Review

Manipulating the *Xenopus* genome with transposable elements

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Abstract

The study of amphibian embryogenesis has provided important insight into the mechanisms of vertebrate development. The frog *Xenopus laevis* has been an important model of vertebrate cell biology and development for many decades. Genetic studies in this organism are not practical because of the tetraploid nature of the genome and the long generation time of this species. Recently, a closely related frog, namely *Xenopus tropicalis*, has been proposed as an alternative system; it shares all of the physical characteristics that make *X. laevis* a useful model but has the advantage of a diploid genome and short generation time. The rapid accumulation of genetic resources for this animal and the success of pilot mutagenesis screens have helped propel this model system forward. Transposable elements will provide invaluable tools for manipulating the frog genome. These integration systems are ideally suited to transgenesis and insertional mutagenesis strategies in the frog. The high fecundity of the frog combined with the ability to remobilize transposon transgenes integrated into frog genome will allow large-scale insertional mutagenesis screens to be performed in laboratories with modest husbandry capacities.

Introduction

The frog *Xenopus laevis* has been used to study early stages of vertebrate development for more than 50 years and continues to be an important model system. The frog lays abundant eggs that are large, develop synchronously, and are easy to manipulate. Genetic manipulation of this tractable model system would further enhance the use of the frog in developmental studies. Two features of *X. laevis* that have hindered genetic studies are the long generation time (1 to 2 years) and the tetraploid genome of this species. The closely related frog *Xenopus tropicalis* shares all the features of *X. laevis* that make this system useful for embryonic manipulation but it develops more rapidly (sexual maturity is reached in 5 to 9 months) and has a diploid genome.

Transposons have widely been used in plant and invertebrate model species to integrate foreign DNA into the host genome. In recent years, these powerful genetic tools have been used in higher vertebrates for transgenesis, insertional mutagenesis, and gene therapy applications. The 'cut-and-paste' DNA transposons are particularly useful for these applications. The *Sleeping Beauty* transposase system was developed in the late 1990s and has been widely used in a range of vertebrate systems. A common ancestor cloning strategy was used to engineer the active transposase enzyme from an inactive form found in teleosts. A genetic toolbox of 'cut-and-paste' DNA transposable elements is now available for use in vertebrates and includes *Tol2* and *piggyBac*.

Here, we review the application of transposable elements to modification of the frog genome. Transposon vectors can be used in the frog for transgenesis and for insertional mutagenesis where enhancer trap and gene trap constructs are used to identify genomic loci involved in developmental processes. Once integrated into the frog genome, the 'cut-and-paste' DNA transposons are targets for remobilization by re-expression of the appropriate transposase enzyme. Transgenic frogs that express the enzyme in the germline can be bred with animals harboring a transposon substrate to generate double transgenic lines where remobilization will occur in the germline in subsequent generations. The high fecundity of the frog can be exploited in these remobili-

zation strategies because each outcross of *X. tropicalis* can generate more than 2,000 offspring.

Xenopus as a developmental genetic model

For more than a century amphibian species have been used as model organisms for the study of vertebrate development [1]. Several features of amphibian embryonic life make these animals useful as models for studying early developmental events. First, the embryos are fertilized outside the mother and are thus accessible for study at the earliest stages of development. Second, the eggs are large and easy to manipulate under low power microscopy. Third, many amphibian species lay vast numbers of eggs, providing adequate numbers for study. Fourth, each cell of the developing frog embryo contains yolk platelets that provide nutrition during prefeeding stages of embryonic life. This allows explanted cells to survive in simple salt solutions for several days and enables study of isolated embryonic tissues and cells. Fifth, *Xenopus* embryos are optically transparent for most of their embryonic life, which allows direct visualization of developing organ systems. Finally, amphibians are tetrapods and their body plan is similar to that of mammals, providing important advantages over other model systems for study of organs that are only present in higher vertebrates.

