

Non-Viral Vectors for Gene Therapy, 2nd Edition
Leaf Huang, Ernest Wagner and Mien-Chie Hung, eds.

2004

***Sleeping Beauty* Transposon-Mediated Gene Therapy for Prolonged Expression**

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I. INTRODUCTION

In the 21st century, we can expect a revolution in the delivery of therapeutics. We can expect genetic medicines that will confer permanent solutions to chronic and acute ailments. How these genetic medicines will be delivered and controlled, without adverse side effects, are the pressing issues facing modern medicine. Gene therapy theoretically represents the best form of treatment for some medical disorders because natural biological products rather than chemicals are employed for their natural function. Delivery of the therapeutic is relatively constant at physiologically effective levels, rather than cycles of high and low concentrations that result from introduction of drugs or other therapeutics at periodic intervals. Conceptually, gene therapy has the potential to provide a marked clinical and economic improvement over infused recombinant protein used in protein-replacement therapies. The essential goal of gene therapy is to provide what all patients want, an improved quality of life. For these reasons gene therapy will become the treatment of choice for disorders such as hemophilia¹. Gene therapy is applicable to both genetic and acquired diseases. In this chapter we review a new vector for non-viral gene therapy, the *Sleeping Beauty* transposon system. This vector combines the advantages of viral vectors, directed integration of single copies of a therapeutic gene, with the advantages of non-viral vectors, the absence of protein factors that can elicit adverse reactions.

A. Non-viral vectors for gene therapy - potential utility and current limitations.

Nature uses two devices for introducing new genetic material into chromosomes of all organisms. The first is viruses, which have evolved elaborate strategies for efficiently introducing their genomes into cells and occasionally into the chromosomes of infected cells. Owing to the high number of potential viruses in the environment and the deleterious aspects of viral infection, most animals have defensive systems to protect their chromosomes from outside intruders. The defenses include acquired immune responses against viral proteins and innate immune responses against selected motifs of viral genomes and/or their transcripts. Nonetheless, due to their efficiencies in gene delivery to cells, they have been used in about 70% of the approximate 1000 gene therapy trials through 2003 (www.wiley.co.uk/genmed/clinical/). The second method is transposons, which have evolved the means to enter chromosomes over such long evolutionary periods that there are few if any host defenses. However, unless facilitated by artificial laboratory techniques, random fragments of DNA that are not transposons, 'naked

DNA', enter genomes at low rates. Delivery of non-transposon DNA by a variety of methods has been the basis of about 30% of gene therapy trials. Thus, the use of either naked DNA or viruses for gene therapy has serious drawbacks. Here is why.

A fundamental component of any gene therapy strategy is the vehicle used for delivery of genes into a cell and into its nucleus for appropriate expression. There are five major barriers in the delivery of genetic material to cells: **(i)** stability of the transgene in the extracellular environment; **(ii)** transfer of genetic material across the cell membrane, **(iii)** delivery of the genetic material to the nucleus without intracellular degradation, **(iv)** integration of the transgenic material into chromosomes so that it can be replicated, and **(v)** reliable expression of the transgene following integration into a genome. Many viruses are good at penetrating some or all of these barriers, but as mentioned, they have other problems. The problems of surmounting these barriers with non-viral DNA have been reviewed^{2,3} and are discussed briefly below and in other chapters in this volume.

Non-viral, DNA-mediated gene transfer has been extensively explored as a means of expressing new genes in cells and tissues and constitutes an alternative with several potential advantages over viral delivery systems. **(i)** Viral vector preparations from cultured mammalian cells come with the risk of contamination by a variety of different infectious agents, including replication-competent virus generated by recombination between virus vector and packaging functions⁴. In addition, the viral particle itself can be toxic, depending on the dose and site of administration. The risks of DNA-mediated delivery, by comparison, are limited to those associated with plasmid preparation from bacterial extracts (endotoxin, etc.) and whatever chemical component is conjugated with the DNA for the purpose of delivery. **(ii)** Viral vector preparations are likely to be more highly immunogenic than DNA-based delivery systems. The best example of this is the acute immune / inflammatory response brought about by adenovirus vector administration and transduction in the liver⁵. **(iii)** DNA-mediated delivery is not constrained by many of the biophysical and genetic limitations of viral vectors, such as genome size and elements required for regulation of expression and replication. **(iv)** DNA-mediated delivery systems are likely to be cheaper, more stable than viral vector preparations, and more amenable to pharmaceutical formulation. **(v)** A further complication in the use of retroviruses^{6,7}, lentiviruses⁸ and adeno-associated viruses (AAV)⁹ may come from their preference for

integrating in or near promoters and transcriptional units, where they may have increased chances of causing adverse effects^{10; 11; 12; 13}.

DNA-mediated gene transfer presents a superior alternative to viral vectors for gene therapy. *In vivo* DNA-mediated gene transfer into a variety of different target sites has been studied extensively. Naked DNA can provide long-term expression in muscle, albeit after injection of relatively large quantities of DNA^{14; 15}. DNA-mediated gene transfer has also been characterized in liver^{16; 17; 18}, heart^{19; 20; 21; 22}, lung^{23; 24; 25; 26}, brain²⁷ and endothelial cells^{28; 29; 30} when administered in association with various cationic lipids, polycations and other conjugating substances^{31; 32}. However, the primary limitation of DNA-mediated gene transfer in these systems is the relatively short duration of gene expression. The “long-term” gene expression that has been observed in muscle and in liver is associated with persistence of the newly introduced DNA in an extrachromosomal form^{14; 15; 17; 18}. The stability of newly introduced DNA sequences can be greatly improved by integration into the host cell chromosome. However, stable integration in tissues after DNA-mediated gene transfer occurs rarely and primarily by random (illegitimate) recombination.

B. Transposons: non-viral vectors that deliver long-term gene expression.

We have developed a new means to achieve stable integration of DNA sequences in vertebrates using the *Sleeping Beauty* (SB) transposon system³³. Since its creation in 1997, *Sleeping Beauty* has been shown to mediate transposition in different cultured cell types^{34; 35} as well as zebrafish embryos^{36; 37; 38; 39; 40}, mouse embryos⁴¹, mouse embryonic stem cells⁴², mouse germ cells^{43; 44; 45; 46; 47}, and in mouse somatic tissues^{26; 48; 49; 50; 51; 52; 53; 54; 55; 56; 57; 58}. *Sleeping Beauty* thus provides a means of achieving chromosomal integration and long-term expression both *in vitro* and in experimental animals, thereby circumventing a primary limitation of non-transposon, DNA-mediated gene delivery for human therapy⁵⁹. The success of the SB system has led to the development of other transposon and transposon-like vector systems, including *Frog Prince*⁶⁰, *Tol2*^{61; 62}, \square C31⁶³, and the retrotransposon L1^{64; 65; 66}. In the sections below, we review the current status of SB transposons for gene therapy.

This review concentrates on the *Sleeping Beauty* transposon system. SB transposons represent a type of mobile element that belongs to the *Tc1/mariner* class of transposons that transpose via movement of a DNA element. *Tc1/mariner*-type transposons comprises almost 3% of the human genome^{67; 68} and therefore are a minority class of transposon species in human and

other vertebrate genomes – retrotransposons comprise most transposons in vertebrate genomes, of which the LINE and SINE families comprise the largest sub-fraction, approximately 33% of the genome (*ibid*). DNA transposons move in a simple, cut-and-paste manner (Fig. 1) in which a precise DNA segment is excised from one DNA molecule and moved to another site in the same or different DNA molecule⁶⁹. The protein that catalyzes this reaction, the transposase, is encoded within the transposon for an autonomous element or can be supplied in *trans* by another source for a non-autonomous element. *Tc1/mariner*-type transposases require a TA dinucleotide basepair for an integration site, a sequence that is duplicated during the integration process. The *Tc1/mariner*-type SB transposon system consists of two components: i) a transposon, made up of a gene of interest flanked by inverted repeats (**IRs**, shown as arrowheads (IR-DR in Fig. 1), and ii) a source of transposase. During *Sleeping Beauty*-mediated transposition, the SB transposase recognizes the ends of the IRs and excises the transposon from the delivered plasmid DNA, and it then inserts the transposon into another DNA site. The transposon structure shown in Fig. 1 is representative of the class of *autogenous* transposons, that is a transposon that encodes an active transposase that directs the movement of the transposon with transposase. To date, no active *Tc1/mariner*-type or SB-like transposase gene has been found in any vertebrate genome although thousands of highly mutated transposase genes have been found in genome sequencing projects. Consequently, all of the ca. 20,000 *Tc1/mariner*-type transposons that reside in human genomes are stable. In contrast, some retroelements are active and do hop occasionally in humans^{70; 71}.

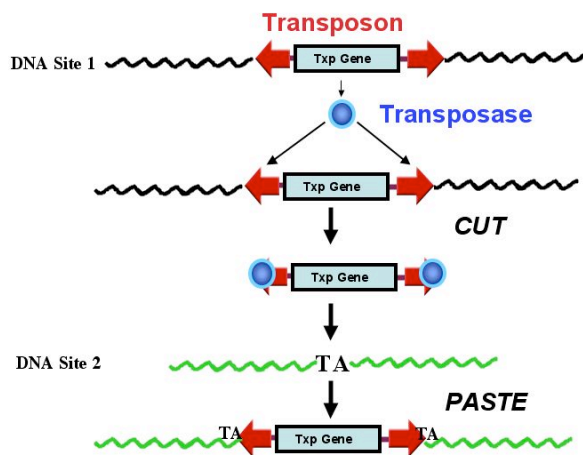


Figure 1. The cut-and-paste mechanism of transposition for a DNA transposon with an active transposase (Txp) gene. SB transposons integrate only into TA-basepairs. After duplication of

the TA site during transposition, TA sequences are formed on each end of the transposon. The inverted red arrows, representing 230 bp each, are the only DNA sequences required by the transposase enzyme for transposition. For gene therapy, the transposase gene is replaced by a DNA sequence that encodes a therapeutic product that could be either a protein or an RNA molecule.

