Gene Transfer into Zebrafish by Sperm Nuclear Transplantation

Suresh Jesuthasan¹ and Sivan Subburaju
Fish Developmental Genetics Laboratory, IMA, 1 Research Link, NUS, Singapore 117604

A technique for fertilizing zebrafish eggs by injection of sperm nuclei is described. Eggs that cleave normally can develop into swimming larvae and give rise to fertile adults. If sperm nuclei are preincubated for 20 min with DNA encoding the green fluorescent protein, transgene expression can be detected in all cells of the embryo. The use of condensed sperm nuclei allows injection with a small bore pipette, which is critical for successful injection of the relatively small zebrafish egg. This technique enables the generation of ubiquitously expressing transgenic zebrafish directly by microinjection. Hence, experiments involving transgenic fish can be completed in days, without the need for growing and breeding founders. This technique may also be used to generate transgenic lines, as transgene expression was visible in the offspring of transgenic founders. The method described here is likely to be applicable to other teleosts, such as medaka and salmon. © 2002 Elsevier Science (USA)

Key Words: transgenesis; sperm; zebrafish; GFP; HuC; histone2B.

INTRODUCTION

The zebrafish has become a widely used organism for studies on vertebrate development as well as a model system for disease (Dooley and Zon, 2000), primarily because of the possibility of obtaining mutations affecting many processes (Haffer et al., 1996). The transparency of the embryo, combined with the accessibility to physical and molecular manipulation, has attracted its use in diverse fields such as heart development (Stainier, 2001), hemato poiesis (Amatruda and Zon, 1999), axon guidance (Beattie, 2000), and visual perception (Baier, 2000). The creation of transgenic zebrafish has further extended the usefulness of this organism. Specific subpopulations of cells in the embryo can be labeled with GFP by using tissue-specific promoters (Goldman et al., 2001; Higashijima et al., 2000; Huang et al., 2001; Ju et al., 1999; Long et al., 1997; Motoike et al., 2000; Park et al., 2000b), and these fish can then be used for detailed developmental analysis of particular cell types and for genetic screens.

Foreign genes can be introduced into the zebrafish germ line by using a variety of methods, such as injection of plasmid DNA into the early embryo (Stuart et al., 1988), transposon-mediated gene insertion (Raz et al., 1998), or retrovirus-mediated gene transfer (Linney et al., 1999). These methods, however, all have the drawback of giving relatively low integration and transmission rates. Integration usually occurs after several rounds of cell division so that expression is mosaic, and only in a small proportion of embryos does integration occur in the germ line (Amsterdam et al., 1995; Bayer and Campos-Ortega, 1992; Lin et al., 1994). Four to eight months are required before fish with ubiquitous transgene expression can be obtained, as injected fish have to be grown to adulthood and screened for germline transmission of the transgene, following which transgenic offspring are raised to adulthood. Experiments involving transgenic fish are thus relatively time consuming.

One approach that circumvents the problem of mosaic expression of foreign DNA is fertilization of eggs by sperm that carry a transgene. Several attempts have been made to modify zebrafish sperm, by coincubation (Khoo et al., 1992) or electroporation (Patil and Khoo, 1996) prior to in vitro fertilization, but none of these has given rise to fish expressing the transgene. In amphibians, a method that has worked successfully is injection of sperm nuclei carrying the transgene. In Xenopus laevis (Kroll and Amaya, 1996) and Xenopus tropicalis (Offield et al., 2000), transgenes are integrated into sperm nuclei which have been decondensed by incubation in egg extract, followed by restriction enzyme-mediated integration (REMI). Large numbers of

¹To whom correspondence should be addressed. Fax: 65-8727007. E-mail: suresh@ima.org.sg.
nonmosaic transgene-expressing individuals can be produced in this way, enabling experiments requiring transgenic animals to be performed without the establishment of stable transgenic lines. Perry et al. (1999) recently reported that transgenic mice can also be produced by intracytoplasmic sperm injection (ICSI), although they did not decondense the nuclei prior to injection.

The zebrafish poses several technical difficulties for the injection of sperm nuclei: the egg is surrounded by a chorion, and the sperm nucleus needs to be placed near the animal pole, where the female pronucleus is localized. Injections thus cannot be as rapid as with Xenopus eggs, which are easier to penetrate and have a more obvious polarity. Also, smaller needle bores are required, as the zebrafish egg is considerably smaller than that of X. laevis. In this paper, a method for fertilizing zebrafish by sperm nuclei injection is described. By preincubating sperm nuclei with DNA, fish with widespread transgene expression can be produced.

