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ARTICLE

# Helper-Independent *Sleeping Beauty* Transposon–Transposase Vectors for Efficient Nonviral Gene Delivery and Persistent Gene Expression *in Vivo*

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**Transposon-based vectors represent promising new tools for chromosomal transgene insertion and establishment of persistent gene expression *in vivo.* Here, we report the development of helper-independent transposon–transposase (HITT) vectors, which contain on single plasmids (i) a *Sleeping Beauty* (SB) transposon containing the transgene and (ii) a SB transposase expression cassette. To obtain an optimal level of transposase expression from HITT vectors, we determined the relative strength of a panel of different promoters in mouse liver and used these promoters to drive transposase expression from injected HITT vectors carrying a human $\alpha_1$-antitrypsin (hAAT) expression cassette flanked by transposon inverted repeats. By correlating promoter strength with stabilized serum hAAT levels, a narrow expression window supporting high-level transposition in the liver was defined. Peak levels of long-term gene expression were obtained with promoters 30- to 40-fold less active than CMV in mouse liver, whereas reduced stable levels of hAAT were detected with both weaker and stronger promoters. Injected HITT vectors induced transposase-dependent insertion of transposon DNA into the genome of at least 5–6% of transfected hepatocytes, generating levels of persistent hAAT expression that were 2- to 4-fold higher than with an optimized two-plasmid approach. In addition, we show that HITT vectors carrying a human factor IX (hFIX)-containing transposon support (i) long-term hFIX expression in normal mice and (ii) partial phenotypic correction in a mouse model of hemophilia B. SB-based HITT vectors represent a major advance in the establishment of persistent transgene expression from nonviral gene delivery systems and should prove useful for gene transfer to tissues or cell types in which transfection efficiencies are low.**

**Key Words: nonviral vectors, naked DNA, gene therapy, liver, *Sleeping Beauty*, transposon**

## INTRODUCTION

DNA transposons are discrete segments of DNA that possess a capacity to migrate and carry genetic information between chromosomal loci. Relics of once-active transposons are found in virtually all organisms and have played major roles in the evolutionary shaping of genomes. The integration machineries of transposable DNA elements consist of an element-encoded *trans*-acting transposase that facilitates DNA excision and reinsertion through interactions with terminal *cis*-elements in the transposon and sequences at the integration site. Based on these attractive features, transposable elements have potential utility for germ-line transgenesis and insertional mutagenesis [16] and recently, as part of nonviral or viral vector systems, have become valuable tools for therapeutic gene delivery with potential to establish persistent, even life-long, transgene expression *in vivo* [7–9].

*Sleeping Beauty* (SB), a member of the Tc1/*mariner* transposon superfamily, was genetically reconstructed from defective fossil genetic elements in salmonid fish genomes [10]. SB transposes through a "cut-and-paste" mechanism catalyzed by transposase subunits that bind two 30-bp direct repeat sequences in each of the terminal 225-bp inverted repeats (IRs) of the transposon and multimerize to facilitate DNA bending and transposon insertion at AT-dinucleotide target sites. The "reawakened" element transposes in a wide range of vertebrates more efficiently than any other tested transposon [11]. However, it remains a possibility that efficient SB transposition



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may require species-specific host factors. So far, conserved high-mobility-group proteins have been identified as cofactors that stimulate binding of transposase subunits to IR direct repeats [12]. Initial findings that SB-based vectors can insert transgenes into cultured human cells showed the potential of SB-supported integration of foreign DNA [10]. Recently, we have documented successful use of a SB transposon system in a nonviral vector system based on codelivery to mouse liver of two plasmids, one carrying the transposon with an embedded factor IX expression cassette and one carrying a transposase expression cassette, resulting in persistent therapeutic expression levels of the factor IX coagulation factor [7]. Similar two-component SB nonviral vector systems have been used to facilitate stable gene delivery of the fumarylacetoacetate hydrolase gene in a mouse model of hereditary tyrosinemia type 1 [9] and to restore permanently expression of laminin 5 protein in epidermal progenitors *in vitro,* allowing corrected cells to regenerate human skin on immunodeficient mice [13]. In addition, we have employed gene-deleted adenoviral vectors to deliver transposon-encoded transgenes [8]. By co-injection of hybrid adeno-SB viral vectors carrying the transposon-tagged factor IX gene and the SB transposase, respectively, persistence of transgene expression could be achieved in actively dividing mouse hepatocytes.

The genomic abundance of transposable elements suggests that regulation of transposition may have played a key role in establishment of a balanced host–parasite relationship, limiting transposon-based insertional mutagenesis. Clearly, such control mechanisms might influence the results of transposon-based gene transfer and may be of great importance for the design of SB-based vectors. Over time, most Tc1/*mariner* transposons have accumulated multiple mutations that have rendered them defective due to either (i) the lack of selection for functionality or (ii) a positive pressure that selects for less active or inactive elements that may participate in regulation of transposition [14]. In support of the latter, missense mutations of the *mariner* transposon *Mos-1* have been suggested to generate dominant-negative "poisonous" subunits that combine with wild-type transposases to reduce transposition [15]. Furthermore, for several Tc1/*mariner* transposons, including Tc1 and SB, it has been suggested that truncated catalytically inactive transposase forms may occupy binding sites and downregulate transposon excision in a competitive fashion [16,17]. Of particular importance for gene delivery applications, studies in flies and *in vitro* with active *Mos-1* and *Himar1 mariner* elements have shown that elevated transposase levels can dramatically reduce the rate of transposition [15,18]. The mechanism for this "overproduction inhibition" remains poorly defined but may involve the generation of less active multimeric transposase complexes [15]. It remains to be ascertained whether one or more of these regulatory mechanisms is operational during SB vector-mediated transposition. However, in *in vivo* plasmid transfection studies, only low doses of plasmid DNA encoding CMV-driven SB transposase (transposon:transposase 25:1) resulted in efficient transposition in mouse liver, whereas higher doses (1:1) led to reduced transposition and limited long-term transgene expression [7]. This inverse relationship between transposase dose and *in vivo* transposition reflects a transposase-based inhibitory effect on the transposition efficacy caused by mechanisms that ultimately result in loss of plasmid DNA (S. R. Yant, J. G. Mikkelsen, L. Meuse, Z. Huang, M. A. Kay, unpublished results).

