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Transposon transgenesis in *Xenopus*

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Abstract

Transposon-mediated integration strategies in *Xenopus* offer simple and robust methods for the generation of germline transgenic animals. Co-injection of fertilized one-cell embryos with plasmid DNA harboring a transposon transgene and synthetic mRNA encoding the cognate transposase enzyme results in mosaic integration of the transposon at early cleavage stages that are frequently passed through the germline in the adult animal. Micro-injection of fertilized embryos is a routine procedure used by many laboratories that use *Xenopus* as a developmental model and, as such, the transposon transgenesis method can be performed without additional equipment or specialized methodologies. The methods for injecting *Xenopus* embryos are well documented in the literature so here we provide a step-by-step guide to other aspects of transposon transgenesis, including screening mosaic founders for germline transmission of the transgene and general husbandry considerations related to management of populations of transgenic frogs.

Keywords

Sleeping Beauty; *Tol2*; transposon; transgenesis; *Xenopus laevis*; *Xenopus tropicalis*

1. Introduction

The aquatic frog *Xenopus* has been a favored model for developmental and cell biology studies for more than five decades. The rapid external development of the early embryo, combined with the large clutch size and ability to induce ovulation throughout the year are important factors that make this model ideal for experimental manipulation. The entire life cycle of *Xenopus* is spent in an aquatic environment, and thus these frogs adapt well to the laboratory and husbandry is relatively inexpensive. The large size of the *Xenopus* oocyte is significant for the harvest of cell-free extracts for biochemical studies and ectopic expression of genes by micro-injection of nucleic acids into oocytes or fertilized eggs is comparatively simple. The most commonly used species of frog used in laboratories throughout the world is the South African Clawed frog, *Xenopus laevis*. While this model species is ideal for early embryological and biochemical studies, genetic manipulation has not been exploited to its fullest potential due to the long generation time (greater than one year) and the pseudo-tetraploid nature of the *X. laevis* genome. A close relative of *X. laevis*, the West African Clawed frog, *Xenopus tropicalis*, is better suited for genetic manipulation as this species is diploid and has a comparatively short generation time (~ six months). The two species share the biological features that have made *X. laevis* an important developmental model system, and the potential

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for genetic manipulation of *X. tropicalis* brings powerful genetics tools to this highly tractable developmental model. An important step in developing *Xenopus* as a genetic model system is the developing strategies to generate transgenic animals that transmit exogenous DNA through the germline. Several strategies have been developed over the last three decades to transform the *Xenopus* genome (Table 1).

1.1. Transgenesis by micro-injection of linear plasmid DNA

The first strategy to produce germline transgenic *Xenopus laevis* was reported more than twenty-five years ago and used a simple linear plasmid DNA micro-injection strategy (1). Etkin and Pearman injected a linear plasmid encoding the SV40 early promoter driving expression of a chloramphenicol acetyl transferase (CAT) reporter into one-cell fertilized embryos and tracked the persistence of the injected DNA throughout development. They estimated that genomic integration of the transgene occurred in approximately 5-10% of the injected animals where the exogenous DNA persisted (~60% of the total injected population). Germline transmission of the transgene was reported in one male out of three that were examined. The progeny of the single transgenic male had different integration events indicating that the germline of the founder was mosaic and different sperm had discrete integration events. Thus, the estimated transgenesis rate using this procedure may be as high as 1-3% (1). We have recently used the same strategy in *Xenopus tropicalis* and have achieved germline transmission in approximately 1% of embryos injected with linear DNA at the one-cell stage (manuscript in preparation). The non-Mendelian frequency of transgenic progeny that we observed in the F1 generation indicates that, as reported by Etkin and Pearman, the germline of the founder frogs is mosaic.

1.2. Restriction enzyme-mediated integration (REMI)

An important advance in *Xenopus* transgenic technology was introduced by Kroll and Amaya with the development of restriction enzyme-mediated integration (REMI) combined with nuclear transplantation (2,3). In this method, decondensed sperm nuclei are manipulated *in vitro* and then micro-injected into unfertilized eggs. The injection procedure activates the egg and normal diploid embryos are produced that express the transgene. As the plasmid integration event occurs very early in development of the nuclear transfer-derived embryos, the resulting animals are also less mosaic than those produced by the linear plasmid injection strategy. Approximately 2-15% of embryos generated by the nuclear transfer technique develop normally and stably express the transgene (3). Modifications to the standard nuclear transfer-REMI technique that simplify the procedure by eliminating the use of restriction endonuclease and sperm decondensation have been reported (4,5). A potential issue with using this strategy in generating transgenic lines for multi-generational studies is that the *in vitro* manipulation of the sperm nuclei may result in chromosomal damage and subsequent mutagenesis.

1.3. I-SceI Meganuclease-mediated integration

The meganuclease method is a variation of the classical linear plasmid integration strategy. The approach uses co-injection of a linearized plasmid transgene that has an engineered meganuclease site(s) together with the meganuclease enzyme (6). The presence of the 'very rare cutter' restriction endonuclease maintains the linearized plasmid in a non-concatamerized state that is thought to promote integration of the plasmid transgene randomly into the genome. The stringency of the *I-SceI* meganuclease enzyme is so high (18 bp recognition sequence) that even complex vertebrate genomes are unlikely to contain many, if any, precise meganuclease sites and thus the target genome is left intact. The Grainger laboratory has reported very efficient transgenesis in *Xenopus tropicalis* (30%) and *Xenopus laevis* (20%) using the meganuclease method (7,8). In addition to generating germline transgenic frogs for

multi-generational experiments, this technology has also been used to study cis-acting regulatory elements in the founder (P_0) generation (9).

