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Effects of Insert Size on Transposition Efficiency of the *Sleeping Beauty* Transposon in Mouse Cells

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Abstract: Transposon vectors are widely used in prokaryotic and lower eukaryotic systems. However, they were not available for use in vertebrate animals until the recent reconstitution of a synthetic fish transposon, *Sleeping Beauty* (*SB*). The reacquisition of transposability of the *SB* transposase fostered great enthusiasm for using transposon vectors as tools in vertebrate animals, particularly for gene transfer to facilitate accelerated integration of transgenes into chromosomes. Here, we report the effects of insert sizes on transposition efficiency of *SB*. A significant effect of insert size on efficiency of transposition by *SB* was found. The *SB* transposase enhanced the integration efficiency effectively for *SB* transposon up to approximately 5.6 kb, but lost its ability to enhance the integration efficiency when the transposon size was increased to 9.1 kb. This result indicates that the *SB* transposon system is highly applicable for transferring small genes, but may not be applicable for transferring very large genes.

Key words: *Tcl*-like, integration, gene transfer, transgenic fish.

INTRODUCTION

Transposable elements can be divided into retrotransposons and DNA transposons according to their mode of transposition (Finnegan, 1989). Retrotransposons move via RNA intermediates and depend on reverse transcriptase, while DNA-mediated transposons move through DNA intermediates by a cut-and-paste mechanism and depend on transposases (Plasterk, 1996). Transposition of DNA transposons requires short terminal inverted repeats (IRs) in *cis* and its encoded transposase in *trans* (Ivics et al., 1997). More common forms of the DNA transposons include the *P* elements of *Drosophila melanogaster*, the *mariner* element of *Drosophila mauritiana*, and the *Tcl* elements from *Caenorhabditis elegans.* The *P* element has been developed into a powerful tool for genomic manipulations in *Drosophila* such as insertional mutagenesis, transposon tagging, enhancer trapping, and enhanced integration of transgenes into the germline cells. Unfortunately, *P* element cannot be widely applied because of its inability to transpose in nonhost species, presumably because of its requirement for specific host factors (Rio et al., 1988; Handler et al., 1993; Gibbs et al., 1994).

Because of the wide phylogenetic distribution of the *Tcl/mariner* superfamily of transposons, it has been proposed that they may not require specific host factors and

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Table 1. PCR Primers and Product Sizes of Stuffing Fragments

Fragment	PCR upper primer	PCR lower primer	Size (bp)
λ1	gggggatccggatggtgatgccgagaactttatg	gggggatcccattgcgtcgctttttgctgtc	1436
λ2	gggggatccggatggtgatgccgagaactttatg	gggggatccgctggcaatatgcgggagattac	3426
λ3	gggggatccggatggtgatgccgagaactttatg	gggggatcccccgtccaagccagagatgac	6903

may be used in heterologous species. However, all the *Tcl/mariner* elements found in vertebrate genomes were defective (Henikoff, 1992; Heierhorst et al., 1992; Goodier and Davidson, 1994; Radice et al., 1994; Izsvak et al., 1995; Ivics et al., 1996; Lam et al., 1996a, 1996b; Liu et al., 1999). This fact has prompted two lines of research: attempts to introduce transposons into heterologous systems and attempts to reconstitute active transposons based on sequences of the vertebrate *Tcl/mariner* elements. The success of these attempts has been demonstrated by transposition of the *mariner* element from *Drosophila mauritiana* in the parasitic flagellate protozoan *Leishmania* (Gueiros-Filho and Beverley, 1997) and the reacquisition of transposability of the synthetic transposon *Sleeping Beauty* (*SB*) (Ivics et al., 1997). The *SB* has been shown to transpose in fish, mouse, and human cells through a precise cut-and-paste mechanism (Ivics et al., 1997; Izsvak et al., 1997; Luo et al., 1998). The reacquisition of transposability of the *SB* transposase fostered great enthusiasm for using transposon vectors as tools in vertebrate animals, particularly for gene transfer to facilitate accelerated integration of transgenes into chromosomes. However, the *SB* was reconstructed based on *Tcl*-like elements, which have a normal size of 1.6 kb. The question remains if *SB* can facilitate transposition of larger molecules. Here, we report the effects of insert sizes on transposition efficiency of *SB.* A significant effect of insert size on transposition efficiency of *SB* was found. The *SB* transposase enhanced integration efficiency effectively for transposons of 5.6 kb, but lost its ability to transpose with a transposon of 9.1 kb. This result indicates that the *SB* transposon system is highly applicable for transferring small genes, but may not be applicable for transferring large genes.