Nonviral vehicles for gene delivery, such as naked DNA, currently attract attention primarily due to good safety profiles, low immunogenicity, easy large-scale production, and low manufacturing costs. However, despite recent advances in the field, nonviral systems often suffer from low *in vivo* transfection efficiencies, which reduce the potency of a transfer technology that requires cotransfection of two plasmids. In continuation of our work with transposon-based gene delivery, we report here the development of helper-independent transposon–transposase (HITT) vectors, which contain on single plasmids (i) a SB transposase expression cassette and (ii) a SB transposon containing the transgene. To obtain an optimal level of transposase expression that did not cause overproduction inhibition, we determined the relative strength of a panel of different promoters in mouse liver and subsequently used these promoters to drive the expression of transposase from injected HITT vectors. By correlating the relative strength of promoters driving transposase production with levels of stable transgene expression, a narrow window of optimal transposase expression was defined. Codelivery of transposase and transposon on single plasmids resulted in increased persistent expression levels of a therapeutic gene. This optimized single-plasmid technology greatly improves and simplifies persistent nonviral gene delivery systems and should prove useful for gene transfer to tissues or cell types in which transfection efficiencies are low.

## RESULTS

### Evaluation of Relative Promoter Strength in Mouse Liver

To develop transposon–transposase vectors containing the components of the SB vector system in a 1:1 ratio on single plasmids, we hypothesized that optimal conditions for transposition could be established using weaker promoters to direct transposase expression. First, we tested a total of 10 promoters for their capacity to direct expression of the human α1-antitrypsin (hAAT) gene from plasmid DNA introduced by high-pressure tail vein injection [19] into livers of adult C57Bl/6 mice. To classify the promoters according to their long-term expression pro-

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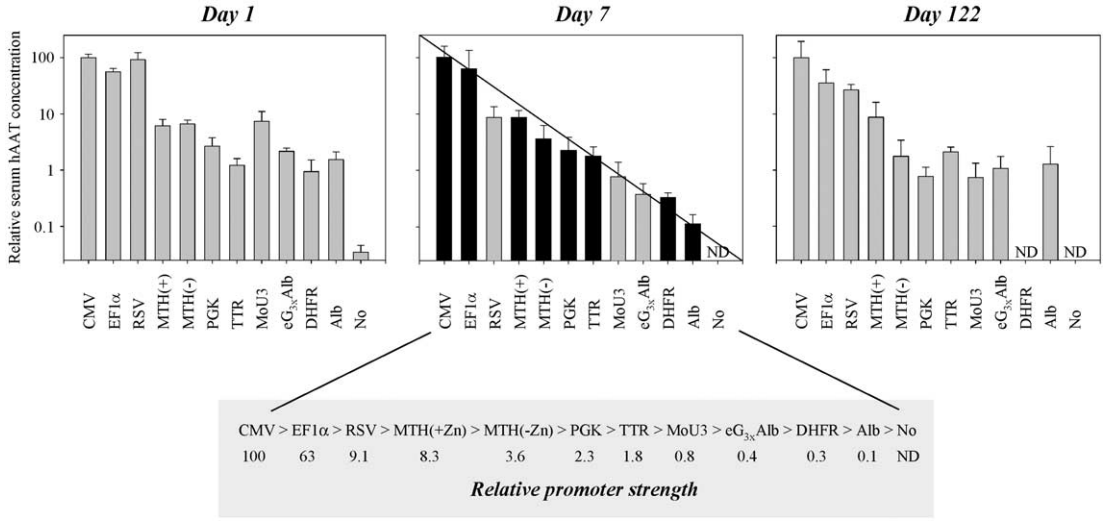


**FIG. 1.** Comparison of serum hAAT expression profiles obtained with different promoters. Mice ($n = 5$ mice per group) were injected at day 0 with 25 μg plasmid carrying the hAAT gene directed by the indicated promoters: CMV (cytomegalovirus), EF1α (elongation factor 1α), RSV (Rous sarcoma virus LTR), MTH (metallothionein), PGK (phosphoglycerate kinase), TTR (transthyretin), MoU3 (Moloney murine leukemia virus U3 region), eG3xAlb (albumin with upstream enhancer), DHFR (dihydrofolate reductase), Alb (albumin), and No (no promoter). Expression from the MTH promoter was induced in one of two groups injected with pBS.MTH-hAAT by adding $ZnSO_4$ (indicated by +) to the drinking water (25 mM final concentration). For all time points, the serum hAAT level relative to that of CMV is shown. Serum hAAT levels for pBS.CMV-hAAT were $5.6 \times 10^5$, $3.6 \times 10^5$, and $9.9 \times 10^2$ ng/ml at day 1, 7, and 122, respectively. For all promoters, hAAT expression levels did not change significantly after day 32; data from day 122 are shown. Mean relative values ± standard deviation are shown. The relative strength of all promoters, determined by serum hAAT levels at day 7 after injection relative to levels obtained with pBS.CMV-hAAT, is shown in the gray box. Based on the distribution of relative serum hAAT levels at day 7, CMV, EF1α, MTH, PGK, TTR, DHFR, and Alb promoters (indicated by black bars) were selected for further studies. Serum hAAT was detected for pBS.No-hAAT only at day 1 after injection. Further details on the tested promoters are given in Table 2. ND, not detectable.

files and relative strength, we monitored serum hAAT levels in treated mice for 3 months after injection. The cytomegalovirus (CMV) promoter was the strongest promoter throughout the experiment, resulting in serum hAAT levels ranging from $5.6 \times 10^5$ ng/ml 1 day after infection to $9.9 \times 10^2$ ng/ml 3 months later. Based on the serum hAAT levels monitored shortly after injection (day 7, Fig. 1) only the human elongation factor 1α (EF1α) promoter came close to matching the strength of CMV. A group of weak regulatory elements (30- to 1000-fold less active than CMV) comprised promoter sequences derived from Moloney murine leukemia virus, murine versions of phosphoglycerate kinase (PGK) and dihydrofolate reductase (DHFR) genes, mouse albumin (Alb and $eG_{3x}$Alb, the latter flanked by an extra enhancer element) and transthyretin (TTR) genes expressed specifically in liver, and the inducible metallothionein promoter (MTH). An intermediate group of promoters (about 12-fold less active than CMV) included the Rous sarcoma virus long terminal repeat (RSV) and MTH induced by inclusion of zinc sulfate in the mouse drinking water.