Tc1/mariner-type transposable elements are ubiquitous in animal genomes and generally can be mobilized in cell-free systems in the presence of their respective transposase enzymes made in *E. coli*^{72;73}, suggesting that they require few, if any, species-specific host factors. The presumed simplicity of this form of transposon made them attractive candidates for use in human gene therapy. However, transposition of SB transposons in cell-free systems has not been demonstrated; it appears that there are host factors that play roles in the transposition process for *Tc1/mariner*-type elements^{74;75;76}. This difference between the SB transposon system and the *Tc1* and *mariner* transposons has not interfered with using SB transposons for gene delivery to vertebrate genomes.

II. THE SLEEPING BEAUTY TRANSPOSON SYSTEM

For the purposes of human gene therapy there are several important facets of using the SB transposon system as a vector that need to be appreciated. 1) SB transposase directs the integration of precisely defined, single copies of a DNA sequence into chromatin (Fig. 2). 2) The integrated gene is stable with respect to expression as a result of the integration, providing long-lasting expression of a therapeutic gene. 3) The transposase elevates the frequency of integration of a desired gene by about 100-fold or more, depending on the transposon, the transposase, the target cells and method of delivery of the system. 4) The SB system is *binary*, meaning that the transposon is *not* autonomous or able to transpose on its own. An appropriate transposase-encoding sequence must be supplied either in *trans* on a second vector³³ (shown in Fig. 2) or in *cis* on the same DNA molecule as the transposon^{35;77;78}. Consequently, there is not a “containment issue” for use of this vector system. 5) The SB transposase is synthetic - it was derived from sequences found in fish genomes (more on this in section B-2) and therefore does not measurably bind to transposons in human or other mammalian cells^{33;79}. 6) Transposons *per se* are not rigorously constrained by the genes they carry or their size although the efficiency of transposition and passage of the plasmids that carry them through cellular membranes (plasma

and/or nuclear, as discussed earlier in Section I-A) appears to decrease with size^{34;80;81}. 7) The transposase has nuclear localization sequences that may enhance translocation of the transposon from the cytoplasm to the nucleus of non-dividing cells⁸². Fig. 2 schematizes the use of SB transposons for gene therapy.

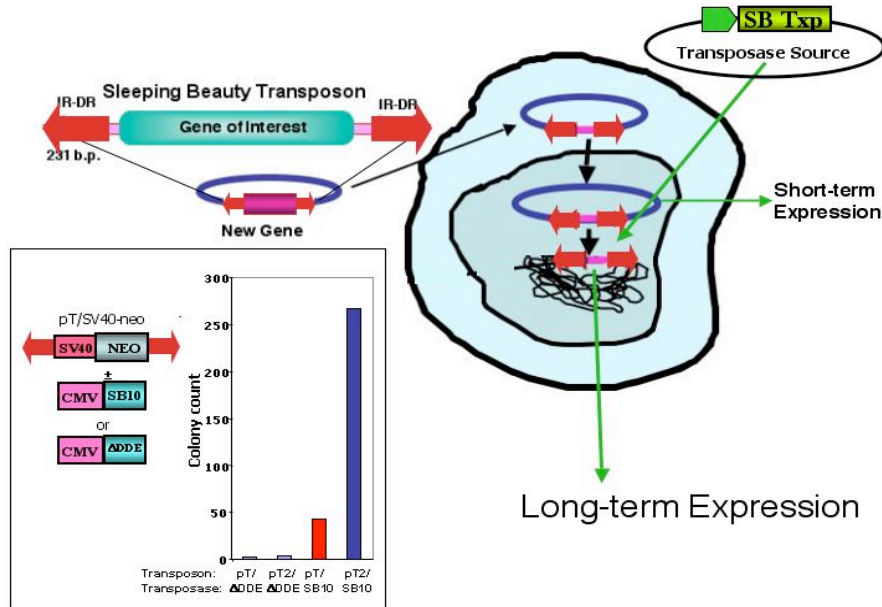


Figure 2. SB transposons for gene therapy. Delivery of a transposon with a transposase-encoding sequence, here shown on two plasmid vectors, can provide long-term expression of the transposed gene compared with shorter durations of expression when the gene remains as an episome in transformed cells in vivo. The inset shows the increase in levels of gene expression as a result of transposition in the presence of SB transposase compared to the levels observed when a defective form (Δ DDE) of the enzyme is supplied³³. T is the original transposon and T2 is an improved version⁸³ as measured by the frequency of G418-resistant HeLa cell colony formation following transposition of an SV40-Neo construct in either of the two transposons and with or without active SB transposase. The numbers of colonies obtained in the Δ DDE experiments are about equivalent to the levels found following delivery without any transposase and represent random, illegitimate recombination into chromosomes.

A. The transposition process.

The transposition-integration process is shown in more detail in Fig. 3. SB transposase cleaves the transposon-donor site at the flanking TA-dinucleotide basepairs in a staggered manner such that three bases, GTC, extend at each 3'-end of the transposon (lines 2 and 3 in Fig. 3). The 3'-ends of the excised transposon invade the target DNA molecule (indicated by the gold

ellipses) at the pair of TA sequences that extend from the 5' - ends that are produced when SB transposase cleaves the target site at a TA-dinucleotide basepair (third line in Fig. 3). This process is called ‘non-homologous end-joining’ and is mediated by cellular cofactors^{74;84}. Integration is completed by repair of the 5-base gaps on both strands. Note that the original TA-dinucleotide basepair target sequence is duplicated on both flanks of the transposon following integration (boxes in line 4 of Fig. 3), the donor DNA’s left and right ends. CAG overhangs on their 3’ ends are brought together with a single A-A pairing in the center that is resolved by one or the other A’s being replaced by a T during DNA repair. As a result, in the most common case, the original TA in the Donor Site is modified to contain a “footprint” TAC(A or T)GTA. Sometimes the repair process introduces more alterations in the donor site, with greater losses of sequence^{69;84}. It should be noted that transposase is not like a restriction enzyme that cleaves DNA molecules into fragments that freely diffuse; rather, the excision-integration reactions are highly coordinated events such that the paste step of transposition follows directly from the cleavage step.

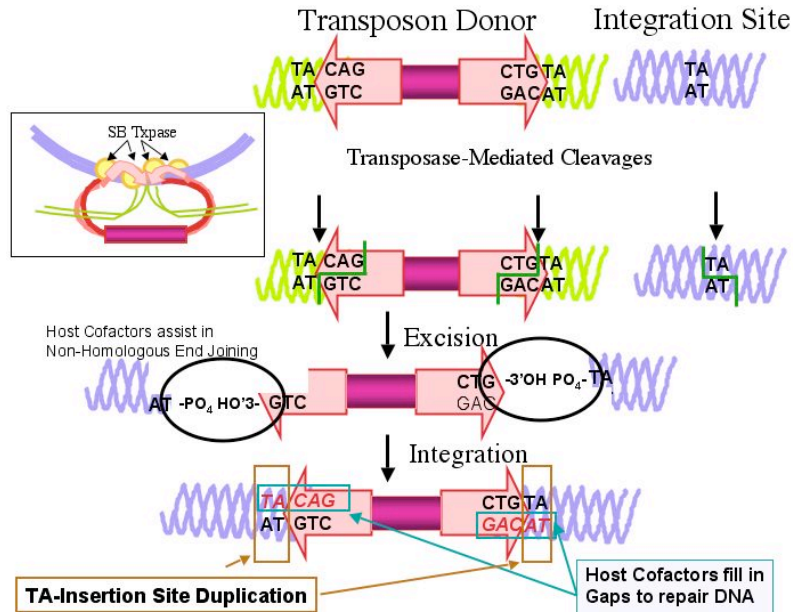


Figure 3. SB-mediated transposition from a donor site (green lines) to an integration site (purple lines). Two SB transposase molecules, shown as yellow circles in the boxed insert on the left, bind on each of the inverted terminal repeat (arrows) to introduce three cleaves – two flanking the transposon (pink structure with inverted arrows representing the inverted terminal repeats) and one in the target integration site (second line). The insert emphasizes that the SB transposase molecules act in concert in a complex of transposon donor and target integration

site. The excision step is shown on the third line with integration occurring by the invasion of the 3'-ends of the transposon joining the exposed TA nucleotides at the integration site (shown in the ellipsoids in the third line). Following ligation of the single strands on each side, DNA repair enzymes fill in the remaining 5-nucleotide gaps (shown in red in the fourth line). TA-target site duplication is indicated in the last line by the boxed TA-dinucleotide basepairs. The transposon-donor sequence is resealed and the single-base mismatch is repaired by cellular enzymes (lower right corner).