1.4. Bacteriophage integrase-mediated integration

The bacteriophage phiC31 infects *Streptomyces* species and integrates its 41.5 kb genome by site-specific recombination using an integrase enzyme encoded by the phage DNA (10). In the endogenous setting, the phage integrase requires a 39 bp sequence in the phage genome (*attP*) and a 34 bp sequence in the bacterial genome (*attB*) for recombination to occur. The precise sequence of the *att* sites is not essential for integration, however changes in the *att* sequences result in decreased recombination activity catalyzed by the integrase. The Carlos laboratory showed that phiC31 integrase can catalyze the stable, non-random integration of a plasmid containing an *attB* site into mammalian genomes (11-13). Integration of the plasmid occurred at specific sites, 'pseudo-*attP* sites', in the genome that shared sequence similarity to the endogenous *attP* site in the phiC31 phage. As the phiC31 integrase does not require host factors to catalyze the integration event, this strategy can be used to integrate *attB*-containing plasmids into any genome that has a 'pseudo-*attP*' site. Allen and Weeks have recently demonstrated that this method can be used to integrate single copy transgenes into the *Xenopus laevis* genome (14-16). Southern blot and GFP expression analysis of stage 46 embryos indicated that integration had occurred in 25-35% of tadpoles, however, the frequency of germline transmission of the integrase-mediated transgenics has yet to be determined.

1.5. Transposon-mediated integration

The class II DNA 'cut and paste' transposons are mobile DNA elements that have been used for a variety of transgenesis applications. These naturally occurring mobile elements consist of a transposase enzyme that recognizes terminal repeat elements that flank a cargo sequence that, in the autonomous element, encodes transposase. As the random excision and reintegration of these elements in the host genome may result in deleterious mutations, transposable elements are under considerable selective pressure and generally exist in the genome as inactive remnants. To be useful as tools for manipulating the genomes of model organisms, the autonomous elements have been engineered to replace the transposase-encoding sequence with a variety of functional sequences, such as fluorescent or enzymatic reporters. The resulting non-autonomous transposons require the transposase enzyme to be supplied *in trans* for excision and reintegration to occur. Transposons have been used extensively in invertebrate systems for many years and have recently been applied to vertebrate transgenesis applications. A major advantage of transposon-mediated transgenesis is that the transgene is incorporated into the host genome without the addition of the bacterial plasmid backbone that may cause transgene silencing. Another significant advantage is that once integrated into the host genome, the transposon is now a substrate for excision and remobilization to a new site in the genome. This feature can be exploited in 'transposon hopping' strategies to generate novel transgenics or for mutagenesis screens. In the case where the primary integration event is mutagenic, remobilization can be used to rescue the phenotype. Another significant advantage of the transposon system is that the precise integration of the mobile element provides an anchor for PCR-based cloning strategies to rapidly determine the genomic sequence flanking the integration event. The cloned flanking sequence can then be used to query genome sequence databases to determine the position of the integration event in the genome and what genes are nearby.

We, and others, have used two transposable elements to modify the *Xenopus* genome. Transposons efficiently integrate into the frog genome and, in the absence of transposase activity, the integration events are stable in the genome for multiple generations (17-21). Like the phiC31 integrase system, transposon-mediated integration strategies generally use synthetic mRNA as a source of the transposase enzyme to catalyze the reaction. As such, the

resulting transgenic founder is likely to be mosaic as during the time it takes to translate sufficient enzyme for catalysis, the rapidly developing embryo may have proceeded to early cleavage stages before integration occurs. The stochastic integration of the transposon in discrete blastomeres may result in a highly mosaic founder animal.

1.5.1. Sleeping Beauty—The *Sleeping Beauty* transposon belongs to the *Tc1/mariner* family of transposable elements and was molecularly reconstructed from an inactive element found in Atlantic Salmon (22). The *Sleeping Beauty* (SB) transposon system has been used extensively in vertebrate cells and model systems for transgenesis, insertional mutagenesis and gene therapy (23-33). In *Xenopus*, co-injection of synthetic mRNA encoding the *Sleeping Beauty* transposase with a plasmid harboring a SB transposon results in efficient integration of the transposon transgene into the genome (19-21). The integration events catalyzed by the co-injection strategy results in enzyme-dependent non-canonical transposition reactions where the SB transposon is present in the genome as small-order concatamers that includes some plasmid vector sequence. Despite the non-canonical transposition mechanism observed using the co-injection strategy, the SB transposon system is efficient with germline transgenesis rates of ~30% observed. While the co-injection strategy results in non-canonical integrations, re-expression of the SB transposase catalyzes canonical transpositions upon remobilization (manuscript in preparation).

