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Mutational Analysis of the N-Terminal DNA-Binding Domain of Sleeping Beauty Transposase: Critical Residues for DNA Binding and Hyperactivity in Mammalian Cells

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The N-terminal domain of the Sleeping Beauty (SB) transposase mediates transposon DNA binding, subunit multimerization, and nuclear translocation in vertebrate cells. For this report, we studied the relative contributions of 95 different residues within this multifunctional domain by large-scale mutational analysis. We found that each of four amino acids (leucine 25, arginine 36, isoleucine 42, and glycine 59) contributes to DNA binding in the context of the N-terminal 123 amino acids of SB transposase, as indicated by electrophoretic mobility shift analysis, and to functional activity of the full-length transposase, as determined by a quantitative HeLa cell-based transposition assay. Moreover, we show that amino acid substitutions within either the putative oligomerization domain (L11A, L18A, L25A, and L32A) or the nuclear localization signal (K104A and R105A) severely impair its ability to mediate DNA transposition in mammalian cells. In contrast, each of 10 single amino acid changes within the bipartite DNA-binding domain is shown to greatly enhance *SB*'s transpositional activity in mammalian cells. These hyperactive mutations functioned synergistically when combined and are shown to significantly improve transposase affinity for transposon end sequences. Finally, we show that enhanced DNA-binding activity results in improved cleavage kinetics, increased *SB* element mobilization from host cell chromosomes, and dramatically improved gene transfer capabilities of *SB* in vivo in mice. These studies provide important insights into vertebrate transposon biology and indicate that *Sleeping Beauty* can be readily improved for enhanced genetic research applications in mammals.

Class II transposons are discrete segments of DNA that have the ability to move within genomes. These elements have been used extensively as genetic tools to explore gene function in different model organisms and have contributed significantly to our understanding of biological systems. The simplest DNA transposons are framed by terminal inverted repeats (IRs), and contain a single gene encoding a transposase that catalyzes the excision of the element from its original DNA context and reintegration into a new locus. This cut-and-paste transposition process can be arbitrarily divided into four major stages: (i) transposase binding to its sites within the transposon IRs, (ii) synaptic complex formation through stable pairing of the transposon ends by transposase subunits, (iii) excision from the donor site, and (iv) reinsertion into a new target site.

Members of the Tc1/*mariner* family of transposable elements are extremely widespread in nature (32). These elements can be transposed in species other than their natural hosts (32), making them increasingly important tools for functional genomics in eukaryotes (17). Until recently, transposon vectors were not available for efficient genetic analyses in vertebrates because the vast majority of elements within vertebrate genomes are transpositionally inactive due to accumulated mutations within the transposon sequence (12, 26). To overcome this problem, a Tc1-like element called *Sleeping Beauty* (*SB*) was genetically reactivated from ancient transposon fossils found within fish genomes (16).

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Each end of the *SB* transposon contains two imperfect direct repeats (DRs) of about 32 bp that serve as binding sites for the SB10 transposase (16). The outer DRs are at the extreme ends of the transposon, whereas the inner DRs are located ~165 bp internal to these sites. In contrast to the Tc3 element from *Caenorhabditis elegans*, in target *SB* elements both the outer and the inner DRs are necessary for efficient transposition (20). SB10 binds less tightly to the outer DRs than to the inner DRs (4), and replacing the outer DRs with inner DR sequences completely abolishes transposition, suggesting that the relative strengths of binding of transposase to the DRs cannot be varied substantially without interfering with the overall reaction (4).

Specific binding to the transposon inverted repeats is mediated by an N-terminal, pairlike DNA-binding domain of the transposase, consisting of two predicted helix-turn-helix motifs (PAI and RED) (21). Although each subdomain contributes to DNA binding, the PAI subdomain plays a more dominant role in specific DNA recognition and cooperates with an adjacent AT hook GRPR-like motif during substrate recognition (21). The PAI subdomain also binds a transpositional enhancer-like sequence within the left inverted repeat of *SB* and mediates the multimerization of transposase subunits via a leucine zipper (21). The function of the RED subdomain, which overlaps with a nuclear localization signal (NLS), is presently unclear (18). The C terminus of the transposase corresponds to the enzyme's catalytic core, which contains a highly conserved amino acid triad, the DD(35)E motif, and is responsible for all the DNA cleavage and strand transfer reactions of transposition (Fig. 1A).

