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A Method for Generating Transgenic Frog Embryos

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1. Introduction

1.1. Summary

The early amphibian embryo has been widely used as a model organism for studying early vertebrate development. This chapter describes in detail a new and very efficient method for generating transgenic *Xenopus* embryos. At the end of the chapter, a new method for fertilizing in vitro matured oocytes is also introduced.

1.2. Background

The elucidation of the molecular basis of pattern formation and differentiation in frog embryos has been hindered by the lack of a system for temporal and tissue-specific expression of wild type and mutant forms of developmentally important genes. RNA injection, the most common transient expression method in *Xenopus*, has been effectively used to study maternally expressed genes. However, since RNAs are translated immediately after injection, this method is unfavorable for the study of zygotic gene products that are expressed only after the midblastula transition. Direct injection of DNA can be used to express genes behind temporal and tissue specific promoters after the midblastula transition. However, in frog embryos this approach has only been marginally successful for two reasons: (1) injected DNA does not integrate into the frog chromosomes during early cell cycles, and therefore, the embryo expresses the genes in a highly mosaic pattern, and (2) many promoters lack adequate temporal fidelity and tissue specificity of expression when the DNA is not integrated into the genome of the frog. To overcome these technical problems, we have developed the nuclear transplantation-based approach to transgenesis

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described in this chapter. The approach enables stable expression of cloned gene products in *Xenopus* embryos, allowing a broader range of feasible experimentation than that previously possible by transient expression methods.

Unlike plasmid injection, transgenesis allows stable, temporally and spatially controlled expression of gene products in desired cells of the *Xenopus* embryo. We have used transgenesis to express genes of interest ectopically, to direct expression of modified gene products which dominantly interfere with the function of their endogenous, wild-type counterparts, and to analyze the spatial regulation of promoters in the embryo ***(1)***. We have been able to obtain large numbers of transgenic embryos readily for these purposes and to interpret reliably the effect of transgene expression without the cell-to-cell variability of expression within an embryo, which plagues many studies using plasmid-injected embryos.

1.3. Overview of Transgenesis Procedure

In the transgenesis approach described here, DNA is integrated into isolated sperm nuclei in vitro, followed by transplantation of the nuclei into unfertilized eggs, thus generating transgenic embryos. Nuclear transplantation of transfected cultured cells was previously used by one of us to produce transgenic *Xenopus* embryos, which expressed promoter-reporter plasmids nonmosaically ***(2)***. However, the cultured cells used as nuclear donors for these transplantations were aneuploid and rarely promoted development of the pseudo-triploid embryos to tadpole stages. To overcome these problems, we now use sperm nuclei to generate transgenic embryos. These nuclei offer many advantages over cultured cell lines. First, since sperm nuclei are haploid, there is no need to destroy the egg nucleus before transplantation to generate a normal diploid embryo. Second, sperm nuclei have been used for many years to investigate the processes of chromosome decondensation, nuclear assembly, and cell-cycle progression ***(3–5)***. These studies have provided us with valuable information regarding the manipulation of sperm nuclei in vitro. Indeed we have discovered that we can introduce DNA into sperm nuclei swelled and decondensed in cell-free egg extracts using restriction enzyme-mediated integration (REMI) ***(6,7)***. When these nuclei are transplanted into unfertilized eggs, we obtain large numbers of normal diploid tadpoles, which develop to advanced stages and express inserted genes at high frequency.

The protocol for *Xenopus* transgenesis described here involves the following steps:

1. Sperm nuclei are incubated with linearized plasmid DNA.
2. After a short incubation, a high-speed interphase egg extract and a small amount of the restriction enzyme used for plasmid linearization are added to the sperm nuclei/plasmid mixture. The extract partially decondenses sperm chromatin, but does not promote replication.

3. After the plasmid-treated nuclei are incubated for a brief period in the interphase extract, the mixture is diluted 50- to 100-fold (or 500- to 1000-fold total dilution of sperm nuclei).
4. Approximately one nucleus is transplanted into an unfertilized egg in a 5–15 nL volume. This procedure is schematically represented in **Fig. 1**.

1.4. Efficiency of Transgenesis Procedure

After transplantation with swelled sperm nuclei, 20–40% of the eggs cleave and develop normally. One person can transplant about 500 sperm nuclei/h to produce several hundred to one thousand embryos in a typical experiment. As with embryos produced by in vitro fertilization, the frequency of normal, advanced development varies somewhat, depending on the overall quality of the eggs; typically, 5–40% of the cleaving eggs develop normally beyond feeding tadpole stages. We commonly obtain 1–2 mo old tadpoles and are currently raising transplantation-derived metamorphosed froglets to sexual maturity.

Embryos from gastrula through tadpole stages derived from sperm nuclear transplantation express plasmids nonmosaically at high frequency. We have used transgenesis to introduce plasmids containing the simian cytomegalovirus (CMV) ***(8)*** or the *X. borealis* cytoskeletal actin (CSKA) ***(9,10)*** promoter into embryos ***(1)***. Transgenic embryos express genes from these promoters in every cell starting at the late blastula and early gastrula stages, respectively, as expected for these ubiquitously expressed promoters. When whole-mount *in situ* hybridization is used to detect plasmid expression, 20–50% of transplantation-derived embryos express CMV and CSK promoter-containing plasmids in every cell. In contrast, embryos injected with these plasmids never express reporter genes in all cells and typically express in only a small fraction (about 5–20%) of cells in the embryo.

We have also made transgenic embryos that express promoters that are spatially restricted ***(1)***. For example, we have used this method to introduce into embryos plasmids containing a muscle-specific actin promoter ***(11)*** linked to chloramphenicol acetyltransferase (pRLCAR) or green fluorescent protein (pCARGFP). We find that 40–60% of tadpoles derived from sperm nuclear transplantations with these plasmids show stable, nonmosaic expression. Expression is restricted to the somites and heart tissue, as expected for this regionally restricted promoter. We have also generated transgenic embryos with plasmid DNA that contains a neural specific β-tubulin promoter driving chloramphenicol acetyltransferase (CAT) (provided by Paul Krieg). These embryos express CAT in the primary neurons of the embryo as expected for this promoter. This correct expression is significant, since embryos injected with plasmids not only express CAT mosaically, but also ectopically, suggesting that integrating DNA into the genome is likely to give better regulation of cloned promoters than expression from nonintegrated DNA.