1.5.2. Tol2—*Tol2* is a member of the *hAT* (*hobo* from *Drosophila*, *Ac* from maize, *Tam* from the snapdragon) family of class II DNA transposable elements and was the first active non-autonomous element identified in a vertebrate species (34-37). *Tol2* was identified due to integration of the mobile element into the tyrosinase gene of the Medaka fish that resulted in an albino phenotype. The *Tol2* transposon system has been used extensively in fish and mammalian systems for transgenesis and gene- and enhancer-trapping strategies (38-45). In *Xenopus*, we have shown that the co-injection of *Tol2* mRNA together with a plasmid harboring a *Tol2* transposon results in efficient transposition of the frog germline (18,46). Shibano and colleagues demonstrated that co-injection of *Xenopus* one-cell embryos with a *Tol2* plasmid with purified *Tol2* protein also resulted in efficient transposition (47). The advantage of injecting purified protein is that enzyme is available immediately to catalyze transposition. Unlike the transposition events generated by the *Sleeping Beauty* co-injection strategy, *Tol2*-mediated transgenesis results in canonical transposition of the *Tol2* transposon into the frog genome (18,46). *Tol2* transposons are stable in the *Xenopus tropicalis* genome and are passed onto progeny at the expected Mendelian frequencies following the F₁ generation. We have recently demonstrated that *Tol2* transposons integrated into the *Xenopus tropicalis* genome are substrates for remobilization. Micro-injection of *Tol2* mRNA into one-cell embryos derived from the outcross of a *Tol2* transgenic frog with a single transposon integration event result in very efficient somatic remobilization of the target transposon: 100% of injected tadpoles had at least one remobilization event in the soma (48). As demonstrated for the co-injection strategy used to generate the founder lines, the remobilization of a *Tol2* transposon resident in the *Xenopus* genome resulted in mosaic animals. Outcross of the ‘remobilized’ frogs demonstrated that the re-integrated *Tol2* transposons are passed through the germline. Although the parental ‘donor’ line had a single integration event, the progeny frequently inherited multiple copies of the transposon indicating that the excision and re-integration events were occurring early in development during the cell cycle, after DNA synthesis but prior to mitosis (48). The demonstration that *Tol2* transposons can be remobilized in the genome is an important step in developing ‘transposon hopping’ strategies in the frog (see ‘*Transposon Hopping*’ in *Xenopus*, section 1.5.4).

1.5.3. Other transposable elements—There are several transposable elements that have been used in other systems that could potentially be applied to the *Xenopus* model. For example,

the *piggyBac* transposon, isolated from the cabbage looper moth *Trichoplusia ni* (49), has been used for transgenic applications in insects and mammals (50-53) and is likely to be useful in the frog as mammalian cell culture studies indicate that the *piggyBac* transposase is more active than either *Sleeping Beauty* or *Tol2* (54). The *Frog Prince* transposon was reconstructed from an ancient inactive transposable element in the frog, *Rana pipiens* (55). This amphibian-derived transposable element may be particularly useful in *Xenopus* as any host factors that may be required for maximal transposition activity are likely to be well conserved between *Rana* and *Xenopus*. The application of multiple transposable elements to the *Xenopus* system will provide valuable molecular tools for genetic applications. For example, transgenic frogs that express a particular transposase enzyme, for ‘transposon hopping’ strategies, can be generated using a different mobile element so that subsequent expression of the integrated transposase will not result in ‘self-excision’.

1.5.4. ‘Transposon Hopping’ in *Xenopus*—‘Transposon hopping’ strategies have been used in fish and mammals for gene- and enhancer-trap screens and cancer gene discovery (45,56-59). The use of double transgenic animals that harbor transgenes for both the enzyme and substrate components of the transposon system dramatically increases the efficiency of ‘transposon hopping’ strategies, as it eliminates the time-consuming micro-injection step and replaces it with a simple breeding strategy. The frog offers some unique advantages for ‘transposon hopping’. First, the high fecundity of *Xenopus tropicalis* routinely provides more than 2000 embryos in each outcross. Second, the long lifespan of the frog, of up to two decades, means that founder frogs are available for many years for embryo collection. Finally, the exquisite embryological features of *Xenopus* make this vertebrate model an ideal candidate for large-scale gene- and enhancer-trap screens and insertional mutagenesis strategies.

2. Micro-injection of fertilized *Xenopus* embryos

The strategies for micro-injecting *Xenopus* fertilized eggs has been well documented in the literature (46,60) and the precise methodology will depend on the type of equipment available in each laboratory. In this section we outline some general methods that we have found useful in our laboratory.

2.1 ‘Batch’ breeding for fertilized egg collection

The most common method used for obtaining eggs for micro-injection is to *in vitro* fertilize expressed eggs with a suspension of sperm harvested from a sacrificed male. We have found that natural matings produce consistently high-quality fertilized eggs and the males can be re-used after a brief rest period (10-14 days). We routinely inject embryos four days per week and at an average cost of ~\$30 per adult *Xenopus tropicalis* male, reusing males by natural breeding also makes fiscal sense. Natural matings are also indicated where transgenic males are used to generate progeny for micro-injection experiments, or other studies, as this strategy allows the animals to be outcrossed many times.