SB mediates transposition in a variety of vertebrate species,

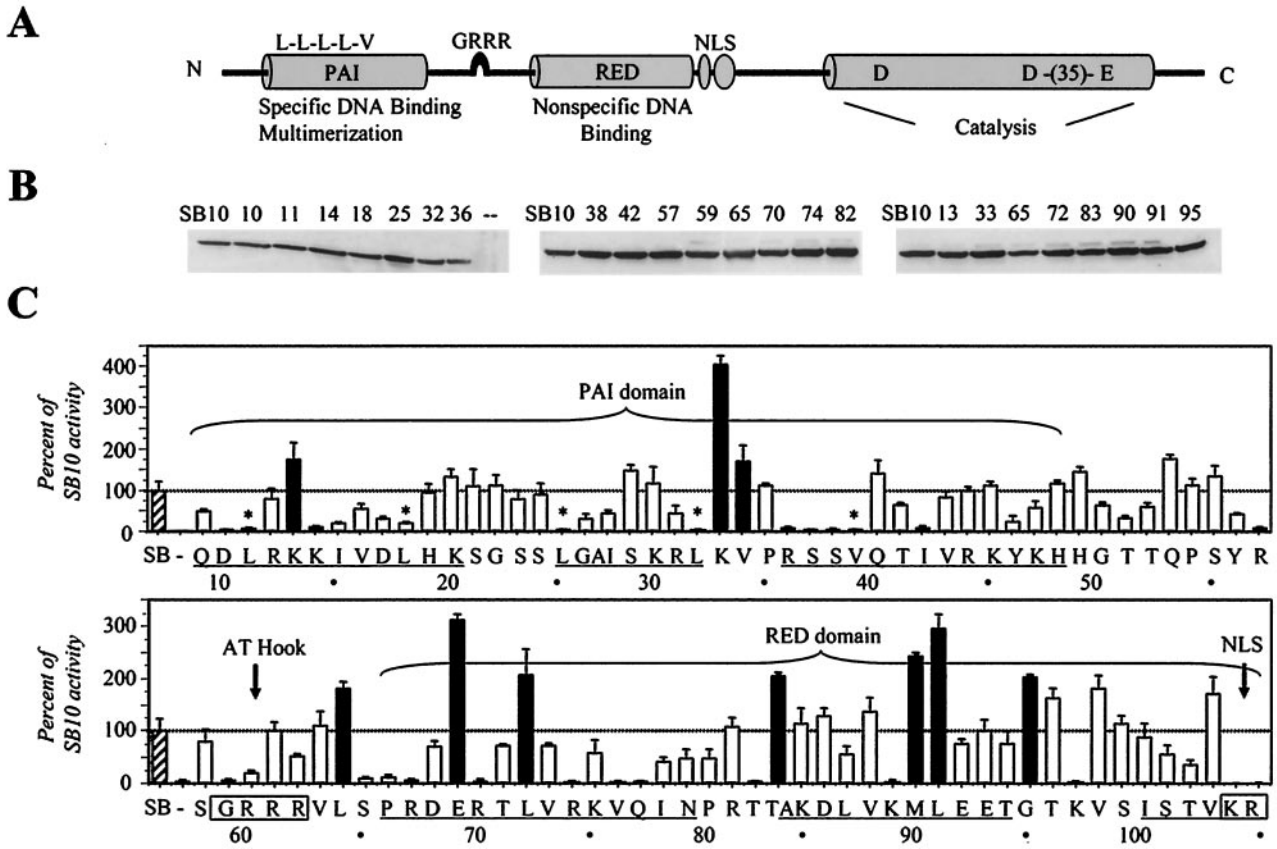


FIG. 1. Effects of amino acid substitutions on the efficiency of *SB* transposition in human cells. (A) Schematic diagram of the SB transposase. Shown are the two parts of the pairlike DNA-binding domain (PAI and RED), the GRRR AT hook motif, the bipartite nuclear localization signal, and the catalytic core containing the conserved DD(35)E motif. A leucine zipper (L-L-L-L-V) shown to be involved in subunit multimerization overlaps the PAI domain. (B) Immunoblot analysis of SB10 and representative mutant transposase proteins expressed by plasmid transfection of HeLa cells. Protein extracts were prepared from cells 40 h posttransfection and subjected to electrophoresis and electroblotting, and the 40-kDa transposase was detected with a polyclonal rabbit antibody to the SB protein. (C) Relative transposition activity of SB10 and mutated SB transposases in HeLa cells. HeLa cells were cotransfected with a plasmid encoding a neomycin-marked transposon (pT/nori) together with a plasmid encoding no transposase (−), the standard SB10 transposase (SB), or a transposase missense mutant. Shown are the transpositional efficiencies of 95 alanine-scan transposase mutants relative to that of SB10, which was adjusted to 100%. The best hyperactive mutants identified after three independent experiments are shown as black columns. Numbers indicate residue positions, asterisks demark the leucine zipper motif, and boxes enclose the AT hook motif and the first two residues of the NLS.

including the mouse germ line (7, 8, 14, 20, 32). In addition, *SB* has shown tremendous success as a therapeutic vehicle for somatic gene delivery in mouse models of human disease (19, 29, 30, 37, 39). Although *SB* is more active than other Tc1/*mariner* family members (8), and its activity can be increased either by codelivery of limiting host cell factors (22, 43) or via methylation of transposon sequences (40), its overall rate of transposition in vitro (27, 38) and in vivo (39) is still insufficient to make *SB* a practical tool for most clinical and basic research applications.

Herein, we analyze the functional importance of the SB10 transposase N-terminal DNA-binding domain by large-scale mutational analysis. We screened 95 missense *SB* mutants for altered transpositional activity in mammalian cells and found that transposase protein is extremely sensitive to mutational inactivation. Four amino acid residues were found to be critical for DNA binding to transposon end sequences. Conversely, we identified 10 mutations within the helices of the bipartite DNA-binding domain that greatly enhanced *SB*'s transpositional activity in human cells. Many of these hyperactive mutations acted synergistically when combined and produced mutant proteins with significantly enhanced affinity for transposon end sequences. These improved end binders supported improved cleavage kinetics from transfected plasmids, increased the frequency of *SB* transposition from mammalian cell chromosomes, and greatly enhanced *SB*'s gene transfer efficiency in vivo in mice. These experiments suggest mechanisms that may have led to the evolution of decreased transposase activity in vertebrate cells, and they demonstrate the potential for improved genetic analyses with transposon-based vectors.

MATERIALS AND METHODS

Recombinant DNA and *SB* mutagenesis. pT/nori and pT/βgeo have been previously described (39). To express wild-type *SB* from a cytomegalovirus promoter, we amplified the SB10 gene from pCMV-SB (16) by PCR using primers 5′-CTCGGATCCATGGGAAAATCAAAAGAAATC-3′ and 5′-GCAGAATT CTAGTATTTGGTAGCATTGCC-3′ and inserted it into the null vector pc-N (39) by BamHI/EcoRI ligation. Alanine substitutions were introduced into the

SB10 gene using mutagenic PCR primers (sequences available upon request), and each mutation was confirmed by DNA sequencing. Hyperactive SB (HSB) clone assignments were made after each stage of mutagenesis according to the mutant(s) which showed the highest overall transposition activity relative to SB10 and pT/neo. HSB1 and HSB2 variants were generated in the second round of mutagenesis, whereas HSB3 and HSB4 were generated in the third and fourth rounds, respectively. For in vitro protein production, the N123 portions of the various SB mutants were isolated by PCR and cloned into the EcoRI/BamHI sites of pGADT7 (Clontech). To generate pT3/MCS and pT3/nori, we amplified the left IR-DR structure from pT/nori by PCR using primers 5′-GCTCTAGA CCTATACAGTTGAAGTCGGAAGT-3′ and 5′-GCGGATCCCCTTGAAAT ACATCCAACGG-3′ and inserted it into pT/MCS (39) and pT/nori by BamHI-XbaI ligation. The pT3/βgeo vector was made by inserting the βgeo cassette from pT/βgeo into pT3/MCS by XhoI ligation.