The South African clawed frog *X. laevis* has been a favored model for developmental biologists for many decades. The entire life cycle of these animals is aquatic, which simplifies husbandry because they can be maintained in simple aquaria and do not need a terrestrial habitat. Unlike many other amphibian species that require seasonal cues for initiation of egg laying, *Xenopus* spp. can be induced to lay eggs throughout the year by simple hormone injections. The embryos are large and are laid in vast numbers. A single female can lay in excess of 1,000 eggs per ovulation and can be induced to lay eggs several times a year. The large egg size and rapid development allows for simple manipulation of gene expression by microinjection techniques. Ectopic expression can be achieved by injection of synthetic mRNAs to achieve early expression of proteins. The timing of protein expression can be delayed by injecting plasmid constructs in which a promoter drives expression of the introduced gene, thus delaying expression until after the mid-blastula transition when zygotic transcription begins. Expression of endogenous proteins can be manipulated using either dominant-negative constructs or by injecting anti-sense morpholino oligonucleotides to achieve a 'knock-down' of the target protein.

The large and rapidly dividing embryos are ideal for embryonic manipulations such as explant and transplantation techniques. Explanted primitive ectoderm (commonly called the animal cap assay) provides a source of pluripotent cells that can be used in a variety of tissue induction assays. The explanted animal caps can be induced with growth factors to differentiate into all cell types found in the embryo. For example, addition of the transforming growth factor-β family member activin can lead to a dose dependent differentiation of the nascent ectoderm to mesodermal and, at high doses, endodermal cell types [2].

Although *X. laevis* is an excellent developmental model and has been used extensively for 'classical' embryologic manipulations, genetic studies in *X. laevis* are not considered feasible. This is because of the long generation time of this species (1 to 2 years) and its tetraploid genome. A genome wide duplication event occurred in *X. laevis* approximately 10 to 40 million years ago [1]. As such, this species maintains four copies of each gene. The combination of these physical attributes makes this species an unsuitable candidate for a genetic model. The advantages of combining the excellent features of the frog for embryonic manipulations and the power of modern molecular genetics has led investigators to identify another candidate frog for genetic analyses. The West African clawed frog, *X. tropicalis*, is a close relative of *X. laevis* and is a true diploid. *X. tropicalis* shares all the features of *X. laevis* that makes this species an excellent embryologic model, but it has the advantages of shorter generation time and diploid genome [3].

X. tropicalis is smaller than *X. laevis*, although it is a genetically similar organism, and it has multiple advantages over *X. laevis* for genetic studies. First and foremost, genetic studies have revealed *X. tropicalis* to be a true diploid, containing ten pairs of chromosomes as compared with 18 pairs for *X. laevis*. *X. tropicalis* adults also reach breeding age faster than do *X. laevis* adults (males 4 to 6 months and females 6 to 8 months for *X. tropicalis* versus about 1 year for *X. laevis*) [4]. Adult female *X. tropicalis* produce smaller eggs (1.0 to 1.3 mm for *X. laevis* versus 0.7 to 0.8 mm *X. tropicalis*) in numbers similar to those with *X. laevis* (1,000 to 3,000 per ovulation), and the eggs can be manipulated in the same manner as *X. laevis* oocytes, including microinjection. In essence, all experiments performed in *X. laevis* can be performed in *X. tropicalis*.

Because both frog species are genetically similar, genes and their regulatory elements, such as enhancers and promoters, can be cross-utilized in both species. Full-length cDNA clones from *X. laevis* and *X. tropicalis* have been analyzed for comparison between species as well as with higher vertebrates [5]. In addition to the experimental data that can be generated using *X. tropicalis*, genetic resources are accumulating [6,7]. Expressed sequence tags (ESTs) have been generated from multiple tissues and developmental stages for both *X. laevis* and *X. tropicalis* and are freely accessible in gene databases such as Genbank. *X. tropicalis* now ranks sixth for the number of ESTs per organism deposited into Genbank as of August 2007 [8]. In conjunction with establishment of cDNA libraries and ESTs, the *X. tropicalis* genome is being sequenced and annotated

to near completion by the Joint Genome Institute and is available to the entire *Xenopus* community. Microarray chips prepared in combination with Affymetrix and the *Xenopus* community have provided a valuable resource to allow rapid analysis of thousands of genes under various experimental conditions. The rapid pace at which these valuable genomic tools have been developed establishes the potential ongoing and future role of *X. tropicalis* in genetic studies.

