Efficient gene delivery and gene expression in zebrafish using the Sleeping Beauty transposon

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Abstract

We used the Tc1/mariner family transposable element Sleeping Beauty (SB) for transgenesis and long-term expression studies in the zebrafish (Danio rerio), a popular organism for clinical disease, vertebrate patterning, and cell biology applications. SB transposase enhanced the transgenesis and expression rate sixfold (from 5 to 31%) and more than doubled the total number of tagged chromosomes over standard, plasmid injection-based transgenesis methods. Molecular analysis of these loci demonstrated a precise integration of these elements into recipient chromosomes with genetic footprints diagnostic of transposition. GFP expression from transposase-mediated integrants was Mendelian through the eighth generation. A blue-shifted GFP variant (BFP) and a red fluorescent protein (DsRed) were also useful transgenesis markers, indicating that multiple reporters are practical for use with SB in zebrafish. We showed that SB is suitable for tissue-specific transgene applications using an abbreviated gamma-crystallin GFP cassette. Finally, we describe a general utility transposon vector for chromosomal engineering and molecular genetics experiments in zebrafish. Together, these data indicate that SB is an efficient tool for transgenesis and expression in zebrafish, and that the transposon will be useful for gene expression in cell biology applications as well as an insertional mutagen for gene discovery during development.

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Introduction

Transposable elements are potential candidates for an efficient gene delivery tool in vertebrate model systems such as zebrafish in part because of their highly successful use in plants and invertebrates. Transposons provide stable gene expression from a single-copy integration event, and these elements can molecularly tag the integration loci to facilitate subsequent molecular analyses (Cooley et al., 1988; Hackett and Alvarez, 2000). In Drosophila, Arabidopsis, tomato, barley, and maize (Osborne and Baker, 1995; Koprek et al., 2001; Parinov and Sundaresan, 2000; Walbot, 2000), transposons have been extremely powerful tools in elucidating gene function through gene tagging and insertional mutagenesis studies.

Invertebrate transposons have been used in zebrafish with modest or no success (Gibbs et al., 1994; Raz et al., 1997; Fadool et al., 1998). Therefore, a search was conducted to find an active vertebrate transposable element. All of the identified fish transposon sequences had been inactivated by mutations acquired throughout evolutionary time (Izsvák et al., 1996). The potential for a vertebrate transposon inspired a systematic point mutagenesis of the consensus sequence derived from the inactive sequences to create an active transposon system known as Sleeping Beauty (Ivics et al., 1997). We report the successful development of this system as a gene transfer tool for zebrafish.

The Sleeping Beauty transposon system (SB) consists of two components: the transposase enzyme and a transposon...
vector containing the terminal inverted repeat/direct repeat (IR/DR) sequences. *Sleeping Beauty* belongs to the Tc1/mariner superfamily of transposons, whose members move by a cut-and-paste mechanism in which the transposase binds the IR sequence, excises the element, and integrates into the target TA dinucleotide. SB has been known to have a wide host range, including human cells (Izsvák et al., 2000), mouse somatic cells (Yant et al., 2000), and the mouse germline (Dupuy et al., 2001, 2002; Fischer et al., 2001; Horie et al., 2001). The SB system was an ideal candidate vector for use in zebrafish.

The SB transposon system was extensively compared in HeLa cells to other transposons and found to be the most efficient available element with known function in vertebrate cells (Fischer et al., 2001). Since then, SB has undergone further technological innovations to improve transposition efficiency (Cui et al., 2002; Geurts et al., 2003). To investigate the efficacy of gene delivery using SB in zebrafish, we analyzed pT-(Ivics et al., 1997) and pT2-(Cui et al., 2002) based transposons for transgene and expression studies in zebrafish. We describe *Xenopus* EF1α/H9251 and H9253- crystallin mini enhancer/promoters and their use with multiple fluorescent proteins as functional transposons for zebrafish.

The use of fluorescent proteins, such as GFP (Chalfie et al., 1994), BFP (Stauber et al., 1998), and DsRed (Matz et al., 1999), facilitated expression analysis of integrated transposons through successive generations in live embryos.

