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Transposon-mediated transgenesis in the frog: New tools for biomedical and developmental studies

Donald Albert Yergeau, Paul Evan Mead

Department of Pathology, St. Jude Children's Research Hospital, 332 North Lauderdale Street, Memphis, TN 38105, USA.

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1. ABSTRACT

The amphibian, *Xenopus laevis* has been an excellent developmental model for over half a century. The large egg size, external fertilization and simple husbandry make this frog an ideal tool to study early vertebrate development. The tetraploid genome and long generation time, however, has hindered the use of *X. laevis* in large-scale genetic efforts. A close West African relative, *Xenopus tropicalis* (also commonly known as *Silurana tropicalis*), overcomes the limitations of *X. laevis* in genetic studies as *X. tropicalis* has a diploid genome and breeding adults can be obtained in six to nine months. We have focused our efforts on developing transposon systems for efficient transgenesis and insertional mutagenesis in the frog. Transposon systems have been used for transgenesis in a wide variety of model organisms. In this review, we will discuss the advantages and limitations of different transposon systems for generating transgenic *Xenopus*. In addition, we will describe strategies for the identification of novel genes through an insertional mutagenesis approach using transposable elements.

2. INTRODUCTION

The South African clawed frog *Xenopus laevis* (*X. laevis*) has been a significant model organism in developmental biology for the past 50 years. *X. laevis* has several features that make this organism an ideal model. First, females do not require seasonal cues for ovulation allowing for simple injection of hormone to stimulate egg production at any time of the year. Second, eggs are externally fertilized, are large and are laid in vast numbers. Eggs can be fertilized *en masse* to generate embryos at synchronous stages of development. Third, *Xenopus* embryos can survive in simple salt solution during early development providing simple visualization of developing organs in the nearly transparent embryos. As each cell contains its own supply of yolk, explanted embryonic tissues can survive for several days in isolation. Early stage embryos heal rapidly after surgical manipulation providing an ideal model for transplantation studies to determine cell fates and potentials. Finally, *Xenopus* are long-lived tetrapods and their body plan is similar to man providing advantages over other model systems in the study of organs found only in higher vertebrates.

With these advantages, *X. laevis* has been used extensively as a model system for the study of early embryonic development. Manipulation of early developmental events has yielded many important discoveries into the molecular pathways regulating vertebrate development. Insights into these molecular pathways have been achieved through a number of biochemical approaches performed in early developing frog embryos. Microinjection of mRNA at early cleavage stages allows for the study of ectopically expressed proteins during early embryonic development. Endogenous protein function can be analyzed *in vivo* with morpholino "knock down" oligonucleotides, dominant-negative constructs, and stably integrated siRNA vectors. Explanted animal cap ectoderm (animal cap assays) provides a source of totipotent cells that can be induced with growth factors to differentiate into numerous cell types found in the developing embryo.

While the use of *X. laevis* in early embryological studies is well established, this species can not be used in large genetic screens due to several limitations. The generation time for adult female *X. laevis* in ideal laboratory conditions is up to one year or more; male frogs reach sexual maturity faster than females (approximately nine months). In addition, *X. laevis* is a pseudotetratploid species due to an ancestral allopolyploidization (1). The combination of a long generation time and a tetraploid genome undermine the use of *X. laevis* as a genetic model. Recently, the West African clawed frog *Xenopus tropicalis* (*X. tropicalis*) has been identified as a potential substitute for genetic studies. *X. tropicalis* is a smaller frog, lays vast numbers of eggs per clutch, and develops more rapidly (six to nine months) than *X. laevis* allowing for multiple generation breeding strategies in a more timely manner. More importantly, *X. tropicalis* is a true diploid (2N = 20 chromosomes) and thus can be used for simple "Mendelian" trait analysis. *X. tropicalis* shares all the embryological features that has made *X. laevis* an important model for early vertebrate development (2).

In recent years, a number of genomic resources have been developed for *Xenopus* (3-5). Extensive expressed sequence tags (ESTs) libraries have been developed and sequenced for both *X. laevis* and *X. tropicalis*. Representative libraries from a variety of frog tissues and developmental stages have been analyzed and clones are readily available from academic and commercial sources. The accumulation of expressed sequence data has led to the development of gene profiling microarray chips by Affymetrix in collaboration with the *Xenopus* community. The *X. tropicalis* genome has been sequenced by the Joint Genome Institute (JGI). Draft assembles of genomic DNA scaffolds have been freely distributed on the world wide web and are a valuable resource for the frog community.