Several features of the natural history of the frog make this tetrapod an attractive model for genetic analyses. The high fecundity of the frog results in many offspring from each cross. *X. tropicalis* colonies are easy to maintain in the laboratory, and the animals will live for approximately two decades in captivity. The long life-span has obvious advantages in maintaining transgenic and mutant founder lines for many years, with the ability to out-cross and back-cross these animals over multiple generations. The ability to generate fertile animals by gynogenesis allows rapid generation of homozygous lines. Haploid *X. tropicalis* embryos can be generated by fertilizing eggs with UV treated sperm. The irradiation of the male gametes results in cross-linking of the genetic material and blocks the contribution of the male DNA to the fertilized egg. The resulting eggs will develop for several days as haploids with only the maternal chromosomes. Gynogenetic diploids can be rescued from haploid embryos by several methods, such as hyperbaric pressure or cold shock, which prevent either exclusion of the polar body or disruption of the mitotic spindle during the first cleavage event [3]. The result of these physical treatments is that the embryo will contain two copies of the maternal chromosomes and is rescued from the early demise that haploid embryos are fated to. The ability to generate gynogenetic diploid animals in large numbers is useful for mapping studies and for decreasing the number of generations (and thus yielding savings in terms of valuable time and space) required for developing homozygous lines. Another feature of the frog that is useful for genetic studies is the ability to sex bias populations of tadpoles to generate either female or male adults. For example, to skew a population of tadpoles toward adult females, estrogens are added to the water before the developmental stage when the germ cells are migrating to the gonad. Feminization of the gonad with estrogen results in the developing frog becoming a female. The ability to separate gonadic sex from genetic sex provides another useful tool for developmental genetic studies.

With the diploid *X. tropicalis* as a new genetic model, a number of laboratories have undertaken forward and reverse genetic mutant screens. Induced mutations can identify critical genes involved in early development. Treatment of mature sperm with *N*-ethyl-*N*-nitrosourea (ENU), a potent alkylating mutagen, has successfully been applied in zebrafish for small [9] and large genetic screens [10]. With the success of the ENU mutagenesis screen in zebrafish and the benefits of these mutations for the study of developmental pathways, several *X. tropicalis* laboratories have undertaken ENU mutagenesis screens. For example, the laboratories of Zimmerman and Stemple [11] recently conducted a pilot ENU mutagenesis screen using *X. tropicalis*. A number of mutant phenotypes was identified, including those of the nervous, hematopoietic, and digestive systems. Although ENU is a powerful tool for obtaining mutants, identification of the genetic lesion induced by ENU is potentially time consuming and often requires extensive positional cloning strategies to uncover the affected gene.

In addition to mutations that can be generated by chemical mutagenesis protocols, naturally occurring mutants have been identified in inbred lines of *X. tropicalis*. The Harland group at the University of California-Berkeley [4] have described three naturally occurring embryonic lethal recessive mutations (*grinch*, *curly*, and *bubblehead*) by inbreeding a Nigerian strain of *X. tropicalis*. *Grinch* mutants exhibit pericardial edema at the onset of heartbeat (about stage 35) and die by stage 48 of development. *Curly* mutants have a characteristic curved tail and die at around stage 40. The *bubblehead* mutation leads to smaller body size, craniofacial defects, and edema, leading to death of the embryos at around stage 40. In addition, carrier adults for each mutation can be crossed with other carriers (for example, *curly* × *grinch*) and compound mutants can be obtained exhibiting characteristics of each natural mutation identified [4]. A gynogenetic screen of wild caught animals performed in the Grainger laboratory at the University of Virginia [12] identified recessive mutant alleles that resulted in defined phenotypes when forced to homozygousity. In the latter study, 42 mutant phenotypes were obtained and include *puffy eye*, *directionless*, and *heartbreaker*. These mutations are excellent models in which to study gene function *in vivo*. However, as with ENU screens, identification of the gene responsible for the mutant phenotype is a laborious process because these mutations may be the result of complex genetic lesions. Nonetheless, these mutants provide an ideal starting point for the establishment of *X. tropicalis* in genomic studies.