We show that SB is an efficient tool for germline transmission and expression from multiple, single-copy insertions in zebrafish. We report the molecular analysis of these
loki, demonstrating a precise integration of these elements onto diverse linkage groups. GFP expression from transposase-mediated integrants (transposants) was found to be Mendelian through the eighth generation. We also describe a general utility transposon vector for use in chromosomal engineering and molecular genetics experiments in zebrafish. These results indicate that Sleeping Beauty is an efficient tool for gene delivery and expression and provides new opportunities for large-scale functional genomics in zebrafish.

Materials and methods

Zebrafish maintenance

Wild-type and bruss zebrafish were purchased from Segrest Farms (Gibsonton, FL, USA). Embryos were raised at 30°C, and spawning was performed as previously described (Kimmel et al., 1995).

Construction of pT- and pT2-based transposon plasmids

The IR/DR transposon sequences of Tanichthys albonubes were inserted into the Smal (New England Biolabs, Beverly, MA, USA) restriction site in pUC19 (Ivics et al., 1997). The XEX-GM2 fragment contains the Xenopus laevis enhancer/promoter XEX (Johnson and Krieg, 1994) fused to the GFP/GM2 open reading frame (Long et al., 1997) and simian virus 40 (SV40) poly (A). Two copies of this fragment were inserted between the IR/DR sequences to create pT/XEX-GM2’2 (used in Fig. 1B).

For construction of pT2/s1EF1α-GM2 (Fig. 1B), a 1342-bp EF1α-GM2-SV40 p(A) cassette was amplified by PCR from pT/XEX-GM2’2 using oligonucleotides 5’/H11032-AGGCATGCAAGCTAGCTTAT-3’ and 5’/H11032-CTTCGCGATATTCCATACCA-3’. The blunted PCR product was cloned into a blunt-filled HindIII site of pT2/HindIII (Cui et al., 2002).

To create pT2/s1EF1α-Cry-GM2 (see Fig. 6A), a 490-bp NheI–NdeI DNA fragment from the Cry1GFP3 plasmid (a kind gift from Marnie Halpern) containing the X. laevis Cry1 promoter fused to GFP was inserted into an NheI–NdeI-digested pT2/s1EF1α-GM2.

The AseI pXEX-BFP cassette (Finley et al., 2001) was blunt cloned into a NotI-linearized and blunted transposon vector (pT/HygR-eGFP) to produce pT/XEX-BFP (see Fig. 4G). The AseI pXEX-RFP cassette (Finley et al., 2001) was similarly blunt cloned into pT/HygR-eGFP to generate pT/ XEX-RFP (see Fig. 4D).

mRNA synthesis and microinjection into zebrafish embryos

SB10-encoding synthetic mRNA was generated from the SBRNAX plasmid as described (Dupuy et al., 2002). One-to two-cell-stage zebrafish embryos were coinjected with 100 ng/μl transposase mRNA and 8.3–16.7 ng/μl of transposon DNA in a 3-nl injection volume at the blastoderm/yolk interface as described (Hyatt and Ekker, 1999).

Genomic DNA isolation and Southern blot analysis

Embryos were harvested by freezing 5–10 days after fertilization and homogenized using a dounce homogenizer in lysis buffer [0.1 M Tris (pH 9), 0.1 M NaCl, 0.05 M EDTA, 0.2 M sucrose, and 0.5% SDS]. A final concentration of 0.1 μg/μl Proteinase K (Qiagen, Valencia, CA, USA) was added to the lysis buffer and incubated at 65°C for 30 min followed by a standard isopropanol precipitation. Genomic DNA from pooled GFP-positive embryos was digested with either NsiI, which is absent in the pT and pT2 vectors, or EcoRV, which cuts pT2 once (Fig. 2A). A 1.6-kb probe specific to XEX-GM2 was used to hybridize the pT
GFP-positive embryos was digested with NsiI for inverse PCR. The 10 bp of the IR/DR sequence (left), the TA dinucleotide insertion site (italics), and the flanking zebrafish genomic DNA sequence (right, bold) are listed.

Southern blot, and the pT2 Southern blots were probed with a PCR-generated, GFP-specific probe of 700 bp.

**Inverse PCR and splinkerette PCR**

Inverse and splinkerette PCR was performed as described (Dupuy et al., 2002). Genomic DNA isolated from GFP-positve embryos was digested with NsiI for inverse PCR. For splinkerette PCR, 500 ng of genomic DNA was digested with SmaI.