2.1.1. Egg collection for micro-injection—The evening before embryo collection, three female frogs are ‘pre-primed’ by injecting 20 units of human chorionic gonadotrophin (HCG; Novarel, Ferring Pharmaceuticals Inc., Parisippany, NJ.) into the dorsal lymph sac located just above the hind leg and placed in a static tank. The following morning, the females are injected again with approximately 200 units of HCG to stimulate egg laying and placed in a 13.25 L polycarbonate tank (45 × 30 × 15 cm; Rubbermaid Commercial Products, Winchester, VA.) containing system water. Three adult male frogs are injected with approximately 100 units of HCG and added to the tank. The three pairs of frogs are left undisturbed for approximately two to three hours. Egg laying will commence approximately thirty minutes after the males grasp the females. Eggs can be gently removed from the tank by collecting them on a small circular

nylon mesh screen (Spectramesh PP500, polypropylene mesh with 500 μ m openings; Spectrum Laboratories Inc., Rancho Dominguez, CA.) cut to fit into a small crystallizing dish (Kimax No. 23000, 90 $\times$ 50 mm). The jelly coat on the eggs will adhere to the mesh and after sufficient embryos have been harvested the mesh circle is placed in a small glass crystallizing dish containing 3% (w/v) cysteine (L-cysteine, non-animal source, Sigma Aldrich, St. Louis, MO.) in system water (pH 8.0). It is important to use L-cysteine (free-base) for dejellying *Xenopus tropicalis* embryos rather than L-cysteine hydrochloride 1-hydrate that is commonly used with *Xenopus laevis*. The free-base form requires less sodium hydroxide to bring the pH of the reducing solution to pH 8.0 and, as *Xenopus tropicalis* embryos are sensitive to high salt concentrations, limiting the amount of sodium hydroxide added to the solution keeps the final salt concentration at acceptable levels. The reducing agent removes the jelly coat from the eggs and after approximately three minutes with gently swirling the mesh circle can be removed free of eggs. The dejellied eggs are then rinsed three times with fresh system water and transferred to a viscous solution of 3% (w/v) ficoll (Ficoll PM 400, Sigma Aldrich, St. Louis, MO.) in 0.5 $\times$ MMR (10 $\times$ Marc's Modified Ringers (10 $\times$ MMR); 1 M NaCl, 20 mM KCl, 10 mM MgSO₄, 20 mM CaCl₂, 50 mM HEPES pH 7.8 and 1 mM EDTA). The viscosity of the ficoll solution helps support the eggs during the injection procedure. Eggs can continue to be collected from the mating tank for several hours. As the eggs are injected, embryos that have already reached early cleavage stages are removed and only single cell embryos are selected for injection.

2.1.2. Micro-injection of *Xenopus tropicalis* one-cell embryos—Embryos in ficoll are transferred to a siliconized glass microscope slide for micro-injection. Embryos are injected with 3 nL of solution containing the desired RNA and/or plasmid DNA. As plasmid DNA is quite toxic to the early *Xenopus* embryo, we limit the amount of injected plasmid DNA to ~50-75 pg. For a ~6 kb plasmid, this equates to ~10 million copies, and as such, is likely in vast excess. If problems with toxicity are encountered, lowering the amount of injected plasmid DNA can be attempted to improve survival. Synthetic mRNA is tolerated at much higher doses than plasmid DNA and we routinely inject 50-250 pg of mRNA encoding the transposase enzyme. Although we have not performed exhaustive dose-response curves for injected plasmid DNA and mRNA, we have observed similar transposition frequencies over the stated range of injected nucleic acids. Large petri dishes (150 mm) are lined with a thin layer of 1% (w/v) agarose and filled with sterile frog system water. The injected embryos are transferred to the agarose-lined plates (~50 embryos per plate in ~50 ml of sterile frog system water) and left undisturbed overnight at 28°C. The following morning, the hatched embryos are transferred to large crystallizing dishes (Kimax 190 $\times$ 100 mm) containing ~2 L frog system water and maintained at 28°C. As the tadpoles grow, they are transferred to larger polycarbonate tanks for rearing to adulthood (see *Tadpole husbandry*, section 3.2.). Transposon-mediated transgenesis results in highly mosaic founder animals (18,19,21), and as such pre-sorting injected embryos based on reporter expression may result in elimination of the germline transgenic founders that do not display robust somatic expression of the transgene. As such, we routinely do not screen the injected embryos for reporter expression, but rather raise all of the injected embryos and determine germline transmission of the transposon transgene by outcross (see *Scoring F₁ tadpoles for transgenic reporter expression*, section 3.2.1.).

2.1.2.1 Preparation of plasmid DNA for micro-injection: We use standard 'midi-prep' scale columns for plasmid isolation for embryo injection (Qiagen Plasmid Midi kit; Qiagen, Valencia, CA.). Plasmid DNA is further purified by phenol:chloroform:isoamyl alcohol (25:24:1 v/v/v) extraction, ethanol precipitation and resuspension in DNase/RNase-free water.