Materials and Methods

Primer Design and Polymerase Chain Reaction

Lambda phage DNA sequences were retrieved from the GenBank, and Oligo software (National Biosciences, Plymouth,) was used to design one upper primer and three lower primers. A *Bam*HI restriction site was included in the 5′ end of primer sequences to facilitate cloning (Table 1). The use of upper primer when coupled to each of the three lower primers yielded fragments of 1436 bp, 3426 bp, and 6903 bp. The λ phage DNA was amplified by using the GeneAMP XL PCR Kit (part N808-0192, Perkin-Elmer, Foster City, Calif.) in a polymerase chain reaction (PCR) of 50 μl containing 200 ng of λ phage DNA, 0.25 mM of deoxynucleotide triphosphate (dNTPs), 1.2 mM $MgCl_2$, 20 μM of upper and lower primers, and 2.0 U of rTth DNA Polymerase XL. Thirty-five cycles of PCR reactions were carried out with 1 minute of denaturing at 94°C, 1 minute of annealing at 61°C, and 5 minutes of extension at 72°C, followed by a final 10-minute extension. An initial denaturation at 94°C for 2 minutes was conducted.

Construction of Plasmids Containing *SB* Transposons of Different Sizes

The PCR products were first extracted once by phenol and once by chloroform, then precipitated by ethanol in the presence of 0.2 M sodium acetate (pH 6.5). The precipitates were resuspended in Tris-EDTA buffer (10 mM Tris, 1 mM ethylenediaminetetra-acetic acid, pH 7.4) and digested by *Bam*HI restriction endonuclease. Following digestion, samples were mixed with 5 μl loading dye and electrophoresed on 1% agarose gel. The λ phage DNA fragments were purified from the gel using the QIAquick Gel Extraction Kit (catalog number 28704, Qiagen, Valencia, Calif.). The purified λ phage DNA fragments with *Bam*HI ends were first cloned into pBluescript SK^+ to take advantage of the color selection. The cloned *Bam*HI fragments were gel purified and cloned into the *Bam*HI-digested pTSVNeo, the plasmid containing the neomycin resistance gene (*neo*) driven by SV40 promoter (Ivics et al., 1997).

In plasmid pTSVNeo, the *Bam*HI site is located downstream of the *neo* gene internal to the right IR element. The plasmid pTSVNeo was digested with *Bam*HI and treated with calf intestine alkaline phosphatase (CIAP). The iso-

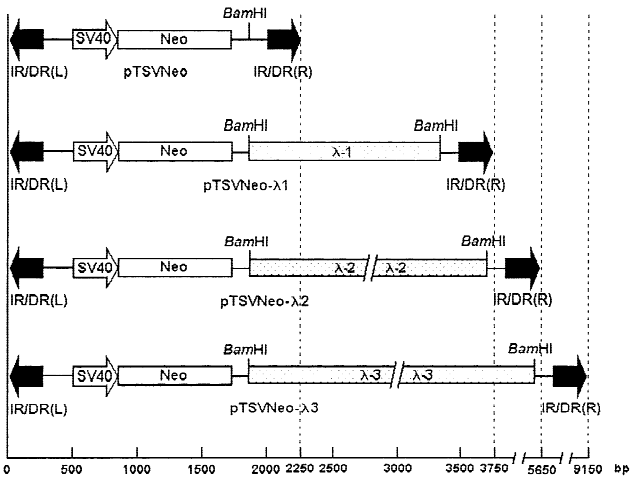

Figure 1. Schematic diagram of the transposon portions of the pTSVNeo, pTSVNeo-λ1, pTSVNeo-λ2, and pTSVNeo-λ3 constructs. The *SB* IR/DR elements are represented as black arrows. The neomycin resistance gene (*neo*) is under the control of simian virus 40 promoter (SV40). Different sizes of stuffing fragments (λ1 in pTSVNeo-λ1; λ2 in pTSVNeo-λ2, and λ3 in pTSVNeo-λ3) were inserted into *Bam*HI site downstream of *neo.* The scale indicates the sizes of the *SB* transposons in base pairs.

lated *Bam*HI fragments of λ phage DNA were ligated to the dephosphorylated vector (Figure 1). After ligation, *Escherichia coli* DH5αF′ cells were transformed with the ligations. Minipreparation of plasmids was used to screen the plasmids harboring the stuffing fragments. The resulting plasmids were designated as pTSVNeo-λ1 (with 1436 bp λ phage DNA), pTSVNeo-λ2 (with 3426 bp λ phage DNA), and pTSVNeo-λ3 (with 6903 bp λ phage DNA). Plasmid DNA was prepared by using the Concert High Purity Plasmid Maxiprep System (Life Technologies, Inc., Rockville, Md.) for transfection experiments.