**EF1α-GM2 photography and imaging**

Male and female transgenic fish homozygous for a single pT/XEX-GM2’2 insertion were individually outcrossed. The resulting progeny were photographed at the following stages: sphere-stage (4 hpf), 3–4 somite (11 hpf), 24 hpf, 48 hpf, 5 dpf, and adult. Embryos were mounted in 2% methylocellulose and photographed under 5× magnification on a Zeiss Axioscope 2 microscope (Carl Zeiss, Thornwood, NY, USA). Images were captured with AxioVision software and a Zeiss AxioCam Digital camera. For GFP detection, a Bandpass GFP filter set with excitation (450–490 nm) and emission (500–550 nm) ranges was used (Finley et al., 2001). Similarly, a Blue GFP Bandpass filter set was used for BFP detection, consisting of excitation (379–401 nm) and emission (435–485 nm) wavelengths. DsRed detection was achieved by using a rhodamine filter set with excitation and emission wavelengths of 546 ± 10 nm and 570+ nm, respectively.

**Results**

**Germline transmission and expression frequency**

We used an EF1α-GFP SB transposon (Fig. 1A) in a transgenesis and expression study (Fig. 1B). In addition to optimized terminal repeat elements (Cui et al., 2002), this vector (pT2/siEF1α-GM2) contains a truncated EF1α-GFP expression cassette to minimize transposon size, a variable which has been shown to be important for optimal transposition in vitro (Izsvák et al., 2000; Karsi et al., 2001; Geurts et al., 2003). Injecting pT2/siEF1α-GM2 alone results in a transgenesis and expression frequency of 5% (n = 20; Fig. 1B). The pT2/siEF1α-GM2 vector co-injected with SB10 mRNA, however, yielded an increased germline transposition and expression rate of 31% (n = 42; Fig. 1B). F0 germline mosaicism rates of 0.3–16% were noted for pT2/siEF1α-GM2 (Fig. 1B) when co-injected with transposase mRNA. In addition to an increase in the transgenesis and expression frequency rate from 5 to ~30%, the transposase mRNA also increases the total number of generated transgenic chromosomes (see below).

We also conducted a transgenesis study using a larger, pT-based transposon (pT/XEX-GM2’2; Fig. 1B). This element represents an extreme comparison to our optimized, pT2-based vector due to its larger size (5 kb), excision context, and original terminal repeat elements. This vector co-injected with SB10 mRNA generated embryos with a 10% transgenesis and expression rate (Fig. 1B), with similar rates of mosaicism (Fig. 1) and copy number observed for the pT2-derived element.

**Expression-based estimate of integration copy number**

Obtaining transgene expression with high fidelity has been a challenge in zebrafish. Expression from retroviruses has been difficult to obtain (Gaiano et al., 1996), or was found to significantly compromise the titer of the recombinant virus (Linney et al., 1999). Expression from plasmid-based injections is highly variable (Udvadia and Linney, 2003), and our experiments were no different in the single line we obtained using standard plasmid injections (Fig. 1). Six different transgenic F0 siblings were analyzed for expression in their offspring. The expression frequency obtained from F1 outcrosses in the pT2/siEF1α-GM2 alone experiments shows non-Mendelian inheritance of expression. Fewer than half of the embryos harboring the transgene had detectable transgene expression. Data from sibling outcrosses produced GFP expressing F2 embryos at an average frequency of 23% with individual crosses ranging from 13 to 36% (data not shown).

In contrast, expression from transposants was found to be robust and Mendelian in nature. Indeed, expression was high enough to allow us to use the segregation of expression as a first estimate of copy number in our transformants (Table 1). To estimate the number of transposon insertions acquired in the germline of founder fish, we calculated the percentage of GFP-expressing F2 embryos collected from an F1 outcross (Table 1). A total of 24 F1 fish from both pT (Table 1A) and pT2 (Table 1B) experiments were analyzed, representing 11 independent founder fish. The percentages of GFP-expressing embryos ranged from ~50 to 100%. We
used these numbers to estimate expression from independent linkage groups. A 50% rate should represent expression from a single linkage group, whereas 96–100% suggests that up to 4 or more independent linkage groups are represented in that particular outcross. Our data indicated a range of insertions in these studies (Table 1, column 6) and the number of loci predicted from this approach was confirmed by subsequent molecular analyses (see below). A rate lower than 50% could reflect epistatic regulation in our non-inbred fish, silencing of the transgene in some insertion sites, or expression from unintegrated episomal DNA.