The model in Fig. 3 raises an obvious question – are the excision and integration steps always coupled? A related question is whether a single transposase molecule can cleave DNA even though it takes four proteins to form an integration complex. The answers are not established for the SB system and there do not appear hard rules for transposons. For instance, whereas Mu and Tn7 select a target site prior to cleavage, Tn10 synaptic complexes can integrate into a target DNA after excision^{85;86}. Our current knowledge of SB transposition is based on separate measurements of excision and integration. The excision step can be semi-quantified by PCR by what is called an *excision assay*^{41;53}. Essentially, PCR primers flanking a transposon will direct the synthesis of a predictable fragment following removal of several thousand bp from the donor site as shown in Fig. 4; sometimes, however, the repair process is not exact and does not produce the canonical footprint shown in the figure. In contrast, the complete transposition process can be estimated by genetic selection experiments. When the two assays were combined to study the effects of various mutations in the transposon, it was found that transposition correlated with excision, as expected from the model shown in Fig. 3⁵³.

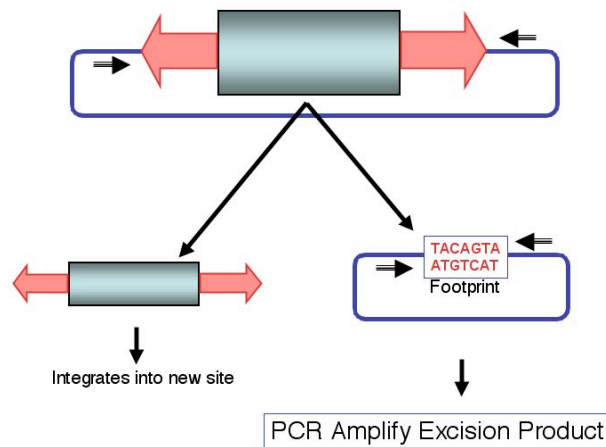


Figure 4. The excision assay for quantifying one step in the transposition process. A pair of primers (black arrow heads) is designed to flank a transposon in a donor molecule, shown here in a plasmid vector (blue line). Following excision of the plasmid, a resealed vector is produced that will be considerably smaller than the donor DNA and often will have a precise, canonical footprint, shown here in the box. The middle A-T basepair shown in the figure could be T-A as well. Following delivery of transposons to a multicellular tissue, each of the integration sites will be unique but the remaining plasmid will be the same so that a single set of primers can record all excision (and correlated integration) events.

There have been no studies with SB transposase to determine if cleavage can occur by monomeric SB enzymes. However, in a related process mediated by the lambda phage integrase, it was found that a single recombinase protein can cleave DNA, but that the enzyme tends to form multimeric complexes in physiological salt solutions, as does SB transposase (Z. Cui and P. Hackett, unpub.), that inhibit activity by single proteins⁸⁷. Thus, at this point the evidence strongly suggests a coupling of excision and integration. Currently, the transposition events in organs of multicellular animals have been evaluated by sequencing insertion sites in chromosomes of treated animals because each cell in which the transposon integrates is a separate event that is non-clonal. This procedure is difficult and is not quantitative. However, the excision assay allows a rapid evaluation of relative level of transposase activity *in vivo* when several methods of gene delivery are used.

B. The origin and development of the SB transposon system.

As noted earlier, *Tc1/mariner* transposons are found in just about every animal genome in which they have been sought, although in most animals in general and in vertebrates in particular their transposase genes appear to be defective⁸⁸. Although the use of these transposons seemed trivial at first, when we, and others, examined the abilities of known, active transposons to move in vertebrate cells, including those from zebrafish and humans, we found marginal transposition activity^{89;90;91;92}(Z. Ivics and Z. Izsvák, unpub.). Consequently, the SB transposon system was constructed based on phylogenetic principles in a 10-step process of site-specific mutagenesis of a salmonid transposase gene that became evolutionarily dormant more than 10 million years ago^{93;94;95}. The *awakened* transposase was named *Sleeping Beauty*³³. The *T* transposon plus SB transposase comprise the SB transposon system. For gene therapy, both components of the SB

system are delivered to cells in plasmids, the transposon and the transposase gene that when expressed can cut the transposon out of the plasmid carrier for reinsertion into a chromosome. The SB transposase gene may or may not be on the same plasmid carrier as the transposon (more on this later). As shown in the insert in Fig. 2, the original SB transposase is able to improve integration from 20 to 40-fold in cultured mammalian cells and about 20-fold in zebrafish embryos^{34;79}. In a head-to-head competition, Fischer et al.⁴⁶ showed that the rate of transposition mediated by the SB transposon system was nearly an order of magnitude higher than those observed for a variety of transposons from nematodes⁹⁶ and flies⁹⁷ in cultured human HeLa cells. In all of these experiments *non-autonomous* transposons were used, *i.e.*, transposons in which the transposase gene was replaced with alternative genetic cargo. The transposase was generally supplied by another plasmid carrying the transposase gene or by mRNA encoding the transposase.

A significant difference exists between the SB transposon and other commonly used members of the *Tc1/mariner* transposon family. SB transposons, called *T_n* (where *n* is the version of transposon, the original was simply *T*³³), contain two ‘repeats’ within each inverted terminal repeat (called IR-DRs for inverted repeats containing direct repeats) compared with the other transposons that contain a single inverted repeat (Fig. 5). Significantly, the original *T* transposon³³ contained DR sequences that varied depending on their position. More recent changes in specific base-pairs within the DR sequences has led to further development of the efficiency of SB transposons with significantly higher transpositional activities^{83;98}, the first being T2 which has consensus inner (Li and Ri) and outer (Lo and Ro) DR sequences.

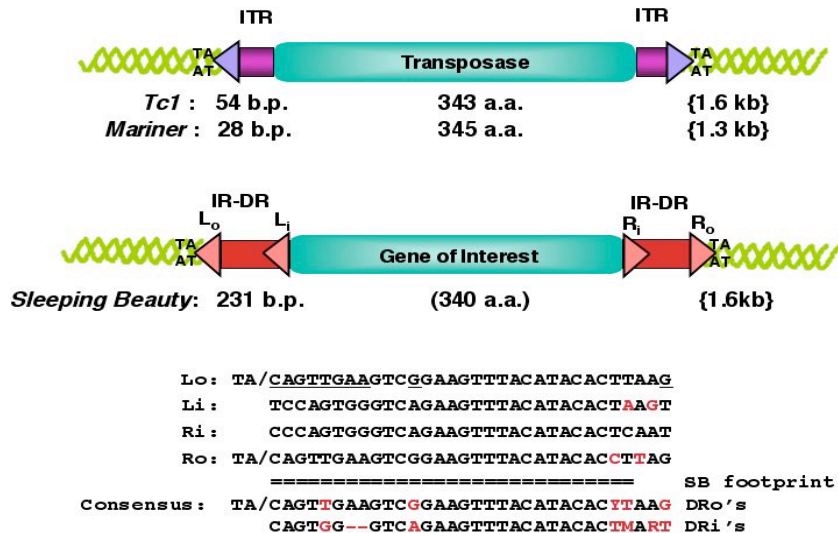


Figure 5. Comparative structures of *Tc1/mariner*-like transposons and SB transposons. ITR, inverted terminal repeat sequence; IR-DR, inverted repeat containing direct repeated sequences. The lengths of the repeat sequences are noted for each transposon as well as the transposase size (or reconstructed size in the case of SB – the transposase is never supplied from a gene flanked by two IR-DR's). The consensus sizes of the transposons are shown in the brackets on the right. The specific sequences of the four DRs in the original SB transposon, *T*, are shown at the bottom along with the consensus sequences that were used to build an improved transposon called T2. The SB footprint refers to the portion of the DR sequence that is protected from DNase hydrolysis when bound by SB transposase³³.

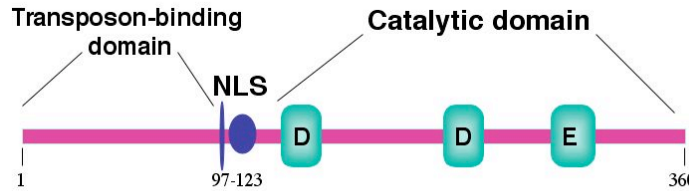
1. Improvements in SB transposons

Analyses in our labs of the DR sequences identified several aspects of the SB transposon system that were unexpected^{53;83}. First, the differences between the inner and outer DR sequences are important – there does not appear to be a universal DR sequence that can be effectively used for the four sites in a complete transposon. Second, SB transposase binds more tightly to the inner DR sequences than to the outer DR sequences. Third, when the outer DR sequences are altered to increase binding affinity of SB transposase, transposition rates dramatically decreased – suggesting that improvements in transposon sequences cannot be evaluated simply on the basis of transposase-DR binding energies. Fourth, the 170-bp spacer sequences between the DRs within an inverted terminal repeat are important – altering them often leads to depression of transposition activity. A ‘transpositional enhancer’ has been identified in the inter-DR sequence⁹⁹ that may facilitate the DNA-bending and pairing that occurs between the two ends of the transposon after four transposase molecules have bound (see inset in Fig. 3). A nucleic-acid-binding protein, HMGB1 has also been shown to elevate rates of transposition, possibly by facilitating binding of SB transposase to the inner DRs and bending the inverted terminal repeat sequences to a form compatible with transposition⁷⁵. All of these data suggest that the process of SB-mediated transposition involves the interactions of several cellular cofactors that together contort the transposon, and probably the new integration site, in a complex manner in concert with four SB transposase molecules^{76;100}. Our findings that maximal binding of transposase molecules to the DNA sites can be deleterious to function is similar to results obtained from analyses of transcriptional regulators. For instance, control and appropriate function is lost when

binding to all sites is too tight at *lac* operator sites^{101;102} and other cases where flexibility and dynamic activity on DNA is required¹⁰³. Two versions of a highly improved SB transposon vector, T2 and T/SA, are now readily employed in the field^{83;98} and more are underdevelopment.