**MATERIALS AND METHODS**

**Sperm Nuclei Preparation**

Testes were dissected from four adult zebrafish (Danio rerio) males which had been killed by immersion in iced water. Testes, which are located on either side of the swim bladder, were removed with fine forceps. Demembranated sperm nuclei were prepared essentially as described by Kroll and Amaya (1996), incorporating the modifications of the Browder lab (http://www.ucalgary.ca/UofC/edweb/virtualembryo/frogs2.html), e.g., omission of protease inhibitors. Either lysolecithin (Sigma L4129) or digitonin (Sigma D5628) was used for demembranation. To check the concentration after washing, nuclei were labeled with Hoechst or Syto11 (Molecular Probes) and counted on a hemacytometer. Aliquots of 10 μl, at a concentration of approximately 100 nuclei/ml, were quick frozen in liquid nitrogen and stored at −80°C. An alternative procedure, where nuclei were demembranated by freeze-thawing (Wakayama et al., 1998), was also tested: nuclei were washed twice in 9 ml nuclear isolation medium (NIM) (Kuretaka et al., 1996) with 5% BSA, once in 1 ml, and finally resuspended in 250 μl before being aliquotted and quick frozen without cryoprotection.

**Transgenesis Mixture**

Plasmid DNA was linearized with a suitable restriction enzyme [NotI for pESG, XbaI for pBOS-H2BGFP (Pharmingen), and EcoRI for ΔEcoHuc-GFP (Park et al., 2000b)], purified with Qiaquick columns (Qiagen), and diluted in sterile water to a concentration of 70 ng/μl. Sperm aliquots were thawed on ice and then mixed by pipetting up and down with a cutting tip (Axygen 10-μl tip). Five microliters of sperm suspension were transferred to a 1.5-ml Eppendorf tube, and 1 μl linearized DNA was added. For experiments with higher amounts of DNA, the stock concentration was increased while the volume added remained at 1 μl. This combination was mixed by pipetting, kept at room temperature (24°C) for 1, 5, or 20 min, then diluted with sperm dilution buffer (SDB): 250 mM sucrose, 75 mM KCl, 0.5 mM spermidine trihydrochloride, 0.2 mM spermine tetrahydrochloride, pH 7.4 (Kroll and Amaya, 1996) or MOH buffer (10 mM KPO4, pH 7.2, 125 mM potassium gluconate, 5 mM NaCl, 0.5 mM MgCl2, 250 mM sucrose, 0.25 mM spermidine trihydrochloride, 0.125 mM spermine tetrahydrochloride) (Offield et al., 2000) to give a final volume of 500 μl. The diluted mixture was kept on ice.

**Injection of Sperm Nuclei**

Female zebrafish were anesthetized with tricaine (Sigma A5040), placed on a clean piece of paraffilm in a petri dish, and gently squeezed to expel mature eggs. Eggs were kept in a mound, and the dish was immediately covered to prevent dehydration. A Pasteur pipette was used to transfer eggs (approximately 50 at a time) to the injection chamber. This consists of V-shaped troughs formed in 1.2% agarose in Hanks’, filled with Hanks’ saline containing 0.5% BSA, to delay activation (Sakai et al., 1997). The troughs were filled so that the eggs were just immersed—there was less than 1 mm distance between the top of the eggs and the bottom of the meniscus. This ensured efficient withdrawal of the injection needle from eggs, as eggs were held back by surface tension of the saline.

Injection needles were made by pulling thin-walled capillaries (Clarks GC100T) on a Flaming-Brown puller (Sutter P87 with box filament; heat = ramp value = 5, pull = 75, velocity = 25, time = 50), and breaking the tips with forceps so that the outer diameter was 10–15 μm; note that sperm nuclei have a diameter of approximately 5 μm. The needles were mounted on a holder, attached to a WPI mechanical manipulator (M3301), and filled from the tip using a Narishige IM 300 microinjector. Filling was monitored with a dissecting microscope (Leica MZ12 with transmitted light base), as sperm nuclei are visible with darkfield illumination. For back-filling, the mixture was drawn into tygon tubing (AAC00001) attached to a yellow tip (Kroll and Amaya, 1996) by using a 20-μl pipet. The tubing was then attached to the capillary and the sperm suspension extruded into the capillary and forced to the tip, using a 200-μl pipet.