### Molecular characterization of T- and T2-based insertions

Southern blot studies with genomic DNA isolated from transgenic embryos were used to analyze the number of integration events and the copy number of each insertion in the pT-based transgenesis experiments. Genomic DNA from embryos was digested with NsiI, a restriction enzyme that does not cut in the transposon vector. Ten insertions were analyzed in this manner and all 10 represented single-copy insertions (data not shown). No head-to-tail concatemers, typically found in plasmid injections (Stuart et al., 1988, 1990; Hackett and Alvarez, 2000) and observed in our plasmid injection generated line (data not shown), were observed in the lines generated from pT/XEX-GM2’2 injections with SB10 mRNA.

We also compared our analysis of insertions with the measured rates of expression (Table 1) to investigate the fraction of expressed transgenes in these studies. Comparison of insertion number with GFP expression data indicates that 70% (n = 10) of integrations express GFP. In one outcross, only a single linkage group was estimated by expression analysis, despite four independent insertions evident on the Southern blot. This could reflect only expression from one transposon, or it could represent multiple, closely linked integrations. Linked insertions have been observed before for many other transposon integrations.

### Table 1

Summary of F₄ expression analysis and Southern blot analysis

<table>
<thead>
<tr>
<th>F₀</th>
<th>F₁</th>
<th>F₂ %GFP</th>
<th>Estimated # linkage groups</th>
<th>Southern analysis copy #</th>
<th>Total # insertions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) pT/XEX-GM2’2 + SB10 mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>100% (46)</td>
<td>5+</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>100% (61)</td>
<td>5+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>94% (47)</td>
<td>4</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>41% (130)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>35% (178)</td>
<td>1</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>41% (166)</td>
<td>1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>E</td>
<td>7</td>
<td>50% (646)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

| B) pT2/αEF1α-GM2 + SB10 mRNA |
| G       | 8       | 70% (725) | 2                         | 3                       | 4                 |
|         | 9       | 52% (164) | 1                         | 2                       | —                 |
|         | 10      | 45% (211) | 1                         | —                       | —                 |
| H       | 11      | 38% (16)  | 1                         | —                       | —                 |
| I       | 12      | 80% (266) | 2–3                       | 3                       | 3                 |
|         | 13      | 50% (364) | 1                         | —                       | —                 |
|         | 14      | 56% (185) | 1                         | —                       | —                 |
|         | 15      | 49% (241) | 1                         | —                       | —                 |
| J       | 16      | 54% (129) | 1                         | 1                       | 2                 |
|         | 17      | 52% (372) | 1                         | 1                       | 1                 |
|         | 18      | 47% (409) | 1                         | 1                       | —                 |
| K       | 19      | 45% (328) | 1                         | 1                       | 1                 |
| L       | 20      | 78% (32)  | 2–3                       | —                       | 2–3               |
|         | 21      | 38% (58)  | 1                         | —                       | —                 |
|         | 22      | 54% (61)  | 1                         | —                       | —                 |
| M       | 23      | 48% (283) | 1                         | 1                       | 1                 |
|         | 24      | 36% (133) | 1                         | —                       | —                 |