Cell Culture and Transfection

Mouse fibroblast *NIH 3T3* cells (ATCC, Manassas, Va.) were cultured at 37°C in a humidified incubator supplied with 5% CO_2 in DMEM medium. The medium was supplemented with 10% fetal bovine serum (FBS), and antibiotic-antimycotic solutions (Life Technologies). One day before the transfection, 5.5×10^5 cells were seeded onto 6-cm plates and transfected with 5 μg of plasmid DNA by using Lipofectin reagent and serum-free opti-MEM medium (Life Technologies) followed the lipofection protocol of Felgner et al. (1987). After 5 hours of incubation with the DNA-lipofection complex, serum-free medium was removed, and cells were shocked with 15% glycerol in phosphate-buffered saline (PBS) for 45 seconds and washed 2 times with PBS. Cells were fed with DMEM medium and returned into the incubator. After 12 hours of growth, DMEM medium was removed, and cells were washed once with PBS, trypsinized, and plated onto 10-cm plates containing complete medium with 400 μg/ml G418 (Life Technologies). Resistant colonies were formed after 2 weeks of selection with G418. Four plates were used for transfection of each *SB* construct, and the experiments were repeated twice.

Data Analysis

At the conclusion of each experiment, medium was removed from the plates. Colonies were stained with 0.1% crystal violet in methanol for 3 minutes, washed extensively with tap water, dried, and scanned by using ScanJet 6100C (Hewlett-Packard, Palo Alto, Calif.). Colonies were counted by visual inspection with each colony marked after counting using a marker. In cases in which high density was encountered, colonies were counted with the assistance of zoom-in function of the computer, or counted by using an Image Documentation System using GelExpert software version 3.5 (NucleoTech Corp., San Mateo, Calif.). SAS statistical package (SAS Institute, version 6.12) was used to conduct analysis of variance (ANOVA), Duncan multiple pairwise comparison test, and to calculate the correlation coefficient (r).

Results and Discussion

Construction of *SB* Transposon Vectors with Different Sizes

To determine the effects of insert sizes on transposition efficiency of *SB* transposon, 3 additional *SB* transposons were constructed using the transposon vector pTSVNeo. This vector contains the selection marker *neo* flanked by the *SB* IR/DR (inverted repeat/direct repeat) elements. Stuffing fragments of different sizes were amplified from bacteriophage λ DNA by PCR (Table 1) and inserted into the *Bam*HI restriction site downstream of the *neo* gene internal to the right IR (Figure 2). The pTSVNeo transposon has a size of approximately 2.2 kb. The resulting plasmids pTSVNeo-λ1, pTSVNeo-λ2, and pTSVNeo-λ3 harbored transposons of about 3750, 5650, and 9150 bp, respectively. These plasmids differ from the original transposon pTSV-

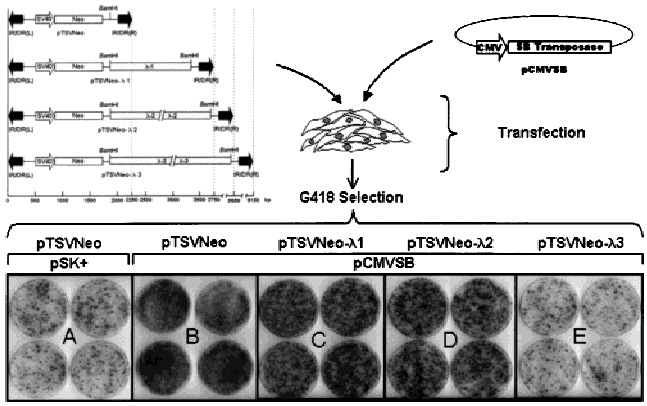

Figure 2. Determination of insert sizes on transposition efficiency of *SB* transposase in *NIH 3T3* cells. *NIH 3T3* cells were cotransfected with different sizes of *SB* transposons (top left corner) and pCMVSB (top right corner) followed by selection with G418. Neomycin-resistant colonies were visualized by crystal violet staining: (**A**) pTSVNeo plus pBluescript SK$^+$ (pSK$^+$); (**B**) pTSVNeo plus pCMVSB; (**C**) pTSVNeo-λ1 plus pCMVSB; (**D**) pTSVNeo-λ2 plus pCMVSB; and (**E**) pTSVNeo-λ3 plus pCMVSB.

