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Gene Transfer into Genomes of Human Cells by the *Sleeping Beauty* Transposon System

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The *Sleeping Beauty* (SB) transposon system, derived from teleost fish sequences, is extremely effective at delivering DNA to vertebrate genomes, including those of humans. We have examined several parameters of the SB system to improve it as a potential, nonviral vector for gene therapy. Our investigation centered on three features: the carrying capacity of the transposon for efficient integration into chromosomes of HeLa cells, the effects of overexpression of the SB transposase gene on transposition rates, and improvements in the activity of SB transposase to increase insertion rates of transgenes into cellular chromosomes. We found that SB transposons of about 6 kb retained 50% of the maximal efficiency of transposition, which is sufficient to deliver 70–80% of identified human cDNAs with appropriate transcriptional regulatory sequences. Overexpression inhibition studies revealed that there are optimal ratios of SB transposase to transposon for maximal rates of transposition, suggesting that conditions of delivery of the two-part transposon system are important for the best gene-transfer efficiencies. We further refined the SB transposase to incorporate several amino acid substitutions, the result of which led to an improved transposase called SB11. With SB11 we are able to achieve transposition rates that are about 100-fold above those achieved with plasmids that insert into chromosomes by random recombination. With the recently described improvements to the transposon itself, the SB system appears to be a potential gene-transfer tool for human gene therapy.

Key Words: gene therapy, inverted terminal repeats, site-specific mutagenesis, transposase

INTRODUCTION

Transgenic DNA must penetrate three biological barriers—the cellular membrane, the nuclear membrane, and chromosomal integrity—for successful gene therapy. Gene delivery to mammalian chromosomes has been achieved using two broad classes of vectors, viral and nonviral [1]. Viruses efficiently deliver genes to target cells for expression as a part of their natural activity [2]. However, mammalian cells have evolved elaborate defenses against viruses and have mechanisms that counteract expression of transgenes [3–6]. Nonviral alternatives to viruses [7,8] are often less effective because integration of single genes into chromosomes requires efficiently penetrating the barriers—the plasma membrane, the nuclear membrane, and chromosomal integrity.

DNA transposons are natural, nonviral vehicles for bringing new DNA into genomes of lower organisms [9]. *Tc1/mariner*-type transposons, initially found in nematodes and *Drosophila,* are widespread in nature and are extremely able to invade genomes, including those of vertebrates [10] and humans [11,12]. *Tc1/mariner* transposons are simple structures consisting of inverted terminal repeats (ITRs) that flank a single transposase gene. Transposase binds at precise sites in each of the ITRs where it cuts out the transposon and inserts it into a new DNA locus (a "cut-and-paste" mechanism; Fig. 1). We found that all of the *Tc1/mariner*-type transposons scattered throughout vertebrate genomes contained transposase genes that are highly mutated, leaving them as repetitive, inactive DNA sequences [13]. Consequently,

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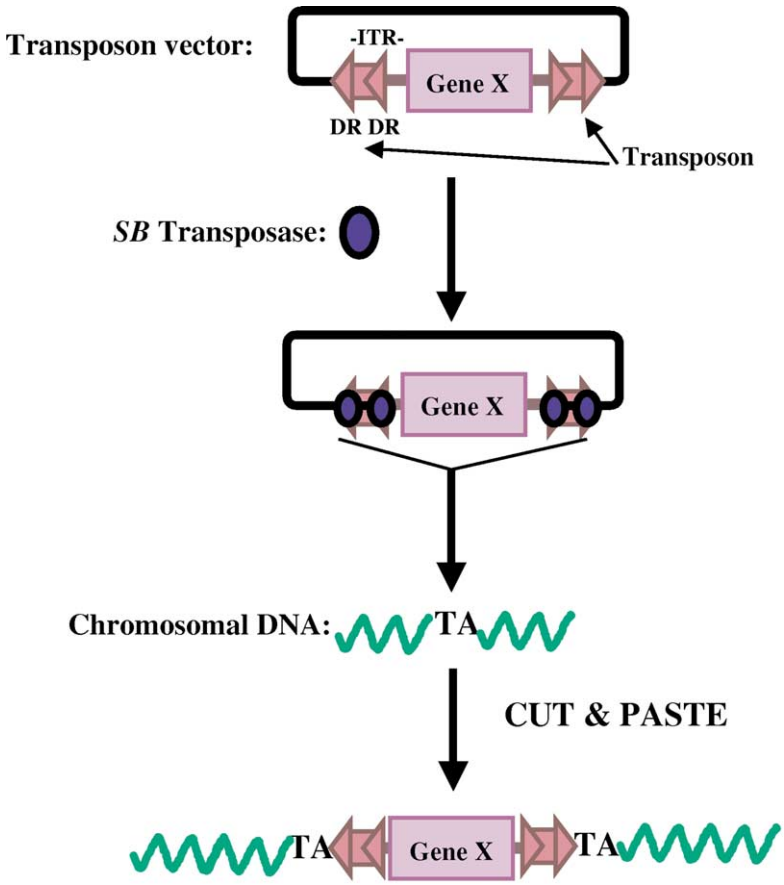

FIG. 1. Cut-and-paste mechanism of *Sleeping Beauty* transposons. For all of our experiments pT/plasmids, where pT designates a *T* transposon in a plasmid, *p,* were used as vectors. *Sleeping Beauty* transposase binds to two direct repeats (called DRs) in each of the inverted terminal repeats (ITRs) of the transposon (shown as arrows), precisely cuts the transposon out of the plasmid, and inserts the transposon into a target DNA, shown as chromosomal DNA. SB transposons, like all *Tc1/mariner*-type transposons, integrate into TA dinucleotide base pairs, which are duplicated on each end of the insertion site.

we resurrected a transposon system from sequences of inactive *Tc1/mariner*-like transposons found in salmonids that we called *Sleeping Beauty* (SB) [14,15]. The system consists of two parts, a transposon and a source of transposase. The SB system uses a transposon with an expression cassette replacing the transposase gene; the transposon can be mobilized when SB transposase is supplied *in trans*.