Transposition assay, immunoblotting, and Southern blot analyses. HeLa cells (5×10^5) were transfected with pT/nori or pT3/nori together with pc-N, pc-SB10, or plasmids encoding individual SB mutants. In some cases, cells were harvested 40 h later, lysed in the presence of a complete protease inhibitor mix (Boehringer), and analyzed by immunoblotting with a rabbit polyclonal antibody to SB transposase (9). To measure transpositional activity, transfected cells were trypsinized 2 days after transfection, diluted in Dulbecco's minimal essential medium containing 600 μg of G418/ml, and growth selected for a period of 2 weeks. G418-resistant ($G418^R$) colonies were counted to determine the amount of *SB*-mediated integration relative to SB10, which was adjusted to 100%. In some cases, $G418^R$ colonies were isolated, amplified, and used to prepare genomic DNA for Southern blot analysis using a radiolabeled *neo* probe (38).

Electrophoretic mobility shift assay (EMSA). Truncated (N123) derivatives of SB10 and mutant SB proteins were generated using the TNT T7 coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Double-stranded oligonucleotides corresponding to the outer and inner DRs from *SB*'s left IR were end labeled using [γ-^{32}P]ATP and polynucleotide kinase. Complexes were formed in a 10-μl volume containing 10 fmol labeled oligonucleotides, 6 μl of in vitro translation product, 1 μg of poly(dI-dC), and binding buffer as previously described (16). For competitions, increasing amounts (1 to 500 nM) of the corresponding unlabeled oligonucleotide were added to the reaction mixture. After a 90-min incubation at 22°C, we added 5 μl of loading dye and resolved each sample on a 5% polyacrylamide gel for 2 to 3 h at 120 V. Binding affinities were determined by measuring the ratio of bound to free radiolabeled probe using a PhosphorImager and normalized according to the amount bound in the absence of cold competitor minus the amount bound at the highest competitor concentration.

Donor cleavage assay. HeLa cells (5×10^5) were transfected with pT/nori together with either pc-N or plasmids encoding transposase. We isolated Hirt DNA 30 h later and assayed for *SB* element donor DNA cleavage by using a previously described PCR-based assay (38). We controlled for potential DNA variations between samples by amplifying a portion of the plasmid backbone using primers 5′-GATGCTGAAGATCAGTTGGGT-3′ and 5′-GCTAGAGTA AGTAGTTCGCCA-3′. PCR products were analyzed on an ethidium bromide gel and quantified using a densitometer. The amounts of excision and repair products generated under each experimental condition were normalized for total DNA content and further estimated by limiting dilution PCR.

Chromosomal transposition assay. We generated three reporter cell lines (15-2, 15-8, and 15-11), each containing a single integrated copy of a neomycin expression cassette disrupted by a hygromycin-marked transposon (38). These cell lines were screened for sensitivity to the drug G418, and 2×10^6 cells from each reporter line were transiently transfected with 20 μg of pc-N, pc-SB10, or pc-HSB3 to promote chromosomal transposition. Cells were growth selected for 3 weeks in Dulbecco's minimal essential medium containing 600 μg of G418/ml, at which time $G418^R$ colonies were counted.

Animal studies. Eight-week-old C57BL/6-*scid* mice were obtained from Jackson Laboratory and treated according to the National Institutes of Health Guidelines for Animal Care and the guidelines of Stanford University. Plasmid DNAs were delivered to mice by hydrodynamics-based transfection as previously described (24, 44). Frozen mouse liver tissue was analyzed for β-galactosidase expression by immunohistochemistry (39).

RESULTS

Transposase mutation and functional analysis in human cells. We performed an alanine scan on the 5′ end of the SB10 transposase gene and screened mutant enzymes for altered activity in human cells using a genetic transposition assay. This screen involved cotransfecting HeLa cells with a donor plasmid encoding a nonautonomous neomycin-marked *SB* transposon (pT/nori) together with a plasmid expressing either the mutated transposases or the standard SB10 protein as a reference control. Cells were then placed under G418 selection, and the numbers of $G418^R$ colonies were compared to the amount obtained in the presence of SB10 as a measure of the relative transpositional efficiency. By Western blot analyses, all constructs encoding mutant versions of the transposase, with the exception of the poorly expressed V39A mutant, showed steady-state levels equivalent to that of SB10 (Fig. 1B and data not shown). Nevertheless, after analyzing a total of 95 different single amino acid substitution mutants for SB, we observed significant variation in the overall integration activity among these mutants (Fig. 1C). For instance, although 37 mutations had no significant effect on the transposition activity of the enzyme compared to SB10, 32 different mutations (D10A, L11A, K14A, I15A, D17A, L18A, L25A, G26A, L32A, R36A, S37A, S38A, V39A, I42A, Y46A, T51A, R57A, G59A, R60A, S65A, P66A, R67A, R70A, R74A, V76A, Q77A, Y82A, K89A, K97A, T102A, K104A, and R105A) reduced transposition frequency to barely detectable levels. In addition, 2 mutations (T41A and G50A) reduced transposition to 65% of SB10, and each of 14 additional mutations reduced activity to 42 to 59% of SB10. We also identified 10 amino acid substitutions that resulted in significant levels of hyperactivity. Compared to that of SB10, mutation K33A improved *SB*'s transpositional activity by 400%, whereas the E69A and L91A mutations each improved its activity by 300%. In addition, one mutation, M90A, increased *SB*'s transposition activity 2.5-fold, whereas six independent mutations improved its activity 2-fold (L72A, T83A, and G95A) or ~1.7-fold (K13A, L64A, and V34A).