Note. (A) Transgenic lines generated from four individual founders in column 1 (B, C, D, E) in the pT screen were analyzed. The founders were identified by the presence of GFP-positive embryos in the F₁ generation. The GFP-positive F₂ embryos (column 2) were isolated, raised, and outcrossed to estimate the number of linkage groups (column 4) containing an insertion by evaluating the percentage of embryos expressing GFP in the F₂ generation (column 3). The numbers in parentheses represent the total number of F₂ embryos analyzed. Embryos from sibling F₂ and F₁ outcrosses were examined by Southern blot to determine the number of integrations (column 5) in each line. Column 6 summarizes the total number of independent insertions obtained from individual founders. (B) Transgenic progeny that stem from seven independent founders listed in the first column (G, H, I, J, K, L, M) in the pT2 screen were similarly analyzed. Column 2 lists the seventeen F₁ fish that were raised in which GFP expression was examined in the F₂ generation (column 3) from the resulting outcross. Based on the GFP percentage in the F₂ generation, we estimated the number of linkage groups (column 4) that have an insertion. Comparatively, a Southern blot analysis (column 5) was performed on the corresponding F₂ embryos. The total number of insertions identified for each founder is listed in column 6.
Fig. 4. Ubiquitous expression of GFP, BFP and DsRed from transposon vectors. A schematic representation of each transposon used to generate the transgenic lines (Tg) is located above each set of fluorescent embryos in (A), (D), and (G). The T2-based vector expressing GFP is under the control of s1EF1α (A), and the T-based vectors expressing DsRed-1 (D) and BFP (G) are under the control of EF1α enhancer/promoter (XEX). Multiple generations expressing GFP (B, C), DsRed (E, F), and BFP (H, I) are shown at 25 hpf (B, E, H) and 5 dpf (C, F, I) along with a wild-type (wt) embryo.
from P-elements (Tower et al., 1993) to Sleeping Beauty in mice (D. Largaespada, personal communication).

For the pT2/s1EF1α-GM2 Southern blot analysis (Fig. 2), genomic DNA was digested with NsiI, which does not cut within the transposon, and EcoRV, which cuts once in the transposon. The EcoRV analysis should produce a very bright signal of 4859 bp if concatemers exist in these animals. Concatemers would also be detected in the NsiI digest.
as a high molecular weight band greater than 10 kb. In both cases, intensities for the bands should be proportional to the number of copies in the concatamer. Transposase-mediated, single-copy insertions would produce bands of varying sizes with intensities less than the single insertion (T) control containing 2 copies of GFP (Fig. 2; lanes 1 and 2). In this investigation, 12 events were analyzed and all reflected single-copy integration events. Taken together, these studies showed that 22/22 insertions from both pT and pT2 transposons were single-copy insertions with $\approx80\%$ ($n = 21$) integrants expressing GFP.

**Insertion-site characterization: direct evidence for transposase-mediated germline integration events**

Inverse and splinkerette PCR were performed for the identification of genomic sequences that flanked the integration sites in these animals. The genomic sequences flanking both left and right sides of transposon insertions were isolated by inverse PCR. To obtain additional sequence from the left side, genomic DNA was digested with Sau3AI for splinkerette PCR. Seventy-eight percent (7/9) of insertions were flanked by TA dinucleotides with an absence of vector sequence, typical of transposase-mediated integration (Fig. 3). One of the integrations contained vector sequence on one side and appeared to utilize a TA approximately 100 bp beyond the IR/DR sequence (data not shown), resulting in a presumptive transposase-mediated mobilization of a 100-bp larger sequence. Together, eight of nine (89%) were transposase-mediated integration events.

**Ubiquitous promoter expression and permanent genetic change: GFP, BFP, and DsRed cassettes**

A large variability in penetrance of expression from transgenes has been reported in zebrafish. For example, GFP appears to be a successful reporter in many research studies (Udvadia and Linney, 2003), while $\beta$-galactosidase has been much more problematic (Bayer and Campos-Ortega, 1992; Culp et al., 1991; Cretekos and Grunwald, 1999; Udvadia and Linney, 2003). The origin of this discrepancy is unknown, especially considering the success at using $\beta$-galactosidase in such diverse systems as mice (Stanford et al., 2001) and *Drosophila* (O’Kane and Gehring, 1987). We were interested in exploring other fluorescent proteins because of their potential utility at stages where the fish exhibit autofluorescence in the wavelengths used to detect GFP and as a separate, distinct reporter in living tissue (Finley et al., 2001).