The SB transposon system has four features that make it attractive as a vector for gene therapy. (1) Both parts of the SB system can be supplied as naked DNA or as DNA (transposon) plus RNA or protein for the sources of transposase. Therefore, the system is likely to have low immunoreactivity. (2) SB transposase, which has a nuclear localization signal, binds to four sites on a transposon, which may facilitate uptake of transposons into nuclei of cells [16]. (3) The transposase catalytically inserts a *single copy of precise sequence* into recipient DNA sequences rather than relying on random integration of variable lengths of DNA. (4) The expression of transposed genes is reliable and long term [17,18], even following passage through the germ line [19–22]. The SB transposon system is nearly an order of magnitude higher for gene transfer into chromosomes of HeLa cells than all other transposons tested [19]. Using both a hydrodynamic injection technique [23,24] and a "gutted" adenovirus, Yant *et al*. [18,25] delivered a factor IX-harboring SB transposon to about 1–5% of hepatocytes in mice, a reasonable goal for effective gene therapy in many cases [7]. Hydrodynamic injection of the SB transposon system has also been used to deliver transgenes to lungs in mice [26]. The stable expression of genes in SB transposons in mouse tissues demonstrates their high potential for gene therapy.

The SB system used in previous experiments was based on the first working version of the system. The usefulness of the SB system depends on its activity and its carrying capacity—how much genetic cargo the transposon can deliver to chromosomes at high efficiency. The overall activity of the SB system for gene delivery depends on (1) the transposon, including its length and the sequences in its ITRs to which SB transposase binds, and the relative geometry of the binding sites [27]; (2) the ease of excision from the donor DNA [28]; (3) the ratio of transposase to transposon [29]; and (4) the activity of the transposase. Here we assess several of these parameters. An in-depth analysis of the structure/function relationship of the ITR sequences has been reported and the transposon sequences have been improved to yield integration rates

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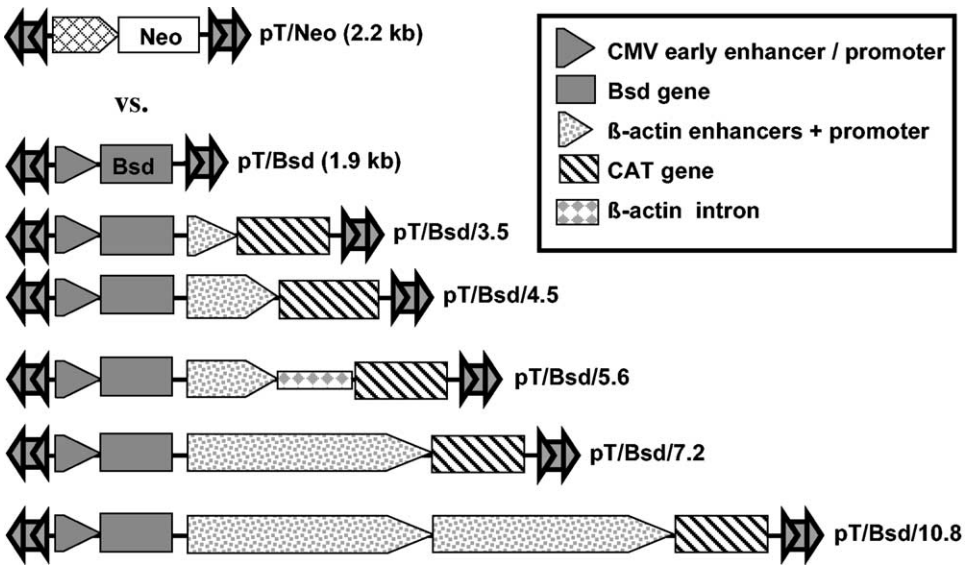

FIG. 2. Transposons with drug resistances used in this study. pT/Neo (2236 bp) was used as a standard for comparison to blasticidin-resistance transposons that are variations of the parent construct pT/Bsd (1901 bp). Fragments of a carp β-actin promoter-driven CAT gene, with the Chinook salmon poly(A) addition sequence, were cloned into pT/Bsd to obtain larger pT/Bsd transposons that have sizes of 3512, 4495, 5626, 7157, and 10,802 bp. The number at the end of each construct designates the total length of each transposon in kilobases.

about three- to fourfold higher than with the original system [27]. Here we report on improvements to the SB transposase to make it more active. Our results suggest that the SB system is a viable vector for delivering genes into genomes of human cells.

RESULTS

In the following sections below, we have examined several important features of the SB transposon system to determine the potential of the system for gene therapy. These include the effects of transposon length on transposition efficiency, the importance of the ratio of transposase to transposons, and the improvements on activity of transposase when selected amino acids are mutated for higher efficiency.

Transposon Carrying Capacity

The carrying capacity of SB transposons is critical for their widespread use in gene therapy. Previous studies [30,31] indicated that transposition efficiency decreases at approximately a logarithmic rate as a function of length. Transposons 10 kb or larger were not integrated into genomes at rates higher with transposase than without. The first study indicated that transposons longer than about 5 kb had low transposition frequencies that dampened enthusiasm for their use with genetic cargo in excess of about 5 kb. However, these conclusions were based on protocols that required active expression of a selectable *neo*^{r} marker for scoring transposition. Because the transposons used in these studies contained "stuffer" DNA fragments from λ phage that is relatively rich in CpG sequences, methylation of the prokaryotic DNA could have attenuated gene expression from the transposon or the sequences could have induced RNA silencing [32]. If so, the transposition rates might have been higher for the longer transposons than the experiments suggested. Consequently, we reexamined the carrying capacity of SB transposons by reducing the potential effects of prokaryotic sequences on transposition and/or expression of the transgene. We constructed pT/Bsd, a 1.9-kb transposon that would confer blasticidin (Bsd) resistance to HeLa cells following integration (Fig. 2). We constructed larger transposons of 3.5, 4.5, 5.6, 7.2, and 10.8 kb by introducing various lengths of stuffer DNA composed of the carp β-actin enhancer/promoter–chloramphenicol acetyltransferase gene (CAT) cassette [33,34]. In all of the transposons the amount of prokaryotic DNA, the *bsd* and *neo* genes, was constant. Equal molar ratios of pT/Bsd (of varying length) and pT/Neo, a 2.2-kb transposon used as an internal standard for transposition activity, were transfected into HeLa cells. Because the various transposon donor plasmids vary in size, a plasmid pGL-1, which has a CMV–GFP cassette, was used as "filler" DNA to maintain a constant amount of the total DNA in all experiments to control for transfection efficiency. We routinely transfected about 50–60% of the cells as measured by transient GFP expression (data not shown). Cells were divided into several culture dishes and grown in medium containing either blasticidin or G418.