DNA-binding activity for single amino acid SB mutants. Since approximately half of the N-terminal missense mutations we analyzed showed a reduced ability to mediate transposition in mammalian cells, we tested whether these mutant proteins could still interact efficiently with transposon end sequences, a necessary first step in the transposition process. To do this, we produced N123 fragments corresponding to the DNA recognition domains of SB10 and mutant SB transposases and then measured the ability of each peptide to interact with double-stranded oligonucleotides corresponding to *SB*'s binding sites by using an EMSA. The results of these analyses showed that the vast majority of these alanine substitution mutants retained significant levels of binding activity with respect to both the inner and outer transposon direct repeats (Fig. 2). This suggests that the major defect in the majority of the transpositionally inactive SB mutants described herein occurs at a step subsequent to transposon end binding, with a few notable exceptions. For instance, the three nonfunctional mutants L25A, R36A, and I42A were each capable of binding the inner DR sequences but were significantly diminished in their ability to mediate binding to the outer DRs (Fig. 2, rows 1 and 2). In addition, the inactive G59A mutant showed a more general defect in the ability to mediate transposon end binding (Fig. 2, row 3). These results suggest that leucine 25, arginine 36, and isoleucine 42 each play a critical role in mediating DNA contacts specifically with the ends of the transposon. Moreover, they suggest that glycine 59 might be involved in mediating critical contacts with conserved portions of the inner and outer

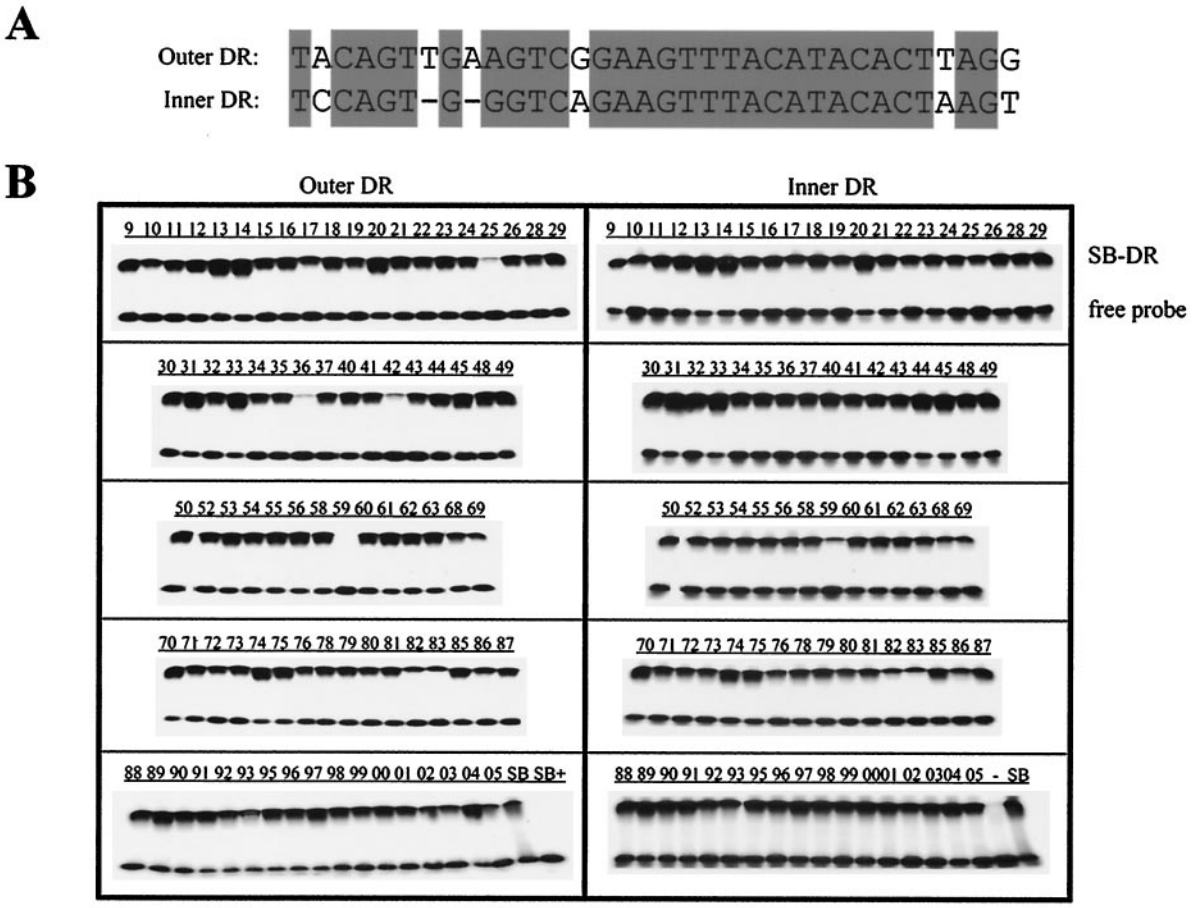


FIG. 2. Effects of single amino acid transposase mutations on transposon DNA-binding activities. (A) Alignment of *SB*'s DR sequences with identical nucleotides shaded in gray. (B) The N123 peptides from SB10 and mutant transposases were complexed with double-stranded radiolabeled oligonucleotides encoding the outer DR (left column) or inner DR (right column) sequences. The numbers represent the amino acid residues in SB10 that were mutated to alanine. Abbreviations: −, no transposase control; *SB*+, SB10-DNA complexes formed in the presence of 500-fold unlabeled oligonucleotide as a specific competitor.