Trangenesis in *Xenopus*

Although the frog has been an excellent model in which to study early aspects of vertebrate development, the ability to create transgenic animals has been lacking. Within the past 20 years, however, molecular techniques have been developed to create transgenic *Xenopus* lines. Germline transgenesis in *X. laevis* was first described by Etkin and Pearman [13] and used a simple microinjection approach to introduce linear plasmid DNA into the fertilized egg. Random integration of the transgene at early cleavage stages resulted in transmission of the chloramphenicol acyl transferase reporter through the germline. Although this technology has proved effective for generating transgenic

progeny in a variety of vertebrate model systems, this method has not been widely used in *X. laevis,* probably because of the highly mosaic integration of the transgene in founder lines and the long generation time for this organism. The mosaicism of the founders precludes analysis of transgene activity in the founder animals, and the lengthy generation time in *X. laevis* results in a 1 to 2 year delay in analysis of the F_1 progeny. In addition, the frequency of germline transgenic animals produced by this method is low (Johnson Hamlet MR, Mead PE, unpublished data).

Another method for generating transgenic *Xenopus* was described by Kroll and Amaya [14] and used restriction endonuclease-mediated integration (REMI). In this approach, linearized plasmid DNA is mixed with sperm nuclei in the presence of the restriction enzyme used to linearize the transgenic construct. Digestion of the sperm DNA causes double strand breaks, enabling integration of the linearized plasmid DNA into the sperm haploid genome. The treated sperm nuclei are then injected into mature oocytes. The injection process activates the egg, the cellular machinery repairs the damaged sperm DNA, and normal development proceeds. One advantage of REMI is the potential to analyze embryos at the founder (P_0) stage, whereas other transgenesis techniques develop chimeric P_0 animals [14]. REMI results in integration of the transgene in the sperm nuclei before fertilization and the resulting embryo is not chimeric. Transgenic *X. tropicalis* [15] have successfully been created using a modified REMI procedure using the gamma-crystallin promoter to drive green fluorescent protein (GFP) expression in the developing lens of *X. tropicalis*. Modifications to the REMI protocol have been developed by various laboratories and have led to higher transgenesis efficiencies [16]. However, several problems arise through the use of the REMI transgenesis techniques. High quality eggs and oocyte extracts for sperm nuclei incubation are required for efficient transgenesis, but they can be difficult to obtain, resulting in low numbers of healthy founders [17]. Furthermore, the presence of the restriction endonuclease and the physical manipulation of the sperm nuclei can result in DNA damage and cause complex genetic lesions in the founder animals.

A modification to the standard linear DNA injection strategy has led to a new methodology for creating transgenic animals. Linear DNA fragments with meganuclease *I-SceI* restriction endonuclease sites are injected into the fertilized egg, together with a small amount of the enzyme. The *I-SceI* meganuclease enzyme, a *Saccharomyces cerevisiae* endonuclease [18], has an 18 base pair recognition sequence and as such will have very few, if any, target sites in even complex vertebrate genomes. *I-SceI* restriction sites are engineered into the plasmid vector containing the transgene and the linearized vector is injected into fertilized eggs together with the *I-SceI* enzyme. In standard transgenesis protocols, such as the one described above [13], linearized DNA injected into the fertilized egg forms large concatamers due to the activity of cellular DNA repair systems. The ligation of the transgene into large concatamers is thought to decrease the efficiency of integration of the transgene into the target genome. The presence of the *I-SceI* meganuclease in the injected sample results in cleavage of the nascent concatamers, thus increasing the pool of linear, single copy transgene substrates for integration into the host DNA. The genomic DNA of the fertilized egg is spared from digestion by the meganuclease because the 18 base pair recognition sequence will occur very infrequently, if at all, in the frog genome. The *I-SceI* system was recently shown to be effective for generating transgenic *X. tropicalis* [17]. Reporter assays in *X. tropicalis* using the *Pax6* promoter driving GFP flanked by two *I-SceI* sites co-injected with meganuclease yields embryos with correct temporal expression of GFP in the developing eyes and later in the spinal cord and brain [17]. The rate of trangenesis (about 30%) using meganuclease is higher than that with REMI (about 2% to 5%) using the same promoter construct [17]. In addition to higher rates of transgenesis, efficient germline transmission of *Pax6* promoter constructs was achieved with the meganuclease system. The *I-SceI* meganuclease is therefore another tool that the *Xenopus* community can utilize to create multigenerational transgenic lines.