2.1.2.2. Preparation of synthetic mRNA for micro-injection: Messenger RNA is *in vitro* transcribed from linearized plasmid DNA using mMessage mMachine kits (Ambion, Austin,

TX.) according to the manufacturers instructions and resuspended in sterile DNase/RNase-free water. We do not recommend using the LiCl method for purification of the final mRNA product as residual LiCl in the injection mix may interfere with normal development of the embryo. After determining the concentration of the mRNA spectrophotometrically, and verifying the integrity of the RNA by gel electrophoresis on denaturing gels, the mRNA is divided into single-use aliquots and stored at -80°C. A fresh aliquot of mRNA is used for each injection experiment and samples are not refrozen.

3. Husbandry

3.1. Frog Husbandry

3.1.1. Water conditions and feeding schedule—The adult *Xenopus tropicalis* are housed in a recirculating aquatic system that is maintained at 26°C; marine salts are added by an automated dosing pump to a final conductivity of 950 micro Siemens (µS). The pH is likewise maintained at pH 7.0 by automated dosing with sodium bicarbonate. Water flowing from each tank is filtered through 21 µm screens and dumped into a central reservoir containing a biofilter to remove nitrogenous waste, then filtered through 20 µm cartridge and activated carbon filters. Following UV sterilization, the water is pumped back to the tanks. The flow rate is adjusted at each tank to achieve approximately two to three complete water changes per hour. Each day, 10% of the total volume of water in the frog system is replaced with fresh reverse osmosis (RO) purified water. The adult frogs are fed three times per week with NASCO frog brittle (Small Nuggets) for Post-Metamorphic *Xenopus* (Nasco, Fort Atkinson, WI, USA). As each tank may contain a different number of frogs, the amount of food added to each tank varies so that the frogs will clear the tank in ten to fifteen minutes. If the animals eat faster than this, then more food is added. If food is uneaten after twenty minutes, the excess is removed with a net.

3.1.2. Colony management and tracking individual frogs—Even in a moderately sized colony, identification of individual animals is a significant challenge. We have adopted two methods for identifying individual frogs. The most simple is individual housing – one frog per tank. The obvious limitation of this strategy is the inefficient use of space. Another important consideration is that frogs housed individually for extended periods of time often fail to thrive compared to siblings maintained in group housing. This is likely due to the environmental enrichment provided by the ‘tank-mates’. This can be overcome in part by housing, for example, an identified male with a small group of female frogs. Alternatively, a transgenic frog harboring a fluorescent reporter can be housed with wild type animals and the transgenic animal can be re-identified by inspecting the frogs for fluorescent reporter expression. Our preferred method is to implant an RFID (radio frequency identification) microchip under the skin of adult frogs (61). This procedure is simple, fast and inexpensive.

3.1.2.1. Implanting chip ID tags in *Xenopus tropicalis*

1. Adult *Xenopus tropicalis* frogs are anaesthetized using isoflurane (1-chloro- 2, 2, 2-trifluoroethyl difluoromethyl ether; Forane, Baxter Healthcare Corporation, IL, USA) (62). For the brief period of time that the frogs need to be anaesthetized for this procedure, we find that isoflurane is superior to other anesthetics commonly used in frogs such as tricaine methane sulphonate (M.S. 222). Isoflurane is faster acting and the recovery time is also faster than that observed with tricaine. Briefly, in a fume hood, the bottom of a 1 liter lidded jar is lined with a paper towel soaked in frog system water and 0.5 ml of Forane is added dropwise to the towel. A frog is added to the jar and anesthesia, determined by the lack of a righting response and a lack of reaction to external stimulus such as a toe pinch, is reached in 10-40 seconds. A lid is placed

on the jar to prevent escape during a potential, albeit brief, excitation phase before anesthesia is reached.

2. Once the frog is anaesthetized, it is removed from the jar and placed on a pre-wetted paper towel and a small (~3 mm) incision is made on the dorsal midline at the base of the head with surgical scissors. A microchip, *mic3*-TAG 64 bit RO (Read Only) embedded in inert resin (microSensys, Germany; www.microsensys.de), is sterilized by soaking in 70% (v/v) ethanol and implanted with forceps under the skin. The small wound does not require sutures or surgical glue and heals rapidly. The frog is then placed in an 8 L tank with approximately 2 cm of frog system water and allowed to regain consciousness. The tank is propped up on a small angle so that the frogs' nose is above the waterline. The animal should be observed frequently during the recovery phase (usually less than fifteen minutes).
3. After the animal has recovered, the tank is filled with 4-5 L of system water and the frog is kept in isolation for several days, with daily water changes, until the wound has completely healed. Before adding the frog back to the aquarium, the presence of the microchip is verified using a handheld reader.

The implanted chip may, over time, move around under the skin on the dorsal surface of the body, however, it is usually simple to find as it causes a small bump. The small chip tags have a short reading range and it is necessary to place the reader directly above the chip in order to read the identification code. The chip can be rescanned at any point in the future by simply placing the frog in a tank with 1-2 cm of system water so that the dorsal surface of the animal is exposed and the position of the chip can be readily identified and read. The unique 16-symbol alpha-numeric code provides a convenient identifier for each tagged frog and information on each animal can be stored in a database for rapid retrieval. In our laboratory, we have developed a simple web-based database that can be accessed from any device with Internet connectivity.