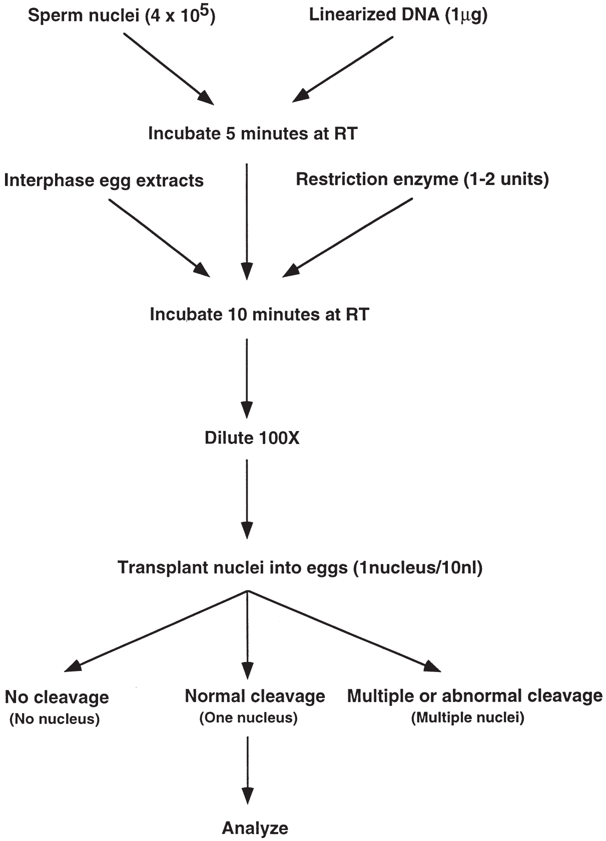

Fig. 1. Overview of transgenesis procedure. Sperm nuclei are incubated with linear DNA for a brief period of time. Interphase egg extracts and a restriction enzyme are then added. The egg extracts partially decondense the chromosomes, and the restriction enzyme very lightly cleaves them. These events facilitate the eventual integration of the linear DNA into the chromosomes. After incubation of nuclei in a mixture of extract, restriction enzyme, and plasmid DNA, the nuclei are diluted, and approximately one nucleus is transplanted per egg. Each activated egg requires a nucleus (or at least the centriole introduced with a nucleus) to divide; therefore, only eggs receiving a nucleus develop into embryos. Eggs that receive more than one nucleus (polyspermic eggs) divide abnormally into multiple cells at the first cleavage division. Embryos developing from monospermic eggs cleave normally during early divisions; only these embryos are isolated and analyzed. Generally, between 20 and 50% of these embryos will be transgenic.

1.5. Analysis of DNA Integration in Transgenic Embryos

We have analyzed genomic DNA from transplantation-derived tadpoles to determine whether early integration of introduced plasmids into sperm or egg chromosomes is responsible for the nonmosaic expression observed ***(1)***. The pCARGFP plasmid was introduced into embryos by transplantation of sperm nuclei, and tadpoles 2.5 wk to 1 mo old were scored for nonmosaic expression of GFP in the somites; the presence and arrangement of pCARGFP were then determined by probing Southern blots of genomic DNA from these tadpoles with a 1-kb probe consisting of GFP sequences from one end of the linearized pCARGFP plasmid. We found that transplantation-derived tadpoles that did not express pCARGFP did not contain the plasmid, whereas each tadpole that expressed GFP contained between 5 and 35 copies of the plasmid/cell. The probe recognized four to eight bands in each GFP-expressing tadpole which were of unique sizes and were not found in other GFP-expressing tadpoles. These fragments represent putative junction points at which pCARGFP was integrated into the genome of each tadpole. Additionally, the probe recognized two common bands in all of the GFP-expressing tadpoles, corresponding to products formed by tandem and back-to-back concatemerization of the pCARGFP plasmid. By comparing the intensity of these bands relative to the putative junction fragments, we estimate that pCARGFP was integrated into the genome as single copies in some instances and as short (two to six copy) concatemers in other instances. Since the plasmid is expressed in all expected cells in the embryo, it is likely that most of these integrations occurred prior to the first cleavage division, assuring that all cells of the embryo would inherit several copies of the plasmid.

1.6. Overview of a New Technique for Fertilizing In Vitro Matured Oocytes

In addition to describing a procedure for making transgenic frog embryos, we are also including a new protocol for using sperm nuclear transplantation to fertilize oocytes that have been matured in vitro. A number of investigators have successfully depleted maternal RNAs from *Xenopus* embryos by injecting oocytes with antisense deoxyoligonucleotides for a message of interest, and then maturing and fertilizing these injected oocytes to produce embryos ***(12–15)***. However, the approaches currently available for obtaining embryonic development from oocytes matured in vitro are quite labor-intensive and technically demanding ***(16)***. The difficulty arises in large part because the surface of matured oocytes must be altered by proteolytic enzymes, and covered with a jelly coat in the oviduct of the female frog in order to become competent for fertilization. When oocytes are matured in vitro, these processes must either be simulated, or the matured oocytes must be reimplanted into the body cavity of a female frog so that these processes occur as it travels back outside through the oviduct of the host female. However, we have found that direct transplantation of sperm nuclei into matured oocytes bypasses these requirements and has allowed us to very simply produce normal embryos from oocytes handled in vitro. A similar approach has also been developed to overcome male infertility in humans ***(17–20)***.

2. Materials

2.1. Sperm Nuclei Preparation

1. 1X Nuclear Preparation Butter (NPB): 250 m*M* sucrose (1.5 *M* stock; filter-sterilize and store aliquots at –20°C), 15 m*M* HEPES (1 *M* stock; titrate with KOH so that pH 7.7 is at 15 m*M*, filter-sterilize, and store aliquots at –20°C) (s*ee* **Note 4**), 0.5 m*M* spermidine trihydrochloride (Sigma S-2501; 10 m*M* stock; filter-sterilize and store aliquots at –20°C), 0.2 m*M* spermine tetrahydrochloride (Sigma S-1141; 10 m*M* stock; filter-sterilize and store aliquots at –20°C), 1 m*M* dithiothreitol (Sigma D-0632; 100 m*M* stock; filter-sterilize and store aliquots at –20°C) (*see* **Notes 4** and **5**).

For steps requiring protease inhibitors, add: 10 μg/mL leupeptin (Boeringer Mannheim 1 017 101; 10 mg/mL stock in DMSO, store aliquots at –20°C), 0.3 m*M* phenylmethylsulfonyl fluoride (PMSF) (Boeringer Mannheim 837 091; 0.3 *M* stock in EtOH, stored at –20°C).