Successful *in vivo* transposition may depend primarily on the transposase concentration obtained shortly after plasmid injection. Based on the distribution of relative serum hAAT levels detected 1 day and 1 week after injection, we selected CMV, EF1α, MTH, PGK, TTR, DHFR, and Alb as candidate promoters to direct transposase expression from SB-based HITT vectors.

### Codelivery of Transposon and Transposase on a Single Plasmid Leads to Persistent Transgene Expression in Mice

To produce *Sleeping Beauty*-based HITT-vectors, we inserted the transposase coding sequence flanked by a short promoter insertion linker (PIL) 227 bp upstream of the left inverted repeat of a transposon (T/hAAT) containing the hAAT gene expressed from an RSV promoter. The two genes were oriented in the same direction. Due to the lack of promoter, this vector (designated pT/hAAT.PIL-SB, Fig. 2A) should provide only leaky expression of the transposase gene. To create a battery of vectors with expected variation in the transposase expression profiles, we introduced the set of promoters into the PIL sequence of pT/hAAT.PIL-SB, generating seven additional HITT vectors (Fig. 2A). Moreover, we included a vector (pT/hAAT.CMV-SB.ds) in which a CMV-transposase expression cassette was inserted downstream of the hAAT transposon.

We investigated whether *in vivo* transposition and persistent gene expression could be achieved from HITT-vector donor plasmids administered to mouse liver by high-pressure injection through the tail vein of C57Bl/6 mice. Our results show that all vectors, except the vectors

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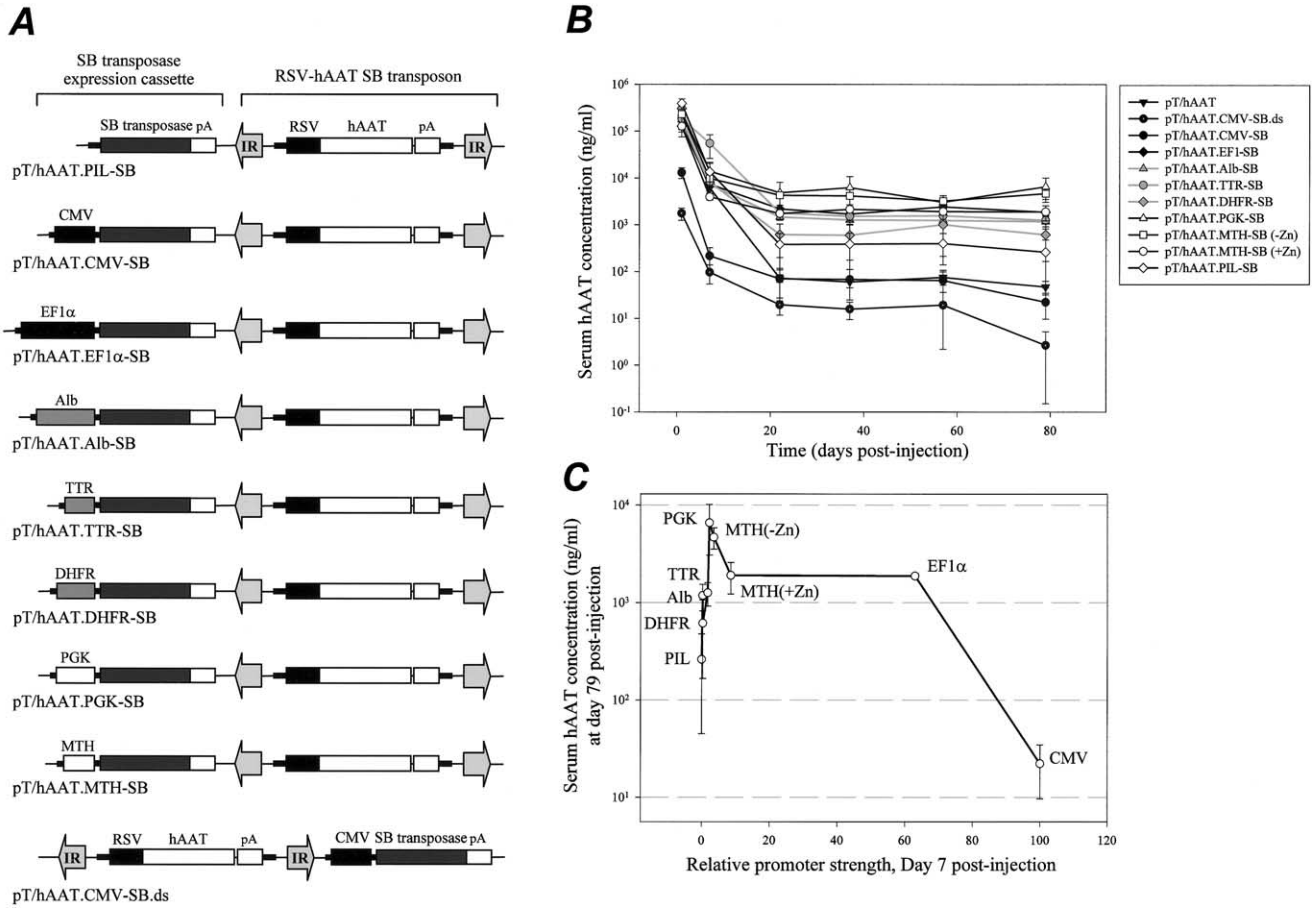


**FIG. 2.** *Sleeping Beauty* transposon–transposase vectors induce persistent transgene expression in mice. (A) Schematic representation of injected SB-based HITT vectors. Plasmids carry the transposon-embedded hAAT and flanking transposase expression cassettes in the same orientation. Promoters indicated by black, gray, and white boxes (strong, weak, and intermediate promoters, respectively) were inserted into pT/hAAT.PIL-SB carrying the promoter insertion linker (PIL) upstream of the transposase coding region. In pT/hAAT.CMV-SB.ds, the transposase cassette was inserted downstream (ds) of the RSV-hAAT transposon. SB, transposase; IR, inverted repeat; pA, poly(A) sequence. (B) Patterns of long-term hAAT expression after administration of SB HITT vectors. Equal molar amounts of indicated vectors were administered to mouse livers ($n = 4$–5 mice per group) by hydrodynamic injection of 30 μg HITT-vector plasmid or 24 μg pT/hAAT into tail veins. The concentration of serum hAAT was followed for 79 days after injection. All HITT vectors, except those with CMV-directed transposase production, induced levels of persistent expression that were improved over the basic level obtained with pT/hAAT. Promoters support transposition and establishment of stable gene expression according to the following hierarchy: PGK > MTH(−Zn) > MTH(+Zn) > EF1α > TTR > Alb > DHFR > PIL > CMV > CMV(ds). For details on promoters, see legend to Fig. 1 and Table 2. Mean values ± standard deviation are shown. (C) Transposition from HITT vectors varies with promoter strength. By relating relative promoter strength (Fig. 1, bottom) with level of persistent hAAT expression after HITT-vector injection (B) an optimal window for transposase expression could be defined.