To achieve efficient and reliable production of transgenic animals, we believe that transposable elements provide the ideal tool with which to create transgenic frogs for large scale genomic studies. Transposons offer several advantages for transgenesis and insertional mutagenesis. A number of laboratories have effectively used transposon based systems, such as *Sleeping Beauty*, *Tol2*, and *phiC31* integrase, to create stable transgenic *Xenopus*. Other reviews in this supplement describe in detail these transposon systems; we briefly discuss the role played by each of these systems in relation to *Xenopus* and discuss the future use of these powerful genomic systems in *X. tropicalis*.

The bacteriophage *phiC31* is a member of the resolvase/invertase family of recombinases that inserts foreign DNA into specific sites within the genome [19]. The *phiC31* recombinase requires two minimal DNA integration sites, namely attB and attP. Upon recombination, two novel sites are created, attL and attR, which prevent remobilization of the inserted DNA fragment in the presence of integrase. In *phiC31* susceptible bacteria, a phage attachment site (attP) is present in the genome and is a target for the integrase enzyme. In vertebrates the precise attP sequence is not present in the genome. Recent studies have demonstrated that there is a limited number of sequence motifs similar to attP sites, and these functional 'pseudo' attP sites allow site-specific integration of *phiC31* to occur [20]. In addition to recognizing site-specific DNA elements and unidirectional integration of DNA, *phiC31* integrase requires no host co-factors for integration to occur and is therefore likely to

function in any organism that contains pseudo attP sites within its genome. Thus, it is a highly attractive system for the insertion of novel DNA elements for gene therapy as well as the creation of transgenic animals [21]. The integrase *phiC31* has been studied in mammalian cultured cell lines [21,22] and *in vivo* animal models [22]. In the frog, Allen and Weeks [23] have shown in *X. laevis* that *phiC31* can effectively integrate a cytomegalovirus promoter driven GFP reporter plasmid into the genome. GFP was first seen at the onset of neurulation (stage 14) and was monitored for 8 days after fertilization. Integration rates for the integrase ranged from 4% to 40%, depending on the amount of integrase mRNA used for injection [23]. Although the *phiC31* integrase results in *X. laevis* are promising, further analysis is needed with this transposon system in both species of frog (*X. laevis* and *X. tropicalis*). For example, germline transmission of the transgenes has not been shown using the *phiC31* system in frogs.

Transposons are autonomous mobile DNA elements found in the genome of many metazoans with no identifiable function. For the developmental biologist, these mobile elements provide powerful genomic tools. The first active vertebrate transposase to be developed was *Sleeping Beauty* (SB), a member of the *Tc1/mariner* superfamily of transposable elements. Activity of this ancient inactive transposition system was restored using reverse engineering. Transposon sequences from related fish species were compared to predict the common ancestral sequence of the active transposase [24,25]. SB has been used with success in multiple vertebrate model organisms, including mouse [26-31] and zebrafish [32,33]. SB integrates its cognate transposon through direct/indirect repeats integrated at random TA dinucleotides in the targeted genome. There is no evidence for sequence site specificity for SB in the genome [28]. It is highly efficient, integrating from one to multiple copies of the transposon into the genome of interest. The random integration of the transposon provides an excellent tool for generating novel insertion events for gene and enhancer trap screens. Recently, Sinzelle and coworkers [34] showed, employing a simple coinjection procedure in *X. laevis*, that the SB transposase can effectively integrate foreign DNA into the frog genome. They demonstrated DNA reporter integration and transmission in the germline to offspring and that by the F_2 generation Mendelian ratios are achieved. Interestingly, they demonstrate that although the SB enzyme is required for integration of the transposon sequences into the frog genome, the integration events are noncanonical and result in inclusion of vector sequences. We have used the SB system in our laboratory and have observed similar noncanonical transposition events in both *X. laevis* and *X. tropicalis* [35] (Yergeau DA, Mead PE, unpublished data). Despite the noncanonical nature of the SB transposition reaction in *Xenopus*, this system offers an efficient method for generating transgenic lines.