2. Improvements in SB transposase

Several studies have been conducted to improve the activity of the SB transposase enzyme^{81;98;104}, the other half of the SB system. Fig. 6 shows the functional domains of the 360-amino acid protein. The first one-third represents a domain has two functions that were thought to be mediated by a leucine zipper motif^{63;95} although this structure is not predicted by a commonly used algorithm¹⁰⁵ and the corresponding region of a related *mariner* family transposase does not form a coiled-coil, as determined by the crystal structure of the N-terminal region of the Tc3 transposase¹⁰⁶. The first is to bind specifically to the DR sequences in the inverted terminal repeats of the transposon. The second role is to bring the two ends of the transposon together to form a synaptic complex (Fig. 3) that invades the target site. The middle, relatively short motif is a nuclear localization sequence (NLS) that is composed of two clusters of basic sequences, thereby making it a bipartite NLS. The bipartite structure of nuclear localizing sequences in transposons kept them from being identified until the one present in SB transposase was uncovered⁹⁵. The third, catalytic domain comprises the carboxy-terminal half of the transposase. Like the N-terminal sequence, it has two functions, one to identify TA-insertion sites and the other to catalyze the three cleavages and one paste reaction of the complete transposition reaction (Fig. 3). The catalytic domain is characterized by the DDE motif, which represents two aspartic acids (D) and one glutamic acid (E) and which is found in all cut-and-paste recombination enzymes such as retroviral integrases, transposases, and phage integrases^{107;108}. Commonly, there are 35 amino acids separating the second D and the E residues. An exception to the DDE rule is the *Mos1* transposase, which has a DDD motif with 34 amino acids separating the second and third aspartic acids; substitution of an E for the third D abrogates enzyme activity¹⁰⁹.



Transposon-Binding Domain

- 1 Transposase molecule binds to each DR sequence
- Coiled-coil structure
- Dimerization activity between two SB transposase molecules

Nuclear Localization Sequence (NLS)

- Bipartite structure

Catalytic Domain

- Mediates cleavage at the ends of the Inverted Terminal Repeats
- Recognizes integration sites at TA dinucleotide basepairs
- Mediates non-homologous end-joining - the "paste" step

Figure 6. Diagram of SB transposase. The three functional domains are identified at the top; the numbers below the structure are the approximate amino acid residue boundaries of the domains. The transposon-binding domain, often called the DNA-binding domain, binds to DRs and can protect the nested sequence from degradation by DNaseI as identified in Fig. 4. The transposon-binding domain also is responsible for dimerization of transposase molecules to form the complex shown in Fig. 3. The NLS sequence comprises amino acids 79-123 and has two clusters of basic amino acids separated by a 10-amino acid spacer. The catalytic domain characterized by the DDE motif is commonly found in all cut-and-paste recombination enzymes.

The combination of functions of the DNA-recognition domain and the catalytic domain present problems for directed mutagenesis to improve the transposase. This is common in transposases – X-ray crystallography of the Tn5 transposon synaptic intermediate complex showed that the domains of Tn5 transposase do not have discrete functions; rather, the amino-terminal domain, catalytic and carboxy-terminal domains all participate in DNA binding, and each plays a role in forming the structure of the synaptic complex¹¹⁰. As noted earlier, one strategy for improvement of SB transposition based on increasing the binding strength of SB transposase to DRs is unlikely to succeed due to the necessity of maintaining the dynamic structures that the transposon passes through during the transposition process. As a result, most improvements have come from further efforts to derive a more accurate consensus sequence based on selected, defunct transposase genes^{81,98} or by systematically substituting a leucine for many amino acids in the polypeptide to determine the effect on transposition¹⁰⁴. Both procedures found several sites that when mutated gave enhanced activity, and both strategies found that

combinations of mutations that enhanced transposition alone did not always act in an additive manner; indeed some combinations canceled each other. All of the assays for enhancement of SB transposase activity were conducted in HeLa cells using the frequency of G418-resistant colony formation as a measure (Fig.2 inset). Assays in other cell types may be useful to detect alternative mutations that lead to higher activity in specific tissues because various SB transposases may not have equal activities in all cells. Consideration of the presumed complex topography of charges in the catalytic center of SB transposase suggests that any simple and directed modification to alter the TA-recognition site has a large probability of altering (reducing) the enzymatic process at the same time. Consequently, development of a site-specific SB transposase¹¹¹ does not appear likely in the near term.

C. Characteristics of the SB transposon system

Features of the SB transposon system important for gene therapy can be divided into two broad categories - limitations on delivery of the complete transposon system into cells of target tissues and limitations on the transposition process. Limitations on delivery of the transposon system are based on the transposon carrier. Most experiments have used plasmids, but one report discusses delivery of the SB system using an adenovirus as the carrier¹¹². Considerations on methods of delivery to cells of specific tissues are discussed in Section III. Limitations on transposition include 1) the size of the transgene in the transposon, 2) the size of the transposon-donor plasmid, 3) the ratio of two components of the SB system, the transposase and the transposon, 4) methylation state of the transposon, and 5) the 'state' of the target cells.

The effect of the size of the transgenic construct, comprising the gene and transcriptional regulatory components, is an obvious concern. Size effects occur at the plasma and nuclear membranes as well as transposition into chromosomes. Three studies to examine the effects of size have been conducted using transposons that carried a selectable marker plus various 'spacer' DNA sequences^{34;80;81}. The first two studies employed prokaryotic sequences and the third used DNA that flanked the carp α -actin gene. In all cases there was a nearly linear decrease of transposition as a function of transposon size, but the decrease was significantly less when the spacer was vertebrate DNA compared to prokaryotic DNA. The results are reconcilable if the prokaryotic DNA was more apt to be methylated and thereby transcriptionally silenced than the eukaryotic DNA spacer that had a lower GC-content. Thus, the rates of transposition are likely to

be higher than indicated by selection for gene expression. The Geurts et al. study indicated that transpositional activity was reduced about 50% when the size of the transgenic construct was about 6 kbp, a size that would accommodate about 85% of cDNAs made to known mRNAs⁸¹. However, if a transposon is flanked by two complete DR elements in an inverted orientation, the ‘sandwiched’ transposon can be mobilized with transgenic constructs as large as 10 kbp⁹⁸.

A second influence on transposition is the size of the transposon-donor plasmid. Plasmid size will affect uptake and transport of the plasmid into and through the cell to the chromosomes. The size also has an effect on the effective separation of the termini of the transposons. The transposon-containing plasmid shown in Fig. 4 is an arrangement where the DNA sequence through the transposon is shorter than the DNA sequence of the plasmid carrier. But, when larger genes are put into the transposon, the DNA sequence length of the plasmid may be shorter than that of the transposon so that the effective separation of the transposon ends is defined by the plasmid. Izsvak et al demonstrated that indeed the closer the transposon ends were, either by engineering the transposon or the plasmid vector, the more effective the transposition³⁴.

Four transposase molecules are required to bring the two ends of a transposon together for the transposition reaction (Fig. 3). As noted earlier (Fig. 6), the transposase molecules can form dimers and tetramers to form the synaptic complex. As a consequence, overexpression of SB transposase leads to inhibition of transposition by quenching the reaction; extra transposase molecules can dimerize with those bound to the DRs to prevent their interactions. Overexpression inhibition was found earlier in *mariner* transposons⁸⁸ and has been shown to occur for SB transposons as well⁸¹. Gene therapy applications of SB transposons must take into consideration overexpression inhibition, which can be accomplished in two ways. The first is to use various ratios of plasmids with either the transposon or the transposase gene. The ratio of transposase to transposon will vary depending on the cell type, the method of delivery, the effects of size on the uptake of the two different plasmids, and the strength of the promoter driving the SB gene. Alternatively, if a *cis* configuration of transposon plus transposase gene is used, then the primary way to vary the ratio of the two components is by choice of the promoter for the SB transposase gene^{78;113}. Thus, different conditions must be tested for different vectors and different target tissues^{48;49;51}.

The methylation state of the transposon appears to be important. The frequency of transposition from one site in chromosomal DNA to another location is more than 100- to 1000-

fold higher in mouse germ cells^{41;43;44;45} than in mouse embryonic stem cells⁴². Apparently, CpG methylation of the transposon, but not necessarily its cargo, enhances SB transposition¹¹⁴. Moreover, removal of CpG sites in plasmids and their accompanying methylation appears to reduce innate immune responses directed against unmethylated prokaryotic plasmids, thereby extending the lifetime of transgene's expression and presence of its encoded polypeptide product^{115;116}. These findings lead to several questions, including: 1) Do those few transposons that integrate do so as a result of being methylated in the nucleus prior to integration? 2) Are there effective ways of methylating the transposon portion of a vector but not its cargo to enhance transposition without inhibiting expression of the transgene? 3) Will methylation of the transposon reduce promoter activities inside the transposon? Answers to these questions may provide means for raising the efficiencies of transposition.

DNA methylation has been invoked as a method for suppression of transposon hopping within genomes^{117;118} based on the findings in plants that most transposons are methylated¹¹⁹ and that abolishing DNA methylation resulted in the activation of transposition^{120;121}. In most cases this appears to involve suppression of retrotransposons. However, methylation is also regulated by dsRNA-mediated mechanisms and via these activities may serve to regulate the expression of transposase genes as well as the methylation state of transposons^{122;123;124}. Because SB transposons do not contain their cognate transposase, dsRNA events to regulate their expression probably do not play direct roles in transposon silencing. However, depending on the insertion site, some, but not all, SB transposons would be silenced as a result of heterochromatin spread¹²⁵.