2. 1X Marc's Modified Ringer (MMR): 100 m*M* NaCl, 2 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 5 m*M* HEPES, pH 7.5. Prepare a 10X stock, and adjust pH with NaOH to 7.5. Sterilize 10X and 1X solutions by autoclaving.
3. Lysolecithin: 100 μL of 10 mg/mL L-α-lysophosphatidylcholine (Sigma Type I, L-4129); dissolve at room temperature just before use. Store solid stock at –20°C.
4. Bovine serum albumin (BSA) (store at –20°C in 1 mL aliquots): 10% (w/v) BSA (fraction V, Sigma A-7906; prepare stock in water, titrate to pH 7.6 with KOH).
5. Sperm dilution buffer (store at -20°C in 0.5-mL aliquots): 250 m*M* sucrose, 75 m*M* KCl, 1 m*M* EDTA (0.5 *M* stock, pH8), 0.5 m*M* spermidine trihydrochloride (Sigma S-2501; 10 m*M* stock; filter-sterilize and store aliquots at –20°C), 0.2 m*M* spermine tetrahydrochloride (Sigma S-1141; 10 m*M* stock; filter-sterilize and store aliquots at –20°C), 1 m*M* dithiothreitol (Sigma D-0632; 100 m*M* stock; filter-sterilize and store aliquots at –20°C).
6. Hoechst No. 33342 (Sigma B-2261): 10 mg/mL stock in dH_2O; store in a light-tight vessel at –20°C.

2.2. High-Speed Egg Extract Preparation

1. 20X Extract buffer (XB) salt stock: 2 *M* KCl, 20 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, filter-sterilize and store at 4°C.
2. Extract buffer (XB): 1X XB salts (100 m*M* KCl, 0.1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$; from 20X XB salts stock solution), 50 m*M* sucrose (1.5 *M* stock; filter-sterilize and store in aliquots at –20°C), 10m*M* HEPES (1 *M* stock, titrated with KOH such that pH is 7.7 when diluted to 10 m*M*; should require about 5.5 mL of 10 *N* KOH for 100 mL; filter-sterilize, and store in aliquots at –20°C) (*see* **Note 4**). Prepare about 100 mL.

3. 2% (w/v) Cysteine (Sigma C-7755): Made up in 1X XB salts within 1 h of use and titrated to pH 7.8 with NaOH. Prepare about 300 mL.
4. CSF-XB: 1X XB salts (100 m*M* KCl, 0.1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$), 1 m*M* $MgCl_2$ (in addition to $MgCl_2$ present in XB salts; final concentration 2 m*M*), 10 mM potassium HEPES, pH 7.7, 50 m*M* sucrose, 5 m*M* EGTA, pH 7.7. Prepare 50 mL.
5. Protease inhibitors: Mixture of leupeptin (Boeringer Mannheim 1017 101), chymostatin (Boeringer Mannheim 1 004 638), and pepstatin (Boeringer Mannheim 600 160), each dissolved to a final concentration of 10 mg/mL in dimethyl sulfoxide (DMSO). Store in small aliquots at –20°C.
6. 1X MMR Prepare as described in **Subheading 2.1., item 2**.
7. 1 *M* $CaCl_2$ (filter-sterilize and store at 4°C).
8. Versilube F-50: Made by General Electric. Can be purchased from Andpak-EMA (1560 Dobbin Drive, San Jose, CA 95133; Tel. [408]-272-8007).
9. Energy mix (store in aliquots at –20°C): 150 m*M* creatine phosphate (Boeringer Mannheim Biochemicals 127 574), 20 m*M* ATP (Boeringer Mannheim Biochemicals 519 979), 20 m*M* $MgCl_2$, Store in 0.1-mL aliquots at –20°C.
10. Pregnant mare serum gonadotropin (PMSG).: 100 U/mL PMSG (367222; Calbiochem, San Diego, CA). Made up in water and stored at –20°C.
11. Human chorionic gonadotropin (HCG): 1000 U/mL HCG (Sigma CG-10 from human pregnancy urine). Made up in water and stored at 4°C.

2.3. Nuclear Transplantation Reagents and Equipment

1. 1X MMR Prepared as described in **Subheading 2.1., item 2**.
2. 2.5% Cysteine in 1X MMR (titrate to pH 8.0 with NaOH). Make up fresh each day.
3. Sigmacote (Sigma SL-2).
4. 100 m*M* $MgCl_2$.
5. 0.4X MMR + 6% (w/v) Ficoll (Sigma Type 400; F-4375) Sterilize by filtration.
6. 0.1X MMR + 50 µg/mL gentamycin (a 10 mg/mL stock solution may be purchased from Gibco-BRL; cat #15710-015). Add 6% (w/v) Ficoll for culturing embryos prior to gastrulation. Culture embryos in 0.1X MMR without Ficoll after gastrulation. Sterilize by filtration.
7. Progesterone (Sigma P-0130; SmM stock in EtOH).
8. Linearized plasmid (200–250 ng/µL): Although we have primarily used plasmid linearized with *Xba*I or *Not*I in transplantation reactions, we have also used *Xho*I, *Bam*HI, and *Eag*I successfully. We think that most enzymes that function in the moderately high salt conditions of the egg extract are likely to work. We commonly purify linearized plasmid for transgenesis using the Geneclean kit by Bio 101, Inc. (cat. no. 1001-200; 1070 Joshua Way, Vista CA 92083; 1-800-424-6101). Plasmid DNA can be eluted in either dH_20 or in TE (10 m*M* Tris-HCl, pH 8.0; 1 m*M* EDTA). If linearized plasmid DNA needs to be concentrated, standard precipitation with 0.1 vol sodium acetate (3 *M* stock; pH 5.2) and 2.5 vol absolute ethanol, followed by a 70% ethanol wash, can be used. We have found that plasmid DNA purified in this manner works well for making transgenic embryos and does not adversely effect embryonic development.

We have used enzymes purchased from Boeringer Mannheim or New England Biolabs for transplantation reactions. Some calibration may be required to determine the optimal amount of enzyme to add to each reaction, since additions of 0.5 µL of undiluted enzyme to reactions can adversely affect the development of nuclear transplant embryos. We generally test several dilutions of enzyme (1:2.5, 1:5; 1:10) to identify a dose that has no apparent deleterious effects on transplant embryo development when compared with embryos produced with no enzyme addition.