3.1.3. Outcrossing transgenic frogs—As described above, we use natural matings to produce tadpoles. When the resulting progeny is not needed for micro-injection, or for observation of tadpoles at early cleavage stages, natural matings are routinely set up late in the afternoon and allowed to proceed overnight. Female and male frogs are ‘primed’ with 200 units and 100 units, respectively, of HCG and placed in pairs in a 7.5 L square polycarbonate tank (8-3/4" × 8-3/4" × 9"; Carlisle square food container) containing 5 L of frog system water. The tanks are covered with a lid and kept at 25°C overnight. The following morning, the adult animals are removed from the tank, the fertilized eggs are rinsed twice with 5 L of fresh frog system water and transferred to a 28°C room for growth (see *Tadpole Husbandry*, section 3.2.). Male frogs can be outcrossed every ten to fourteen days and female frogs regenerate eggs in approximately eight to ten weeks.

3.2. Tadpole Husbandry

Tadpoles are raised in static tanks containing frog system water that is drawn from a recirculating system. *Xenopus tropicalis* tadpoles are kept in a warm room at 28°C and are fed three times per day with a suspension of Sera Micron powdered food (Heinsberg, Germany) in system water. The Sera Micron suspension is prepared in a 250 ml plastic squeeze bottle to facilitate feeding. The tadpoles are housed in large polycarbonate tanks at a density of 5-10 tadpoles per liter of water. For maximal growth rates, one-half of the water volume is removed each week and replaced with fresh frog system water. An air stone is added to each tank to improve oxygenation by gentle bubbling. Approximately two weeks after metamorphosis (~six weeks post fertilization), the young froglets are moved into the recirculating frog system. We have observed that moving post-metamorphic animals to the adult system too soon after metamorphosis results in a significant loss of young animals. Post-metamorphic frogs are fed

daily with Zoo Med Aquatic Frog & Tadpole Food Micro Pellets (Zoo Med Laboratories, San Luis Obispo, USA).

3.2.1. Scoring F_1 tadpoles for transgenic reporter expression—Embryos injected with a cocktail of transposon substrate plasmid and mRNA encoding the transposase enzyme are reared to adulthood and outcrossed to determine germline transmission of the transgene and to establish transgenic lines for further study. Where fluorescent reporters are expressed under the control of strong promoters that drive widespread expression of the transgene, the simplest method for screening the F_1 tadpoles is direct observation under a dissecting fluorescent microscope. Screening large populations of tadpoles individually is usually not practical as it may take several hours to screen a single moderately-sized outcross of 2,000 tadpoles. We use two methods for screening large populations of tadpoles; visual inspection of groups of tadpoles and PCR analysis of pooled genomic DNA samples.

3.2.1.1. Batch scoring tadpoles for transgenic reporter expression: Tadpoles can be screened efficiently in small batches of ten to twenty animals. A tank of tadpoles (~stage 40 (63)) is transferred to a small crystallizing dish containing frog system water and 0.015% (w/v) tricaine methane sulphonate. Small groups of tadpoles are placed in each well of nine-well glass plates (Pyrex, Spot Plate, 9 Depression, 85 × 100 mm) and scored for fluorescent protein activity under a dissecting fluorescence microscope. Each group is counted under bright-field view for total numbers and under fluorescence illumination so that the frequency of transgenic tadpoles in the outcross population can be determined. Groups containing transgenic tadpoles can be rapidly resorted and selected individuals transferred to fresh system water without anesthetic to regain mobility.

3.2.1.2. Genomic PCR analysis for the presence of the transgene in F_1 progeny: In some cases, screening large numbers of tadpoles for reporter expression is not practical. For example, when the transgene drives low-level expression of the reporter or drives reporter expression in a small subset of cells in the embryo, analyzing large populations for reporter expression may be very time consuming. Likewise, when many founders are being screened at one time, observing thousands of F_1 progeny from each founder is not practical. In these cases, we use PCR genotyping of populations of F_1 tadpoles to identify founders. Genomic DNA is prepared from pools of F_1 tadpoles and PCR primers specific for the transgene are used to amplify each sample. Using known transgenic and control wild type tadpoles, we have determined that we can robustly identify a single transgenic tadpole in a pool of 100 tadpoles using the genomic PCR strategy.

3.2.1.2.1. Preparation of genomic DNA from pools of F_1 tadpoles: Progeny at swimming tadpole stage (~stage 37-40) are anesthetized with tricaine (0.015% (w/v) in system water), pooled into groups of 100 and placed in 15 ml polypropylene tubes. Excess liquid is removed from the tube with a transfer pipette and the tadpoles are resuspended in 2 ml DNA extraction buffer (20 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% SDS) containing 0.1 mg/ml proteinase K (recombinant PCR grade, Roche Diagnostics GmbH, Mannheim, Germany) and digested overnight at 56°C. Excess RNA is removed (optional) from the samples by adding 1,000 units of RNase A (Sigma Aldrich, St. Louis, MO.) and digesting at 37°C for one hour. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 (v/v/v)) is added to each sample at room temperature and mixed gently for five to ten minutes on a nutator (Adams Nutator Mixer). After centrifugation, the aqueous upper phase is transferred to a 1.5 ml tube and DNA is precipitated on ice by adding a one-tenth volume of 3 M sodium acetate pH 5.2 and equal volume of isoamyl alcohol. After five minutes on ice, the DNA is recovered by centrifugation (16,110 × g, 10 minutes at room temperature) and washed with 0.5 ml of 70% ethanol. After centrifugation (16,110 × g, 5 minutes at room temperature), the ethanol is

removed by aspiration and the pellet is air-dried for five to ten minutes. The DNA is resuspended in 200 µl sterile water overnight at 4°C. The overnight resuspension is important to ensure that the entire sample is hydrated and in solution. The DNA concentration is determined by absorbance at 260 nm.