We measured transposition efficiency as the ratio of colonies that were resistant to blasticidin compared to G418 (Fig. 3A). There was an approximately inverse linear relationship between transposon length and transposition frequency for transposons between 1.9 and 7.2 kb (Fig. 3B). SB transposase mediated the delivery of 5.5-kb transposons half as efficiently as 2-kb transposons. At 10.8 kb, a size at which transposition rates in the other studies were nil, we observed a residual enhancement of integra-

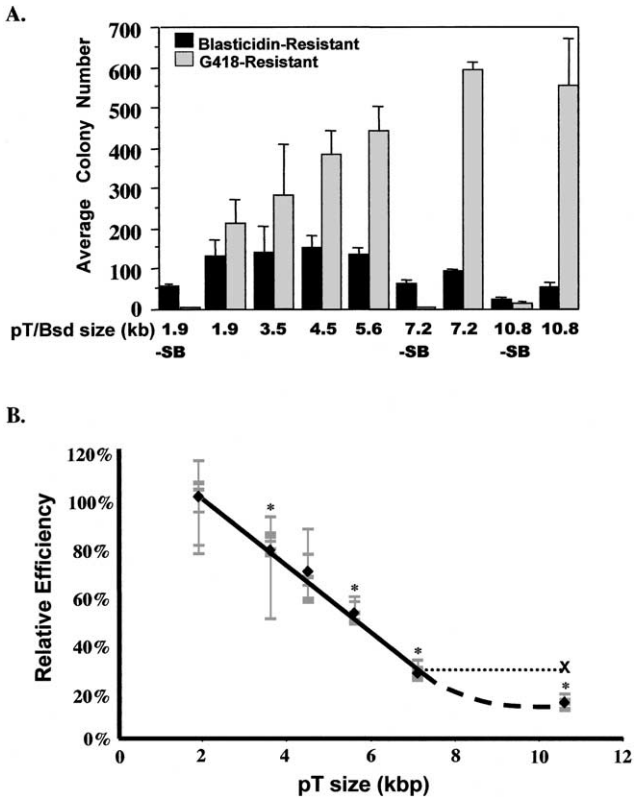

FIG. 3. Effects of transposon size on transposition. (A) Equal molar numbers of the pT/Bsd transposons were cotransfected with 500 ng pT/Neo, 500 ng pCMV-SB, and pGL-1 as filler DNA into HeLa cells in equal molar numbers ($\sim 3.7 \times 10^{11}$ total transposons per transfection) and 3×10^4 were replica-split into either 800 μg/ml G418 or 100 μg/ml blasticidin. The numbers of G418-resistant colonies were scored after 14 days, whereas Bsd-resistant colonies were counted after 19 days of selection. pT/Bsd, pT/Bsd/7.2, and pT/Bsd/10.8 cotransfections were tested with and without pCMV-SB. At least three independent experiments were run for each construct. Standard errors are indicated for each average. (B) Relationship of transposition efficiency to transposon size. The black diamonds denote the average efficiencies compared to pT/Neo, adjusted to 100% for pT/Bsd. Individual efficiencies per experiment are shown by hash marks on the range bars. The "X" value for the 10.8-kb plasmid is a corrected value based on the suggestion that pT/Bsd10.8 has a lower transfection efficiency than pT/Bsd (1.9 kb). Asterisks indicate colony-forming values for each point that are *t*-test statistically different ($P < 0.05$) from the value of the point to its left, e.g., pT/Bsd3.5 compared to pT/Bsd1.9, pT/Bsd5.6 compared to pT/Bsd4.5.

tion by SB transposase as demonstrated by transfections done with and without SB transposase (Fig. 3A). For the 10.8-kb transposons we verified that the amplification in integration rates was due to transposition rather than an enhanced rate of recombination by examining the junction sequences of several *bsd* transgenes. Three of the four insertions had the specific junction fragments expected for transposition and flanking TA sites, indicative of transposition [10] rather than random recombination (data not shown). We noted that the background level for the 10.8-kb transposon without added SB transposase is about half that of the smaller constructs. Random recombination of the *bsd* gene into HeLa chromosomes should be influenced not by the size of the plasmids but by transfection of the plasmids into cells, suggesting that the observed transposition value for the 10.8-kb transposon may be lower than the actual transposition rate. Correcting for the apparent decrease in uptake of the pT/Bsd10.8 plasmid is shown in Fig. 3B by the dotted line. Thus, our results indicate that (1) the size–efficiency curve for transposition is not linear for transposons longer than about 7 kb and (2) SB transposase confers a significant advantage for gene delivery even for long genes, an important consideration for gene therapy.