DR sequences, although an essential role for this residue in ensuring the proper folding of the transposase N terminus cannot be ruled out.

Transposase and transposon hyperactivity. Here we tested whether combinations of hyperactive mutations result in an additive or a synergistic effect. For these purposes, we engineered each of seven hyperactive mutations (K13A, E69A, L72A, T83A, M90A, L91A, and G95A) into the K33A hyperactive mutant individually. Analysis of these double mutants showed that the K33A mutation acts synergistically with both L72A and G95A, whereas most other combinations of hyperactive mutations had an additive effect on transpositional activity (Fig. 3B, bars 3 to 18). However, a transposase containing all seven hyperactive mutations was found to be nonfunctional in human cells (Fig. 3B, bars 19 to 20), suggesting that certain combinations of hyperactive mutations can adversely affect the integration capabilities of the enzyme. Nevertheless, we were still able to generate additional enzymatic improvements by carefully excluding any amino acid changes that resulted in a net decrease in transposase activity (Fig. 3B, bars 21 to 30). In the end, we were able to successfully develop a series of transposase variants with markedly improved integration capabilities, including four hyperactive SB mutants (HSB1 through HSB4) that could support transposition frequencies improved as much as ninefold compared to that of the original SB10 protein.

To test whether transposition frequencies could be further increased by combining our hyperactive transposases with a hyperactive transposon, we generated a new transposon vector, pT3/nori, containing (i) an extra transpositional enhancer region via duplication of the left IR/DR structure and (ii) an additional flanking TA dinucleotide (Fig. 3A, right). Each of these modifications was independently shown to improve transposition rates in previous reports (4, 21). Indeed, use of this vector with SB10 produced transposition frequencies that were twofold higher than with the original pT/nori vector (Fig. 3B, bars 1 to 2). Moreover, this hyperactive element supported superior transposition frequencies with each of the hyperactive SB mutants, resulting in 14-fold more efficient transposition with pT3 and HSB3 than with the standard pT/SB10 system.

We studied the average number of transposon insertions per HeLa cell genome following stable transfection with either the traditional or the hyperactive *SB* system. By Southern blot analysis, the original pT/SB10 system was found to produce an

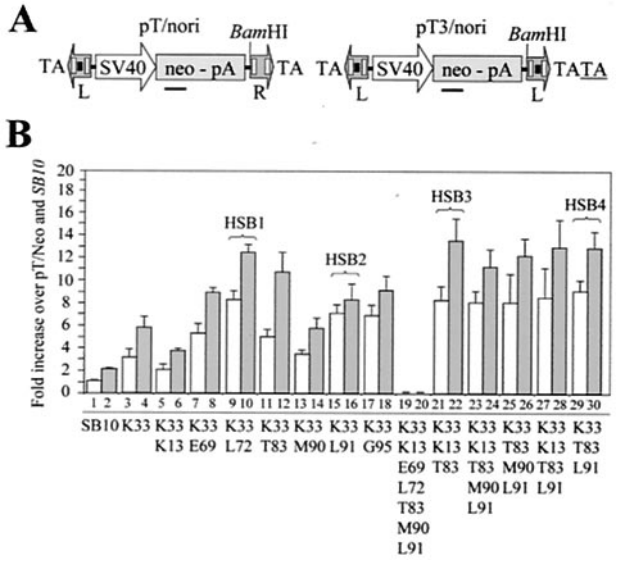


FIG. 3. Effects on the frequency of *SB* element transposition when using hyperactive mutations in combination and in the presence of a hyperactive transposon. (A) Schematic overview of the first-generation (pT/nori) and hyperactive (pT3/nori) neomycin-marked transposon vectors. The pT3-derived vector contains an extra transposition enhancer domain via duplication of the left IR-DR structure and has an extra TA dinucleotide flanking the 3′ end of the element (underlined) to promote increased excision. (B) Transposition frequencies in HeLa cells using traditional SB10/pT and hyperactive transposase and transposon components. The effects of hyperactive mutations in combination are shown relative to those of the SB10 transposase. Gene transfer frequencies for each transposase used in combination with either pT/nori (white bars) or pT3/nori (shaded bars) are shown.

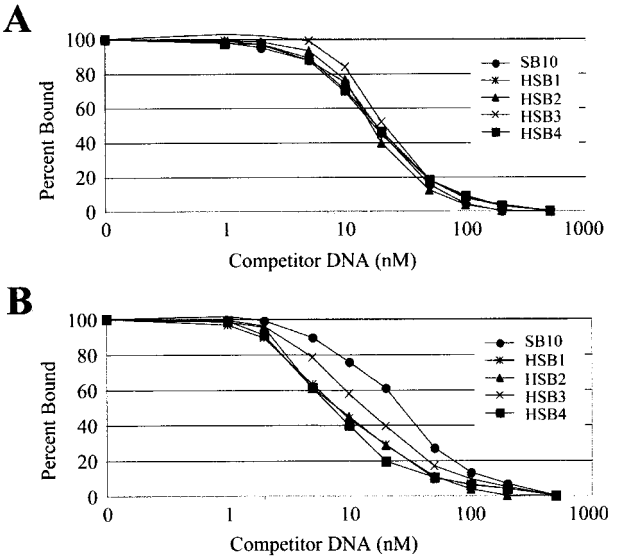


FIG. 4. Comparison of the relative binding affinities of SB10 and HSB mutants for transposon binding sites. SB-N123 peptides for SB10 and hyperactive transposases were complexed with double-stranded radiolabeled oligonucleotides encoding the inner (A) or outer (B) DR sequences. Protein-DNA complexes were formed in the presence of increasing amounts of the corresponding unlabeled oligonucleotide. The binding percentage was determined using a PhosphorImager and normalized according to the amount of probe bound in the absence of any competitor minus the amount of probe bound at a 500 nM concentration.

average of one to two transposon insertions per genome, whereas this value was increased approximately threefold with the hyperactive system, with two $G418^R$ clones each showing 10 independent insertions (data not shown).