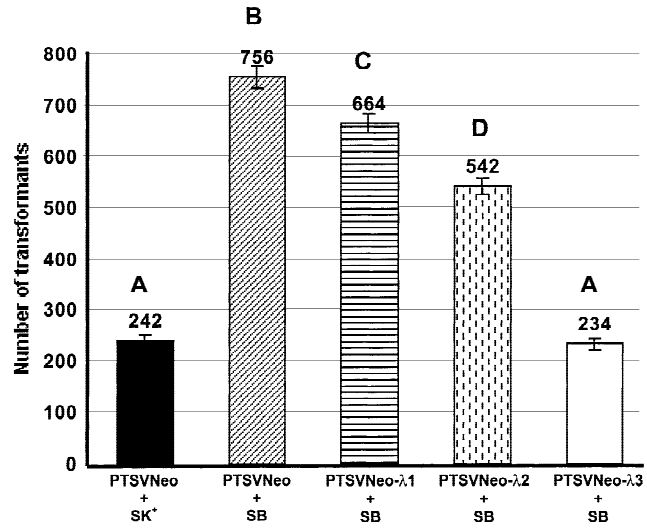

Figure 3. Quantitative presentation of the effects of insert sizes on transposition efficiency of *SB* transposase in *NIH 3T3* cells. Numbers of transformants were averages of colony numbers from 8 plates of 2 transfection experiments. Letters above the histogram bars indicate statistical groupings.

Neo only in the sizes of their stuffing fragments. In all these plasmids, *neo* gene was driven by the simian virus 40 promoter/enhancer. These plasmids were used to determine the effects of insert sizes on *SB* transposition efficiency in mouse cells.

Determination of Insert Size Effects on *SB* Transposition Efficiency

The *SB* system consisted of a transposon vector harboring the genes of interest and the *SB* transposase to be provided in *trans* through an expression plasmid, pCMVSB. The expression of the *SB* transposase is driven by the cytomegalovirus (CMV) promoter (Ivics et al., 1997). The transposition of the *SB* transposons depends on cotransfection of the transposon with the *SB* expression plasmid. Therefore, the transposons of different sizes were cotransfected with pCMVSB into *NIH 3T3* cells by lipofection. The hypothesis was that if the transposase enhances transposition of the transposons, increased numbers of neomycin-resistant colonies would result. To test this hypothesis, the transfected cells were selected with the antibiotic G418 for 2 weeks until the formation of discrete colonies. The transfection of transposon vector alone without the cotransfection with the *SB* expression plasmid was used as a control. As expected, cotransfection of the *SB* expression plasmid pCMVSB with pTSVNeo enhanced the number of resistant colonies more than 3-fold in *NIH 3T3* cells as compared with the control ($P < .01$, Figure 3).

Transformation efficiency of the transposon constructs was inversely correlated with the insert sizes ($r^2 = -.98$). As the insert sizes increased, the transformation efficiency decreased. As shown in Figure 3, insertion of a stuffing fragment of 1.4 kb (pTSVNeo-λ1) resulted in a statistically significant reduction of 12% ($P < .01$). Similarly, insertion of a 3.4-kb stuffing fragment (pTSVNeo-λ2) led to an additional statistically significant reduction of 16% in transformation efficiency. Insertion of a 6.9-kb stuffing fragment abolished the enhancement in transformation efficiency by the *SB* transposase. These results suggest that the *SB* transposon system is highly sensitive to increases in insert sizes, and that there is an upper limit in transposon size for efficient transposition. Thus the *SB* transposase may provide enhanced integration rates for transposons up to 5.6 kb, but its application for larger transposons may be limited. The *SB* transposon system can be used for gene transfer experiments involving large numbers of eukaryotic genes, particularly for expression systems using complementary cDNAs. Considering that most of the eukaryotic cDNAs are smaller than 5 kb, we conclude that insert size limitation of *SB* transposon system will not greatly affect its application for gene transfer studies, but an upper limit should be considered for using the *SB* transposon vectors containing large inserts.

This research is the first to test the effect of insert size on transposition efficiency of the *SB* transposase using *NIH 3T3* cells. In the mouse cell line, the transposition efficiency of the *SB* transposase was not as high as shown in HeLa cells (Ivics et al., 1997). The lower efficiency in the mouse cells could result from the host cell differences. *NIH 3T3* mouse cells are immortalized cells, whereas the human HeLa cells are transformed cells. Transformed cells are readily leaving culture plates and their relanding could magnify the initial transformation rates.

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