Overexpression Inhibition and Optimization of the Transposon-to-Transposase Ratio

There are four transposase-binding sites in an SB transposon—the two direct repeats (DRs) in each ITR bind transposase molecules (Fig. 1). Our model for transposition indicates that the transposase molecules can bind to each other in a crisscross manner to juxtapose the two ends of the transposon [27]. This model predicts that the transposition rate should rise as the ratio of transposase (SB) to transposon (pT) increases—up to a point. When the ratio of SB to pT exceeds about 4 to 1, the efficiency of transposition should decrease due to quenching [29] of the transposases bound to the ITRs. Binding of free SB transposase molecules to those bound to the ITRs would prevent the juxtaposition of the transposon ends, which is required for mobilization. Izsvak *et al*. [30] used different promoters to drive expression of SB transposase and did not find evidence that overexpression of SB inhibited transposition over the 17-fold range of expression they tested. Accordingly, we tested the effects of transposition over a much broader range, from almost 17:1 to 1:33 of pT to SB plasmids, a 560-fold range. We transfected 30, 100, or 500 ng of pT/Neo with 30, 100, 300, 500, or 1000 ng of pCMV-SB, using pGL-1 to maintain a constant level of total transfectable DNA. The data shown in Fig. 4 demonstrate the dramatic inhibitory effect observed with the higher doses of SB. At 1000 ng of pCMV-SB, the resistant colony formation approached background for all three concentrations of pT/Neo. These results are consistent with overexpression inhibition. When 30 or 100 ng of pT/Neo was used, 100 or 300 ng of pCMV-SB, respectively, yielded the highest colony formation, giving the same ratio of pT:SB of 1:3. At the highest pT/Neo level of 500 ng, the maximal level of transposition occurred at 100 ng of pCMV-SB, a 5:1 ratio. At this lowered dose of transposase, the number of G418-resistant colonies was about 6-fold higher than that seen with 100 ng of pT/Neo plus 300 ng of transposase.

The dramatic effect of lowered transposition efficiency at higher SB doses suggested that transposition at a very high rate might be cytotoxic. We examined

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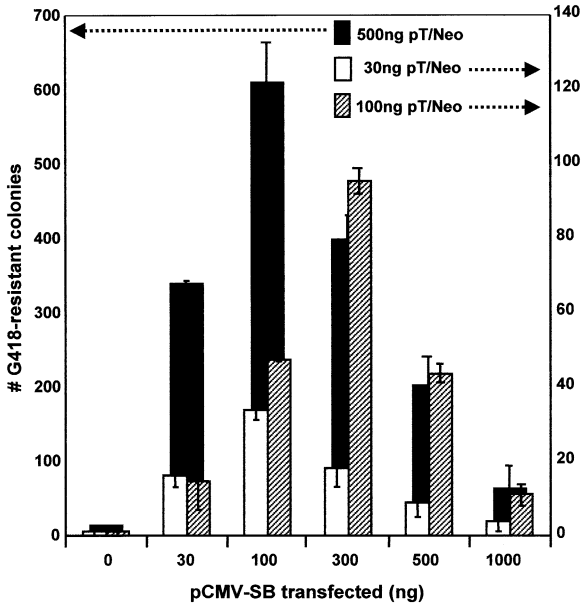

FIG. 4. Effects of pT:SB ratios on transposition efficiency. 30, 100, or 500 ng of pT/Neo was cotransfected with 0, 30, 100, 300, 500, or 1000 ng of pCMV-SB. pGL-1 was added as filler DNA to maintain a constant amount of DNA per transfection. The results of the 30 and 100 ng of pT doses are measured on the right ordinate, while the 500 ng of pT dose results are measured using the left ordinate.

this possibility by determining the average number of inserted transposons per genome. We hypothesized that the sizes of the colonies might be indicative of the numbers of transposons per genome if insertional mutagenesis were to lower the fitness of the cells and increase its generation time. Hence, we selected large, >2 mm diameter; medium, 1–2 mm diameter; and small, <1 mm diameter; colonies from which we isolated high-molecular-weight DNA. There was no significant difference in the number of inserts in smaller compared to larger colonies. However, there was a difference in the numbers of transposon inserts as a function of SB dose when the starting concentration was kept at 500 ng of pT/Neo. Thus, transfection with 500 ng of pT/Neo and 100 ng of pCMV-SB, which is at the peak of gene transfer, had an average of about 3 inserts/genome, whereas doses of transposase of 500 and 1000 ng yielded an average of about 1.1 and 1.2 inserts per genome, respectively (Fig. 5). Colonies smaller than 1 mm diameter were difficult to grow and often did not have detectable bands on the Southern blots, suggesting that if the colonies were G418 resistant (rather than "feeder colonies") then the inserts might have been unstable.

Improvements to SB Transposase

The original SB10 sequence was constructed from consensus active and inactive *Tc1/mariner* transposase sequences from a variety of metazoans [15]. We sought to improve the transposase by further modifications of the amino acid sequence based on a phylogenetic comparison with active *mariner* transposases (Fig. 6A). In total, we made 14 amino acid changes by site-directed PCR mutagenesis and tested them in the cell-culture transposition assay. Figure 6B shows the results of all changes that resulted in an improved activity as well as one representative change that gave diminished activity (P54N). The combination of the T136R, M243Q, WA253HVR, and A255R changes was incorporated into a new transposase, SB11, which en-