containing a CMV-directed transposase gene (CMV-SB), induced high hAAT expression immediately after DNA administration (>$10^5$ ng hAAT/ml serum; Fig. 2B). For mice treated with CMV-SB-containing vectors, the serum hAAT levels 1 day after injection were 1–2 logs lower, indicating that the high levels of transposase produced from these vectors triggered a loss of plasmid DNA, thus impairing both hAAT production and downstream transposition events. In the remaining mouse groups (except for mice treated with pT/hAAT.PIL-SB), the levels of stable serum hAAT, reached 3 weeks after injection, were significantly higher (20- to 150-fold) than in the control group injected with transposase-deficient pT/hAAT ($P < 0.05$). These findings demonstrate that SB HITT vectors can provide both sufficient transposase and sufficient transposon substrate for efficient insertion of the transgene into chromosomal DNA and thereby facilitate persistent gene expression.

### A Narrow Window of Transposase Expression Provides Optimal Conditions for HITT-Vector-Based Transposition

Although most vectors raised the persistent serum hAAT levels well above that of the control group, the potency of

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individual vectors varied according to the promoter directing expression of the transposase. Peak levels of hAAT expression (4700–6600 ng hAAT/ml serum) were obtained with HITT vectors in which the MTH and PGK promoters directed expression. Statistical analyses showed no significant difference between these two groups ($P = 0.4$), whereas significantly reduced stable levels of hAAT were monitored with both weaker (TTR, Alb, DHFR; $P = 0.002$, $P = 0.0006$, and $P = 0.001$, respectively, for comparison with pT/hAAT.MTH-SB-treated group) and stronger (EF1α and zinc-induced MTH; $P = 0.004$ and $P = 0.02$, respectively) promoters (Fig. 2C). Surprisingly, even leaky expression from the promoterless transposase cassette (PIL) could support transposition, albeit with low efficiency, resulting in long-term hAAT levels that were increased 6-fold relative to the pT/hAAT-treated control group. However, the serum hAAT means of these groups did not differ significantly ($P = 0.06$). By correlating the estimated relative strength of the various promoters driving transposase expression with serum hAAT levels obtained 3 months after injection of HITT vectors, we defined a narrow window of transposase expression supporting the highest level of integration of vector-encoded transposons (Fig. 2C). According to this correlation, expression levels that were 30- to 40-fold lower than CMV-driven expression provided optimal conditions for transposition in the liver. Notably, among the weaker promoters even very small differences in promoter activity had profound effects on the gene insertion efficiency. In addition, an estimated 2-fold induction of the MTH promoter in the presence of zinc was sufficient to affect insertion of the transposon negatively, with statistical significance ($P = 0.02$). It appears, however, that the transposition reaction was not equally sensitive to higher concentrations of transposase since the very dramatic effect of overproduction inhibition seen with CMV was not observed with EF1α-directed transposase expression. Together, these findings suggest that a wide transposase dose range may support transposition from SB HITT vectors but that *in vivo* efficiency depends on balancing transposase expression in a fashion that eliminates negative effects caused by either insufficient transposase production or mechanisms of overproduction inhibition.

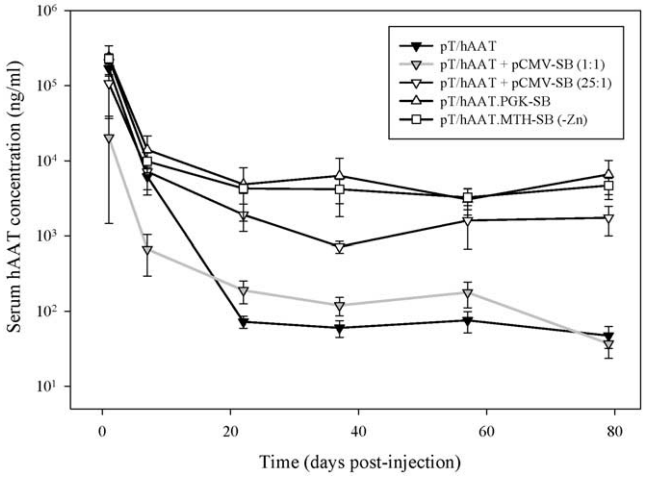


**FIG. 3.** Improved transgene transposition with SB HITT vectors. Serum hAAT levels in mice receiving 24 μg pT/hAAT, 30 μg pT/hAAT.PGK-SB, or 30 μg pT/hAAT.MTH-SB (data from Fig. 2C) compared with 24 μg pT/hAAT + 20 μg pCMV-SB (1:1 ratio based on hAAT and SB transposase gene copy numbers) and 24 μg pT/hAAT + 0.8 μg pCMV-SB (25:1). When necessary, pUC19 "stuffer" DNA was included to ensure that each mouse was injected with the same total amount of DNA (44 μg). Gene copy numbers determined amounts of DNA in the two-component injections; hAAT and SB transposase genes were injected in equal copy numbers for HITT vector and 1:1 two-component injections. Average values ± standard deviation are shown. $n = 5$ mice per group.

