of the seven fish in which analysis was extensive enough to have seen them.

Would the frequency of germ-line transmission we observed be high enough to allow us to generate mutations by

injecting DNA into zebrafish eggs? Although it will not be possible to answer this question until the number of integrations needed to generate a mutation is known, if one assumes that this frequency is the same in fish as in mice (one mutation in 20 integrations; refs. 21 and 29), then we estimate that in theory one or two people could generate 400–500 mutant fish per year. In practice, however, it would be difficult if not impossible to achieve this rate without further technical advances.

It would be helpful if the mutagenizing DNA carried a reporter gene that was expressed in the offspring of the founder fish. This would facilitate screening their haploid F_1, since one could be certain of having examined embryos with putative insertions. It also would be extremely helpful if reporter gene expression could be detected in living embryos, both to aid in the process of screening and to limit the raising of fish to the F_1 fish that inherited the injected DNA. A reporter gene that conferred a visible phenotype (for example, pigmentation) or a selectable phenotype or one that produced a product that could be detected with a live stain would make the detection of transgenic animals rapid enough. The use of promoter trap constructs so that only fish with mutagenizing integrations would be raised could also help to make the approach feasible (20, 30).

A further issue is whether the insertion of injected DNA causes major disruptions at the site of integration in zebrafish DNA, as has been found to occur in mice (24, 29). Such disruptions can make it difficult to identify and to clone the gene that has been mutated by the insertion. For this reason, it would be highly desirable if an integration system could be introduced into the zebrafish egg, causing the injected DNA to integrate in a reliable configuration. A number of well-studied integration systems might ultimately prove useful for this purpose, including retroviral and *Ty* core particles, *P* elements, various plant transposable elements, and Tn*10*.

Note Added in Proof. Carl Fulwiler, Harvard University, also has observed a high frequency of germ-line transmission of DNA sequences injected into fertilized zebrafish eggs (personal communication).

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