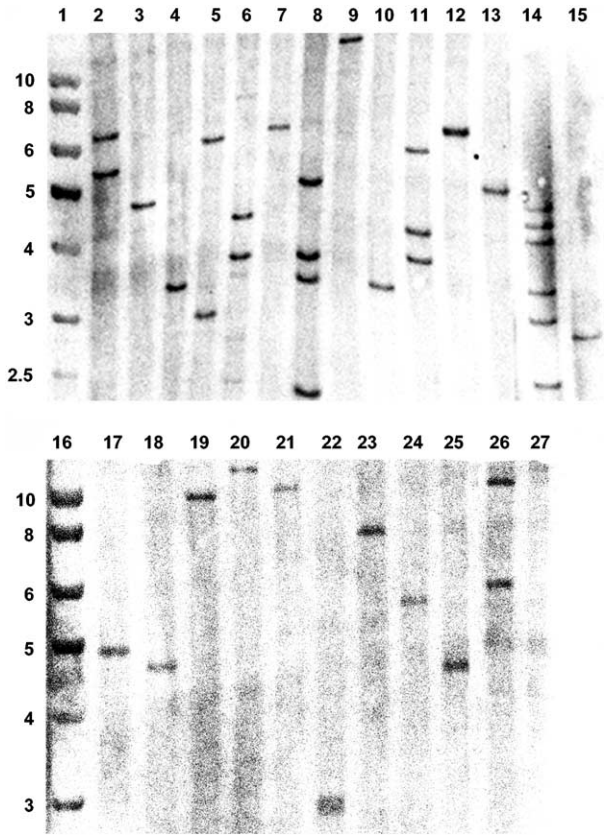

FIG. 5. Representative Southern blots of integrated transposons as a function of pT/Neo and pCMV-SB transfections into cultured HeLa cells. G418-resistant colonies were selected and divided into three groups: large colonies (L), >2 mm diameter; medium colonies (M), 1–2 mm; and small colonies (S), <1 mm diameter. DNA was isolated as described in Ivics *et al.* [15] and treated with restriction endonuclease *Sst*I and the fragments were separated by polyacrylamide gel electrophoresis. 5 μg of digested genomic DNA was loaded into each lane as follows. DNA from five separate HeLa transfections, A1 and A2, containing 500 ng of pT/Neo + 100 ng of CMV-SB; B1 and B2, with 500 ng of pT/Neo + 500 ng of CMV-SB; or C1, with 500 ng of pT/Neo + 1000 ng of CMV-SB: lanes 2–7, 14, 15, 17–21 and 27, large colonies; lanes 8–11 and 22–25, medium colonies; lanes 12, 13, and 26, small colonies. Lanes 1, 16, markers; 2, A1-L1; 3, A1-L3; 4, A1-L4; 5, A2-L2; 6, A2-L3; 7, A2-L4; 8, A1-M1; 9, A1-M2; 10, A2-M1; 11, A2-M4; 12, A1-S1; 13, A1-S2; 14, A2-L1; 15, B1-L1; 16, markers; 17, B1-L2; 18, B1-L3; 19, B2-L1; 20, B2-L3; 21, B2-L4; 22, B1-M4; 23, B2-M2; 24, B2-M3; 25, B2-M4; 26, B2-S2; and 27, C1-L1.

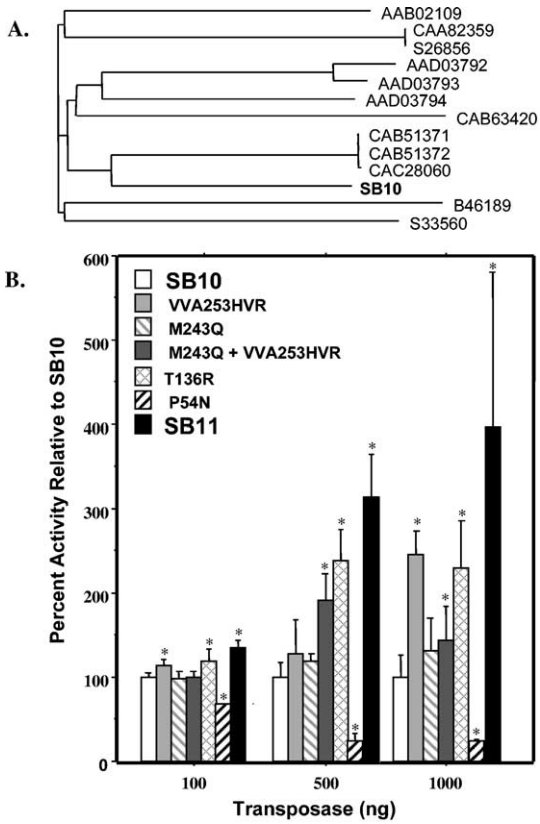

FIG. 6. (A) Phylogenetic tree representation of active *mariner* transposases aligned to assign possible amino acid changes based on a consensus of conserved regions. (B) Transposition activities of mutated SB10 transposases. Original SB10 and mutant transposases were cotransfected in various amounts, 100, 500, or 1000 ng, along with 500 ng pT/Neo. Colony counts for three independent experiments were obtained 14 days posttransfection in G418 selection. pGL-1 was added as filler DNA to maintain a constant amount of DNA per transfection. Transposition efficiency for SB10 transposase was adjusted to 100% for each transposase dose and mutant transposases are shown as relative activity compared to SB10 at their respective doses. Asterisks indicate values that are *t*-test statistically different from the SB10 + pT values ($P < 0.05$) at each given level of transposase.

hanced transposition of T/neo about threefold. The P54N change in the DNA-binding domain of SB transposase resulted in a three- to fourfold decrease in transposition activity and consequently was not incorporated into SB11. The P54N substitution may have increased the binding strength of the SB transposase to its binding sites on the transposon, a change that would be expected to lower transposition frequency [27]. The combined increase in transposition with the positive amino acid substitutions is about the same improvement in transposition as seen with SB10 and an improved transposon, *T2* [27]. When the improved SB11 transposase was used with the improved transposon, *T2,* we did not see any further increase over that achieved with just one of the improved components of the transposon system (Fig. 7, right-hand entry).