Injections were carried out with the Narishige injector by using a pressure of 3–5 psi and time of 100 ms. Sperm nuclei were injected into the animal pole region of the egg. Eggs were penetrated about 50–100 μm from the micropyle, which is visible under brightfield illumination, then rotated so that the tip was near the microcapillare prior to injection. After a batch of eggs were injected, they were transferred with a Pasteur pipette to a 9-cm petri dish with 20 ml E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) and then placed in a 28°C incubator. After 5 h, nonleaving embryos and those obviously retarded in epiboly were removed.

**Microscopy**

Brightfield imaging of embryos was carried out on a Leica DM LFS microscope using a 10× objective, with a Princeton Instruments Micromax camera driven by IPLab software (Scanalytics). Fluorescence microscopy was carried out on a Zeiss Axioskop with a BioRad 1024 laser scanning confocal microscope, or on a Zeiss Axiosvert with a Zeiss LSM 510 confocal microscope, with a 10× dry or 25× immersion objective. A 488-nm laser was used to excite GFP. Hoechst was imaged with a multiphoton microscope (Zeiss), using a 760-nm laser line.
Injection of Plasmid DNA

Linearized DNA was injected at a concentration of 70 ng/μl into the animal pole region of embryos at the one-cell stage, using a gas pressure injector. Approximately 1 nl was injected per embryo.

Fixation and Nuclear Labeling

Embryos were fixed in 2% formaldehyde in PBS for 15 min at room temperature, then rinsed in PBS and stored at 4°C. This light fixation prevents zebrafish cells from autofluorescing. Nuclei were labeled by incubating the embryos for 5 min in 1 μg/ml Hoechst.

### RESULTS

#### Fertilization of Zebrafish Eggs by Sperm Nuclear Transplantation

Zebrafish sperm nuclei were demembranated by using lysolecithin or digitonin, or by freeze-thawing in NIM with BSA. In all cases, damage to the plasma membrane was suggested by two observations. First, no tails could be seen when treated sperm were examined on a compound microscope. Second, only a minority of sperm nuclei were labeled when incubated in 1 μg/ml Hoechst prior to freeze-thawing, lysolecithin treatment, or digitonin treatment, whereas after treatment, all nuclei were fluorescent (Fig. 1).

To establish the conditions for injecting sperm, nuclei were first diluted 1:100 in sperm dilution buffer and back-filled into capillaries. A series of injections was made into the injection chamber containing Hanks’ saline, and the injection volume was adjusted such that each pulse gave, on average, one nucleus, as detected under darkfield optics. Surprisingly, no nuclei could be injected after approximately 15 min. By observing a capillary loaded with Syto-11-labeled nuclei under a fluorescence microscope, it was found that nuclei settled and became attached to the surface of the capillary over this period. Even when capillaries were coated with Sigmacote or gelatin, nuclei flow

### TABLE 1

<table>
<thead>
<tr>
<th>No. injected</th>
<th>No. cleaving</th>
<th>Normal cleavage</th>
<th>Normal at 24 h</th>
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<tbody>
<tr>
<td>110°</td>
<td>28</td>
<td>3</td>
<td>3</td>
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<tr>
<td>130°</td>
<td>50</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>61°</td>
<td>39</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>90°</td>
<td>63</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
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Note. Results of four different injections, using nuclei that were lysolecithin-treated, digitonin-treated, or freeze-thawed. Abnormally cleaving embryos have three or more blastomeres after first division, indicating polyspermy.

### FIG. 1

Sperm nuclei before (A, B) and after (C, D) demembranation. (A, C) Brightfield images, showing sperm heads on a hemacytometer. (B, D) Fluorescence images, showing Hoechst labeling of nuclei. Only a few nuclei are labeled prior to demembranation, whereas all nuclei are labeled after treatment with lysolecithin. Scale bar, 50 μm.

### FIG. 2

Injection method. (A) A schematic diagram of the injection set-up. (B) An egg with an injection needle near the micropyle (arrowhead).
eventually stopped. To circumvent this problem, two different approaches were tested. First, nuclei were suspended in buffer with 6% polyvinylpyrrolidone (MW 360000; Sigma 5288) and back-filled into capillaries. The increased viscosity of this medium prevented nuclei from settling, and a reliable rate of nuclear injection could be obtained for over 1 h. Second, nuclei were filled at regular intervals from the tip, under a dissecting microscope. The second method was found to be simpler and more efficient, as clumps of nuclei which blocked the needle could be avoided.