The autonomous *Tol2* transposable element, a member of the hAT (*hobo* of *Drosophila*, *Ac* of maize, and *Tam3* of snapdragon) family of transposons, was identified as the genetic lesion in a naturally occurring albino mutation in the teleost medaka (*Oryzias latipes*) [36]. The *Tol2* element was the first functional DNA-based transposon system to be identified in vertebrates. The *Tol2* transposon encodes a functional transposase enzyme that can catalyze the mobilization of the entire transposon. Genetic manipulation of the *Tol2* element was used to derive a non-autonomous system for use as a genetic tool in vertebrate cell lines and in transgenesis [37,38]. The enzymatic activity of the natural element was deleted from the transposon so that self-mobilization was no longer possible. Reporter genes were cloned into the transposon element and transposase activity was supplied in *trans* by co-transfection with the cloned transposase sequence. Several laboratories have used the *Tol2* non-autonomous element in zebrafish for enhancer trap [39,40] and gene trap screens [41]. The Kawakami group [42] first demonstrated that *Tol2* transposase could excise a *Tol2* transposon from a plasmid vector in frog embryos with high efficiency. They note there are differences in the excision pattern between frog and zebrafish suggesting species-specific host factor interactions in teleosts and vertebrates with the *Tol2* transposon element.

Our laboratory has used the *Tol2* transposon system for integration of reporter constructs in both *X. laevis* and *X. tropicalis* [43]. We have demonstrated stable integration of a *Tol2* transposon containing a minimal EF-1α promoter driving expression of a GFP reporter into several *X. tropicalis* founders [43] (Yergeau DA, Mead PE, unpublished data). Mendelian ratios of GFP positive embryos were achieved by the F_2 generation through out-crossing of the *Tol2* GFP positive founders. This indicates that the initial integration events occurred at early cleavage stages in the founder line and resulted in chimerism in the germline of the founder. We have found that *Tol2* can integrate one to multiple copies into the genome of the frog [43]. Polymerase chain reaction based methodologies have been used to clone the integration sites of the *Tol2* transposons, and the flanking sequences align precisely to *X. tropicalis* genome sequence scaffolds. Sequence analysis of the *Tol2* integration sites also indicated that these were true transposition events, because an eight base pair target site duplication flanking the transposon could readily be identified.

Another transposon based system is *piggyBac*, originally identified in moths [44]. Although it has not been tested to date in *Xenopus*, *piggyBac* has been shown to be more efficient than SB and *Tol2* in mammalian cell culture [45]. An amphibian specific transposase, *Frog Prince*, was derived from an inactive transposon identified in the frog *Rana pipens* [46] and has been shown to be able to integrate a reporter construct into mammalian tissue culture cell lines. *Frog Prince* has not been tested vigorously *in vivo* to

determine whether germline transmission is feasible in the frog. These two transposon systems may provide additional tools for the generation of transgenic *Xenopus* for large scale insertional mutagenesis screens.

Insertional mutagenesis screens (trap vectors)

Transposon systems have been used to generate transgenic lines of frogs that express reporter genes, such as GFP, under the control of tissue specific enhancer and promoter elements. In addition to standard transgenesis, these integration systems will provide excellent methodologies for large-scale insertional mutagenesis screens in the frog. Novel genetic loci can be identified using transposon vectors that harbor gene or enhancer trap constructs that identify genes close to the integration site. Enhancer trap vectors contain minimal promoter elements that drive expression of a reporter gene and can be used to identify gene regulatory sequences in the regions that flank the integration site. The activity of the endogenous enhancer element is determined by tissue restricted expression of the reporter minigene. Enhancer trap *Tol2* transposon vectors have been used in zebrafish to generate lines of fish with novel expression patterns. The advantage of enhancer trap constructs is the development of tissue restricted reporter lines, which are invaluable for a variety of experimental approaches such as fate mapping and transplantation studies. In addition, this approach allows the potential to identify the endogenous gene that is controlled by the trapped enhancer. However, because enhancer elements can act over large distances, it is unlikely that the enhancer trap integration event will be mutagenic.