A major question that has been unanswered since the first report on the SB transposon system is why relatively few cells that take up the transposon and express it within a few days actually support transposition. For example, in our common assays with HeLa cells, about 10^6 to 10^7 transposon molecules are delivered per cell and about 80% of cells take up and express the DNA, yet at most only 3% of the cells can be recovered following genetic screening for expression of the transgene³³(J. Bell, A. Geurts et al., unpub.). In mouse tissues the reduction seems about the same, even though the ratios of transposon plasmid to transposase plasmid may be as much as 20:1, depending on the experiment^{48;49;50}(E. Aronovich, unpub.). Because a large fraction of cells transiently express the transgene contained in the transposon, it is clear that SB transposons can enter the nuclei of most cells, as can transposase with its NLS sequences, so that nuclear entry does not appear to be a key barrier. Factors mentioned above, including

methylation status and effective ratios of transposon to transposase, which change as the number of transposase transcripts accumulate in a cell, may affect the overall rates of transposition. Nevertheless, on a per vector basis, transposition is a highly improbable process, opening the possibility that the ‘state’ of the target cells may be important. At present, despite its clear importance, we have little understanding of what ‘state’ really means and how it might vary in the cells from one tissue to another. More work is needed to understand this issue. It may involve subtleties of the cell cycle beyond simply the disappearance of the nuclear membrane, which does not appear to be a critical barrier for entry of SB transposons into nuclei.

D. Integration-site preferences of SB transposons

Based on mapping of nearly 2000 transposon-integration events in either mouse or human genomes from cultured cells and tissues in mice, transposons integrate almost randomly, equally into exons, introns and intergenic sequences as a function of their lengths^{41;43;44;45;47;56;126;127}; unlike retroviral^{6;7}, lentiviral^{7;8} and AAV⁹ vectors, which preferentially integrate nearby promoter elements and transcriptional units (Fig. 7a). Although on a macro-scale SB transposons seem to integrate in a nearly random fashion, some *Tc1/mariner*-type transposons are known to prefer ‘hotspots’ that consist of particular sequences flanking the invariant TA integration site¹²⁸. Consequently, the flanking sequences of integration sites for SB transposons have revealed a consensus AYATATRT or (AT)₄ simple sequence palindrome^{47;56;126}. One feature of such AT-rich sequences is that they are highly deformable¹²⁶. However, in a genome like that of the mouse that is 58%(A+T), roughly 30% of the dinucleotide basepairs may be TA, corresponding to about 2x10⁹ integration sites per diploid genome. With so many sites, it can be difficult to find preferential integration sites that might vary from the TA palindrome. Consequently, Liu et al.¹²⁹ looked for preferential sites within limited sequences such as plasmids. They found that there were highly favored sites that did not match the consensus but did have high deformation coefficients (Fig. 7c). In particular, they found that these sites exhibited characteristic alternating higher and lower angles of rotation between adjacent basepairs, a greater spacing between the TA base pairs and a tilting of the basepairs at the TA integration site. These same characteristics are found in the TA palindromes. In these analyses, there was little concern for relative accessibility of the DNA sequences to transposition, a consideration that probably plays a role in genomes with variations in the degrees of sequence condensation between active and transcribed chromosomal regions.

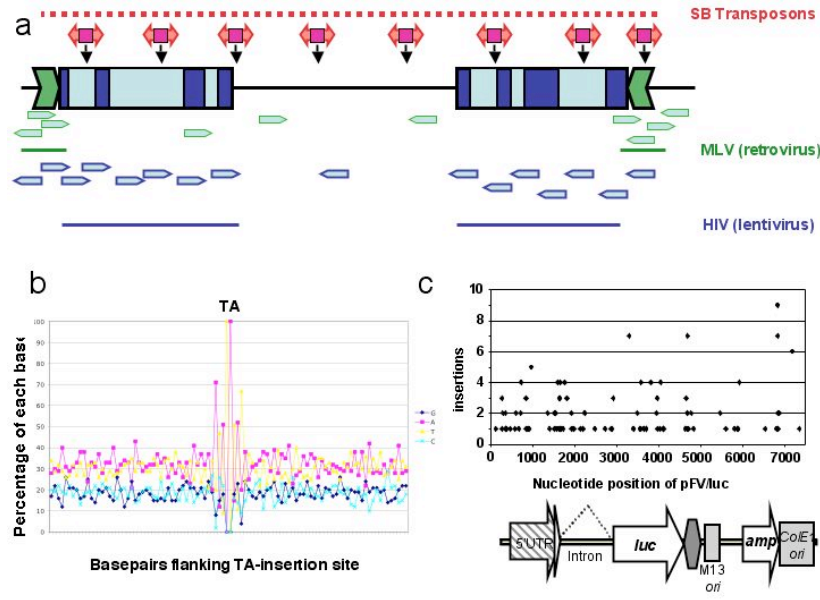


Figure 7. Integration-site preferences of SB transposase. (a) Integration preferences are shown for SB transposase (red double-sided arrows and dotted lines), retroviruses (green arrowheads and lines) and lentiviruses (blue arrowheads and lines). The preferences of the three classes of vectors are shown by the horizontal lines (dotted for SB transposons) with clustering indicated by the symbols. Separating transposons and viruses is a schematic of chromosomal DNA with transcriptional motifs shown as the green chevrons and transcriptional units shown by the dark blue (exons) and light blue (introns) boxes. (b) Percentages of bases over 50 bp on each side of TA-insertion sites in mouse genomes; the TA insertion sites in the center of the chart are invariant (G. Liu and Y. Horie, unpub.). (c) Preferential TA-insertion sites in the pFV/luc plasmid. Integration sites with two or more hits vary from an expected Poisson distribution; 29% of the total hits were between base pairs 6815 and 6854, which comprises less than 0.5% of the plasmid¹²⁹.

Although a full description of preferred sites is ongoing, the following can be concluded at this stage: 1) By and large transposition appears to be far more random for transposons than for many viral vectors. 2) SB transposase does not appear to have a distinct preference for exons, introns or transcriptional regulatory motifs such as enhancers and promoters. 3) There are preferred sites, but they appear to be fairly common and are not simply sequence based. In a sense this is a bit surprising because one might expect that given the millions of potential AT-integration sites, the first sites encountered by a transposon-transposase complex might result in integration. Recent work suggests that heterochromatin is preferentially located at the periphery

of mammalian nuclei and that transcriptionally active chromatin is located more toward the interior¹³⁰. As transposon integration does not occur preferentially (or non-preferentially) in heterochromatin, other factors such as chromatin structure and assorted factors affect the probabilities of integration. Over all, it is possible that the lack of integration preference by transposons may cause fewer adverse effects due to insertional mutagenesis than by viruses. However, the random nature of SB integration makes this vector a useful agent for insertional mutagenesis, as discussed next.

E. Applications of the SB transposons for gene discovery

SB transposons have been used for insertional mutagenesis in mice and fish. In zebrafish, SB transposons have been used for functional genetic studies following their introductions into early embryos^{36;37;39}. Transposons also have been used in medaka for the same purposes^{131;132}. In mice the most productive random screens have employed strains that express SB transposase and have resident SB transposons that can be remobilized in the germ line to produce animals with new insertions in every cell. The reported rates of hopping vary from about 0.1 new insertions per offspring to about 2 new insertions per offspring, depending on the numbers and locations of the resident transposons^{41;43;44;45;46;47}. All of the studies have shown that most, but not all, of the remobilized transposons tend to hop to a site relatively close (10 Mbp) to their origin, thereby allowing for saturation mutagenesis of particular regions of the genome. A database of locations of transposons in the mouse genome has been established¹²⁷. The data suggested that there are only about 10^4 transposition sites/genome that can be efficiently hit following remobilization from a single source in the germ cells of a given seed mouse⁴⁴, a number that is far lower than the number of potential TA sites, estimated to be about 10^6 , as discussed earlier. This suggests that about 100 founder mice would suffice for saturation insertional mutagenesis of the mouse genome using the strategy of transposon remobilization.

From the perspective of gene therapy, there have been two quite significant findings. First, no offspring with dominant mutations have been discovered following germline remobilizations, unless the new mutations were backcrossed to homozygosity^{44;47}. However, the number of total published and characterized insertional events is very low – less than 200 at the time of this review. Second, there are no reports of abnormalities in the mice that express the SB transposase, either ubiquitously or from a germ cell-specific promoter, even if these mice also

harbor transposon transgenes. It is unclear if a similar degree of transposon insertion site complexity exists in non-germline tissues of doubly transgenic transposase plus transposon mice. But if a similar complexity does exist, then it suggests that of the approximately 2×10^4 different *Tc1/mariner*-type transposon insertions that might be present in any one tissue, no dominant mutations that would result in neoplasia or other observable phenotypes have been recovered. In fact, so far, no dominant germline mutations have been recovered by germline mutagenesis using SB vectors either. Nevertheless, recent work from the Largaespada lab shows that appropriately designed SB transposon vectors can accelerate cancer in genetically susceptible mice via an insertional mutagenesis mechanism in which gain-of-function, dominant mutations are induced (Collier et al., unpublished observations).