DNA-binding affinities of the hyperactive SB mutants. Previous work with the bacterial element Tn*5* has indicated that transposase hyperactivity may originate from an enhanced affinity of the transposase for the transposon IRs (45). Therefore, we measured the affinities of SB10 and mutant SB proteins for the ends of the element by EMSA. The results of these analyses revealed that there was no change in the binding affinity of any of the proteins for the inner DRs (Fig. 4A). Interestingly, each of the four HSB mutants showed significantly stronger binding to the outer DR sequences than SB10, with HSB1, HSB2, and HSB4 all showing a three- to fourfold greater affinity for these outer DR sequences and a twofold higher affinity for HSB3 (Fig. 4B). Therefore, mutations that alter *SB*'s DNA-binding capabilities also enhance its transpositional activity in mammalian cells.

Donor DNA cleavage activity. To study the cleavage activity of the mutant proteins, we isolated plasmid DNA from HeLa cells transiently transfected with transposon donor and transposase-expressing constructs and subjected these DNAs to PCR using primers flanking the donor element. In contrast to control cells expressing either no transposase or three transpositionally inactive SB mutants (Fig. 5, lane 1 or 3 to 5, respectively), cells expressing the SB10 transposase supported the production of a 271-bp product of excision and double-strand break repair (Fig. 5, lane 2). This product was cloned and sequenced, and was found to contain a TA-flanked 3-bp (C[T/A]G) footprint at the excision site, which is consistent with *SB*-mediated DNA cleavage activity (38). Interestingly, when we compared the relative amount of excision and double-strand break repair product made under each experimental condition, we found that the HSB mutants all displayed substantially elevated donor DNA cleavage activity in HeLa cells relative to SB10 (Fig. 5B, lanes 2 and 6 to 8). We estimated the amount of these products by limiting dilution PCR and found approximately three- to fourfold higher cleavage activity for the HSB mutants than for SB10 (data not shown). Therefore, improved binding to the ends of the transposon can significantly enhance more downstream events in the transpositional reaction, including the rate at which a target *SB* element is excised in vertebrate cells.

Chromosomal transposition. In contrast to the efficient *SB* transposition from extrachromosomal plasmids, the frequency at which chromosomally located *SB* elements are mobilized in mouse embryonic stem cells and human HeLa cells is exceptionally low ($\sim 10^{-6}$ events per transfected cell) (27, 38). We therefore tested whether transposase hyperactivity could improve *SB* mobility from mammalian cell chromosomes. To do this, we used a stably integrated excision reporter construct to permit G418 selection of *SB* element chromosomal excision events following transient transposase expression (Fig. 6A). We generated three reporter lines, each containing a single copy of the excision reporter construct integrated at different genomic loci (data not shown), and selected for G418-resistant

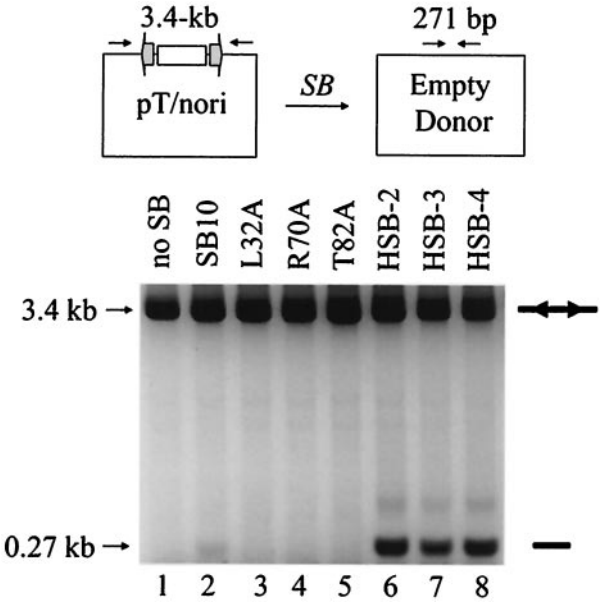
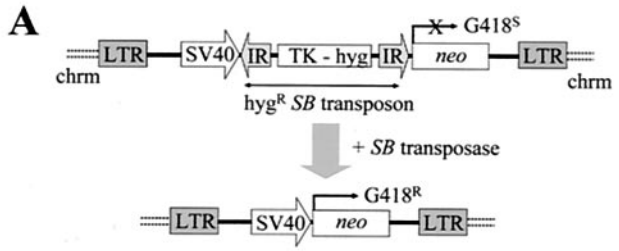


FIG. 5. Effects of amino acid substitutions on donor DNA cleavage activity in human cells. A transposon donor plasmid was transiently transfected into HeLa cells either alone or in combination with a transposase plasmid, and the DNA was isolated 30 h later for PCR analyses with primers flanking the donor element. Donor plasmids that have undergone *SB*-mediated transposon excision and double-strand break support the amplification of a 271-bp product. The amount of *SB* element excision produced in the presence of inactive (lanes 3-5) and hyperactive (lanes 6 to 8) *SB* mutants is shown relative to that produced in the presence of SB10.