9. Agarose-coated injection dishes: 2.5% agarose in dH_2O is poured into 35-mm or 60-mm Petri dishes. Before the agarose solidifies, a well template (a rectangular square of Dow-Corning Sylgard 184 elastomer) is laid onto it. After the agarose has solidified and the Sylgard templates have been removed, 1X MMR is poured into each dish to prevent dehydration. The dishes are then wrapped in parafilm and stored at 4°C (weeks to months) until use.
10. Transplantation needles: 30-µL Drummond micropipets (Fisher, cat. #: 21-170J) are pulled to produce large needles with long, gently sloping tips (**Fig. 2**). A micropipet (1 mm wide; 8 cm long) is first heated in a Bunsen burner flame and drawn by hand to make the bore of the needle (200–400 µm wide). This drawn pipet should be 10–15 cm in length and should remain fairly straight when held by one end. To produce a gently sloping needle tip, this pipet is drawn again. We use a gravity-driven needle puller for this: the upper end of the needle is fixed in a brace, the center of the needle bore of the drawn pipet is placed within a small heating coil, and a weight is attached to the lower end of the needle. The gravity driven pullers we have used are home-built and about 10–20 yr old, but similar vertical pullers are commercially available from Narishige (i.e., Model PB-7). The second pull can also be performed with a horizontal needle puller available from Sutter Instrument Co. (Model P-87; Flaming/Brown micropipet puller) using settings like those used to make other injection needles. In limited trials of the Sutter puller using a standard setting, we have found that the needles produced had a steeper slope near the tip and were slightly more difficult to use than those drawn with our vertical puller; however, settings can probably be adjusted on this and other commercially available pullers to produce long, gently sloping tips that will work well for transplantation. Needles are clipped with a forceps to produce a beveled tip of 60–75 µm diameter (*see* inset in **Fig. 2**), using the ocular micrometer of a dissecting microscope for measurement.
11. Transplantation apparatus: We have found most commercial injection apparatuses commonly used for RNA and DNA injections unsuitable for nuclear transplantation. This is largely due to the difference in needle tip size. Flow through the 5–10 µm needle tips used for fluid injections can be controlled at fairly high pressures. However, with standard air-injection systems, we have been unable to obtain the extremely low positive pressure, and gentle, controlled flow required to deliver an intact nucleus in a small volume (10–15 nL) through the 50–70 µm tips of nuclear transplantation needles. Oil-filled injection systems (Drummond) are likely to work, since they are based on a positive displacement mechanism that should not be affected by the tip size of the needle. At this writing, though,

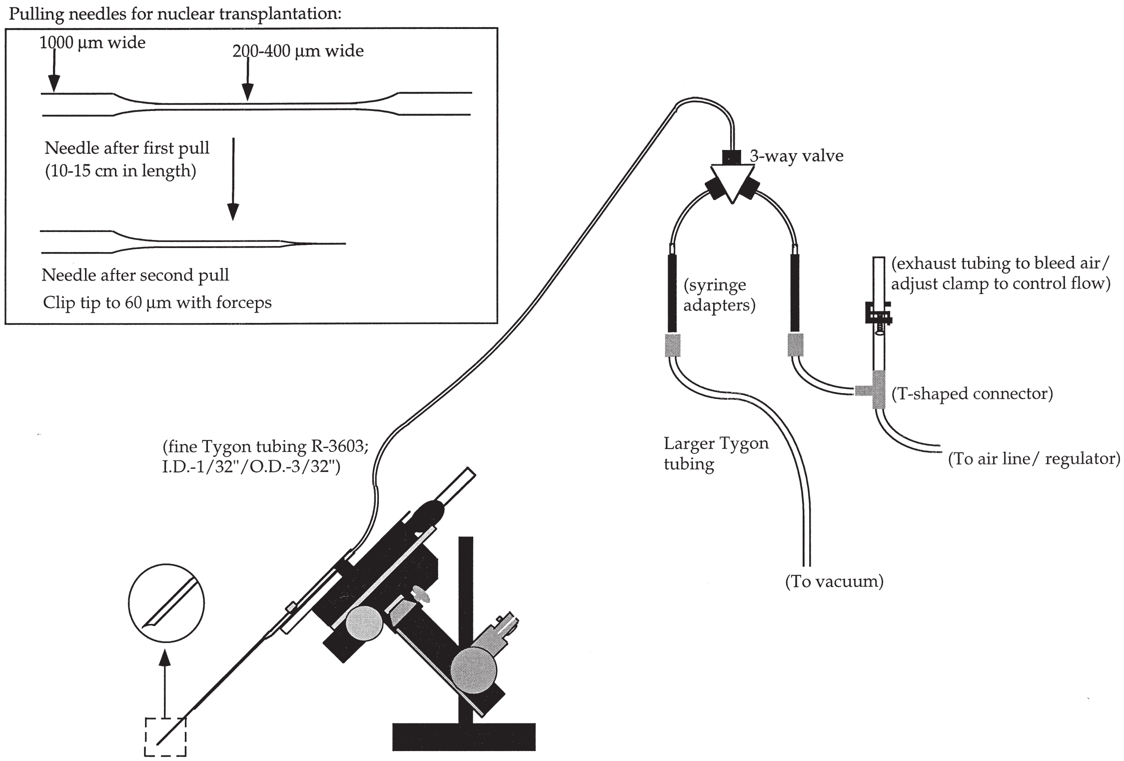

Fig. 2. Diagram of injection apparatus. A pressure regulator is set up on the house air, and a line connecting the regulator is split with a T-shaped connector into an exhaust tube and line to a three-way valve. Another line connects the house vaccuum to the three-way valve. Finally, another line connects the three-way valve to the needle. By adjusting the valves in the three-way valve, the air pressure, and the clamp on the exhaust tube, one can very finely control the level of positive or negative pressure going into the needle. The rectangular inset shows how the needle should appear after the first and second pulls. The circular inset shows how the point of the needle should appear after it is clipped.

we have not tried one of these injection apparatuses for nuclear transplantations. Instead, we will describe how to make a home-made air injection apparatus that works extremely well for nuclear transplantions on a large scale and that costs very little (approx $200).

The transplantation apparatus that has given us the most success is shown in **Fig. 2**. A line connects the house vacuum outlet to a three way valve. Another line connects the house air outlet to a T-connector that splits the air flow into an exhaust line and another line connecting to the three way valve. Finally, another line connects the three way valve to the needle. For fine control of the positive pressure into the needle a screw clamp is placed on the exhaust line. Screwing down on this clamp increases the positive pressure into the system, while opening the clamp decreases the positive pressure. Negative pressure is established by opening slightly the valve (on the three way valve) connected to the house vacuum line. A more rough adjustment of positive pressure also can be obtained by opening or closing the valve (on the three way valve) connected to the house air line. By using a combination of these adjustments, we are able to obtain a very slow, controllable flow through a 50–70-μm needle. As flow is continuous, transplantations can usually be done more rapidly than injections of RNA or DNA, since it is only necessary to move from egg to egg to deliver nuclei. Parts needed to build the transplantation apparatus shown in **Fig. 2** are listed in **Table 1**.

Alternatively, a transplantation apparatus like the one shown in **Fig. 3** can be constructed. For this apparatus, a large, air-filled Hamilton Syringe (30 cc Multifit Interchangeable syringe with Luer-Lok tip; Fisher) is connected to a length of Tygon tubing. A metal plunger removed from a Syringe Microburet (Model # SB2; Micro-metric Instrument Co., Cleveland, OH) is used to control injection of the nuclei. We have found this apparatus usable although it is not controlled as easily as the one shown in **Fig. 2**.