### SB HITT Vectors Facilitate Transposase-Dependent Transgene Gene Insertion and Higher Levels of Stable Gene Expression Than an Optimized Two-Plasmid Vector System

Based on a two-plasmid SB vector system, we previously found that a transposon:transposase 25:1 plasmid ratio using a CMV-driven transposase provides optimal conditions for therapeutic transposition *in vivo* [7]. It is likely, however, that ideal conditions in this setup were created only in a subset of the transfected cells and that transposition in the remaining transfected cells suffered from suboptimal plasmid ratios or even lack of transposase-encoding plasmid. To test whether HITT vectors with adjusted transposase expression could improve transposon-based gene transfer, we compared the long-term hAAT expression profiles of the most efficient vectors (pT/hAAT.PGK-SB and pT/hAAT.MTH.SB) with the optimized two-plasmid approach in which transposase expression was directed by a CMV promoter. Our data confirmed that a 1:1 ratio of pT/hAAT and pCMV-SB supported transposition only to a limited degree, whereas usage of 25-fold less transposase plasmid led to 50-fold higher levels of persistent hAAT expression ($P = 0.001$) (Fig. 3). In addition, hAAT expression after HITT-vector administration stabilized at levels that were 2.7- to 3.8-fold higher than levels obtained with a 25:1 two-plasmid injection ($P = 0.04$ (PGK) and $P = 0.009$ (MTH)), demonstrating the increased potential of these vectors to facilitate stable gene transfer.

To demonstrate that HITT-vector function was dependent on transposase activity, we introduced an inactive transposase containing a point mutation in the C-terminal catalytic D,D(35)E motif into PGK- and MTH-based HITT vectors. As shown in Fig. 4A, these vectors (pT/hAAT.PGK-mSB and pT/hAAT.MTH-mSB) both failed to induce prolonged transgene expression in sharp contrast to their functional-transposase counterparts, resulting in a 100-fold difference in final serum hAAT levels between mutant and active transposase groups ($P = 0.04$ (PGK) and $P = 0.0004$ (MTH) for comparison of mouse groups

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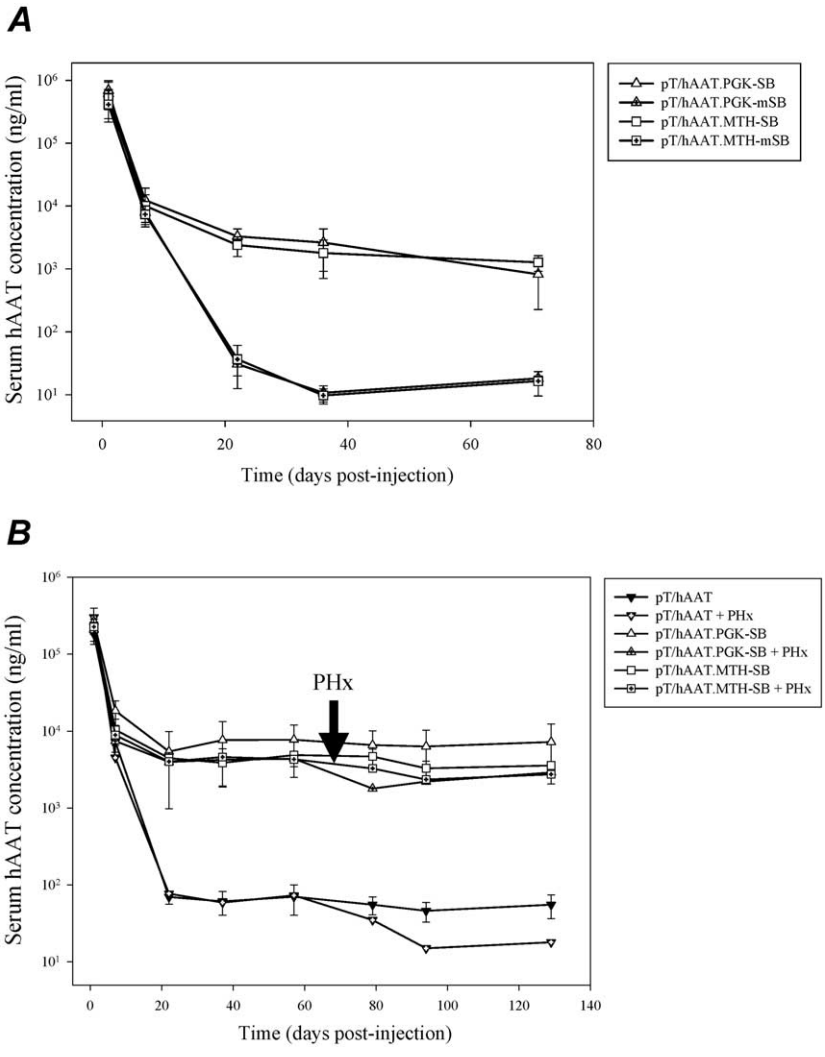


**FIG. 4.** Persistence of transgene expression is induced by transposase-dependent gene insertion. (A) Comparison of long-term stability of hAAT expression in mice ($n = 4$ mice per group) injected with SB HITT vectors harboring active (SB) or inactive (mSB) transposase. Animals received 30 μg of HITT vectors indicated. Mean values ± standard deviation are shown. (B) Serum hAAT levels after induced liver cell division in HITT-vector-treated mice. Mice ($n = 5$ per group) treated with 24 μg pT/hAAT, 30 μg pT/hAAT.PGK-SB, or 30 μg pT/hAAT.MTH-SB either were subjected to partial hepatectomy (PHx) at day 70 after DNA injection ($n = 2$, symbols marked with +) to facilitate loss of extrachromosomal DNA or did not undergo surgery ($n = 3$). The persistent high serum hAAT levels even after partial hepatectomy demonstrate that integrated copies of the hAAT gene and not putative episomal hAAT-encoding DNA account for the persistence of gene expression. In the absence of transposase (pT/hAAT), we could detect a significant reduction in serum hAAT, indicating loss of a significant portion of episomal pT/hAAT due to hepatocyte cell division. Mean values ± standard deviation are shown for groups that did not undergo surgery, mean values are shown for pairs of animals subjected to partial hepatectomy. Black arrow indicates time of partial hepatectomy.

treated with HITT vectors encoding wild-type and mutant transposase). To verify that the transposon carried by the HITT vector was indeed inserted into liver genomic DNA, we subjected mice treated with pT/hAAT.PGK-SB, pT/hAAT.MTH-SB, or pT/hAAT to partial hepatectomy ($n = 2$ for each group). By removal of 2/3 of the liver, hepatocyte proliferation and a derived loss of extrachromosomal DNA were induced as shown before [20,21]. Results show that the hAAT expression levels in HITT-vector-treated mice were unaffected by liver regeneration and persisted throughout the test period, indicating that transgene expression was directed from integrated versions of the gene (Fig. 4B). In contrast, a marked drop in gene expression, indicative of plasmid DNA loss, was observed in control mice receiving pT/hAAT. In summary, our data suggest that HITT vectors efficiently serve as a combined source for transposase production and substrate for transposon release, inducing transposase-dependent transgene integration and stable expression. In comparison to a two-plasmid approach, codelivery of the vector components on single units may create improved conditions for transposition in mouse liver by providing equal transposon–transposase ratios in all transfected cells.