B

Expressed protein:	*Frequency of SB element transposition*		
	line 15-2	line 15-8	line 15-11
GFP	ND	ND	ND
SB10	5.9×10^{-5}	$<1 \times 10^{-6}$	2.0×10^{-5}
HSB-3	2.5×10^{-4}	5×10^{-5}	1.2×10^{-4}

FIG. 6. Effect of transposase hyperactivity on chromosomal transposition rates. (A) Genetic assay for detecting rare chromosomal transposition events. A neomycin resistance gene (*neo*) under the control of simian virus 40 (SV40) promoter is inactivated by the insertion of a nonautonomous *SB* element containing a hygromycin resistance (hyg^R) gene driven by the thymidine kinase (TK) promoter. This construct is packaged into a lentivirus and randomly integrated as a single-copy provirus into the genomes of infected HeLa cells. Transient expression of active SB transposase in these cells results in excision of the *SB* element and activated expression of the *neo* gene, resulting in G418 drug-resistant growth. LTR, human immunodeficiency virus type 1 long terminal repeat; chrm, chromosome. (B) Transposition frequencies in HeLa cells as determined using SB10 and hyperactive transposase. Three different reporter cell lines were each transiently transfected with plasmids encoding green fluorescent protein (GFP) as a control, SB10, or HSB3. Cells were growth selected in G418 for 3 weeks, and the resulting colonies were fixed, stained, and counted. ND, none detected.

growth following transfection with plasmids encoding green fluorescent protein as a control, SB10, or HSB3. Although the original SB10 enzyme induced chromosomal transposition at the expected frequency, the HSB3 mutant increased this frequency four- to fivefold in each of the three reporter cell lines (Fig. 6B), demonstrating that hyperactive transposases may facilitate increased mobilization of transposable elements from mammalian cell chromosomes.

Hyperactivity in mice. We tested whether improvements defined in a transformed human cell line were also applicable in vivo by studying *SB* transposition in the livers of adult mice. Standard and hyperactive transposon plasmids containing β-galactosidase-marked elements (pT/βgeo and pT3/βgeo, respectively) were delivered to the livers of immune-deficient mice together with plasmids encoding no transposase, SB10, or HSB2 (24, 44). Regardless of transposase expression, approximately 25% of mouse hepatocytes expressed β-galactosidase on day 2 (three mice per group) (data not shown). However, on day 42, only those animals which received transposase plasmids showed any significant levels of β-galactosidase expression, which is consistent with *SB*-mediated transposition into the mouse genome (39) (Fig. 7, bars 1 to 4). Interestingly, while the pT3-based vector supported 1.5-fold higher in vivo transposition than the pT vector when used in combination with SB10, use of the pT3 vector with HSB2 did not significantly improve on the in vivo activity achieved with the pT/HSB2 combination. These results are consistent with the findings previously reported by Geurts et al. (9) and indicate that combining increases in transposase-based activity with enhanced terminal repeat-based activity may not necessarily result in an additive effect under certain experimental conditions. Nevertheless, mice expressing HSB2 still showed persistent reporter gene expression in 57% of transfected mouse hepatocytes over a 6-week time period, which was significantly higher than the 8% frequency observed with the SB10 enzyme (Fig. 7, bars 5 to 6). These results demonstrate a sevenfold increase in the transposition frequency of the hyperactive transposase in somatic cells of an adult mammal.

DISCUSSION

For the present study, we screened 95 different SB element transposase mutants for altered activity using a quantitative mammalian cell-based genetic assay. In the process, we have identified 32 residues within the transposase that are critical for in vivo mobilization of target elements in human cells. These inactivating mutations localize to portions of each of the six N-terminal DNA-binding helices, a putative AT hook believed to mediate DNA contacts with the minor groove of A-T base pairs (1, 21), a leucine zipper motif previously implicated in transposase-transposase interactions (21), and the transposase bipartite nuclear localization signal (18). Consequently, this largely diverse class of inactive SB mutants should prove useful in investigating the molecular processes that regulate

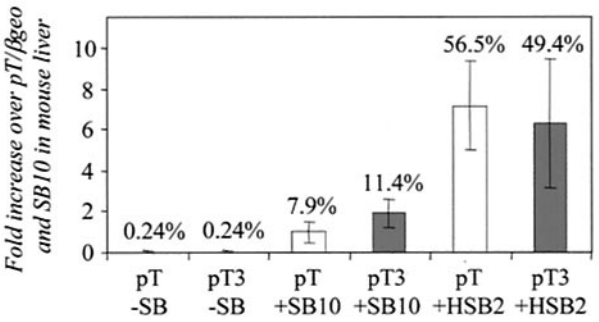


FIG. 7. Comparison of transposition activities in mouse liver following administration of standard pT/SB10 and hyperactive *SB* systems. C57BL/6-*scid* mice (four to five per group) were injected via the tail vein with 1 μg of plasmids encoding no transposase, SB10, or an improved transposase (HSB2) together with 25 μg of either pT/βgeo (encoding a β-galactosidase-marked transposon) or the improved pT3/βgeo version. Mice were sacrificed 6 weeks later, and their livers were sectioned and stained for β-galactosidase expression to determine the mean number of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-positive hepatocytes observed under each experimental condition (shown ± standard deviations). The transposition efficiency for SB10 transposase plus pT/βgeo was adjusted to 100%, and other combinations are shown as relative activities. Noted above each bar are the percentages of transfected mouse hepatocytes that remained X-Gal positive 6 weeks later.

DNA transposition in vertebrates. In addition, we identified four amino acids (leucine 25, arginine 36, isoleucine 42, and glycine 59) that substantially contribute to transposase binding to the transposon ends. These results indicate an important role for both the PAI subdomain and the AT hook motif in regulating transposase DNA binding and suggest that leucine 25 likely plays an important role in mediating both DNA binding and transposase subunit interactions in the cell (21). Collectively, these mutational studies experimentally confirm the locations of predicted functional domains within *SB* and suggest that SB and other Tc1/mariner family transposases (25) possess very little functional redundancy, an intrinsic feature that may have significantly contributed to the inactivation of this family of transposable elements in nature (11).

In the course of our mutational analyses, we also identified at least 10 single amino acid mutations in SB that result in significant levels of hyperactivity in mammalian cells. Interestingly, many of the hyperactive mutations described herein also produced a synergistic effect when combined, suggesting that additional mutants might be even more active. Indeed, mutations within portions of the transposase besides the ones described herein (9, 42) could be expected to improve additional features of the transposase besides DNA binding and rate of DNA cleavage, such as the efficiency of target site capture or *SB*'s overall strand transfer capabilities. Ultimately, however, future studies will likely require more robust methods to enrich for improved transposases in mammalian cells, and various strategies to do so are presently in development.