We characterized a variety of expression cassettes during development and after multiple generations to ascertain the reliability of expression from SB-mediated transposition events. For this analysis, we evaluated three fluorescent proteins as transgenic markers under the control of the EF1$\alpha$ enhancer/promoter in T- and T2-based expression cassettes. Previously, we reported that coinjection of three fluorescent proteins (GFP, BFP, and DsRed) can be detected using filter sets specific for each, thereby allowing simultaneous, multicolor labeling of zebrafish embryos (Finley et al., 2001). We examined the properties of the fluorescent proteins in SB transposon vectors transmitted through the germline. Expression from integrated GFP transformants became detectable between seven- to eight-somite-stage (data not shown) and provided bright expression with a good signal-to-noise ratio at 25 hpf (Fig. 4B). After 5 dpf, the signal-to-noise ratio decreased due to an increase in background fluorescence, but was stable and especially robust in the lens (Fig. 4C). The RFP transposon produced high signal-to-noise expression beyond 25 hpf (Fig. 4E) that was constant and showed a robust signal-to-noise expression level at 5 dpf (Fig. 4F). BFP expression was apparent by 25 hpf (Fig. 4H) and was also detectable at 5 dpf, where autofluorescence is visible in the wt eye (Fig. 4I). In the BFP transgenic fish, the BFP signal was best observed around 25 hpf (Fig. 4H) after which it diminished over the next 4 days (Fig. 4I). All reporter genes were analyzed for expression after multiple generations. No decrease in expression level in successive $F_2$-$F_8$ generations was observed (data not shown).

**Ubiquitous, constitutive, and maternal expression of an SB transposant line**

We carried out a detailed time course characterization of reporter gene expression to test the utility of SB as a permanent transgenic marker in zebrafish. To follow the GFP expression under the EF1$\alpha$ enhancer/promoter (pT/XEX-GM2’2), embryos collected from homozygous male and female outcrosses were systematically examined for GFP expression at several developmental time points (Fig. 5). Embryos from a homozygous female outcross showed strong, maternally derived GFP expression at the sphere stage (4 hpf, Fig. 5B). Embryos produced from a male outcross gave no detectable expression at sphere stage (Fig. 5D) but did show GFP expression at the three- to four-somite-stage (Fig. 5F) that continued to become stronger throughout somitogenesis. All transgenic embryos exhibited robust GFP expression at 24 hpf (Fig. 5H) and 48 hpf (Fig. 5J). By 5 days of development, expression in the lens remained especially bright (Fig. 5L). GFP expression was also stable and ubiquitous in adult fish (Fig. 5N, compare with wt, Fig. 5M).

**Tissue-specific promoter expression**

We also evaluated expression from tissue-specific promoters in SB transposons. For this, we constructed an abbreviated $\gamma$-crystallin GFP transposon using a lens-specific expression cassette from the X. laevis $\gamma$-crystallin gene (Offield et al., 2000). We used this reduced promoter to minimize the size of the transposon. This truncated $\gamma$-crystallin construct ($\gamma$-Cry) was inserted into a pT2-based vector
to achieve tissue-specific GFP expression in transgenic embryos. As shown in Fig. 6, expression from the shorter γ-crystallin promoter cassette (T2/γ-Cry-GM2) resulted in robust and eye-specific expression in injected (Fig. 6B and C) and transgenic (Fig. 6D) embryos. These data show that transposants using ubiquitous and tissue-specific promoters can be generated by using the SB transposon system.

**Generation of general utility transposon vectors**

We have constructed a series of pT2-based vectors for use in a variety of applications (Fig. 7). These plasmids contain transgenic marker cassettes (either EF1α-GFP or γ-crystallin-GFP) for identifying transgenic fish. The constructs contain a unique cloning site for insertion of a promoter of choice, a gene of interest, or other genetic elements. The marker cassettes are modular in nature, offering full options for exchange of promoter, reporter, or poly(A) signal for specific applications in transgenic animals. These SB vectors are designed to provide a valuable resource with multipurpose functionality and are readily available upon request (http://beckmancenter.ahc.umn.edu/).

**Discussion**

**Gene transfer in zebrafish**

Previously, plasmid injections into the zebrafish embryo have been the method chosen to generate transgenic embryos expressing a particular gene of interest. Germline transmission frequencies resulting from plasmid injections are highly variable (reviewed in Udvadia and Linney, 2003). Plasmid injections also have a tendency to integrate as concatemers (Stuart et al., 1988, 1990) and have been shown to produce variegated expression (Stuart et al., 1990) or no expression from an integrated transgene (Stuart et al., 1988; Culp et al., 1991; Hackett and Alvarez, 2000). Similarly, expression was not achieved from the initial versions of the retrovirus (Lin et al., 1994; Gaiano et al., 1996). Subsequently, examples of detectable reporter gene expression from retroviral insertions have emerged (Linney et al., 1999). Even though the retrovirus facilitates rapid cloning of genes (Amsterdam et al., 1999), it is hindered by potential limitations in size (Udvadia and Linney, 2003), regulatory sequences (Linney et al., 1999), complexities in preparation (Gaiano et al., 1996; Udvadia and Linney, 2003), and safety concerns (Hackett and Alvarez, 2000). Alternatively, I-SceI meganuclease-mediated transgenesis approaches shown to be successful in Medaka (Thermes et al., 2002) may be a viable approach in zebrafish. To our knowledge, SB represents the first reproducibly efficient gene delivery and expression methodology suitable for large-scale functional genomics studies in zebrafish.