Sperm nuclei were then injected into the animal pole region of eggs. Removal of chorions with proteinase K was unnecessary, and was in fact found to lead to premature activation. Eggs could be easily orientated and penetrated by using needles with a tip of 10–15 μm (outer diameter), if they were held in agarose wells and immersed in saline (Fig. 2). After injection, eggs were transferred to E3, with minimal transfer of the high-salt buffer, and this allowed activation without any leakage of cytoplasm.

Out of 38 eggs that cleaved normally in four separate experiments, 24 were morphologically normal after 24 h (Table 1). All embryos that had more than two cells after first cleavage, indicating polyspermy, were retarded in epiboly and failed to develop normally. Twelve embryos that appeared normal at 24 h were grown up. Eight of these survived to adulthood, and when crossed to one another gave rise to normal embryos. Fertilization by intracytoplasmic sperm injection thus allows normal development of zebrafish.

**Gene Transfer by Sperm Nuclear Transplantation**

Sperm nuclei were incubated with linearized plasmid DNA prior to injection, to determine whether this would allow the generation of nonmosaic transgenic fish. Two plasmids were tested: pESG, which contains GFP driven by the Xenopus EF-1α promoter (Johnson and Krieg, 1994), and pBOS-H2BGFP, which contains eGFP fused to histone, regulated by the human EF-1α promoter (Mizushima and Nagata, 1990). When sperm nuclei were incubated with 70 ng of either plasmid for 20 min at room temperature prior to injection into eggs, almost all resulting embryos (50 out of 56 for pBOS-H2BGFP; 54 out of 56 for pESG) contained GFP-positive cells. While most expressed GFP in a mosaic fashion, a proportion of these embryos (10 for pBOS-H2BGFP and 15 for pESG) were completely green and morphologically normal. Confocal microscopy indicated that all cells fluoresced (Figs. 3A and 3C); this was confirmed by lightly fixing embryos and labeling nuclei with Hoechst (Fig. 3E). In embryos injected with linearized plasmid after natural fertilization, in contrast, expression was always mosaic (n = 65 for pESG and 85 for pBOS-H2BGFP) (Figs. 3B and 3D). Fluorescence was detected in nuclear-transplanted embryos at 7 days postfertilization, apparently in all cells. In these embryos, and also in those imaged at 5 or 24 h postfertilization, some cells appeared to fluoresce more brightly than others. Variable expression in different cells within single embryos has been seen in stable zebrafish transgenic lines of Xenopus EF-1α-GFP (data not shown) and in tilapia lines with carp β-actin-LacZ (Rahman et al., 2000). Both lines were obtained by plasmid DNA injection, indicating that nonuniform expression is not an artifact of sperm-mediated transgenesis.

When sperm nuclei were incubated for 1 or 5 min with 70 ng pBOS-H2BGFP prior to dilution in SDB, no GFP-positive embryos were obtained (n = 40 embryos; Table 2). Similarly, inefficient gene transfer was seen when embryos obtained by natural fertilization were injected with DNA at a concentration of 0.14 ng/μl, which is the final concentration of DNA in the injection buffer for effective sperm-mediated gene transfer. When higher amounts of DNA were used, specifically 240 ng of pBOS-H2BGFP with 5 μl sperm nuclei for 20 min, all normally cleaving embryos (14 out of 69) appeared to express GFP ubiquitously at 50% epiboly. However, none of them were morphologically normal at 24 h postfertilization, despite appearing normal during early cleavage stages. With 5 min incubation in 240 ng pBOS-H2BGFP, no normal embryos were seen after 24 h, although 7 out of 10 normally cleaving embryos expressed the transgene in a mosaic fashion.

To test whether sperm-mediated gene transfer could be used to give cell type-specific expression, sperm nuclei were...
incubated with a plasmid containing eGFP fused to a 3.2-kb element upstream of the zebrafish Huc gene, which was reported to give neural-specific expression (Park et al., 2000b). Fish generated by sperm-mediated gene transfer expressed GFP in the nervous system (Fig. 3F, in a pattern similar to that of Huc mRNA as shown by in situ hybridization (Kim et al., 1996; Park et al., 2000a), and also to previously published stable transgenic lines (Park et al., 2000b). Unexpectedly, the embryos also expressed GFP in the enveloping layer during epiboly, and in some muscles cells at 24 h postfertilization. This ectopic expression was also seen in fertilized eggs injected with plasmid DNA at 50 ng/μl, indicating that the promoter used here is somewhat leaky. The level of fluorescence in embryos obtained using sperm-mediated transgenesis appeared lower compared with those obtained by plasmid injection into fertilized eggs. One reason for this may be that embryos obtained using sperm-mediated transgenesis have a lower copy number of the transgene.