3.2.1.2.2. PCR screening: Genomic DNA prepared from pools of tadpoles is used in PCR reactions with primers specific to the transposon transgene. A set of control primers, designed to amplify a defined region of genomic sequence is used as a control for DNA recovery and must be included for every sample. Each PCR reaction contains 100 ng of genomic DNA template and 25 pmoles of each primer. We routinely use HotStar Taq polymerase for genomic PCR analysis and follow the instructions recommended by the supplier (Qiagen, Valencia, CA). PCR amplification is as follows: 95°C, 15 minutes to activate the polymerase and denature the sample DNA, followed by 35 cycles of 95°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds. A final extension of five minutes at 72°C is added at the end of the 35th cycle. The annealing temperature will vary depending on the primer set used. We typically design primers with an annealing temperature of ~55°C so that different reactions can be run simultaneously in the same PCR machine. PCR products are resolved on 1.2% (w/v) agarose gels in 1× Tris acetate EDTA buffer containing 0.2 µg/ml ethidium bromide. Using this method, many thousands of tadpoles can be screened in several hours. This method will also give an estimate of the transposition frequency in a single founder. For example, if a single pool out of a total of twenty pools of 100 tadpoles each shows a PCR product with the transgene-specific primers, then the frequency of transgenic progeny from that founder will be approximately one transgenic tadpole per 2,000 progeny. While this is an estimate of the transgenesis frequency, the data is useful for determining how many progeny from subsequent outcrosses of a given founder will need to be screened visually to identify the transgenic progeny. Positive pools of genomic DNA can also be analyzed using EPTS LM-PCR to clone the integration site(s) of the transposon transgenes (46). Frequently, a round of nested PCR amplification will be necessary to amplify the specific integration site when performing EPTS LM-PCR from a pooled genomic DNA sample.

4. Anticipated results with transposon-mediated transgenesis

4.1. Transgenesis rates

We have observed similar rates of transgenesis when using either the *Tol2* or *Sleeping Beauty* transposase systems with germline transgenic frequencies ranging from 20-30% or more. As the integration events are occurring randomly during early cleavage stages, the founder animals are mosaic for the transposon transgenes. Consequently, the frequency of germline transgenics observed in the F₁ generation does not usually correspond to the expected Mendelian frequencies for dominant alleles. In rare instances, the frequency of transgenic animals in the F₁ generation can be as low as, or less than, 1%. As such, screening large populations by the PCR method described above (see *PCR screening*, Section 3.2.1.2.2.) is advised when transgenic progeny are not immediately identifiable in the outcross population. The non-Mendelian frequency of transgenic animals in the F₁ populations is due to mosaicism of the founder germline and the expected Mendelian frequencies are observed in subsequent generations. With the *Tol2* system, we also frequently observe founder animals that have multiple (six or more) independently-segregating transposon alleles. Outcross of these founders can result in higher than expected frequencies (>50%) of transgenic tadpoles in the F₁ population. Transgenesis frequencies greater than 50% in the F₁ generation is an immediate indicator that the founder has multiple transposition events that are passed through the germline. Individual alleles from these founder lines can be derived by serial outcross of the progeny.

4.2. Tissue-restricted expression of transposon transgenes

Transposon transgenesis can be used to generate frogs with tissue-restricted expression of reporter proteins to study the development of specific organs or tissues. Tissue specific expression of fluorescent reporters can be achieved by using distinct promoter and enhancer elements to drive reporter expression in the transposon construct. For example, we have used the *flk1* (VEGFR2) promoter and first intron enhancer to drive tissue specific expression of green fluorescent protein (GFP) in vascular endothelial cells using the *Sleeping Beauty* system (Fig. 1 (20)). Tissue-restricted reporter expression can also be achieved by ‘enhancer trapping’ with transposons that harbor minimal ubiquitous promoter elements. For example, using a *Tol2* transposon with a 279 bp fragment of the *Xenopus* EF-1 α promoter driving GFP expression (*Tol2XIG* (38)) we have identified transgenic lines with distinct GFP expression profiles (18,48). Fig. 2 depicts the expression profiles of two *Tol2XIG* transgenic lines, *handlebar* (*hbr*) and *jovan heat* (*joh*). The *hbr* line has widespread expression of the GFP reporter (Fig. 2a) and was used as a substrate for remobilization of the single inherited transposon by injecting one-cell progeny of this line with synthetic mRNA encoding *Tol2* transposase (48). One of the novel integration events produced by this injection-based remobilization strategy is shown in Fig. 2b. The *joh* tadpoles have intense GFP expression in the developing kidneys (Fig. 2b, c) whereas the transposon donor line (*hbr*) did not (compare Fig. 2a and b; white arrows). Thus, remobilization of the same *Tol2XIG* transposon from one site in the genome to another resulted in a profound change in GFP expression (48). The integration site for the *joh* allele is near the *HNF1 β* gene that is expressed at high-levels in the developing *Xenopus* kidney (64). It is likely that *cis*-acting regulatory elements that control the endogenous expression of the *HNF1 β* gene are influencing the expression of the *Tol2XIG* transposon that integrated ~46 kb away. We have noted that this integration site variation of reporter gene expression is not limited to transposons that harbor small promoter elements. We have observed the same phenomenon with virtually all promoter/enhancer elements that we have used to date, including the *flk1* promoter/enhancer transposon depicted in Fig. 1 that encodes approximately 6 kb of *Xenopus flk1* regulatory sequence (20). This indicates that transposon transgenes are susceptible to the activity of nearby *cis*-acting regulatory elements and are also likely to be influenced by local chromatin environments. To date, we have not observed transposon gene silencing and the reporter gene expression appears to be consistent over several generations.