3. Methods

3.1. Transgenesis Method (see Note 3)

3.1.1. Sperm Nuclei Preparation

We have generally followed the standard protocol of Murray ***(4)***, but have omitted the protease inhibitors leupeptin and phenylmethylsulfonyl fluoride from many steps to avoid transfer into the final mixture, which is diluted for egg injections.

1. Dissect and isolate the testes from a male:
   a. Anesthetize a male in a bucket containing a liter of 0.1% Tricaine (MS222, aminobenzoic acid ethyl ester, Sigma A-5040) and 0.1% sodium bicarbonate for at least 20 min (immersion of the animal in ice water for 20 min may also be used), and pith it.
   b. Cut through the ventral body wall and musculature, and lift the yellow fat bodies to isolate the two testes, which are attached to the base of the fat bodies, one on each side of the midline.

Table 1
Part for Transplantation Apparatus[a]

Company	Product	Catalog number	Price	Phone/address
Newport Corp. (This may not be necessary; if your house air pressure is fairly low, you can attach the tubing directly to house air.)	Air regulator/filter	ARF	$79.00	1-800-222-6440 1791 Deere Ave. Irvine, CA 92714
Western Analytical Products, Inc. (They are the US distributor for Omnifit Ltd. Phone: 01223-69841 Fax: 01023-61106 51 Norfolk St. Cambridge CB12LE)	Three-way valve	001102	$105.51	1-800-541-8421 25407 Blackthorn Murrieta, CA 92563
	Trivalve caps (mixed colors)	001310	$5.25	
Fisher	Hoffman open-side tubing clamps	05-875A	$20.00 for 10	
	T-shaped connectors	15-319C	$10.44 for 12	
	Tygon tubing 1/32 in.	12-169-1A	$4.10	
	Tygon tubing 3/16 in.	14-169-3B	$7.50	
Fine Science Tools, Inc.	Precision micromanipulator MM33	25033-10	$690.00	1-800-521-2109 373-G Vintage Park Dr. Foster City, CA 94404
	Magnetic base	25810-00	$84.95	

[a] A base somewhat heavier and more stable than the magnetic model available from Fine Science Tools was previoulsy available from Brinkman. To our knowledge, however, a comparable product is no longer available either from Brinkman or from other manufacturers; we have had a copy built by the local machine shop.

c. Remove the testes with dissecting scissors, and place them in a 35-mm tissue-culture dish containing cold 1X MMR.
   Rinse the testes in three changes of cold 1X MMR and two times in cold 1X NPB, removing any attached pieces of fat body or debris with a fine forceps. Take care not to puncture the tissue pouches, since this releases the sperm.

2. Move the cleaned testes to a dry 35-mm tissue-culture dish, and macerate the tissue well (until clumps are no longer visible to the naked eye) with a pair of clean forceps.

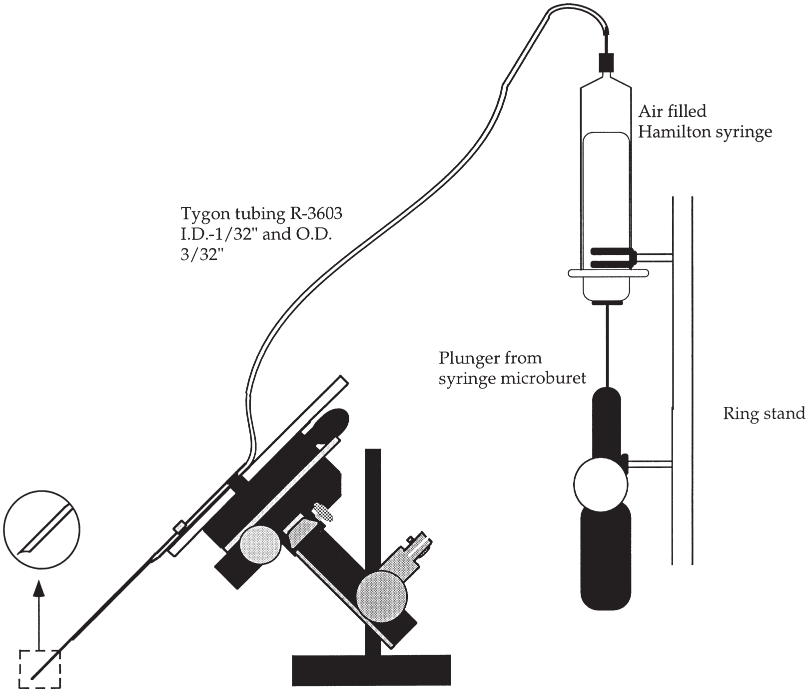

Fig. 3. Diagram of alternative injection apparatus. A line connects the needle to a 30-mL Hamilton syringe that is held by a clamp to a ring stand. The glass syringe plunger is pushed in using a microburet that is also clamped to the ring stand. A slow, controlled flow of liquid through the needle can be obtained by leaving a large cushion of air inside the syringe between the glass plunger and the end attached to the tubing. The circular inset shows how the point of the needle should appear after it is clipped.

3. Resuspend the macerated testes in 2 mL of 1X NPB by gently pipeting the solution up and down through a fire-polished, truncated Pasteur pipet with an opening of about 3 mm in diameter.
4. Squirt the sperm suspension through two to four thicknesses of cheesecloth placed into a funnel, and collect the solution into a 15-mL tube (we use round bottom polypropylene tubes; Fisher, cat. #: 14-956-1J). Rinse the forceps and dish with 8 mL of 1X NPB, and force this through the cheesecloth into the 15-mL tube. With a gloved hand, fold the cheesecloth and squeeze any remaining liquid through the funnel into the 15-mL tube.
5. Pellet the sperm by centrifugation at 1500*g* for 10 min at 4°C (we use a Sorvall HB-6 or similar swinging bucket rotor fitted with the appropriate adapters). Resuspend sperm in 8 mL NPB and repellet by centrifugation at 1500*g* for 10 min at 4°C.
6. Resuspend pellet in 1 mL NPB with a cut plastic pipet tip, warm the suspension to room temperature, and add 50 µL of 10 mg/mL lysolecithin. Mix gently and incubate for 5 min at room temperature.

7. Add 10 mL cold 1X NPB + 3% BSA (with protease inhibitors; 1:1000 dilution of leupeptin and PMSF stock solutions) to the suspension, mix gently by inversion, and centrifuge at 1500*g* for 10 min at 4°C. Decant the supernatant.
8. Resuspend the pellet in 5 ml cold 1X NPB + 0.3%BSA (no protease inhibitors), mix gently by inversion, and repellet as before.
9. Resuspend the pellet in 500 µL of sperm dilution buffer, and transfer suspension into a 1.5-mL Eppendorf tube. Count the sperm density using a hemacytometer (Fisher, cat. #: 02-671-5): dilute a small amount of the concentrated sperm 1:100 in sperm dilution buffer, and add 1µL of 1:100 Hoechst stock to visualize the sperm heads under a fluorescence microscope. For a 1:100 dilution of our sperm stock, we typically obtain counts of 75–125 ($\times 10^4$ cells/mL). At this concentration, the undiluted stock contains 75–125 sperm/nL. If your sperm stock is substantially less concentrated (i.e., a count of <50 for a 1:100 dilution), repellet the sperm and resuspend in a smaller volume of sperm storage buffer. Sperm can be stored at 4°C and used for transplantations for up to 48 h.