Two important considerations in comparing the activities of SB10 and SB11 are their relative expression levels and lifetimes. Differences in either the expression level or the stability of SB10 compared to SB11 due to the changes in their amino acid sequences would confuse our conclusions about the relative activities of these two enzymes. Consequently, we examined both the expression levels and the stabilities of SB10 and SB11 in transfected HeLa cells by measuring the levels of transposase protein over time following inhibition of translation with cycloheximide. Expressed in similar amounts (Figs. 8A and 8B), the half-life of SB11 transposase was approximately the same as that of SB10, about 80 h in tissue culture (Fig. 8C and data not shown). An alternative examination of the half-lives of the two transposases by Western blotting of cultures over time without use of cycloheximide also indicated that the lifetimes of SB10 and SB11 were indistinguishable (B. Kren, personal communication). SB11 consistently migrated slower than SB10 in our polyacrylamide gels, with an apparent shift in mobility of ~1

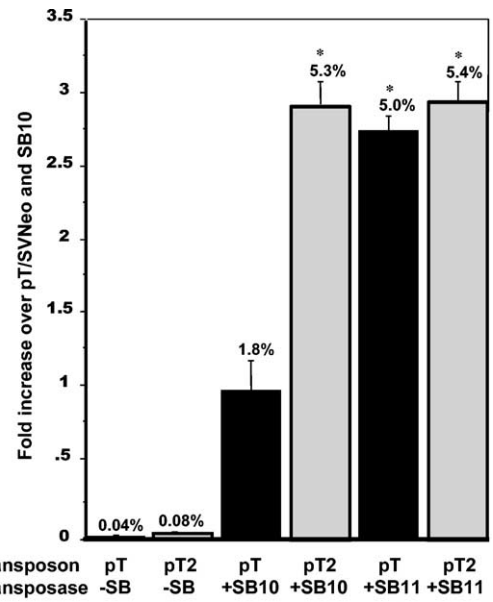

FIG. 7. Comparison of transposition activities of improved SB transposases and transposons. 500 ng pGL-1, original SB10, or improved SB11 transposase was cotransfected with 500 ng pT/Neo or the improved pT2/SVNeo. Colony counts were obtained 14 days posttransfection under G418 selection. Transposition efficiency for SB10 transposase plus pT/Neo was adjusted to 100% and other combinations are shown as relative activities Standard errors are indicated for each set of conditions. Noted above each bar is an estimate of the percentage of cells transfected that received pT/Neo and were G418 resistant. Percentages were determined by the average number of resistant colonies of the total number plated into selection, based on a 60% transfection efficiency and 0.66 plating efficiency for these experiments (data not shown). Asterisks indicate values that are *t*-test statistically different from the SB10 + pT values ($P < 0.05$).

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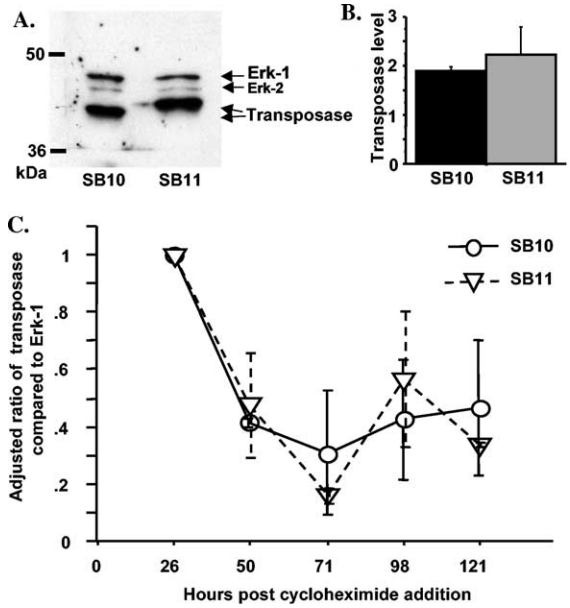

FIG. 8. Expression levels and stabilities of SB10 and SB11 transposases. Plasmids containing CMV-SB10 and CMV-SB11 were transfected independently into HeLa cells. 72 h posttransfection, 100 μg/ml cycloheximide was added and lysates were prepared every 24 h for Western blotting with antibodies against SB transposase and Erk-1 for a control. (A) Western blot showing relative expression levels and mobilities of SB10 and SB11 compared to the control Erk-1 (MAPK1 at 43 kDa) before cycloheximide addition. (B) Quantitative comparison of the expression levels of each transposase compared to Erk-1. (C) Plot of the presence of SB10 and SB11 as individual ratios of transposase/Erk-1 over time.

kDa, presumably because three of four substitutions replaced hydrophobic residues with positively charged residues, T(136), V(253), and A(255) to R; H, and R, respectively. This changes the predicted overall charge at pH 7.0 from (+)50.94 for SB10 to (+)53.03 for SB11 and increased the molecular mass from 39.5 kDa for SB10 to 39.7 kDa for SB11. Nevertheless, the amino acid substitutions had no apparent effect on the lifetime of the transposase.

DISCUSSION

Sleeping Beauty has opened the possibility for efficient, nonviral gene delivery for human gene therapy. To be effective, the transposon system needs to be capable of integrating cDNA coding regions and regulatory motifs for appropriate expression in targeted tissues. The average protein-coding sequence in humans is about 1300 nucleotides and about 80% of human cDNAs are less than 7 kb [11], suggesting that most human cDNAs could be efficiently integrated using the SB transposon system. This is not the case for many viral vectors. For instance, whereas adeno-associated virus can accommodate the small coding regions such as that for factor IX (1497 bp) for human gene therapy, the 6996-bp factor VIII cDNA is too large. The SB transposon system does not appear to have hard size limitations. Our results that transposons larger than 10 kb can transpose differ from those of others [30,31]. This may be due to differences in experimental design, including the content of the tested transposons. Their use of CpG-rich sequences for expanding the length of the transposon may have led to an additional reduction in apparent transposition frequency due to an increasing loss of transgene expression as the CpG-rich content increased in the larger transposons. Moreover, it is clear that transfection of larger plasmids is lower than that of smaller DNAs, which will further lower the level of G418-resistant colony formation regardless of transposition rates.