With the accumulation of genomic resources available for *X. tropicalis*, several labs have undertaken forward- and reverse-genetic studies using *X. tropicalis*. Chemical mutagenesis by the alkylating mutagen ethyl nitorsourea (ENU) has proven to be successful in the zebrafish and in the fruit fly. ENU randomly mutagenizes the sperm's genetic material by single nucleotide conversion creating point mutations. Two *X. tropicalis* groups are using ENU in small-scale mutagenesis projects to identify novel genes required for early development (6). Several mutants identified show developmental defects in the digestive, neural or hematopoietic systems. Another method to uncover novel genetic pathways is to study naturally occurring mutations found within frog populations (7). Inbreeding of laboratory stocks of *X. tropicalis* has revealed embryonic lethal recessive mutations characterized by disorganized body plans. A gynogenetic screen performed by the Grainger laboratory on wild-caught *X. tropicalis* frogs has identified several mutant alleles when bred to homozygousity (8). Gynogenesis allows for the rapid generation of homozygous animals. In frogs, gynogenetic diploid progeny can be rescued from haploid embryos by either cold shock or hyperbaric pressure (9, 10). Haploid embryos can be generated by *in vitro* fertilization of eggs with UV- or chemically-treated sperm (11). Irradiation results in cross-linking of the paternal DNA and, although the sperm are viable and can activate the egg, the genetic material can not contribute to the activated egg. The resulting haploid embryos develop to early tadpole stage but are not viable beyond swimming tadpole stages. Suppression of polar body exclusion or disruption of the first mitotic spindle results in rescue of a diploid genome where all of the genetic material is of maternal origin. Although ENU mutagenesis and identification of natural mutants has proven to be useful in generating aberrant phenotypes, identifying the mutant locus affected can be labor-intensive requiring positional cloning strategies. Nonetheless, these mutants provide a strong starting point for the use of *X. tropicalis* in genetic studies.

3. TRANSGENESIS IN THE FROG

The inability to create transgenic frogs has been a detriment to the use of *X. laevis* in genetic studies. A number of techniques to create transgenic frogs have been developed in the past two decades to incorporate reporter genes into the frog genome. Larry Etkin and coworkers were the first to successfully generate transgenics by injecting linear DNA, in this case a chloramphenicol reporter gene, into *X. laevis* embryos (12). The injected embryos were mosaic and founder animals carrying the transgene were generated in limited numbers. With a long generation time in *X. laevis,* combined with the highly mosaic founders, the linear DNA injection method proved to be an inefficient tool for the creation of transgenic lines.

In order to overcome the limitations of using linear DNA injected into embryos, Kroll and Amaya developed a novel integration strategy termed restriction endonuclease mediated integration (REMI) (13, 14). REMI uses linearized DNA mixed with sperm nuclei in the presence of the restriction enzyme used to digest the transgene construct. Restriction digestion of the sperm DNA causes double-stranded breaks allowing for the integration of the linearized plasmid into the sperm genome. The manipulated sperm nuclei are then injected

into oocytes where penetration of the needle activates cell division in the egg. The main advantage of REMI is the creation of founder animals containing the integrated transgene whereas previous linear DNA injections resulted in mosaic animals that must be outcrossed in order for the transgene to be studied. REMI has been used successfully in *X. tropicalis* for transgenesis. *X. tropicalis* lines expressing GFP under the control of the lens-specific gamma-crystallin promoter have been developed using a modified REMI technique (15, 16). Although effective for transgenesis in *Xenopus*, REMI produces low numbers of founder animals and is dependent on the quality of reagents used in the procedure. Furthermore, the manipulation of the sperm nuclei by a restriction endonuclease can produce unwanted DNA damage and cause genetic lesions in founder lines.

A novel restriction-endonuclease assisted method for transgenesis, Meganuclease, has been adapted in the frog. Meganuclease restriction sites are cloned into the construct flanking the transgene to achieve linearization of the donor vector. Meganuclease *I-SceI* is a very rare cutter and has an eighteen base pair recognition sequence, as such the probability of an exact match (1 in 4^{18} = 1 site in 6.87 x 10^{10} base pairs) occurring in a vertebrate genome is very low (a typical vertebrate genome contains ~1-3 x 10^9 base pairs). Plasmid DNA harboring a transgene construct is linearized with the meganuclease enzyme and injected together with the *I-SceI* enzyme into fertilized one-cell embryos. Injection of linear DNA into vertebrate cells results in rapid self-ligation of transgene construct to form high-order concatamers. The formation of high-molecular weight concatamers is thought to decrease the efficiency of transgene integration in the host genome. The presence of the meganuclease enzyme maintains the transgene construct in the linear form, thus promoting integration of the low-molecular weight donor DNA without digesting the host genome. The meganuclease method has been used successfully in zebrafish (17, 18), medaka (19, 20) and *Xenopus* (21-24). While the meganuclease method overcomes many of the problems encountered with standard linear DNA or REMI strategies, plasmid DNA sequences may integrate along with the transgene and may result in transgene silencing.

To overcome the deficiencies of existing strategies for transgenesis in frogs we, and other laboratories, have begun to investigate DNA transposon systems for frog transgenesis (25). Transposons are mobile genetic elements with the ability to integrate foreign DNA elements randomly into a target genome (26). DNA-based "cut-and-paste" transposons are bipartite systems comprised of a DNA substrate that is flanked by direct and indirect repeat elements and an enzyme (transposase) that binds to the repeat elements and catalyzes the excision and re-integration of the substrate DNA. The cargo of naturally occurring DNA transposons encodes the transposase enzyme that catalyzes the mobilization of the entire element and derivatives that share the terminal inverted repeats. As such, the "cut-and-paste" DNA transposons are self-contained "autonomous" mobile elements. The presence of active endogenous transposable elements in the genome poses a potential threat to genome integrity as random remobilization may result in disruption of critical sequences. Transposon sequences are thus under selective pressure to be immobilized by the host to avoid loss of genomic integrity. Mutation of either the transposase enzyme, or the repeat elements recognized by the enzyme, will result in loss of transposition activity. For experimental purposes, the transposase encoding sequence can be replaced with any DNA element, including mini-genes encoding fluorescent or other reporter proteins. The manipulated transposon is a "non-autonomous" element and transposase enzyme must be supplied *in trans* to achieve transposition into the host genome.