Two embryos with widespread expression of GFP under the Huc promoter, and four with GFP under the control of the Xenopus EF-1α promoter, were grown up. One from each group survived to adulthood, and when crossed to wild-type fish, gave rise to offspring expressing GFP. Approximately 20% of the progeny of the pESG fish (10 out of 51) expressed GFP in all cells. In the case of the HuC-GFP fish, approximately 50% of the progeny (72 out of 149; 2 different crosses) expressed GFP in the nervous system (Fig. 3G; similar expression was seen in all GFP-positive embryos). These observations establish that transgenes introduced by sperm-mediated gene transfer can be transmitted through the germ line.

**DISCUSSION**

This paper demonstrates that a teleost egg can be fertilized by injection of sperm heads and that sperm-mediated gene transfer can be achieved simply by preincubating the sperm nuclei with DNA. Fertilization by sperm nuclear transplantation has been described in a number of other animals, including rhesus monkeys (Chan et al., 2000), mice (Perry et al., 1999), and Xenopus (Kroll and Amaya, 1996; Offield et al., 2000), but some modification to these techniques was required for successful application to zebrafish. In contrast to the methods used originally for X. laevis and X. tropicalis, sperm nuclei were not decondensed prior to injection into zebrafish. This allows successful injection into the relatively small zebrafish egg. When large bore pipettes were used, cytoplasm leaked out of the zebrafish egg, and normal development was not seen. Also, the technique described here uses a gas pressure injector, which is a standard piece of equipment in most zebrafish labs, in contrast to the pump device used in Xenopus transgenesis (Kroll and Amaya, 1996), or the piezzo-driven injector used in mice transgenesis (Perry et al., 1999).

Nonmosaic expression of GFP requires prolonged incubation of the transgene with sperm nuclei prior to injection. Incubation for 5 min or less is insufficient, as is injection of similar concentrations of DNA into already fertilized eggs. This suggests that some form of association must occur between DNA and sperm nuclei prior to fertilization so that DNA is retained in all cells. Excessive amounts of DNA appear to be detrimental to the embryo, so a careful balance of DNA concentration and incubation time is required for ubiquitous transgene expression and normal development.

It is possible that the transgene is integrated during early cleavages, and this is supported by the observation that transgene expression can be seen in progeny of injected eggs. Previous attempts to use sperm-mediated transgenesis in zebrafish have been unsuccessful, as no expression of transgenes was seen when intact sperm was coincubated or electroporated with DNA prior to fertilization, even though the presence of foreign DNA could be detected by PCR (Muller et al., 1992; Patil and Khoo, 1996). The critical step for expression thus appears to be use of sperm nuclei, rather than intact sperm.

Perry et al. (1999) have reported a similar approach to transgenesis of mice. Similar to what was found here, they obtained embryos with widespread expression of reporter genes. They also observed transgene integration into the genome and germ line transmission, indicating that restriction enzymes are not essential for integration. Surprisingly, they could obtain widespread transgene expression with only 1 min incubation of sperm nuclei with DNA, which is not possible with zebrafish. One reason for this difference may be that zebrafish sperm nuclei have different properties compared with mouse sperm nuclei that render association with extraneous DNA less efficient. Alternatively, there might be less time for integration to occur in the zebrafish embryo, as early cleavages occur much more rapidly compared to the mouse. Recently, Sparrow et al. (2000) reported that prolonged incubation of Xenopus sperm nuclei in DNA also allows efficient transgenesis without the need for decondensation.
Sperm-mediated transgenesis in zebrafish will allow transgene experiments without the need for establishing stable lines. With cell- or tissue-specific promoters, it will be possible to misexpress genes in any given cell type or region of the embryo, to test their function. This will be useful in cases where stable lines cannot be created, for example, where misexpression causes lethality. This technique can also be used for promoter analysis, or for creating gene traps, as has been done in X. laevis (Bronchain et al., 1999). Additionally, since transgene expression can be seen in offspring, this technique might be useful for generating transgenic lines.

In summary, this paper establishes that sperm-mediated gene transfer can be used to obtain nonmosaic expression of foreign DNA in zebrafish. With experience, it is possible to inject around 150 eggs per hour. Modification of the technique by other laboratories should aid in making this approach more efficient and allow wider use of transgenic experiments. This technique should also, in principle, be applicable to other teleosts, such as medaka.

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