3.1.2. High-Speed Extract Preparation

This protocol is an adaptation of Murray ***(4)***. Briefly, a crude cytostatic factor (CSF) arrested egg extract (cytoplasm arrested in meiotic metaphase) is prepared. Calcium is then added to allow the extract to progress into interphase, and a high-speed spin is performed to obtain a purer cytoplasmic fraction. Cytochalasin is omitted from the protocol, since carryover of cytochalasin into the final extract used for sperm incubations interferes with normal development of transplant embryos. Use of high-speed rather than crude cytoplasmic extracts is advantageous, because high-speed extracts promote swelling of added sperm nuclei (and some chromatin decondensation), but do not promote DNA replication. Replication of sperm DNA incubated in these extracts occurs after transplantation of the nucleus into the egg rather than in the extract. High-speed extract can be stored frozen in small aliquots (at –80°C) and thawed before use.

1. Prime 8–12 female adult *X. laevis* about 24 h prior to HCG injection by injecting 25–100 U of PMSG into the dorsal lymph sac. Maintain at room temperature. The evening before the extract preparation begins, inject each frog with 500–800 U HCG, and place 2 frogs/container into 2 L 1X MMR. Since one frog with lysing or activating eggs can compromise the whole extract preparation, we prefer to separate the frogs into pairs for the ovulation. The frogs are then placed at 15–18°C overnight (12–14 h). On the next morning, the egg quality from each container is screened before mixing all the eggs and starting the extract preparation. All the eggs released from a frog that lays mottled, lysing, or dying eggs are left out of the extract preparation.
2. All solutions should be prepared before beginning the extract preparation, since the procedure should be carried through all steps promptly once it is initiated; opti-

mally, the high-speed spin should begin within 45–60 min of dejellying the eggs. Gently expel eggs manually from each frog into a large dish of 1X MMR, and collect unbroken eggs with even pigmentation. Good eggs can also be collected from the 1X MMR in the frog buckets. Total volume of eggs should be 100 mL or greater before dejellying.

3. Remove as much MMR as possible from the eggs. Dejelly eggs in 2% cysteine in XB salts (no HEPES/sucrose). Add a small amount at a time, swirl eggs, and partially replace with fresh cysteine several times during dejellying. Remove broken eggs with a pipet during dejellying. Dejellying can be performed separately for different batches of eggs, and batches that show breakage or egg activation are discarded.
4. Wash eggs in XB (with HEPES/sucrose). We use about 35 mL for each wash, and do four washes.
5. Wash eggs in CSF-XB with protease inhibitors. We do two 25-mL washes.
6. Using a wide-bore Pasteur pipet, transfer eggs into Beckman ultraclear tubes. For these volumes, we typically use 14 × 95 mm tubes (cat. no.: 344060; Beckman, Fullerton, CA 344057). If multiple tubes will be used, try to transfer an equal volume of eggs per tube. Remove as much CSF-XB as possible, and replace with about 1 mL of Versilube F-50.
7. Spin in a clinical centrifuge at room temperature for about 60 s at 1000 rpm (150*g*) and then 30 s at 2000 rpm (600*g*). Eggs should be packed after this spin, but unbroken. Versilube should replace the CSF-XB between the eggs, and an inverted meniscus between the Versilube and displaced CSF-XB should be clearly visible. Remove the excess CSF-XB and Versilube, and then balance the tubes.
8. Spin the tubes in rubber adapters for 10 min at 16,000*g* at 2°C in Sorvall HB-4 or similar swinging bucket rotor to crush the eggs. The eggs should be separated into three layers: lipid (top), cytoplasm (center), and yolk (bottom). Collect the cytoplasmic layer from each tube with an 18-gage needle by inserting the needle at the base of the cytoplasmic layer and withdrawing slowly. Transfer cytoplasm to a fresh Beckman tube on ice. If large volumes of darkly pigmented eggs are used, the cytoplasmic layer may be grayish rather than golden at this step. After a second spin to clarify this extract, it should be golden.
9. Add protease inhibitors to the isolated cytoplasm (do not add cytochalasin); recentrifuge the cytoplasm in Beckman tubes for an additional 10 min at 16,000*g* to clarify, again using a swinging bucket rotor. Collect the clarified cytoplasm as before. Expect to get obtain 0.75–1 mL cytoplasm/batch of eggs collected from one frog.
10. Add 1/20 vol of the ATP-regenerating system (energy mix). Transfer the clarified cytoplasm into TL100.3 thick-wall polycarbonate tubes (Beckman 349622). Tubes hold about 3 mL each and should be at least half full.
11. Add $CaCl_2$ to each tube to a final concentration of 0.4 m*M*; this inactivates CSF and pushes the extract into interphase. Incubate at room temperature for 15 min and then balance for the high-speed spin.
12. Spin tubes in a Beckman tabletop TL-100 ultracentrifuge in a TL100.3 rotor (gold top; fixed angle) at 70,000 rpm for 1.5 h at 4°C.

13. The cytoplasm will fractionate into four layers, top to bottom: lipid, cytosol, membranes/mitochondria, and glycogen/ribosomes. Remove the cytosolic layer from each tube (about 1 mL if 2–3 mL were loaded into the tube) by inserting a syringe into the top of the tube through the lipid layer. Transfer this fraction to fresh TL-100 tubes, and spin again at 70,000 for 20 min at 4°C.
14. Aliquot the high-speed cytosol supernatant into 25-μL aliquots in 0.5-mL Eppendorf tubes. Quick-freeze aliquots in liquid nitrogen, and store at –80°C until use. We typically obtain 1–2 mL of high-speed cytosol from preparations of this scale. Sperm nuclei should be incubated in an aliquot of extract and stained with Hoechst as previously described to determine whether extract is effective. If active interphase extract has been prepared, nuclei should swell visibly (thicken and lengthen) within 10 min of addition to extract at room temperature.