Because SB transposase activity is often provided from its gene, as discussed in the following sections, there is a chance that some SB genes will integrate into genomes of targeted cells, resulting in expression at a low rate. Early studies on the SB system indicated that there is a high degree of binding specificity by SB transposase for the DRs of SB transposons but not for related transposons, even those of other species of fish³³. This specificity, and the requirement for a specific stoichiometry⁸¹, presumably four transposase polypeptides per transposon, would account for the lack of detected remobilization of endogenous transposons in cells expressing SB transposase. There is no evidence to suggest that SB transposition will be accompanied by recombination or deletion events at an integration site¹³³.

In contrast to SB-type DNA transposons, there are millions of retrotransposons in human and other mammalian genomes. In humans, about 100 of these retrotransposons are active due to active reverse transcriptase/integrase genes. They apparently can direct new insertions in about 12% of individuals^{65;134;135}. In contrast to retrotransposons, all known DNA transposons in mammalian genomes have been silent for the past 50 million years. Thus, *retrotransposition* is a natural phenomenon in humans that has been associated with induction of genetic disease, e.g. hemophilia¹³⁶, and thereby can be considered a base line against which to estimate potential adverse effects from the relatively few cells that will take up one or two copies of an SB transposon following gene therapy.

III. APPLICATIONS OF *SLEEPING BEAUTY* TRANSPOSONS FOR GENE THERAPY

DNA-mediated gene delivery holds great promise in its potential for therapeutic application, as attested by the different chapters in this volume. In some cases, a beneficial therapeutic outcome may be anticipated after a transient burst of expression from newly introduced gene sequences. Perhaps the best example of the utility of such short-term gene transfer and expression is in the development of DNA vaccines¹³⁷, in which case such short-term expression can elicit an effective immune response against a DNA-encoded antigen. However, for many other applications a more extended period of expression following introduction of new sequences will be required in order to achieve a therapeutic benefit. The most demanding circumstance would be in the treatment of a genetic deficiency disease, in which case indefinite expression of the newly introduced gene is sought. Such extended or indefinite expression of newly introduced DNA could be brought about by one of three different approaches: (i) Maintenance of the newly introduced DNA in an extrachromosomal form. This requires stability of the plasmid in the cellular setting, lack of cell division (if copy number per cell is to remain constant), or the ability of the vector element to replicate. (ii) Tethering of the DNA element to the endogenous chromosome, thus promoting its replication and maintenance. (iii) Integration of the DNA into the host cell chromosome, thus allowing its stable maintenance and, perhaps, expression by relying on the cellular machinery for this purpose. As described earlier, *Sleeping Beauty* mediates chromosomal integration of non-viral DNA and therefore provides the potential for extended and even indefinite expression in a gene therapy setting.

The utility of the SB system for achieving long-term expression in animals was supported by experiments in zebrafish and in transgenic mice, as described earlier in Section I.B. However, more pertinent to the potential application of SB for gene therapy is its activity when introduced into somatic tissues. As the transposon component of the SB system consists of DNA, and the transposase component thus far has been provided as a transposase-encoding DNA, delivery of the SB system to somatic tissues is subject to the same constraints as any of the non-viral DNA delivery methodologies thus far reported. In addition, there may be additional constraints that are specific to the SB transposon system, such as the requirement for an optimal molecular ratio of SB transposase to transposon as detailed above. For these reasons, testing of the SB transposon system in somatic tissues has thus far been carried out using the most effective means of non-viral DNA delivery to these tissues. In this section we review the current status of applying the SB transposon system to achieve stable gene transfer and expression in somatic

tissues of the mouse as a model for gene therapy, emphasizing key concepts in the study of transposition *in vivo* that have been addressed in this work. We also present a consideration of safety concerns in the potential clinical application of the SB transposon system.

A. SB-mediated gene expression in the liver

The ability of SB to mediate stable, long-term expression in mouse tissues was initially reported in the seminal work by Yant *et al*⁴⁸, in which extended expression of α 1-antitrypsin as a reporter in normal C57BL/6 mice and of human clotting factor IX as a therapeutic gene product in factor IX-deficient mice was demonstrated. These studies took advantage of the newly discovered “hydrodynamics”-based method for delivery of DNA to the mouse liver^{138;139}, a technique that has since become widely used for the study of gene transfer and expression in the liver even though the mechanism of uptake is poorly understood^{140;141}. In addition to demonstrating transposase-dependent long-term expression of gene products secreted into the blood stream, Yant *et al*⁴⁸ also used a plasmid rescue technique to recover *NEO* transposon sequences from the liver after co-delivery with a transposase-encoding plasmid, thus providing molecular demonstration of the ability of SB to mediate transposition in the liver.

As mentioned above, it is anticipated that the overall level of long-term gene transfer achieved using non-viral delivery approaches is likely to be much lower than that observed using viral delivery systems. As shown by the work of Yant *et al*, Factor IX deficiency has been an excellent model system for gene therapy studies because a therapeutic benefit can be achieved if only a small fraction (1%) of the normal level of gene product is observed in the blood stream of the treated animal⁴⁸. Another approach that can be taken in gene therapy studies where a relatively low level of gene transfer is anticipated is to employ a strategy whereby the selective outgrowth of genetically corrected cells is anticipated. Montini *et al* used the FAH-deficiency model of inherited tyrosinemia to demonstrate selective outgrowth of hepatocytes that had been genetically corrected by hydrodynamics-based delivery of a FAH-encoding SB transposon⁴⁹. A 20-fold increase in the frequency of FAH-corrected hepatocytes was observed when an SB-transposase-encoding plasmid was co-delivered along with FAH-encoding transposon. Because this system results in the clonal outgrowth of FAH-positive cells in the liver of FAH-deficient mice, they were able to genetically characterize transposition events not only by recovery of

transposon junction sites by linker-mediated PCR but also by Southern blot hybridization, thus providing a quantitative molecular assessment of SB-mediated transposition in the mouse liver.

The ability of the SB system to accommodate long sequences of DNA⁸¹ allows it to be used for genes, or their derivatives such as the B-domain-deleted version of FVIII^{142;143;144} for gene delivery to the liver. Recently, Ohlfest *et al.*⁵⁸ showed that a B-domain-deleted version of the FVIII gene could be delivered to livers of FVIII-deficient mice for correction of hemophilia A and expressed for several months at a constant level. As in other previous studies employing viral vectors^{145;146;147}, a major problem with immune responses to secreted FVIII was encountered that could only be avoided by either immunosuppression of the mutant mice or pre-tolerization with recombinant FVIII within 24 hours of birth.

Although this chapter is primarily devoted to the application of SB to non-viral gene transfer, SB also provides the potential to contribute an integrating function to viral vectors that do not integrate as a part of their normal replicative cycle. The mouse liver is highly transducible by vectors based on human adenovirus type 5. Yant *et al.*¹¹² found that SB transposons could be transduced using helper-dependent adenoviruses, but reported that effective transposition required excision of the transposon substrate from the adenovirus vector into a circular form, mediated in this case by Cre recombinase. The constructs used for this study required circularization in order to form a complete and expressible transgene, but this approach prevented control experiments in which the circularization step was omitted. Nonetheless, these results demonstrated the effectiveness of this strategy in combining the efficiency of adenovirus-mediated gene delivery with the integrating function of the SB transposon system to achieve integration and long-term expression in the liver.

Applying the SB system for gene transfer and integration into somatic tissues *in vivo* faces the challenge of delivering both transposon and transposase components to the same cells in a setting where the frequency of transfected cells may be limited. Under these circumstances, what makes the most sense conceptually is to deliver both the transposon and the transposase-encoding sequence to the cells of the liver on the same plasmid (i.e., *in cis*). We found that this approach was, in fact, required in order to achieve efficient gene transfer into cultured HuH7 human hepatoma cells^{35;52}. However, early *in vivo* studies testing such plasmids in which the SB transposase was regulated by the relatively strong CMV early promoter/enhancer yielded lower levels of long-term expression than when the transposon and transposase-encoding sequences

were delivered on separate plasmids (P. Score et al., in prep). Mikkelsen *et al.*⁷⁸ screened a series of promoters exhibiting a range of activities after transient delivery into the liver, and found that elements such as the human phosphoglycerate kinase promoter, providing a low to moderate level of activity, were most effective in providing expression of the transposase component in mediating long-term expression in recipient animals. Similarly, we have found that the human ubiquitin C promoter, which is much less active in the liver than CMV in our hands, is much more effective in providing expression of the transposase component for SB-mediated transposition and long-term expression in the liver (P. Score et al., in prep). These results are consistent with previous observations demonstrating “overexpression inhibition” for *Tc1/mariner*-type transposons^{88:148}, including SB⁸¹, and serve to define conditions where gene delivery, integration and expression can more efficiently be achieved using the SB system.

While accumulation of the evidence described above has supported the effectiveness of the SB system for long-term expression in the liver, there are numerous studies that have emerged reporting high level and long-term gene expression in the liver after delivery of plasmids, *i.e.*, without the benefit of a transposon^{17:18;149}. In our experiments, we observed that gene expression became extinguished in most animals injected with a reporter transposon in the absence of transposase-encoding plasmid, in some cases long-term expression was observed in these control animals. These results made us wonder: in our animals injected with both transposon plasmid and transposase-encoding plasmid, how much of the observed expression is due to transposition and how much is due to maintenance of the plasmid as an extrachromosomal element or, potentially due to random recombination? We therefore devised a strategy whereby induced expression of Cre recombinase in the liver mediates excision of the promoter from a reporter transposon *unless* the reporter transposon has been excised from the plasmid by SB, segregating it from one of the LoxP sites and rendering it non-functional for Cre-mediated recombination¹¹³. We found that induction of Cre recombinase resulted in a two-log-fold decrease in gene expression unless SB-encoding plasmid was co-delivered along with the reporter transposon. These results indicated that most of the expression observed in these experiments is associated with SB-mediated transposition.