There is one anomaly in the data shown in Fig. 3A. As the pT/Bsd vectors increase in size, the transposition rate of pT/Neo, which we used as an internal standard, increased. This was unexpected. There may be two contributing factors. First, as the pT/Bsd plasmids increase in size, their efficiency of transfecting HeLa cells may decrease—this appears to be the case for pT/Bsd10.8. As a result, there would be fewer competing transposase-binding sites from pT/Bsd vectors, allowing more transposase to interact with pT/Neo constructs, thereby enhancing the odds of its transposition. Second, we used pGL-1 as a control for total mass amount of transfectable DNA in our experiments. pGL-1 has a CMV promoter that might have altered the overall expression of SB transposase from pCMV-SB by competing for transcription factors. As we have shown in Fig. 4, the ratio of SB transposase to transposon affects the efficiency of transposition. Regardless of the causes of this anomaly, the data clearly support the hypothesis that the SB transposon system can deliver large genetic constructs to human chromosomes. In support of our findings, efficient remobilization of large SB transposons resident in chromosomes of mouse tissues has been observed (Junji Takeda personal communication, D. Largaespada *et al.,* unpublished).

An important criterion for effective gene therapy is sufficient chromosomal integration activity. Four changes in the amino acid sequence of SB10 transposase improved transposition about 3-fold, which corresponds to an integration rate about 100-fold above background recombination rates. Optimization of the ITR sequences and of the sequences flanking the transposon in the donor plasmid, pT2 [27,28], improves transposition another 3-fold. All together, we expected these changes would result in about a 10-fold improvement over the standard transposon system used in most of the experiments in this report. However, the data in Fig. 7 suggest that we have hit a limit of about a 3-fold enhancement when all improvements are combined. We do not understand this apparent limitation, which corresponds to about 3% of the transfectable HeLa cell population (E. Aronovich and J. Bell, unpublished data), in our assay system. Whatever the

reason, transposition appears to be very inefficient in tissue culture compared to cells of whole animals. For instance, whereas initial tests indicated a relatively low rate of remobilization of SB transposons of about 2×10^{-5} transposon events per mouse ES cell [17], the rates of transposition in offspring of mice harboring transposons and expressing SB transposase are between 0.2 and 2.0 remobilizations per pup [19–22], an increase of nearly 100,000-fold. The T2/SB11 system should be even more active. We have shown in Fig. 4 that the relative amounts of transposon and transposase are important for optimal transposition. By incorporating a transposase gene on the same plasmid as the transposon [35], it will be possible to adjust in each cell the relative ratios of the two components by appropriate choice of promoter for the transposase gene. The *cis* constructs that have been tested ([35]; Betsy Kren, unpublished) are more efficient at gene transfer than using two constructs. Ratios of transposase to transposon can be regulated by enhancer/promoter strength.

In addition to their greater efficiency at directing integration of genes into genomes than naked DNA alone, transposons are delivered with precise borders in single units for each mobilization. In contrast, random recombination of naked DNA often results in integration of concatamers, which have a propensity of being repressed over time [36,37]. Concatemers are not transposed at a measurable frequency.

A third criterion for a gene-therapy vector is safety. Human diploid genomes have about 28,000 *mariner*-type transposons but none have an active transposase gene [11]. Mobility-shift experiments done with the SB transposase on its natural target sequence, salmonid transposons, and related transposons in zebrafish indicated that there was no detectable binding to the heterotropic species. Nevertheless, it is possible that even residual binding of SB transposase to endogenous human transposons could mobilize them at an exceedingly low rate to elicit a cytotoxic effect. However, no one has reported any unexpected toxicities in transgenic mice that constitutively express SB transposase (our unpublished observations and personal communication from J. Takeda). Nevertheless, the duration of transposase activity should be kept as short as possible. We presume that in the future the SB transposase activity will come from transfer of SB protein bound to transposons to form transpososomes. This will further curtail possible binding of SB transposase to human sequences. The other safety issue is insertional mutagenesis. SB transposons appear to integrate more or less randomly in mammalian genomes [19–22,28,38,39]. The insertional consequences of SB transposons should be similar to those for any other insertional vector and less consequential than retroviruses that have double sets of enhancers in each of their long terminal repeat sequences. As a further safety feature for SB transposons, we are including insulator elements [40] to protect endogenous chromosomal genes from inactivation by the transgene enhancers (P. Hackett, unpublished data).

The best gene-therapy vectors will be those that can be targeted to specific tissues or cell types. While the plasmids harboring the transposon and/or transposase have no signals for targeting to specific organs or tissues, conjugating plasmids with modified DNA-condensing agents such as lactosylated polyethylenimine can direct DNA to specific cell types such as hepatocytes [41]. With its ability to integrate genes of variable size leading to long-term expression, the SB transposon system has great potential for gene therapy to ameliorate both acute and chronic disorders [42–44].

Materials and Methods

Construction of test transposons. The maps for plasmids pFV3CAT, pCMV-SB, and pT/Neo can be found through our Web site, http://www.cbs.umn.edu/labs/perry/; for this article we have shortened the official designation from pT/SVNeo to pT/Neo. The *Bgl*II–*Eco*RI fragment of pCMV-Bsd (Clontech, Palo Alto, CA) was cloned into pT/BH between *Bgl*II and *Eco*RI to give pT/Bsd. Cutting with *Sal*I, Klenow fill-in, and religation destroyed a *Sal*I site outside the transposon ITR-R to give pT/Bsd(−*Sal*I). A linker containing *Not*I and *Xba*I sites, made by annealing two oligos, 5′-AATTCGCGGCCGCTCTAGA-3′ and 5′-ACGTTCTAGAGCGGCCGCG-3′, leaving staggered ends compatible with *Eco*RI and *Hin*dIII, was cloned into an *Eco*RI/*Hin*dIII restriction site of pT/Bsd(−*Sal*I) to give pT/Bsd(−*Sal*I+*Xba*I). The 3.7-kb *Xba*I fragment of pFV3CAT [26], containing 1.1 kb of β-actin promoter/upstream sequence and intron 1 driving the CAT gene and a polyadenylation signal from the Chinook salmon growth hormone gene, was cloned into the *Xba*I site of pT/Bsd(−*Sal*I+*Xba*I) to give pT/Bsd/5.6. About 1.1 kb of the intron sequence from pT/Bsd/5.6 was deleted by *Age*I restriction and then religated to give pT/Bsd/4.5. The upstream β-actin promoter sequence was partially deleted to about 250 bp, from *Eco*RI to *Stu*I, by *Eco*RI restriction and Klenow treatment followed by *Stu*I restriction and religation to give pT/Bsd/3.5. A 3.5-kb *Sal*I fragment of upstream carp β-actin promoter sequence from the p*Sal*I/*Sal*ICAT [30] was cloned into the *Sal*I site of pT/Bsd/3.5 to give pT/Bsd/7.2, and two tandem fragments were cloned in to give pT/Bsd/10.8.