**Known constraints on using SB-based transposons**

The efficiency of SB-mediated transposition has been shown to be inversely proportional to transposon size (Geurts et al., 2003; Izsák et al., 2000). We considered this in our pT2-based vectors for zebrafish. Nevertheless, the 5-kb transposon (pT/XEX-GM2’2) effectively transposed in zebrafish and resulted in a transgenesis and expression frequency of 10% (Fig. 1). This study represents a lower estimate of transposition rate for the generation of lines harboring comparably sized transposable elements. Extrapolation from cell culture data (Geurts et al., 2003) suggests that even larger elements can be readily mobilized in zebrafish.

**SB use is facilitated by fluorescent protein technologies in fish**

We are interested in developing high throughput assays for transgenesis and gene tagging studies in zebrafish. Hence, we chose to take an expression approach rather than a PCR-based strategy for screening. Expression assays facilitate all aspects of generating and using transgenic fish, from rapid screening of the injected embryos through successive propagation of the established lines. Fluorescent screening is relatively inexpensive with many available hardware options, from a fluorescent dissection microscope to a recently available fluorescent embryo sorter (Union Biometrica, Somerville, MA, USA). Fluorescent protein expression technology thus facilitates the generation, use, and propagation of lines and opens the door to semiautomated screening procedures.

**Chromosomal engineering by SB**

The formation of multicopy concatemers and complex sequence rearrangements (Cretekos and Grunwald, 1999; Culp et al., 1991; Stuart et al., 1988, 1990) after standard plasmid integration transgenesis methods result in poor quality recipient chromosomes. Transposons, however, provide effective gene transfer with clean integration of a single cassette. The SB transposon system offers the opportunity for F₁ or F₂ expression-based screening procedures using tissue-specific expression of fluorescent proteins as a method of high throughput screening. Retroviruses function as insertional mutagens in zebrafish (Golling et al., 2002), but they require a labor-intensive, F₂ generation screening effort. Alternative strategies have been developed, such as enhancer trapping in *Drosophila* (O’Kane and Gehring, 1987) and 5’- and 3’-gene-trapping approaches in mouse ES cells (Niwa et al., 1993; Zambrowicz et al., 1998; Stanford et al., 2001), which in principle can be used to prioritize and potentially enrich for insertions within genes prior to the third generation of animal propagation. These expression-based insertion-site context approaches would also generate many nonlethal insertion lines for cell biology, cell lineage,
Fig. 6. Tissue-specific expression from SB transposons. Coinjection of pT2 transposons (A) containing GFP under the control of an abbreviated X. laevis γ-crystallin promoter (see methods) and SB10 mRNA produces embryos with eye-specific GFP expression in F₀ embryos at 48 hpf. (B) Lateral view. (C) Dorsal view. An F₁ embryo expressing GFP specifically in the eye at 48 hpf is shown in (D).

Fig. 7. Versatile pT2 transposon vector. Diagram of the pT2-based vector available for multipurpose use. The restriction enzyme sites available for cloning of a gene or promoter of interest, or alternative reporter genes, are listed. The current vector allows GFP to be used either as a ubiquitous transgenic marker under the EF1α promoter or a lens-specific transgenic marker under the γ-crystallin promoter and is approximately 2 kb in size. {BsaBI} indicates BsaBI site inactivated by methylation in common E. coli hosts.
and other developmental biology applications. These data show that expression from transposed genes can come from both tissue-specific as well as constitutive transcriptional regulatory motifs. Thus, in addition to the utility of the SB transposon for transgenesis studies, the Sleeping Beauty transposon system is a new tool for functional genomics applications in the zebrafish.

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