A number of transposon systems have been identified, including *Sleeping Beauty*, *Tol2* and *piggyBac* and have been successfully used for transgenesis in vertebrate model systems (27). In this review, we will describe several of these systems in more detail as well as discuss other methods for insertion of exogenous DNA into the genome of the frog. In addition, we will discuss discovery of novel genetic elements through insertional mutagenesis approaches.

3.1. *Sleeping Beauty*

In the late-nineties, Ivics and colleagues reconstructed an ancient inactive *Tc1/mariner*-like transposable element found in salmonoid fishes and termed this element *Sleeping Beauty*. Using genomic sequence data for twelve *TcE* elements from eight fish species, Ivics and colleagues reverse-engineered the transposase to restore both DNA-binding and catalytic activity to the enzyme (28). *Sleeping Beauty* is a "cut-and-paste" DNA transposase that integrates its transposon, flanked by direct/indirect repeats, randomly at TA dinucleotide sites within the genome. *Sleeping Beauty* is an efficient transposase, integrating from one to multiple copies of the transposon into the genome (29). *Sleeping Beauty* has been used in a number of organisms including the frog (30, 31), zebrafish (32, 33), mouse (34-36), rat (37) and in human cell culture lines (28, 38). The ability of *Sleeping Beauty* to function in a range of vertebrates indicates that host factors required for enzymatic activity are conserved across wide evolutionary boundaries.

Recently, Sinzelle *et al* (2006) investigated the function of the *Sleeping Beauty* transposon system in the frog *X. laevis* using a simple microinjection approach (30). They showed that the *Sleeping Beauty* transposase can efficiently integrate a GFP reporter cassette flanked by transposon direct/indirect repeats into founder frogs. The authors show germline transmission of the transgene to the offspring and that second generation lines achieve Mendelian ratios (30). Interestingly, the authors suggest that integration of the transposon into *X. laevis* was via a non-canonical transposition reaction that resulted in the incorporation of vector sequences. Nonetheless, this study provides evidence that *Sleeping Beauty* is functional in *X. laevis*. We have investigated the ability of *Sleeping Beauty* to incorporate exogenous DNA reporter constructs into the frog and we also observed the non-canonical inclusion of vector sequence into the genomes of both *X. laevis* and *X.*

tropicalis (Yergeau and Mead, unpublished observations). Our laboratory has recently generated a Flk1-GFP transgenic *X. laevis* line by *Sleeping Beauty* mediated transposition (31). Green fluorescent protein driven by the *X. laevis* Flk1 (VEGFR2 or KDR) promoter was seen in the developing vasculature, recapitulating the expression pattern of the endogenous gene. This transgenic line provides an ideal model to study early vascular development in the embryo. Despite the non-canonical transposition of the transposon into the frog genome, *Sleeping Beauty* provides an efficient tool to generate transgenic frogs.

3.2. *Tol2*

Tol2 was identified as the genetic lesion within the tyrosinase gene of an albino mutant Medaka fish (*Oryzias latipes*) (39). *Tol2* (Transposon element *Oryzias latipes* 2) was the first fully active autonomous cut-and-paste transposable element identified in a vertebrate species (40, 41). The autonomous element contains a catalytic transposase that can efficiently integrate its cognate transposon into the genome. Since autonomous elements are not preferred genetic tools due to the presence of an active transposase, a non-autonomous element was engineered without a functional transposase. This allows the transposition of exogenous *Tol2* elements to be combined with transposase *in trans*, and thus regulate the mobilization of the element. Several laboratories have used the *Tol2* non-autonomous element for enhancer trap and gene trap screens in zebrafish (42-45). Insertional mutagenesis by integration of the *Tol2* transposon has recently been demonstrated in zebrafish (46). Kawakami and co-workers demonstrated that co-injection of a plasmid harboring a *Tol2* transposon with synthetic *Tol2* transposase mRNA resulted in efficient excision of the transposon from the donor plasmid (47).