### Quantitative Evaluation of SB HITT-Vector-Directed Transposition in Vivo

To monitor the efficiency of HITT-vector-directed transposon insertion in the genome of mouse hepatocytes *in vivo,* we injected HITT vectors with transposon-encoded cytoplasmic β-galactosidase (β-gal) into immune-deficient SCID mice. Two months after plasmid administration, livers were harvested and stained for β-gal expression to determine the percentage of hepatocytes with stable gene expression. As reported previously, estimates of vector integration frequency based on expression of cytoplasmic β-gal may be lower than the actual numbers

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due to instability and reduced detection of β-gal [21,22]. However, in correlation with the hAAT expression data, we observed variations in persistence of gene expression, depending on the promoter driving transposase expression. In mice treated with pT/RSV-βgeo.CMV-SB, β-gal expression persisted in 0.08 + 0.03% of hepatocytes compared to less than 0.04% in animals that received a transposase-deficient vector (pT/RSV-βgeo). When we administered pT/RSV-βgeo.PIL-SB 0.27 + 0.09% of the hepatocytes maintained stable expression, providing additional evidence that leaky expression of the transposase was sufficient to induce transposition. More importantly, HITT vectors with PGK- and MTH-directed transposase expression provided the best conditions for transposon insertion, resulting in stable β-gal expression in 0.92 + 0.27% and 2.20 + 0.96% of mouse hepatocytes, respectively. Based on the assumption that 40% of mouse hepatocytes are transfected by high-pressure tail vein plasmid injection, these values suggest that optimized SB HITT vectors induce transposition in a minimum of 5–6% of the transfected hepatocytes.

**Orientation of Expression Cassettes Is Not Critical for HITT-Vector Function**

It appears from the relationship between transposase promoter strength and efficient HITT-vector transposition that even minor adjustments of expression may affect the level of persistent gene expression obtained after plasmid administration. To explore further means of fine-tuning *in vivo* transposase production, we created "bidirectional" (bid) HITT vectors in which the transposase expression cassette was oriented in the direction opposite that of the transposon RSV-hAAT cassette. We hypothesized that smaller variations in gene expression due to possible interference between closely located promoters might affect transposase production and result in changes in transposition. We first compared expression profiles of pairs of HITT vectors harboring the PIL-, CMV-, and Alb-directed transposase gene in opposite orientations (Fig. 5A). For the CMV vectors, the effect of overproduction inhibition was not immediately evident for the bidirectional vector, as serum hAAT 1 day after plasmid injection was more than 1 log higher for pT/hAAT.CMV-SB.bid than for pT/hAAT.CMV-SB ($P = 0.002$). However, as indicated by similar levels of long-term hAAT ($P = 0.9$), this difference did not result in increased levels of hAAT gene insertion. Based on these observations, we propose that the initial transposase expression from the two vectors differs, perhaps due to promoter interference, but that subsequent abundant production of transposase from pT/hAAT.CMV-SB.bid leads to a delayed onset of overproduction inhibition and reduced transposition. For vectors with Alb-directed transposase expression, hAAT expression levels did not differ statistically ($P = 0.4$). Interestingly, transposition from vectors in which the transposase gene was flanked only by the promoter insertion linker, PIL, depended on orientation of the gene (Fig. 5A), resulting in final levels of serum hAAT that were statistically different ($P = 0.03$). We believe that transposition from pT/hAAT-.PIL-SB occurred as a result of leaky transposase expression driven by RSV or elements in the vector backbone and that the genetic design of pT/hAAT.PIL-SB.bid did not similarly allow expression of transposase, resulting in a 20-fold reduction in stable hAAT expression. Therefore, at the extremes of transposase expression, it appears that orientation-derived expression differences that are expected to be small can in fact affect the transgene expression profiles. In an effort to explore possible effects on the most efficient HITT vectors, we generated pT/hAAT.PGK-SB.bid and pT/hAAT.MTH-SB.bid and compared them with their unidirectional counterparts. As shown in Fig. 5B, we could not detect significant differences in the hAAT expression profiles between these four vectors ($P = 0.9$ and $P = 0.5$ for comparisons of PGK and MTH vectors, respectively). In summary, our data support the notion that orientation-related variations in gene expression may influence vector function in cases in which transposase expression is leaky or very strong but that such variations are minimal and not sufficient to affect transposase production and transposition from high-efficiency SB HITT vectors.

**HITT Vectors Facilitate Long-Term Expression of Human Factor IX and Partial Correction of Mouse Hemophilia B Phenotype**

To test the therapeutic potential of codelivery of transposon and transposase on single plasmids, we created a panel of HITT vectors containing a transposase-embedded human factor IX (hFIX) cDNA expressed from an EF1α promoter (T/EF1α-hFIX). The hFIX expression profiles of these vectors confirmed that PGK- and MTH-directed transposase expression provided more optimal conditions for transgene transposition and establishment of persistent gene expression than leaky and CMV-driven expression (Fig. 6). Hence, 5 months after plasmid injection, serum hFIX levels were 3.2 ± 2.7 and 12.4 ± 9.6 ng/ml for CMV and PIL vectors (both at levels that were significantly different from the pT/EF1α-hFIX-treated group; $P = 0.03$ and $P = 0.02$, respectively) and 141 ± 20 and 257 ± 173 ng/ml for PGK and MTH vectors, respectively, reaching concentrations that were more than 1000-fold increased compared to the group of mice treated with the control vector ($P = 0.01$ and $P = 0.0001$, respectively). Notably, transposition was also efficiently supported by levels of transposase expressed from the weak albumin promoter (Alb), resulting in a high stable level of serum hFIX (127 ± 72 ng/ml) that did not statistically differ from hFIX levels obtained with PGK ($P = 0.7$) and MTH vectors ($P = 0.2$). To ascertain whether the stable serum hFIX levels obtained with the most powerful HITT vectors were therapeutic in an animal model of hemophilia B, we administered pT/EF1α-hFIX.PGK-SB or its transposase-