The results described above provide considerable support for activity of the SB transposon system for mediating gene insertion and long-term expression in the liver. There are many challenges to be faced. Currently, the most effective way of delivering transposon and

transposase-encoding DNAs is by rapid, high-volume, tail-vein injection in mice^{138;139}. While it may be argued whether DNA can be delivered directly into the hepatic circulation under increased pressure for therapeutic purposes in a large mammal, including humans¹⁵⁰, it is clear that the hydrodynamics-based DNA delivery technique does provide an accurate technique to evaluate effects of genes that are delivered to the liver. Nevertheless, evaluation of the effectiveness of the SB transposon system after delivery to the liver under more clinically applicable conditions is necessary. Additionally while the hydrodynamics-based gene transfer approach may have provided a sufficient level of gene transfer and expression for preclinical treatment of hemophilia B⁴⁸, hereditary tyrosinemia⁴⁹ and hemophilia A⁵⁸, a higher level of gene transfer may be necessary for treatment of other diseases, such as metabolic disorders in which the effectiveness of transposon mediated gene expression is likely to be cell-autonomous. Reports of using retroductal delivery of plasmids in a mixture of various agents that prolong the lifetime of the circular DNA molecules before uptake have been reported^{151;152}. Tests are ongoing in our labs for the efficacy of ligand-conjugated DNA condensing agents⁵² and nanoparticles/nanocapsules for delivery of transposons to liver.

In addition to increased delivery of transposon and transposase-encoding DNAs to the liver, effectiveness of the transposon system may be increased by providing improvements in the activity of SB transposase and in the ability of SB transposons to serve as substrates for transposition, as described earlier in Section II.B. Tests of improved SB transposon systems are underway in several laboratories that seek to treat liver diseases with non-viral vectors.

B. SB-mediated gene expression in the lung

One of the most effective methods of non-viral gene delivery to the lung is by intravenous injection of DNA complexed with linear polyethyleneimine¹⁵³. Belur *et al.*⁵⁰ used this technique to demonstrate long-term expression of luciferase transposons in mouse lung when provided with a source of SB transposase. Transposase, in this case, was provided either by co-injection of an SB-transposase encoding plasmid or by injection of luciferase transposon into transgenic mice expressing SB transposase. At least a 100-fold increase in expression was observed in comparison with control animals injected with transposon alone, and immunohistochemistry studies indicated a high proportion of the transduced cells were type-2 alveolar pneumocytes. Liu *et al.*²⁶ further demonstrated that expression could be directed to

endothelial cells of the lung by using the endothelial-1 promoter to regulate transgene expression in the context of an SB transposon delivered as a PEI complex. Both of the studies described above delivered DNA to the lung after intravascular injection. The potential for gene delivery through the airway remains an alternative for SB transposons. Airway delivery of SB, which has been previously reported¹⁵³, could thus provide the means for a longer lasting, stable gene therapy for cystic fibrosis. Other complexes could be tested for gene transfer in other specific cell types of the lung, for potential treatments by SB-mediated gene transfer.

C. SB-mediated gene expression in hematopoietic cells

The integrating capacity of the SB transposon system is its key asset for treatments that involve extensively dividing hematopoietic and lymphoid cell populations. Non-viral gene transfer techniques have been developed for cultured hematopoietic cells and preliminary results from transposition studies in established lymphoid cultures (Jeff Essner et al, unpub.) have been encouraging. Non-viral, DNA-mediated gene transfer in primary hematopoietic cells has been limited to myeloid-erythroid colony-forming cells following electroporation^{154;155}. There has been one preliminary report of SB-mediated gene transfer into primitive hematopoietic stem cells, in a mouse model of Fanconi anemia complementation group C¹⁵⁶. This work took advantage of the selective outgrowth of stem cells genetically corrected by transposition with a FANC-C encoding transposon. Gene transfer into stem cells was demonstrated by transplantation into irradiated secondary transplant recipients with subsequent genetic analysis for presence of the FANC-C transposon. Such transposition events may have been rare in this case, so future studies must focus on more effective means of DNA delivery in order to effectively apply the SB transposon system to mediated gene transfer into hematopoietic stem cells.

D. SB-mediated gene expression in tumors

Virus-mediated gene transfer has been used to treat tumors in murine models and in some clinical trials¹⁵⁷. Retroviral and adenoviral vectors have been effective gene transfer vehicles for this purpose however there are some disadvantages associated with their use¹⁵⁸. Many viral vectors are highly immunogenic which diminishes the efficacy of repeated administration and raises biosafety concerns^{27;159;160}. In addition, although most viral vectors are designed to be non-replicating, recombination events have been reported yielding undesired effects¹⁶¹. Traditionally,

viral vectors have been seen as much better than plasmid-based vectors due to their ability to support long-term expression of the transgene and an overall higher gene transfer efficiency².

However, the SB transposon system was designed for providing prolonged expression without using viral vectors and consequently may be useful for cancer gene therapy. The use of genes for cancer therapy requires special consideration since depending on the vector system used, one seeks to achieve long-term expression for an acute disease. However, there are several situations in which long-term expression would be desirable for cancer gene therapy. First of all, it might be possible to achieve a life-long cure that would depend on life-long expression or if not life-long, an effective therapy might require prolonged expression over many months. Prolonged expression is desirable if the gene therapy is used to prevent tumor recurrence, which might occur years after initial therapy. Delivery of appropriate genes to a tumor and/or tumor-associated stromal or endothelial cells could theoretically affect a long-term cure and prevent local recurrence. Systemic therapy for suppression of metastases is another potential application of cancer gene therapy, but may be more difficult to achieve since the vector, or its product, must be delivered throughout the body. In either situation, some genes will be more effective, if stable long-term expression is achieved.

Three main approaches for cancer gene therapy have been proposed^{157;158}: anti-angiogenesis genes, *e.g.*, endostatin, angiostatin¹⁶²; cell suicide induction using enzymes that activate a prodrug, *e.g.*, Herpes Simplex Virus thymidine kinase (HSV-TK)^{163;164} or which activate intrinsic apoptotic pathways (*e.g.*, p53 or Bax); and delivery of genes that promote an immune response to tumor cells (*e.g.*, GM-CSF, IL-2). Several studies have shown that constant low-level delivery of anti-angiogenesis inhibitor is more effective than cycling or bolus dosing^{165;166;167}. This may be due to the fact that tumors continuously make pro-angiogenic factors that must be counteracted by the continuous presence of anti-angiogenic factors. Some gene therapies for tumor cell killing, such as HSV-TK activation of the pro-drug ganciclovir, act only on dividing cells¹⁶⁸. Since not all cells within a tumor are dividing at any one time, particularly migratory/metastatic cells, such therapies must be delivered long-term to be effective. Ultimately, the choice of therapeutic gene used depends on the type and anatomic location of the tumor being targeted, the percentage of cells that can be successfully transduced or transfected with the vector being used, and the clinical situation being addressed (*e.g.*, front-line or

consolidation therapy). One paper addresses the potential of SB for cancer gene therapy in terms of the percentage of cells that can be transfected⁵⁷.

SB was tested for gene-transfer and long-term expression in xenografted human glioblastoma cells growing in nude mice⁵⁷. In these experiments, transposon vectors expressing either the Neo, luciferase, or GFP genes were used with SB10 or the catalytically-inactive transposase DDE, Fig. 2). The plasmid DNAs were complexed in polyethyleneimine (PEI) and injected directly into xenografted tumors growing subcutaneously in nude mice. Two weeks after injection all tumors that did not receive active SB transposase had lost detectable luciferase expression. At four weeks, a very noticeable increase in luciferase expression was observed in tumors that received CMV-driven SB at a 1:20 ratio of SB to luciferase transposon plasmid. One explanation for increasing expression over time would be clonal expansion of cells that harbor transposon insertions. Indeed, when the explanted treated cells were plated in G418, about 8% of the clonable U87 cells were resistant to G418, while none of the tumor cells injected with the catalytically inactive transposase yielded G418-resistant colonies. Ten of ten cloned insertions from these G418-resistant clones demonstrated that the insertions were the result of transposition events into TA dinucleotide sites in the human genome. These data establish that tumors growing in vivo can be targets for long-term gene transfer and expression using SB, but that so far, only about 10% of the tumor mass might be successfully transfected long-term. The potential advantages that SB has in terms of reduced immunogenicity and better scalability should also apply to the treatment of cancer. However, given the lethal outcome of the cancers being considered for treatment by SB, a different risk/benefit analysis is certainly in order.

E. Safety issues for transposon-mediated gene therapy

SB shares safety issues in common with many other gene therapy vectors including unintentional induction of innate or adaptive immune responses and insertional mutagenesis. The published literature on safety testing for SB is scant. Clearly, this is an important area for future research.