Our laboratory has used the *Tol2* system for integration of GFP-containing constructs into *X. tropicalis*. We co-injected plasmid DNA containing a green fluorescent protein mini-gene driven by a ubiquitous promoter (*Xenopus* EF-1alpha) together with messenger RNA encoding *Tol2* transposase (48). The co-injection strategy resulted in efficient generation of chimeric founder lines that passed the transgene through the germline at a rate of 30-40%. The chimerism of the founder animals was highlighted by the non-Mendelian numbers observed in the F1 generation. Outcross of subsequent generations resulted in the expected Mendelian inheritance ratios for the dominant reporter alleles. The chimerism of the founders was most likely due to integration of the transposon into individual blastomeres at early cleavage stages of development. Integration into blastomeres that are not fated to contribute to the germline results in green founder animals that fail to pass the transgene onto the next generation. As demonstrated in the zebrafish, we observed multiple independently-segregating transposon alleles in individual *Xenopus tropicalis* founder lines (48). Serial outcross of the founders was used to segregate individual transposon alleles ((48); Yergeau, Kuliyev and Mead, unpublished data). The rate of germline transgenesis achieved with *Tol2* was similar to that observed with *Sleeping Beauty* in the frog. Unlike *Sleeping Beauty*, however, *Tol2* appears to use a canonical transposition mechanism in the frog (48). PCR-based strategies were used to clone the *Tol2* integration sites in several founder lines and indicated that a standard integration mechanism was used to insert the transposon into the frog genome. For example, the precise boundaries of the transposon terminal repeat arms were maintained and the expected eight base pair target site duplication was identified flanking the transposon (48, 49). Thus, we have demonstrated that *Tol2* provides an efficient platform for transposon-mediated transgenesis in the frog. Co-injection of purified *Tol2* transposase protein with a plasmid harboring a *Tol2* transposon results in efficient mobilization of the transposon in frog embryos (50).

3.3. *piggyBac*

Another transposable element identified in invertebrates is *piggyBac*. Found in the genome of the cabbage looper moth, *Trichoplusia ni*, the *piggyBac* transposon has been shown to integrate efficiently into *Drosophila* (51, 52), mammalian cell lines (53-55) and the mouse (53). Comparison of *Sleeping Beauty*, *Mos1*, *Tol2* and *piggyBac* in mammalian cell lines shows that *piggyBac* is more active than *Sleeping Beauty*, *Mos1*, and *Tol2* when tested in excision assays performed in HEK293 cells (56). In the mouse, the coding region of the *piggyBac* transposase has been codon-optimized resulting in a more active enzyme when compared to the native enzyme (57). *piggyBac* gene trap technology combined with the *cre-lox* system has been developed to perform gene trap mutagenesis in the mouse providing a simple platform for gene discovery (58). In the platform presented by Wu *et al*, the *cre* recombinase system allows for rescue of the inactivated gene by removing the mutagenesis gene trap cassette. To our knowledge, *piggyBac* has not been tested in the frog. A recent report, however, described the identification of a family of *piggyBac*-like transposons in *Xenopus* species (59). The presence of a related transposon family in *Xenopus* suggests that any host factors that may be required for transposition are present in frog cells.

3.4. Additional tools for integration of foreign DNA into *Xenopus*

3.4.1. phiC31 integrase

The bacteriophage phiC31 is a recombinase of the resolvase/invertase family that inserts DNA into specific sites within the genome. Two minimal DNA integration sites, *attB* and *attP*, are required for efficient integration into the genome. In bacteria, a phage attachment site (attP) is present in the genome and is the site of integration for the integrase. After recombination, the *attB* and *attP* sites are lost, creating novel sites *attL* and *attR*. The creation of these two new sites prevents remobilization of the inserted DNA element even in the presence of active integrase. In higher organisms, imperfect attP sites ("pseudo" attP) are present in limited numbers in the genome (60, 61). In addition to the site specificity of integration of DNA into the genome, the integrase requires no host co-factors. Thus, genomes containing "pseudo" attP sites can be used as substrates for

integration of attB-containing vectors. This feature allows the phiC31 integrase system to be used as a tool for gene therapy and for the creation of transgenic animals. The phiC31 integrase has been tested in mammalian cell lines and *in vivo*. PhiC31 integrase was shown to be effective in the frog *X. laevis* for the creation of transgenic animals (62, 63). The authors show efficient integration of a GFP reporter into the genome of *Xenopus laevis*. In addition, the authors show tissue specific expression of GFP under the control of the gamma-crystallin lens specific promoter. Germline transmission of the phiC31 integrated GFP transgene, however, has not been demonstrated *in vivo* in the frog *X. laevis*. Currently, the phiC31 integration system has not been systematically studied in the diploid *X. tropicalis*. Nonetheless, phiC31 integrase provides another method for the creation of transgenic frogs.

3.4.2. *Minos* and *Frog Prince*

In addition to the transgenesis tools previously shown by our group and others to insert DNA into the frog genome, two novel transposon systems, *Minos* and *Frog Prince*, have been developed in other model systems that could have application for frog transgenesis. Originally found in *Drosophila hydei, Minos* is a transposable element from the *Tc1/mariner* family with 255-bp terminal inverted repeats surrounding the active transposase (64, 65). *Minos* has been shown to stably integrate exogenous DNA into the genomes of several organisms including *Drosophila melanogaster* (66, 67), the medfly (*Ceratitis capitata*) (68), *Ciona intestinalis* (69-71), mammalian cell lines (72) and in the mouse germline (73). Like other members of the *Tc1/mariner* family, *Minos* integrates randomly in the genome at T/A dinucleotides. *Frog Prince* is another transposable element originally discovered in the frog *Rana pipiens* (74). This amphibian transposon has been to shown to effectively integrate a reporter construct into multiple vertebrate cell culture lines. Both *Minos* and *Frog Prince* have not been vigorously tested for germline transmission in *Xenopus.* These two transposon systems provide additional tools for functional genomic analysis in *Xenopus*.