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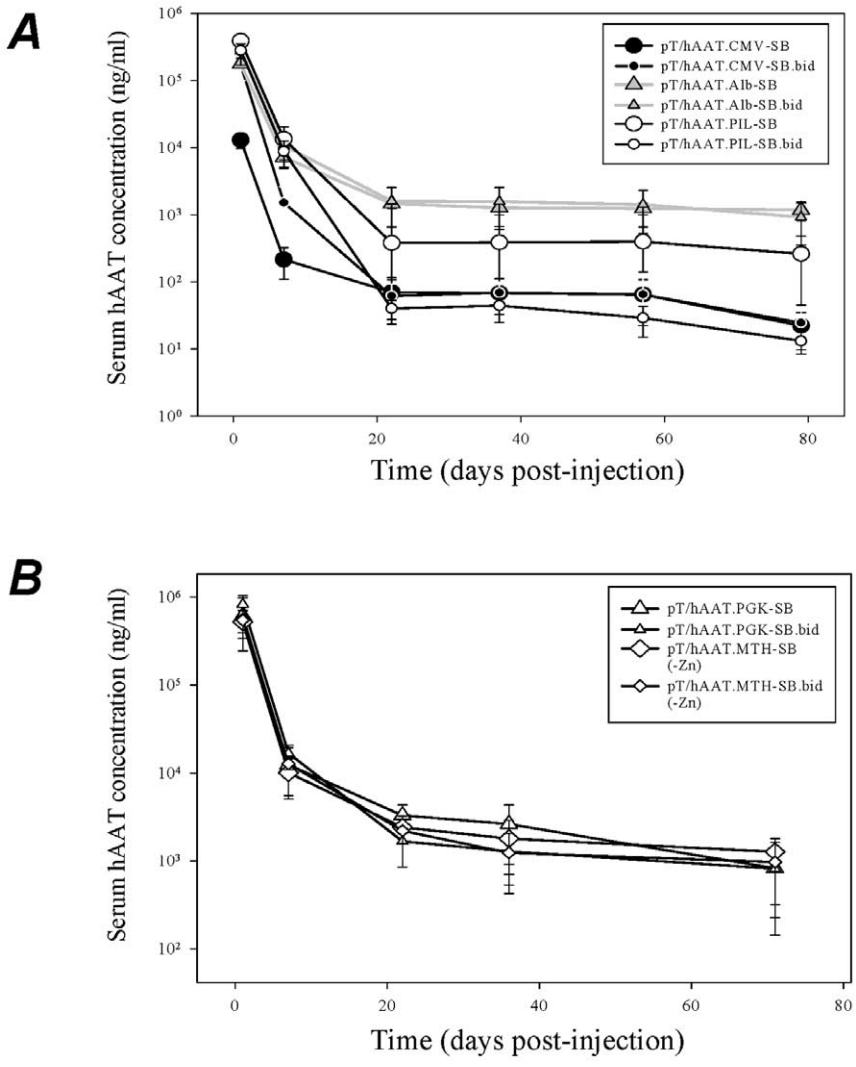


**FIG. 5.** Effects of the orientation of expression cassettes on SB HITT-vector function. HITT vectors carrying the transposase expression cassette in orientation opposite to that of the transposon-embedded hAAT cDNA (constructs labeled with bid for "bidirectional") were compared to constructs with both expression cassettes oriented in the same direction. Plasmids (30 μg) were injected into the tail vein of mice and the serum hAAT concentration was monitored over time. (A) Comparison of HITT vectors with CMV-, Alb-, and PIL-directed transposase expression ($n = 5$). The expression profiles of the "unidirectional" vectors (indicated with large symbols), presented previously in Fig. 2B, are included for direct comparison. (B) Comparison of HITT-vector-based PGK- and MTH-directed transposase production ($n = 4–5$). Profiles of pT/hAAT.PGK-SB and pT/hAAT.MTH-SB, shown earlier in Fig. 4A, are included for easy comparison. Mean values ± standard deviation are shown.

mutated counterpart, pT/EF1α-hFIX.PGK-mSB, to the liver of hemophilic mice and determined serum hFIX concentration after 110 days (Table 1). We could not detect hFIX in the serum of mice receiving the mutated version of the HITT vector, whereas mice injected with pT/EF1α-hFIX.PGK-SB had hFIX levels ranging from 187 to 322 ng/ml. In accordance, the blood clotting capacity varied significantly between the groups as determined by clipping the tails and monitoring the time required for clotting to halt bleeding (bleeding time). Whereas clotting could not be detected in pT/EF1α-hFIX.PGK-mSB-treated mice (bleeding times >30 min), mice injected with pT/EF1α-hFIX.PGK-SB stopped bleeding 18 to 22 min after tail clipping. In a control group of normal mice bleeding times ranged from 10 to 14 min. We conclude that a single treatment with a HITT vector optimized for transposase expression can establish persistent therapeutic levels of hFIX and result in partial correction of the bleeding disorder in hemophilia B mice.

## DISCUSSION

For a broad range of clinical applications, the success of therapeutic gene delivery depends on the persistence of high levels of transgene expression. Among the viral vector systems, stability of expression of vector-encoded genes can be accomplished by transduction of target cells with retroviral vectors that integrate into the genome through actions of the virus-encoded integrase. Recently, hybrid vectors that combine the integration capacity of retroelements [23,24] or DNA transposons [7] with the high infectivity of adenoviruses have been shown to stabilize gene expression. Traditionally, nonviral gene vehicles have suffered from the inability to integrate their