5. Conclusions

Transposon-mediated integration strategies have been successfully employed to generate transgenic lines in both *Xenopus laevis* and *Xenopus tropicalis*. The ability to rapidly clone the transposon-mediated integration events by PCR amplification of the flanking sequence is an important advantage provided by this system over other integration strategies. The demonstration that transposon transgenes stably integrated into the frog genome are efficient substrates for remobilization is an important step in developing ‘transposon hopping’ strategies in this highly tractable developmental model system. The high fecundity and long lifespan of *Xenopus* frogs, combined with the advantages of classical embryological manipulation of the embryos, makes this an ideal model for large-scale gene trap, enhancer trap and insertional mutagenesis screens.

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Fig. 1.

Transposon transgenes harboring tissue-restricted promoters can be used to label specific structures in the developing tadpole. Fluorescence photomicrograph of an albino transgenic *Xenopus laevis* tadpole (stage 51) harboring a *Sleeping Beauty pT2flk1GFP* transposon transgene (20). The *flk1* (VEGFR2) promoter/enhancer drives expression of the reporter in vascular endothelial cells and GFP can be readily observed in the blood vessels of the developing tadpole. The image shows a close-up view of the right side of the dorsal anterior region of a *pT2flk1GFP* tadpole; the right eye is indicated by the white arrowhead (50× magnification).

Fig. 2.

The integration site of the transposon influences the expression of the transgenic reporter. Two transgenic *Xenopus tropicalis* tadpoles (**a** and **b**), each harboring a single copy of a *Tol2* transposon encoding an EF-1 α promoter driving expression of GFP (*Tol2XIG* (38)), are shown. Dorsal view, anterior is facing the lower-right corner of the frame. The eye is labeled with a white arrowhead. **a.** The *handlebar* (*hbr*) locus (scaffold 98, base pair position 2851245 (98:2851245)) has intense, widespread GFP expression at stage 47. The single-copy *Tol2XIG* transposon in the *hbr* line was remobilized by micro-injecting fertilized one-cell embryos with *Tol2* transposase mRNA to generate a series of novel integration patterns. **b.** The *jovan heat* (*joh*) line was generated by remobilization of the *hbr* locus (48). The new integration site

(512:565147) in *joh* results in robust expression of the GFP reporter in the developing kidney that was not present in the parental *hbr* line. White arrows indicate the left pronephros in the *hbr* and *joh* embryos. The remobilization event in the *joh* line resulted in transposon integration near the HNF1 β gene; the integration site is ~46 kb from the HNF1 β gene (48). HNF1 β is highly expressed in the developing *Xenopus* kidney (64). **c.** High levels of GFP expression are maintained in the *joh* pronephros throughout development. A dorsal view (10 $\times$ magnification) of a stage 50 *joh* embryo is shown with the anterior facing to the left. The white arrowhead indicates the right eye. The insert shows a magnified view (60 $\times$ magnification) of the right pronephros (boxed).

Table 1

Comparison of the transgenic methodologies used to modify the *Xenopus* genome. References for each technique are provided in the text. ND = not determined.

Transgenesis method	Germline frequency	Strengths	Potential weaknesses
Linear DNA	$\leq 1\%$	Simple Can use any plasmid construct	Low frequency of integration Mosaic germline Plasmid sequences included in transgene
REMI	$\geq 15\%$	Good for analysis of founder (P_0) animals Can use any plasmid construct	Complex methodology <i>In vitro</i> manipulation of sperm nuclei might cause mutations Plasmid sequences included in transgene
Meganuclease	$\geq 20\text{-}30\%$	Simple Good for analysis of founder (P_0) animals	Plasmid sequences included in transgene
phiC31 integrase	ND	Simple Limited number of integration (pseudo- <i>attP</i>) sites in genome Large insert capacity	Non-random integration Plasmid sequences included in transgene
Transposons	$\geq 20\text{-}30\%$	Simple Integrated DNA does not include plasmid vector sequences Can excise and/or reintegrate transposon transgenes	Mosaic integration at early cleavage stages May not be suitable for analysis of transgene expression in founder (P_0) animals