Hyperactive transposase mutants have been previously described for a number of different transposable elements, including Tn*5* (36, 45), Tn*10* (33), P element (2), *Himar1* (23), and most recently *SB* (9, 42). In the case of the two *SB* reports, single amino acid substitution mutations were introduced into the C-terminal catalytic domain of the SB transposase based on phylogenetic comparisons with other active transposases. These mutations were subsequently combined into a single active form containing either six (9) or two (42) amino acid changes, which ultimately improved transposition approximately three- or fourfold, respectively. Unfortunately, attempts to further increase this efficiency were ultimately unsuccessful (9), which suggested a potential limit to further enhancements using this type of experimental approach. In contrast, the mutations described in the present report localize exclusively to the N terminus of the transposase and result in a ninefold enhancement in *SB* transposition, making it the most active vertebrate transposase described to date.

Herein, we also describe the first in-depth investigation into the mechanism(s) leading to transposase hyperactivity in mammalian cells. This study remains an important first step since information gained from these and subsequent investigations might ultimately facilitate the rational development of better genetic tools for vertebrates. Nevertheless, it is important to note that, based on studies with other hyperactive transposases, the mechanisms leading to *SB* hyperactivity are likely to be diverse. For instance, in the case of the P element, it has been postulated that hyperactivity might result from a reduced sensitivity of the mutant transposase to cellular kinase activities (2). In light of these findings, we note that the SB transposase does contain several conserved phosphorylation target sites for casein kinase II ([S/T]XX[D/E]) in its N terminus (18), and in at least one instance, mutations within a conserved threonine residue within these sites (i.e., T83A) results in significantly enhanced *SB* transposition. These data raise the distinct possibility that *SB* activity might be actively curtailed by components of host cell signaling pathways. Nevertheless, a role for phosphorylation in the regulation of DNA transposition in mammalian cells will need to be addressed experimentally in future studies to fully clarify this important issue. In addition, studies with the bacterial element Tn*5* suggest that hyperactivity might be due in part to the reduced affinity of the transposase for an inhibitor protein (35). Although cellular proteases may in theory generate truncated forms of the transposase that could ultimately interfere with *SB* transposition, there have been no reports to date that would support such a pathway for *SB*. Moreover, although *SB* appears to be strongly inhibited at high enzyme concentrations in vitro (9) and in vivo (28, 39), recent studies suggest that these mutant HSB enzymes are still sensitive to overproduction inhibition (S. Yant and M. A. Kay, unpublished data), suggesting that another mechanism(s) must be involved.

Very little is known about the complex nature of the steps involved in the assembly and activation of the synaptic complex for *SB*. Interestingly, most of the hyperactive mutations defined in this report localized to the RED subdomain portion of the bipartite DNA-binding domain, suggesting that it could play an important role in these processes. Based on functional studies with the Tc1 and Tc3 transposases (3, 34) and protein comparisons made by computer modeling (31), this region of the transposase likely functions in nonspecific DNA interactions. Interestingly, two of the three mutations implicated in *Himar1* hyperactivity also map within a nonspecific DNA-binding region of the transposase (23), suggesting that enhanced transposase activity may be due in part to increased affinity for DNA in general. However, this is unlikely to be the case since

none of the HSB mutants showed any detectable increase in nonspecific DNA-binding activity in control EMSA studies using random oligonucleotides (J. Park and M. A. Kay, unpublished data). Nevertheless, we have found that our hyperactive mutants bind much more strongly to the outer DRs and show increased cleavage activity compared to that of the SB10 enzyme. Although hyperactive mutants of the Tn*5* transposase have in fact been attributed to an increase in the affinity of the transposase for the transposon IRs (45), a previous report has shown that *SB* binds much more tightly to the inner DRs than to the outer DRs and that interchanging these sites results in severely impaired transpositional activity (4). Nevertheless, our finding that transposase hyperactivity can originate, at least in part, through enhanced DNA-binding activity suggests that alternative explanations for previous findings (4) may require further consideration. Alternatively, it is possible that the mutations we introduced into the transposase cause conformational changes in the enzyme that favor its assembly and/or cleavage activation within the synaptic complex. Although this latter contention will certainly need to be explored in future studies, such a hypothesis is most consistent with our findings and is supported by a recent investigation into *SB*'s DNA-binding capabilities (21).

The Tc1/*mariner* family elements are being developed for use in a variety of model organisms and hold great promise to extend the utility of transposon-based genetic analyses available for *Escherichia coli* and *Drosophila melanogaster* to vertebrate species. Recently, the *SB* and Tc1-like *Minos* elements have been applied towards insertional mutagenesis screens and germ line transformation in the mouse (5–8, 14, 15, 41). Such genetic studies require labor-intensive methods that could be significantly eased by more active transposases. To this end, we have shown that improved *SB* mutants increase the rates of transposition from mammalian cell chromosomes and retain significant levels of hyperactivity in vivo in the mouse. Alternatively, these mutants could be used to more effectively investigate cellular DNA repair pathways that respond to transposition-induced DNA damage in vertebrates, especially those involved in nonhomologous DNA end joining (22, 38). Moreover, based on previous work with Tn*5* (10), the use of these hyperactive mutants might aid in the establishment of an in vitro transposition system for *SB*, which does not presently exist. Such a system would help define the optimum conditions under which transposition can take place and would certainly provide a better understanding of the biochemistry involved in *SB* transposition. Finally, the use of these hyperactive variants, either alone or in combination with limiting host cell factors (22, 43), should greatly facilitate transposon-based RNA interference delivery for improved functional genomics (13) and might assist in the transposition of larger-sized elements (9, 20), which would prove especially useful for many gene therapy applications.

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