*3.1.3. Transgenesis by Sperm Nuclear Transplantation into Unfertilized Eggs (see **Notes 1–3**)*

1. Inject two to four adult female frogs in the dorsal lymph sac with 500–800 U HCG, and incubate at 15°C for 12–16 h before transplantations.
2. Set up injection area: Coat inside of transplantation needles with Sigmacote to prevent shearing of sperm nuclei flowing through the needle (needles can be coated 10 min to several months before use). Attach approx 1 cm Tygon tubing (R-3603 1/32 in.; Fisher, cat. #: 14-169-1A) to the end of a plastic pipetman (200 μL) tip, and use the pipetman to draw up Sigmacote; then attach the other end of the tubing to the injection needle. Depress the pipetman plunger to force Sigmacote through the needle until a few drops emerge from the tip, and then release the pipetman plunger to withdraw most of the solution. Rinse needle with water before using for transplantations.
3. Adjust the transplantation apparatus. Fill a Petri dish with water, attach a needle to the tubing and micromanipulator, and establish a very low positive needle pressure through the needle. To do this, set the air flow valve to partially closed (so that it can be opened further), and then open the vacuum valve until liquid is drawn into the needle. When the needle is filled to the wide bore, partially close the vacuum until the flow is either stopped or just slightly outward owing to the partial air flow pressure. The pressure should be so low that it should not be possible to see the meniscus moving at all.

   When you are finished adjusting the system and are ready to load for transplants, put a finger over the exhaust tube to discharge the liquid from the needle, back-load the needle, and add positive pressure to the needle just slightly to begin injecting. This is done either by increasing the air flow or screwing done on the clamp fitted on the exhaust tube.
4. Fill agarose-coated injection dishes with 0.4X MMR + 6% Ficoll.
5. Set up a reaction. Sample reaction (~1:10 dilution of sperm stock): 4 μL sperm stock (~4×10^5 nuclei) and 5 μL linearized plasmid (150–250 ng/μL). Incubate for 5 min.
6. Add: 0.5 μL of an ~1:5 dilution of *Xba*I or *Not*I, 2 μL 100 m*M* $MgCl_2$ (add to 5 m*M* final at all steps to aid enzyme action), and 25 μL high-speed extract.

7. Mix the reaction by gentle pipeting (using a clipped yellow tip). Incubate for 10 min at room temperature; sperm will now be visibly swelled if diluted into Hoechst as before and observed with a 10X–20X objective.
8. While sperm are swelling in reaction mixture, collect eggs from individual frogs and dejelly them in 2.5% cysteine hydrochloride in 1X MMR (pH 8.0 with NaOH).
9. Under the dissecting microscope, inspect the eggs released from each frog for general health (eggs with even pigmentation and that remain round after dejellying). Draw the healthiest eggs into a wide-bore Pasteur pipet and transfer them to the square space in the injection dish. We generally fill the square space with eggs such that no space is left between the eggs. After about 5 min in 0.4X MMR + 6% Ficoll, the eggs will pierce easily.
10. Dilute the sperm into sperm dilution buffer (SDB) at 1:25–1:100 (such that the final dilution is 1:250–1:1000 or a concentration of 1–2 sperm/10–15 nL injection volume). For some enzymes, such as *Not*I or *Xba*I, add $MgCl_2$ to 5 m*M* to aid enzyme action.

    Before removing sperm from the stock tube or from the dilution used for injection, **always mix thoroughly** with a cut yellow tip, since sperm will rapidly settle out of the suspension.
11. Use a piece of Tygon tubing attached to a yellow tip (as previously described for Sigmacoting needles) to draw up the dilute sperm suspension and back-load the needle. Reattach the needle to the micromanipulator, and turn the air pressure up just slightly so that solution begins to flow from the needle tip (seen under the microscope as a schlearing solution of a different density). Owing to the low air pressure, solution will flow out of the needle only when the tip enters the liquid.
12. Transplant sperm nuclei into unfertilized eggs. The rate of flow should be robust enough that the needle does not reverse flow or clog with cytoplasm during injections and slow enough to be manageable. At the flow and injection rates we generally use, about 10-nL vol is delivered in each injection, so a 1:500–1:1000 dilution of the original sperm stock allows approximately one sperm to be injected in that volume. Move the needle fairly rapidly from egg to egg, piercing the plasma membrane of each egg with a single, sharp motion and then drawing the needle out more slowly. The angle of the needle should be perpendicular to the membrane surface (rather than glancing) to avoid tearing the plasma membrane.

    A hole about the diameter of the needle tip should be visible on the egg and should remain open for about 5 s after injection; when the flow is too low, the hole created in the egg by the needle instantly closes over after injection and little or no volume is delivered. When the flow is too rapid, the surface of the egg near the injection site may ripple or the site of injection may expand in size significantly. If the needle becomes clogged with cytoplasm, bring the tip to the air-liquid interface of the dish. Sometimes the surface tension of the interface removes the cytoplasm plug in the end of the needle. If a needle tip is too narrow, or if it becomes partially clogged with debris during transplantations, the injected nuclei will be damaged during transplantation, and haploid embryos will result.

Haploid tadpoles have shortened trunks and tails, are thicker than normal throughout the trunk region with a “pigeon-chested” appearance, and often have heads and tails that curl toward the dorsal side; these tadpoles will live for a while, but usually become edemic and die around the time of feeding ***(21)***.

You can determine whether your sperm dilution and the flow rate used for injections were appropriate by watching the first cleavage of the transplanted eggs. If few of the eggs received a nucleus, the frequency of cleavage will be low; one-fifth to one-third of our transplantations typically result in normally cleaving embryos. If too much volume was injected into the eggs, they may also fail to cleave; in this case, the animal hemisphere pigmentation may appear mottled or “marbleized,” or have other signs or unhealthiness owing to overinjection. Eggs that were injected with more than one nucleus will divide at the time of first cleavage abnormally into three or four (or more) cells. Many of these embryos will develop to blastula stages, but most fail during gastrulation; in some, a region of the embryo will fail to cellularize and die. Eggs injected with multiple nuclei that do gastrulate usually do so abnormally; typically, blastopore closure is incomplete, resulting in embryos that form two wings of somites and neural tissue on each side of the exposed yolky tissue lying in the center of the trunk. This type of gastrulation failure is common to stressed or unhealthy embryos (particularly embryos derived from “soft” eggs).

13. When the cleaving transplant embryos have reached the 4- to 16-cell stage, gently separate them from uncleaved eggs and move with a wide-bore Pasteur or Spemann pipet to a separate dish of 0.1X MMR + 6%Ficoll + 50 μg/mL gentamycin. We commonly culture transplanted embryos in 6- or 12-well tissue-culture dishes with about 10-embryos/well, since culturing embryos at high density can compromise their health. It is also important to remove dying embryos promptly, since they also can compromise the health of their siblings.
14. When embryos are around stage 12, media is replaced with 0.1X MMR + 50 μg/mL gentamycin without Ficoll. Because of the large needle tip used for transplantations, embryos often develop large blebs at the site of injection. These blebs occur when cells are forced out of the hole left in the vitelline membrane at the injection site, but they generally do not affect development. The blebs usually fall off on their own at the neurula or tailbud stages, but they can be removed manually once the embryos have reached the late blastula stage.