Gene trap vectors differ from enhancer traps in that they do not contain a functional reporter mini-gene. The simplest version of the gene trap is a construct that contains a splice acceptor sequence upstream of the reporter sequence. Integration of this vector into an actively transcribed locus can result in generation of a fusion transcript that contains the reporter gene and the endogenous gene. Several laboratories have successfully developed transposon based gene trap studies in the mouse [28] and zebrafish [32,33]. Splice acceptor gene trap vectors have also been used successfully in frog. Bronchain and coworkers [47] used a REMI strategy to stably integrate splice acceptor gene traps into the frog genome and identified several novel genes. Although splice acceptor gene trap vectors can function to identify transcriptionally active genes, the efficiency of these trap vectors is low because the integration event must occur downstream of a functional splice donor site and the resulting fusion must result in the reporter gene being in frame with the upstream exons. Because the activity of the reporter gene depends on the activity of the endogenous gene, identification of the targeted loci may also be complicated either by very low expression levels of the trapped gene or by a narrow window of transcriptional activity during development. If the endogenous gene is expressed for a brief time during development, then the expression of the reporter gene may potentially be missed. Although the efficiency of this strategy is low, an advantage of this system is that the integration event is likely to be mutagenic. The intragenic integration and the generation of a fusion transcript of the reporter with the endogenous gene increase the likelihood that the insertion event will disrupt the activity of the targeted gene. Another advantage of this approach is that the integration site and the gene that is targeted can easily be identified by using 5'-rapid amplification of cDNA ends (RACE). This becomes an advantage when sequence analysis of the DNA flanking the transposon integration site provides no direct information on the trapped gene. For example, if the integration event occurs in a repeat region it may be impossible to determine the locus that is targeted. Conversely, if the trap vector has integrated into a gene dense region of the genome, then it may be difficult to identify the actual gene that is trapped because splicing may occur with exons that reside hundreds of kilobases away.

A related strategy for gene trapping is the polyadenylation (polyA) trap vector [48]. A polyA trap vector contains a promoter that drives expression of a reporter gene but lacks a functional polyadenylation signal in the 3' untranslated region. In the absence of a functional polyadenylation signal the nascent transcript is unstable and does not result in expression of the reporter protein. A splice donor site is engineered at the 3' end of the reporter gene such that functional polyadenylation signals can be 'trapped' following integration of the vector into a functional gene. The advantage of this approach is that the expression level of the reporter does not depend on the transcriptional activity of the endogenous gene. All that is required for activity of the polyA trap reporter is the sequestration of a function polyadenylation signal. As such, both active and inactive gene loci can be identified using this strategy. As with the splice acceptor gene trap described above, the trapped loci can be identified using 3'-RACE strategies. To increase the mutagenic potential of the polyA trap vector, a gene inactivation cassette can be cloned upstream of the reporter mini-gene. A splice acceptor signal followed by a functional 3' untranslated region sequence cloned at the 5' end of the reporter mini-gene will result in premature termination of the endogenous gene [48].

Remobilization of transposons

A characteristic of DNA based transposon systems, such as *Tol2* and SB, is that a transposon integrated into the genome is a substrate for remobilization by the transposase enzyme. The high fecundity and long lifespan of *X. tropicalis* make this model organism an excellent candidate for transposon remobilization based insertional mutagenesis screens. A transposon integrated into the host genome is stable but can

be induced to remobilize ('hop') in the presence of the transposase enzyme. The simplest strategy for re-expression of the transposase is microinjection of mRNA encoding the enzyme into fertilized eggs harvested from a transposon transgenic animal. Founder lines of *X. tropicalis* females containing the transposon can be stimulated to produce large numbers of eggs and, once fertilized, the one cell zygotes can then be injected with synthetic mRNA encoding the transposase for remobilization in the offspring.

This approach has successfully been used in zebrafish to remobilize a *Tol2* enhance trap transposon and resulted in multiple novel expression patterns in the injected progeny [39,40]. The approach is technically feasible in the frog, and large numbers of eggs for microinjection can be collected from a single *X. tropicalis* female. However, it is time consuming and labor intensive. Ideally, the microinjection should be completed before the first cell division of the embryo. *X. tropicalis* embryos develop quickly, thus decreasing the time available to inject prior to the first cell division. Remobilization at early cleavage stages with resulting chimeric embryos and out-crossing the progeny will be required to isolate individual remobilized transposon integration events. Nonetheless, simple microinjection of the transposase provides a starting point for transposon remobilization strategies in the frog.