4. STRATEGIES TO UNCOVER NOVEL GENETIC ELEMENTS WITH TRANSPOSABLE ELEMENTS

4.1. Insertional Mutagenesis

The use of transposons as vehicles to generate transgenic lines in frogs is a powerful technique for the study of gene function. Transposons carrying gene specific promoters or enhancers linked to fluorescent reporters can provide useful information on the expression of the gene. Study of these regulatory elements can provide essential information on the regulation of the gene, patterns of developmental expression and *in vivo* visualization of organogenesis. In addition, due to the random nature of integration, transposons can be used as insertional mutagens where integration results in disruption of sequences critical for gene expression. Enhancer or gene trap vectors can used to identify novel genetic elements not previously uncovered by standard biochemical approaches.

Transposons can efficiently incorporate gene trap or enhancer trap constructs into the *Xenopus* genome. Simple gene trap constructs use a splice acceptor site upstream of a reporter gene and rely on intragenic insertion in the correct reading frame to generate a fusion protein with the endogenous targeted gene. The efficiency of simple gene trap vectors is low due to the low probability of the transposon trap integrating in the correct orientation and reading frame of the targeted gene. Also, expression of the fusion protein is dependent on the endogenous promoter and trapped genes that are expressed at low levels, for a narrow time frame during development or in only a small subset of cells may easily be missed in a visual screen for fluorescent marker gene expression. A more efficient method for gene trapping that overcomes many of the problems inherent with simple gene trap construct is the polyadenylation trap vector. Polyadenylation (or polyA) trap vectors are bi-functional constructs that disrupt endogenous gene expression by insertion of a premature transcription termination and polyadenylation cassette. A reporter gene, driven by a ubiquitous promoter, is followed by a splice donor cassette. The lack of a functional polyadenylation signal at the 3'-end of the reporter cassette results in an unstable mRNA and no reporter gene expression (75). Intragenic insertion of the polyA trap (pAT) vector results in splicing between the reporter splice donor and an endogenous splice acceptor and will thus provide the endogenous polyadenylation signal to the fusion transcript and generation of a functional reporter mini-gene (see Figure 1). As the expression of the reporter gene is not dependent on the endogenous expression of the trapped gene and is driven by the ubiquitous promoter, identification of "trapped" tadpoles by monitoring reporter gene expression is comparatively simple. PolyA trap vectors have several advantages over standard splice acceptor gene trap constructs. As described above, expression of the reporter gene is not dependent on the expression of the endogenous gene, thus avoiding difficulties associated with identifying trapped loci that are expressed at low-levels or in small subsets of cells during narrow windows of development. Insertion of the splice acceptor-polyA element can result in premature truncation of the endogenous gene product and thus the pAT vector is likely to be more mutagenic than a standard gene trap construct. As expression of the reporter gene is driven by the promoter element within the trap vector, reporter gene expression is independent of the reading frame of the endogenous gene and requires only the acquisition of a functional polyadenylation signal by mRNA splicing. As such, the efficiency of the pAT vector to identify functional gene loci is superior to that of the standard splice acceptor gene trap. A potential problem with the pAT vector is that, as expression of the reporter is independent of the regulatory elements controlling the endogenous gene, no information on the expression pattern of the trapped locus is gained using the polyA trap vector outlined in Figure 1. This limitation can be overcome in at least two ways. First, once the endogenous locus is identified using standard PCR strategies, see below, anti-sense riboprobes to the endogenous gene can be used for *in situ* hybridization studies to reveal the expression pattern of the trapped locus. Second, a second reporter gene can be included in the trap