As with other non-viral systems for gene therapy, delivery of naked DNA might provoke the innate immune response¹⁶⁹, resulting in an adjuvant-like effect and making an immune response against the encoded transgene more likely¹⁷⁰. Indeed, a serious safety concern is that delivery of genes via the SB system will provoke an immune response and formation of

inhibitory antibodies to a gene product that is benefiting the patient via enzyme replacement therapy. Thus, the form of the plasmid that is delivered, the quantity of DNA delivered, and complexing agents used must be studied carefully. Hypomethylated CpG dinucleotides are recognized by the Toll-like receptor 9 (TLR9) and are potent adjuvants¹⁶⁹. Methylation of the transposon and transposase-encoding plasmids may help to prevent activation of the innate immune system, and subsequent adaptive immunity to the transgene-encoded protein. This has been demonstrated in gene-transfer experiments using naked DNA¹¹⁶. The effects of methylation of plasmids on SB transposition¹¹⁴ were addressed earlier in section II.C. The effect that SB transposase protein itself has on the immune response is currently unknown. Presumably, transposase will be recognized as a foreign protein and, depending on the cell-types that express the SB protein, an immune response may be generated. Not only might such a response prevent repeated administration of SB vectors, there might be concern that an autoimmune response against endogenously expressed *Tc1/mariner*-type transposase proteins or peptides (if they exist) might be provoked. Current efforts are underway to determine immunological responses to SB transposase in mice that constitutively express the protein. Nevertheless, this issue requires more study.

In common with integrating vectors such as lentiviruses, MLV, and AAV, SB causes chromosomal integration of vector DNA. As described earlier, SB does not prefer to integrate near promoters or within genes, as do the retroviral vectors. Instead, SB integration at TA dinucleotides is similar to what one would expect by chance^{44,47,126}. Nevertheless, given a large number of genomic integration events, many will land near or within genes, including those genes that might trigger cancer if mutated appropriately. Studies are underway to determine if lifelong somatic transposition of chromosomally resident transposon vectors, deliberately designed to activate or inactivate genes, will cause cancer in mice. It will be important to determine whether promoters used to drive transgene expression can activate endogenous genes as effectively as MLV long terminal repeats. Of course, a Moloney-based vector used to treat X-linked SCID did cause leukemia in patients via an insertional mutagenesis mechanism¹⁷¹. The use of insulator elements¹⁷², which could prevent enhancement of endogenous promoters, might ameliorate risk due to insertional mutagenesis. However, another form of mutagenesis risk would be from gene truncation due to promoter or splice acceptor insertion into cancer genes. Another important issue is the likely number of unique insertion events that will occur over time. If the

SB transposase plasmid is delivered as a gene on a plasmid, then there is some potential for achieving the unintended long-term transposase expression. The result of long-term expression of SB transposase in a cell that harbors one or more transposon vectors could be ongoing transposition and many different insertion events. Research into the relative risk due to this effect could be accomplished using SB transposase transgenic mice⁴⁵. Alternate methods for delivering transposase are highly desirable. Clearly, it is possible to deliver transposase as *in vitro*-transcribed mRNA in microinjected embryos⁴¹. Whether SB transposase mRNA can be practically delivered in a gene therapy setting is unclear. Another possibility would be to deliver transposon DNA together with purified SB transposase protein. However, active purified SB transposase has not been reported. Theoretically, it is possible that an ideal stoichiometry between transposon, transposase, and required co-factors could be pre-assembled and delivered to cells as one unit, the “transposasome”, that would maximize efficiency while maintaining safety.

For both sources of risk described above, it behooves the gene therapist to determine the lowest possible dose of vector that will confer a beneficial clinical outcome. The use of less vector plasmid should reduce the risk of an immune response and possibly, by limiting the number of total insertion events, may reduce the risk of mutagenesis. Thus, improvements in the activity of the SB system are an important aspect of SB safety research.

IV. FUTURE DIRECTIONS

A. Delivery methods for the SB transposon system.

Transposon-mediated gene delivery is in its infancy compared to the use of viral methods. The challenges for using transposons to correct genetic diseases are identical to those for all of the other methods of non-viral gene delivery – efficacious transfer with minimal undesirable side effects. The challenge of delivery to specific organs and tissues has commanded considerable interest, as discussed here and elsewhere in this volume. The use of a variety of DNA ‘coatings’ that are decorated with cell-specific ligands has and will continue to allow improvement of targeting of all types of non-viral vectors, including transposons¹⁷³. For instance, PEI conjugated with galactose has been used to target SB transposons to mouse liver following injection into the tail vein⁵². Other methods, including nanoparticles and nanocapsules are becoming available for gene therapy^{174;175}. The greater use of tissue-specific and cell type-

specific promoters that can be regulated will compensate for misdirected vectors. Improvements on both fronts are ongoing in many labs and they can be incorporated into protocols that employ SB transposons.

B. Efficiency and evolution of the SB transposon system.

A second area of activity is improvement of the efficiency of SB transposition based on improvement of the transposase enzyme. As discussed in Section II.B.2, the combination of target-site recognition motifs embedded in the cut-and-paste catalytic domain is a major impediment to rational design of a site-specific SB transposase. Our experience is that simple addition of amino acid sequences to the termini of SB transposase generally have inactivated the enzyme completely (unpublished) so that the addition of targeting sequences is unlikely to be successful. Until a clear three dimensional structure of the SB transposase is developed, all improvements will come from random mutagenesis with either a genetic selection screen or laborious testing of each derivative. The amino-terminus of the Tc3 transposase of *C. elegans* is the only crystal structure of a *Tc1/mariner*-type transposon that has been published and this structure includes only the transposon-binding sites and not the catalytic domain.

C. Safety Issues for the SB transposon system

The most formidable challenge is the issue of random integration^{176;177;178} based on the results from the French X-SCID clinical trial in which two cases of a leukemia-like syndrome apparently resulting from insertional activation of the LMO2 oncogene were reported¹⁷¹. There are two ways to avoid the *observed* problems of random or semi-random integration – the use of site-specific integrases, and blocking enhancer activity within the therapeutic vector from activation of endogenous genes. The *Streptomyces* □C31 phage integrase appears to direct integration of the transgenic construct into a relatively small number of sites in human chromosomes that resemble its normal recognition site^{63;179;180}. However, potential problems associated with this integrase, including chromosomal deletions mediated by the integrase, have not been thoroughly investigated. The second strategy, to use border/insulator elements to block enhancers in vectors from activating chromosomal genes is discussed below.

For many gene therapy applications, long-term expression of genes is essential. Transcriptional silencing of retroviruses poses a major obstacle to their use as gene therapy vectors and border elements have been proposed as a solution¹⁸¹. Consequently, a number of investigators have incorporated border elements into their constructs to block methylation of the

therapeutic gene^{182;183;184}. However, none of these studies have addressed the blocking of enhancer effects of the transgenic constructs on endogenous chromosomal genes.

How might border/insulator elements be used? Although the DNA in nuclei is often envisaged as a tangled mess of chromosomal fibers, domains of activated genes appear to be structurally divided by sequences called matrix attachment regions (MARs) or scaffold attachment regions (SARs)^{185;186}. The sequences of MARs proximal to different genes are not identical. MARs appear to be able to ensure proper expression of transgenes in terms of tissue specificity and timing but do not seem to protect genes against repression when integrated into some regions of chromosomes^{187;188}. Insulators are a second type of DNA sequence that appear to function by a different mechanism than do MARs to alleviate position effects in transgenic animals^{189;190;191}. The 5' constitutive DNaseI hypersensitive sites from the chicken β -globin locus control region is the most studied insulator element^{192;193;194;195;196}. This insulator has several motifs that confer either of two properties. The first property is to act as a barrier to block heterochromatinization by spread of methylation that can permanently shut down expression of transgenes. The second property is an enhancer-blocking activity mediated by a site that binds a protein known as CTCF. CTCF binding to DNA blocks enhancers neighboring a transgenic construct from influencing the activity of the transgene^{197;198}.

All of the evidence described here serves to emphasize the following common features of border elements. First, they are active only when part of chromatin. Second, none of the border elements alter the expression levels of genes in transient assays. Third, neither MARs nor insulators alter tissue-specificity of transgene expression. Essentially MARs appear to block enhancer activity, but they do not exhibit silencing activity. Many insulators have the ability to block both silencing and enhancing activities. However, boundary/insulator elements have a role in establishing domains of open chromatin characterized by global changes in histone modifications. As a result, the effects of incorporating boundary/insulator elements randomly in human chromosomes are unknown. For instance, random integration of an insulator-flanked vector could lead to repression of a tumor suppressor gene if the vector integrated between a critical enhancer and the gene's transcriptional unit. Future studies must therefore consider the implications of widespread insertion of such elements as a safety precaution.

D. Conclusion.

Since its creation in 1997, the SB transposon system has been on a rapid course of

development for employment as a vector for non-viral gene therapy. Although problems and questions remain, within the first seven years of its appearance, there have been 22 papers on its basic properties, 21 papers on its activities in mice and about half a dozen papers on its use in other vertebrates. When combined with developments for delivery of various forms of non-viral constructs, this record suggests that the SB transposon system is on a relatively rapid adaptation pathway for use in gene therapy as a vector that combines the advantages of viral vectors - high rates of chromosomal integration and integration of single copies of a therapeutic gene, with the advantages of non-viral vectors - the absence of protein factors that elicit adverse responses.

V. ACKNOWLEDGEMENTS

We thank the Arnold and Mabel Beckman Foundation for support of our work and all members of the Beckman Center for Transposon Research for a long history of contributions of ideas and results. We are especially grateful to Dr. Elena Aronovich and for careful many suggestions and proofreading of the manuscript and to Kirk Wangenstein for proofing. The authors were supported by NIH grants 1PO1 HD32652-07 (PBH and RSM), R43 HL076908-01 (PBH and RSM), 1RO1-DA014764 (DAL), and 1RO1-DA14546-01 (SCE).

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