3.2. Fertilization of In Vitro Matured Oocytes by Nuclear Transplantation

1. Prime female adult *X. laevis* with 50 U PMSG, and leave at 18°C for about 48 h before isolating oocytes. Techniques for obtaining ovary tissue, isolating oocytes by manual defolliculation and culturing and injecting oocytes have been described elsewhere ***(16,22)***. Maintain oocytes in 1X modified barth’s saline (MBS) + 1 mg/mL (w/v) BSA, and inject and manipulate as desired. Alternatively, oocytes can be maintained in oocyte culture medium (OCM). Prepare fresh for each experiment. This medium is preferable if oocytes will be cultured for an extended

period (>24 h) before maturation. Transfer defolliculated oocytes to fresh media, and change these media several times to remove traces of yolk and debris.

2. Prepare sperm nuclei as previously described (**Subheading 3.1.1.**)
3. Add 1–5 µm progesterone to oocytes maintained in MBS + 1 mg/mL BSA to begin maturation.
4. Determine when sperm nuclei should be transplanted. A general rule to follow is that oocytes should be ready for fertilization in about 2X the amount of time taken to get from progesterone addition to germinal vesicle breakdown (GVBD; appearance of white spot in the animal hemisphere). We typically add 5 µm progesterone in the evening after defolliculating oocytes (5–7 PM), incubate oocytes at 18°C overnight and during the next day, and inject sperm 20–25 h after progesterone addition (*see* **Note 2**).

   The most common mistake made is not allowing oocytes sufficient time after maturation before injecting the sperm nuclei. Oocytes must be able to respond to pricking by a needle with a vigorous cortical contraction before sperm are transplanted, or no development will occur. Even after oocytes first become responsive to pricking, they are probably not fully competent to support embryonic development immediately and should be incubated an additional 3–4 h at 18°C. Since there is probably quite a bit of variability between batches of oocytes from different frogs and between frogs from different colonies, the optimal timing should be determined by prick-activating a small number of test oocytes at several times during the incubation period to determine when they become responsive.
5. Dilute and transplant sperm nuclei as described in the transgenesis protocol. There is no need to swell the nuclei in interphase extract. We have used slightly lower dilutions of sperm than are used for transgenesis for this protocol (such that two to three sperm may be deposited into some eggs) and have done these injections in 0.4X MMR without Ficoll. Use a 40–60 µm wide needle tip to transplant the sperm as described for transgenesis. When successful, oocytes should pierce very easily for injection, and membrane texture should not seem at all rubbery. There should be a normal cortical contraction in the animal hemisphere after activation, and the injected, matured oocytes (eggs now) should look and later cleave like fertilized eggs. When testing this method, approx 25% of the in vitro matured oocytes developed into blastula-gastrula stages. Of these, the majority developed into tadpoles, and were apparently morphologically normal and raised for months.

4. Notes

4.1. Factors Affecting the Success of Nuclear Transplantation-Based Transgenesis

1. Egg quality is a major factor that contributes to the level of postgastrula development, which is obtained from sperm nuclear transplantation. To obtain good postgastrula development, eggs must be generally healthy. In particular, they should have even pigmentation and should be firm enough to hold their shape well after dejellying. In addition, it is important that they do not become acti-

vated before they are injected with nuclei. When egg quality is poor, a fraction of the embryos will show morphogenetic defects, resulting in incomplete blastopore closure during gastrulation. This problem is often compounded by expressing genes at high levels during the gastrula stages. Therefore, embryos expressing genes from the CMV promoter are more likely to show nonspecific gastrulation defects than embryos expressing genes from strong promoters that are turned on after gastrulation.

2. Transplantations should be performed and eggs incubated after transplantation at temperatures no higher than 22°C. We have found that transplantation and early incubation of activated eggs at elevated temperatures (24-25°C) lowers the frequency with which plasmids are expressed in batches of nuclear transplantation-derived embryos. Embryos in these batches also frequently express plasmids in only one-quarter to one-half of the expected cells. We believe that acceleration of the first cell cycle, which occurs at 24–25°C, may give these embryos inadequate opportunity to integrate introduced plasmids prior to first cleavage, thus resulting in more chimeric and nonexpressing embryos.
3. It may be important to note that although this technique is very efficient and workable, it involves several steps; all of which are critical for its success. Therefore, we suggest that anyone trying to learn the technique does so in steps, rather than all at once. For example, one should first learn to isolate sperm nuclei and transplant them into eggs. Once this can be done successfully, resulting in normal development, then one can determine whether sperm nuclei, swollen in extracts, gives normal development. If swelling of sperm in extract has no adverse effects on the level of development obtained after transplantations, one can add plasmid and enzyme to the reaction, thus reconstituting the whole transgenesis procedure.
4. Dilution drastically changes the pH of HEPES, making it impossible to pH the stock directly.
5. For each sperm nuclei prep, it is most convenient to make about 30 mL of 2X NPB form stock solutions. Then use this 2X stock to make all subsequent solutions (i.e., 1X NPB + protease inhibitors, 1X NPB + 3% BSA, and so forth).

4.2. The Frog as a Mouse

6. The transgenesis procedure described here compares very favorably with those developed for mouse or zebrafish. Embryos expressing plasmids nonmosaically can be obtained in high numbers directly, and since the embryos are not chimeric, breeding of animals is not required. In addition, the cost of studies involving transgenic frog embryos will be considerably lower than that required for similar studies in mammals. In fact, the frog may prove useful for the study of regulation of mouse promoters. Limited studies have shown that mouse promoters are regulated appropriately in other systems and vice versa ***(23–28)***. Therefore, initial promoter mapping and analysis may be done more easily and effectively in the frog than in the mouse.

4.3. Future Prospects in the Frog: Knockouts and Genetics

7. Except for studies where gene function has been inhibited by the expression of dominant negative mutations ***(29–31)*** or maternal mRNAs where degraded following injection with oligonucleotides ***(12–15)***, it has been difficult to inhibit the function of genes in the early embryo specifically. In the future, we also hope to combine transgenesis with antisense ***(32–34)*** and ribozyme ***(35–37)*** technologies in order to deplete specific gene products from *Xenopus* embryos.

   The advantages of the frog system are numerous, but one major disadvantage is that it has not been exploited at the genetic level. The method for transgenesis we have developed can be adopted for an insertional mutagenesis scheme. Since *Xenopus laevis* is pseudotetraploid and has a long generation time, we suggest using *Xenopus tropicalis*, which is diploid and has a generation time of around 4–6 mo ***(38)***. For similar reasons, *Xenopus tropicalis* will also be the species of choice for doing targeted mutations.

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