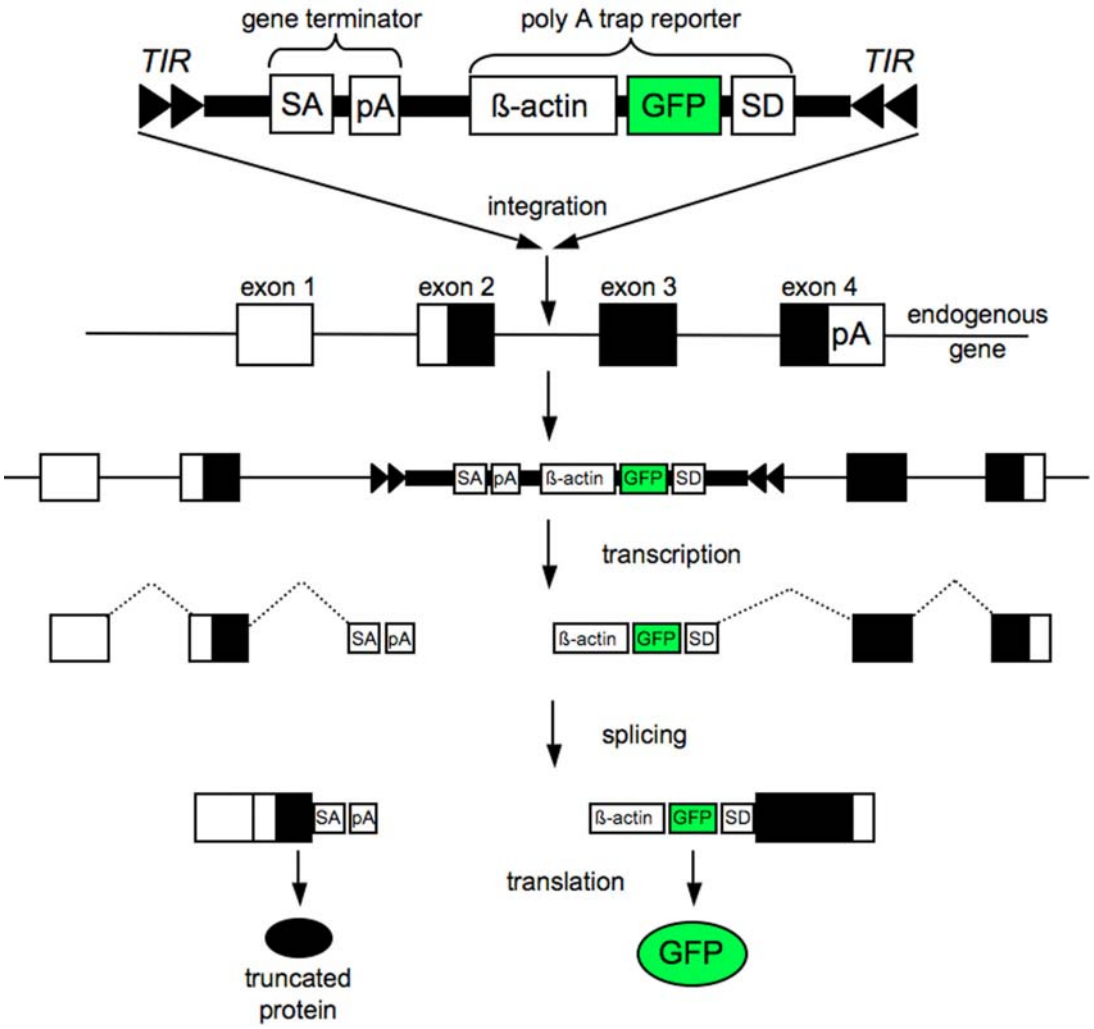

Figure 1. Schematic diagram of a pAT transposon construct and its functional elements. Integration of the transposon into a gene results in trapping of the polyadenylation sequence and stabilization of the GFP-encoding mRNA produced by the minimal ubiquitous (ß-actin) promoter. The splice acceptor and polyadenylation signal within the gene terminator cassette at the 5' end of the construct, results in premature termination of the targeted endogenous gene. Disruption of the endogenous gene may result in a mutant phenotype when the targeted tadpoles are breed to homozygousity. Boxes indicate exons and black shading represents coding sequences. Terminal repeats flanking the transposon are represented by black triangles. Intronic sequences spliced out of the nascent transcripts are represented by dashed lines.

vector within the gene terminator cassette (see Figure 1) between the splice acceptor and the polyadenylation signal. Integration of the trap transposon in the correct reading frame relative to the trapped locus will result in a fusion peptide encoding the second reporter. Expression of the second reporter will thus reveal the expression pattern of the endogenous gene. The inclusion of a second reporter will increase the total size of the trap construct and may influence the choice of transposon system for integration of the polyA trap vector. *Tol2* is ideal for these applications as *Tol2* efficiently and randomly integrates constructs over a wide range of cargo sizes (76, 77).

Enhancer traps (ETs) have been used with great success in several developmental organisms including the zebrafish and Medaka (78). Enhancer trap vectors were developed to identify the regulatory regions of endogenous genes located within the genome of interest and contain a minimal promoter driving expression of a reporter gene. Integration of the ET transposon near regulatory elements that control the tissue-specific or developmental expression of a gene can override the minimal promoter and confer tissue-restricted expression to the reporter gene. ET vectors can be used to identify genes, and their regulatory elements, that are expressed in developmentally- or tissue-restricted patterns. As enhancers and suppressors can act over large distances and are position independent, ET cassettes are likely to be more efficient than gene trap vectors in identifying gene loci. For the same reasons, ET transposons are less likely than gene trap vectors to be mutagenic as integration of the functional ET transposon may be outside the gene. There are, however, several

advantages of enhancer trap constructs. One is the creation of tissue-restricted reporter lines for fate-mapping studies. Targeted genes or tissues could be visualized *in vivo* allowing for the study of the trapped gene during development. The use of fluorescent reporter genes, such as GFP (Green Fluorescent Protein) in the trap constructs allows direct observation of the trapped locus in living embryos. For this reason, fluorescent reporters are generally preferable to enzymatic reporters, such as ß-galactosidase where fixing and processing of the embryos is required to visualize the reporter expression. Second, tissue-specific expression of the reporter can be used to mark a tissue for transplantation studies. *Xenopus* has long been used for the study of cell labeling and these reporter lines would provide another valuable tool to study cell movements during development. Third, the enhancer gene trapped can be easily identified using genomic resources and PCR based methodologies and these vectors provide unique tools to uncover genes essential for tissue development.