Cell culture and transposition assays. HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% *Characterized* fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, and 1X antibiotic–antimycotic (Gibco BRL, Carlsbad, CA). Cells (3×10^5) were plated on 60-mm dishes 24 h before transfection. Qiagen column-prepped plasmid DNA (Qiagen, Valencia, CA) was transfected with TransIT-LT1 (Mirus, Madison, WI). Twenty-four hours posttransfection, the medium was changed to remove remaining transfection reagents, and 48 h posttransfection, cells were split into selective medium. G418-resistant colonies were obtained after 12 days selection with 800 μg/ml G418 (Mediatech, Herndon, VA). Blasticidin-resistant colonies were obtained after 20 days of selection at 100 μg/ml blasticidin (ICN Chemicals, Irvine, CA). After selection, colonies were fixed with 10% formaldehyde, stained with methylene blue, air-dried, and counted.

Mutagenesis of SB10 to create SB11. We used the following sequences, listed as GenBank accession numbers, to obtain consensus amino acids for each position in *Tc1*/*mariner*-like transposases: AAD03792, AAD03793, AAD03794, CAA82359, S26856, CAB51371, CAB51372, CAC28060, AAB02109, S33560, B46189, and CAB63420. For SB(M243Q) construction, the Transformer site-directed mutagenesis kit from Clontech was used with a 5′-phosphorylated Trans oligo *Ssp*I/*Eco*RV, 5′-CTTCCTTTTTC-GATATCATTGAAGCTTT-3′, and the M243Q primer 5′-GGTCTTCCAA-CAAGACAATGACC-3′. Following denaturation of the template pCMV-SB, a single round of T4-polymerase extension from annealed primers

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created heteroduplex double-stranded DNA containing both the mutation and the conversion of a unique *Ssp*I site to an *Eco*RV site on one strand. The reaction was sealed by addition of T4 DNA ligase before digestion with *Ssp*I restriction endonuclease to remove parental plasmid. Transformation into *mutS* (repair-deficient) *Escherichia coli* amplified the mutated strands. The parental strands were counterselected after isolation of plasmids by cleavage with *Ssp*I. After sequencing, the *Sac*II fragment, containing the mutant SB(M243Q) open reading frame, was then subcloned back into pCMV-SB to create pCMV-SB(M243Q). Additional mutations to pCMV-SB(M243Q) were made via a PCR–mutagenesis strategy using primers designed to amplify the plasmid and generate overlapping 12- to 16-bp homologous ends containing the mutations. The following primers were used: T136R, 5′-TTTGCAAGAG-CACATGGGGACAAAGATCGTACTTTTTG-3′ and 5′-ATGTGCTCTTG-CAAACCGTAGTCTGGCTTTCTTATG-3′, and V253H and A255R, 5′-AACTACAGAGACATCTTGAAGCAACATCTCAAGACATC-3′ and 5′-TTTTCTCACGTGTTTGGAAGTATGCTTGGGGTCAT-3′.

Once amplified, polymerase chain reactions were digested with *Dpn*I to remove template DNA. PCR products were transformed into TOP10 F competent cells (Invitrogen, Carlsbad, CA) and homologous recombination by the bacteria produced the desired products. After sequencing, the amplified coding sequence was subcloned back into pCMV-SB as described above to generate the final vector pCMV-SB11 without PCR-induced mutations.

Western blotting of SB transposases and analysis. HeLa cells were plated at ~80% confluency on 100-mm dishes and transfected in duplicate with 8 μg pCMV-SB (SB10) or pCMV-SB11 along with 2 μg pRL-TK, a *Renilla* luciferase-expressing plasmid (Promega, Madison, WI), as a control for transfection efficiency. Twenty-four hours posttransfection, medium was changed, and at 48 h, the cells were equally split among six 100-mm plates. At 72 h, lysates from one representative plate (0 h, Fig. 8) were collected in lysis buffer (50 mM Tris–CI, pH 7.4, 250 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% NP-40, 1 mM $NaVO_4$, 1 mM Na_2PO_4) and 100 μg/ml cycloheximide was added to the remaining five plates from each experiment. Lysates were subsequently collected every ~24 h for 5 days. Forty micrograms of total protein lysate was run on 8% polyacrylamide gels, transferred to Immuno-blot PVDF membrane (Bio-Rad, Hercules, CA), and probed with rabbit polyclonal antibodies for both SB transposase and Erk-1 (Cat. No. sc-93; Santa Cruz Biotechnology, Santa Cruz, CA). A second probing with horseradish peroxidase-conjugated donkey anti-rabbit Ig (Cat. No. NA9340; Amersham Pharmacia, UK) and detection with Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, IL) revealed the expression of the proteins in the cell lysates. Luciferase readings were quantified from a sample of the 0 h lysate to determine transfection efficiency using the Dual-Luciferase Reporter Assay System (Promega) substrate for *Renilla* luciferase. Protein levels were quantified by digitally measuring the intensity of Western blot signals electronically scanned into the NIH Image 1.63 densitometry program (NIH, USA) from an autoradiogram. Levels of transposase were compared to levels of Erk-1 for each sample at each time point. Protein levels were adjusted for transfection efficiency as determined by the luciferase activity per microgram of protein at the 0 h time point (data not shown). Statistical analyses for this and all other experiments were performed using Statview 5.0.1 (SAS Institute, Cary, NC).

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