The potential problems with the microinjection approach can be resolved by using an *in vivo* remobilization strategy. Expression of the transposase *in vivo* can be achieved by generating transgenic lines that express enzyme under the control of specific promoter and enhancer elements. Transgenes that direct expression of the transposase in the germline of the frog can be used to achieve remobilization in double transgenic animals that carry both the transposase and the transposon substrate. Expression of the transposase in the developing gametes results in mobilization of the transposon substrate. If this strategy is performed in the male germline, potentially millions of sperm with novel reintegration events can be produced. This strategy has been used extensively in the mouse, in which double transgenic 'seed' males are out-crossed to wild-type females and novel reintegration events are scored in the progeny [31,49,50].

The frog is an ideal organism in which to apply this *in vivo* transposon remobilization approach. First, the clutch size of *X. tropicalis* is very large, and an out-cross of a double transgenic seed frog with a wild-type female can yield up to 3,000 offspring. The double transgenic male frogs can be out-crossed at least once per week, resulting in the potential to generate vast numbers of offspring to score for novel integration events. Second, the lifespan of the frog is long and an individual double transgenic seed frog will survive and produce offspring for more than a decade. Third, because oogenesis occurs throughout the lifespan of the female frog, germline remobilization in the female is also feasible in the frog. Maintenance of maternal stores of the transposase mRNA in the developing oocyte, however, may result in continual remobilization events after fertilization and may result in chimeric embryos. For this reason, targeting expression of the transposase in the male germline may be the preferred strategy for *in vivo* remobilization in the frog. Finally, because the remobilization events have occurred in the gametes before fertilization, the resulting embryos will not be chimeric and will thus allow analysis of the novel integration events in the progeny, and time consuming out-crossing strategies will not be required.

Conclusion

Insertional mutagenesis strategies in the frog using DNA based transposon systems will provide a mechanism for identifying developmentally regulated genes and will provide important reagents for the *Xenopus* community. Gene and enhancer trap transgenic animals with tissue specific expression patterns can be used in a variety of cell and tissue transplantation studies in the frog and will also provide tools for detailed fate mapping studies. Cells labeled with fluorescent reporter genes can be isolated using fluorescence activated cell sorting and used in combination with gene expression microarrays to identify tissue specific genes throughout early development.

The frog has been an important model for uncovering fundamental developmental pathways. The recent push to bring modern molecular techniques to the frog will allow the power of modern molecular genetics to be applied to this well established developmental model system. DNA based transposon systems provide important advantages for integration of novel genetic elements for both transgenesis and insertional mutagenesis strategies. The high cargo capacity of transposon systems such as *Tol2* allow large and complex transgenic constructs to be inserted into the frog genome. Multifunction gene and enhancer trap vectors can be developed that will increase the utility of the trapped loci. For example, gene and enhancer trap vectors that direct expression of Gal4-upstream activator sequence (UAS) binary systems will provide useful tools for manipulating the targeted cells (for review [51]). This binary system has been used extensively in *Drosophila* and successfully applied in REMI mediated transgenic *Xenopus* [52]. A founder frog with a specific transposon integration event harboring a GAL4-UAS binary reporter transposon can be interbred with other transgenic lines that carry functional proteins under the control of UAS elements. In this way, specific constructs can be expressed in the targeted cells during embryonic development. Examples of potentially useful proteins to express in this binary system include other fluorescent reporters (such as red fluorescent protein [RFP] or yellow fluorescent protein [YFP] for cell labeling and lineage tracing), proteins that target the cell for cell death (such as enzymes that convert prodrugs to cytotoxic compounds for

cell/tissue ablation studies), or proteins that protect cells from apoptotic death (such as Bcl2 to study the consequence of maintaining specific cell types that are normally fated to undergo programmed cell death).

The study of amphibian embryos has provided important insight into the mechanisms of vertebrate development. Combining modern molecular genetics with the simplicity of embryonic manipulations in the frog will make this already valuable system a more powerful model for elucidating vertebrate development at the molecular level.

Competing interests

The authors declare that they have no competing interests.

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