4.2. Transposon Remobilization

A unique feature of the transposon system is that the integration of a "cut-and-paste" transposon into the frog genome provides a substrate for the transposase for remobilization. The transposon integrated into the founder animal is stable and is passed to the offspring in Mendelian ratios. This stable transposon can be induced to remobilize, or "hop", to another site within the genome with re-expression of the transposase. There are two ways to introduce the transposase to the transposon substrate in the frog genome, through microinjection or by out-crossing the transposon "substrate" line to a transgenic frog expressing the transposase under the control of a promoter driving expression of the enzyme in the germline. Microinjection of transposase mRNA into embryos containing the resident transposon has been used successfully in zebrafish (42). Multiple unique expression patterns that differ from the parental GFP expression profile were seen after injection of *Tol2* mRNA into transgenic progeny. This microinjection strategy can be easily adapted to *X. tropicalis*. Since *X. tropicalis* females can be induced to produce eggs at any time of the year and lay large numbers of eggs, many hundreds of fertilized eggs can be microinjected with transposase mRNA for remobilization in a single day. This microinjection approach, however, is time consuming and labor intensive. Microinjection of the transposase must occur prior to the first cellular division in order to achieve maximum remobilization. Excision and re-integration of the resident transposon at early cleavage stages will result in a mosaic animal and the individual re-integration events would need to be separated by serial outcross of the resulting frogs. *X. tropicalis* embryos divide rapidly and the eggs must be injected immediately following fertilization, thus limiting the time frame available for the micro-injection step. Even with these limitations, microinjection of the transposase into transposon-harboring embryos provides an ideal starting point for remobilization strategies. Remobilization of resident transposon transgenes may be more efficient than the plasmid-mRNA co-injection strategy used to generate founder lines as injected plasmid DNA is toxic to the developing embryo and thus limits the amount of substrate that can be injected. In contrast, injected mRNA is tolerated at much higher concentrations and injecting mRNA alone will lead to higher survival rates amongst the injected zygotes.

Remobilization can also be achieved using an *in vivo* breeding approach. Transgenic animals harboring a transposon substrate can be interbred with transgenic animals that express the transposase enzyme in the germline. Generation of transgenic animals expressing the transposase under the control of a promoter that drives expression of the transgene in the germline can be easily achieved through transposon-mediated transgenesis. For example, a *Sleeping Beauty* transposon can be used to generate a transgenic frog that expresses the *Tol2* transposase. Double-transgenic frogs, harboring both the substrate transposon and transposase enzyme transgene, can be generated using simple breeding strategies. The double transgenic "seed" males are raised to adulthood and outcrossed to wild type female frogs. During spermatogenesis in the "seed" male, the expression of the transposase enzyme from the transgene results in remobilization of the resident transposon in the developing gamete. As remobilization occurs prior to fertilization, the resulting embryo will not be mosaic and thus eliminating the requirement for further outcross to isolate individual transposon alleles. The developing progeny of the outcross of the "seed" males and wild type females are scored for reporter gene expression and tadpoles with interesting expression patterns are selected for further analysis. Figure 2 outlines a typical breeding strategy for an *in vivo* remobilization screen. The *in vivo* remobilization strategy has been used successfully in mouse (35, 36, 79, 80) and rat (37, 81) model systems. The frog provides the ideal model to perform this *in vivo* breeding-mediated remobilization strategy due to the high fecundity and long life span of this vertebrate model. For example, a double transgenic "seed" male animal can be outcrossed to a wild type female once a week producing up to 3,000 embryos. As remobilization has occurred during early spermatogenesis, many millions of unique sperm with novel integration events can be produced. Over the two-decade life span of the frog, many millions of progeny with unique transpososition events can be generated by a single "seed" animal. The breeding-mediated remobilization strategy eliminates the time-consuming and labor-intensive micro-injection step from the insertional mutagenesis strategy and provides a platform for large-scale transposon gene and enhancer trap screens.

The advantage of insertion mutagenesis strategies over other potential mutagens such as chemically-induced mutation and radiation, is the ability to rapidly identify the site of the targeted locus using simple PCR-based cloning strategies. The presence of a heritable tag inserted in the genome provides an anchor for PCR cloning approaches (48, 49), including linker-mediated PCR and rapid amplification of cDNA ends (RACE) protocols. The draft sequence of the *X. tropicalis* genome has provided megabase-long scaffolds of annotated sequence to rapidly identify the genes flanking the transposon insertion site (48, 49). In addition to the rapid identification of the integration

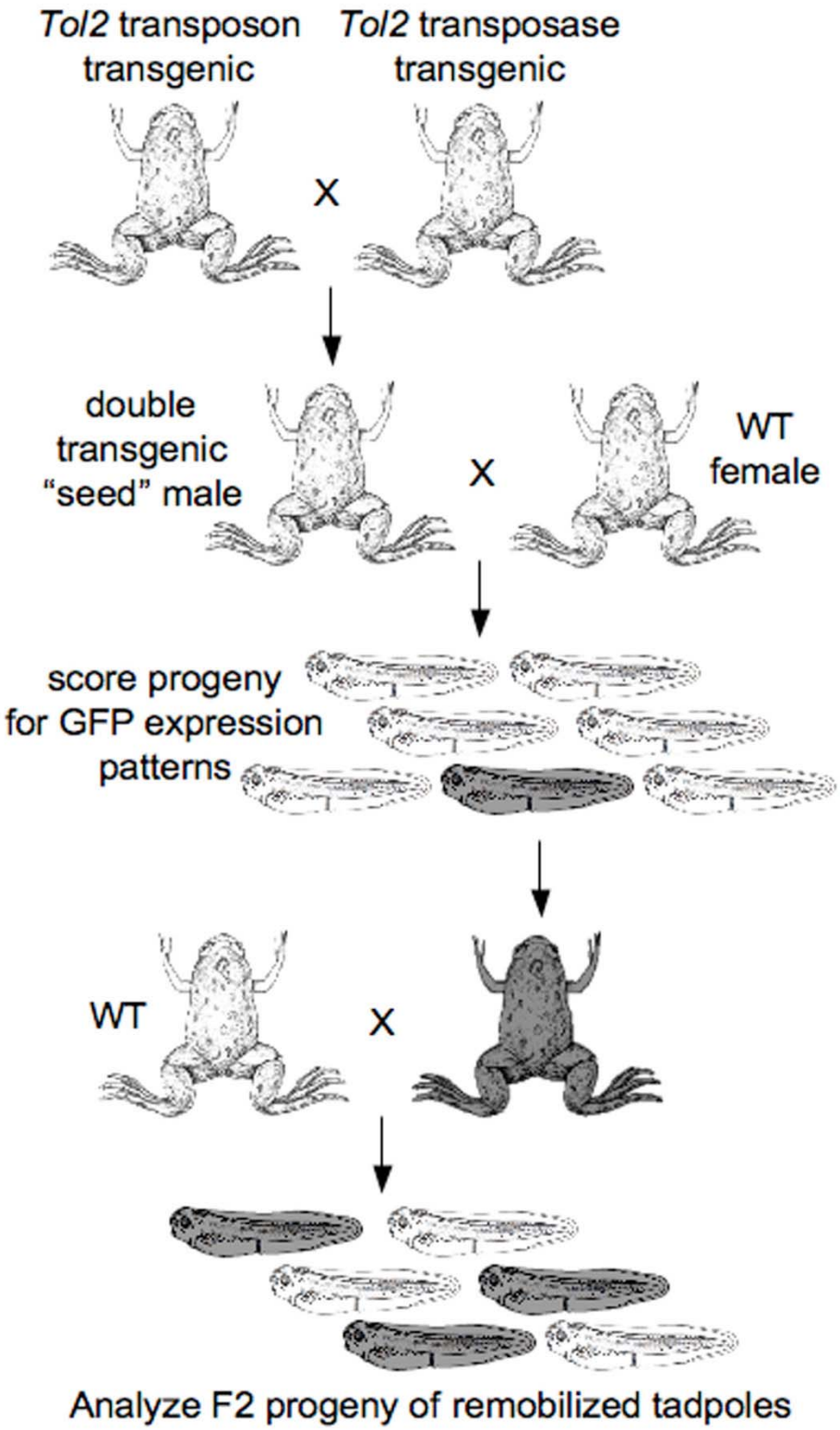

Figure 2. Multi-generation *in vivo* transposon remobilization breeding strategy. Double transgenic "seed" males are produced by intercross of transgenic lines harboring a transposon substrate and a transposase mini-gene that drives expression of the cognate enzyme in the germline. Progeny from the outcross of the double transgenic "seed" males with wild type females are scored for novel GFP expression patterns. Selected tadpoles are raised to adulthood and outcrossed to establish individual lines and to maintain sufficient progeny for further experimental analysis.

sites, transposon-based systems offer a convenient strategy for verification of the mutagenesis. For example, if a pAT transposon results in disruption of a gene and a mutant phenotype, remobilization strategies can be used to restore the wild type locus, thus verifying that the integration event caused the observed phenotype.

5. PERSPECTIVE

The use of amphibian embryos in classical developmental experiments such as cell labeling and transplantation studies has provided many important insights into early vertebrate development. The ability to create transgenic animals through transposase-mediated transposition in the frog provides an invaluable tool for the study of endogenous genes *in vivo*. Transposons have the ability to carry various genetic cargos including gene trap, enhancer trap and mutagenesis cassettes and are easily inserted into the genome by its specific transposase. Combining classical developmental experiments with modern molecular genetic techniques will increase the utility of the amphibian model system in developmental biology.

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Abbreviations: pAT: polyadenylation trap, ET: enhancer trap

Key Words: *Xenopus laevis*, *Xenopus tropicalis*, transgenesis, transposon, *Sleeping Beauty*, *Tol2*, Review

Send correspondence to: Paul E. Mead, Department of Pathology, 332 North Lauderdale Street, Memphis, TN 38105, USA, Tel: 901-495-4226, Fax: 901-495-5947, E-mail: paul.mead@stjude.org

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