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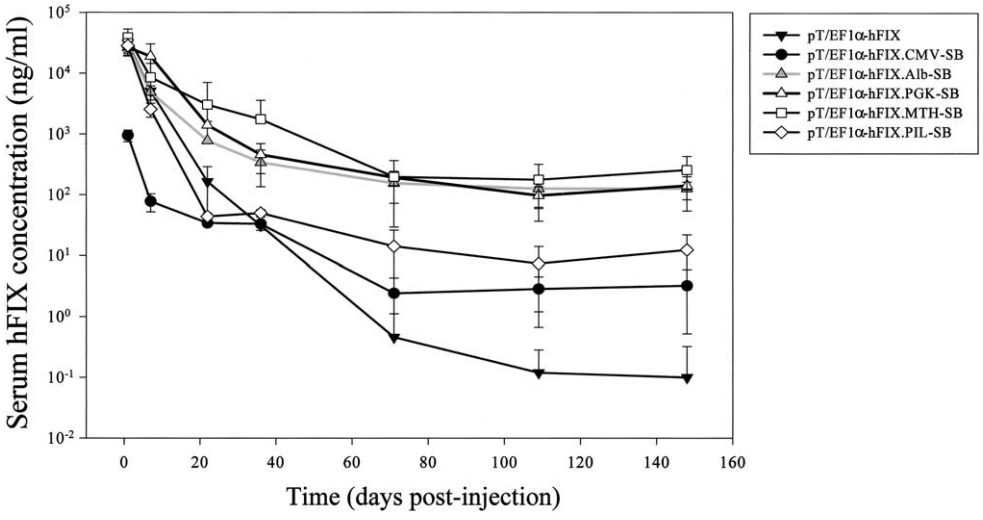


**FIG. 6.** Transposition from SB HITT vectors induces long-term expression of hFIX. Animals received equal copy numbers of the hFIX-tagged transposon: 35 μg pT/EF1α-hFIX + 7 μg pUC19 or 42 μg pT/EF1α-hFIX-derived HITT vector plasmid harboring different transposase expression cassettes as indicated were injected into the mouse tail vein ($n$ = 5 per group of mice). Serum hFIX concentration was monitored over time by serum ELISA. Mean values ± standard deviation are shown.

cargo gene into chromosomal DNA, leading to only transient and short-term expression of the gene of interest. However, with the use of DNA transposons that are active in mammalian cells, new tools are being developed based on efficient transposase-directed gene excision from donor plasmid and insertion into genomic acceptor sites. Here, we report the development and therapeutic use in adult animals of a single-plasmid vector system that carries on the same plasmid *trans*-factors and *cis*-elements facilitating gene insertion and long-term expression. The integration machinery, derived from the *Sleeping Beauty* DNA transposon, consists of (i) a transposase expression cassette and (ii) the transgene flanked by the transposon inverted repeat sequences required *in cis* for transposition. To avoid an inhibitory effect of transposase overproduction, we chose to produce the transposase from a series of promoters within the single-plasmid vector and were able to relate promoter strength with the ability to mediate transposition in the context of the HITT-vector plasmid. Since the tested HITT vectors were roughly the same size, we believe that all vectors were transfected with equal efficiency as suggested by similar levels of serum hAAT monitored shortly after injection of plasmid DNA (Fig. 2B). Hence, we propose that differences in the capacity of HITT vectors to induce long-term gene expression in mouse liver strictly depend on the choice of promoter directing transposase production. We present a vector system that is optimized for use in mouse liver and offers

**TABLE 1:** Partial phenotypic correction in hemophilia B mice treated once with HITT-vector plasmids

| Group | Mouse strain | Vector treatment | Mouse | hFIX (ng/ml) Day 110 | Bleeding times (min) |
|---|---|---|---|---|---|
| 1 | C57Bl/6 | No DNA injection | 1 | ND | 14 |
| | | | 2 | ND | 13 |
| | | | 3 | ND | 10 |
| 2 | Hemophilia C57Bl/6 | 42 μg pT/EF1α-hFIX.PGK-SB "active transposase" | 4 | 322 | 20 |
| | | | 5 | 187 | 18 |
| | | | 6 | 303 | 19 |
| | | | 7 | 221 | 22 |
| 3 | Hemophilia C57Bl/6 | 42 μg pT/EF1α-hFIX.PGK-mSB "inactive transposase" | 8 | UD | >30 |
| | | | 9 | UD | >30 |
| | | | 10 | UD | >30 |
| | | | 11 | UD | >30 |
| | | | 12 | UD | >30 |

ND, not done; UD, undetectable by ELISA.

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a level of transposase expression balanced between insufficient production and overproduction.

The optimal level of transposase expression may vary between cell types and species due to differences in the influence of cellular host cofactors on transposition and variations between quiescent and dividing cells. In support of the latter, inhibitory effects of the transposase are not clearly evident in dividing cells in culture, perhaps due to a "diluting" effect of cell cycling, reducing the cellular level of transposase [11,25], although very high doses of transposase plasmid relative to transposon plasmid cause inhibition of transposition in culture [26]. In addition, differences in activities of transposase-directing promoters in various tissues and cell types will affect the transposon–transposase ratio in transfected cells and according to our data affect the overall level of transposition. Although the molar ratio of transposon and transposase is constant for all cells transfected with one particular HITT vector, the transfection efficiency obviously will have great impact on the total cellular transposase production and an effect on downstream inhibitory or stimulatory effects on transposition. In the case of the mouse liver, our data suggest that smaller differences in the initial uptake of HITT-vector plasmid obtained in different lines of experiments (indicated by different initial hAAT levels; compare day 1 time points for PGK- and MTH-based HITT vectors in Figs. 2B and 4A) may affect transposition and downstream stable levels of transgene expression. We therefore stress that vectors creating optimal conditions for transposition in liver may not necessarily be the perfect choice in other tissues, and estimates of optimal transposase production should be made to design the best possible SB HITT vector according to transfection efficiencies and promoter strength. Based on our findings, we suggest that promoters that are 30- to 40-fold less active than the CMV promoter in the particular tissue are included among promoters screened for ideal transposase production.

As many as 40% of the mouse hepatocytes are transfected *in vivo* by hydrodynamic plasmid injection into the mouse tail vein [7,19]. Such efficient DNA uptake makes the mouse liver an ideal target organ for SB-based gene delivery even for a two-plasmid delivery strategy [7]. Yet, by administering transposase and transposon on a single plasmid we were able to improve stable transgene expression, suggesting that the equal ratios of the vector components provided in all transfected cells may further enhance transposition. In contrast to the mouse liver, other tissues of clinical relevance are not as easily transfected *in vivo,* rendering a two-plasmid SB transfer system inefficient and clinically impotent due to the reduced codelivery of transposon and transposase components. We predict that SB HITT vectors will prove useful for *in vivo* nonviral gene transfer to cell types that are less transfectable *in vivo*. Studies of *in vivo* gene transfer to cell types such as skin progenitor cells, bone marrow stem cells, and myoblasts should help clarify whether HITT-vector transposon-based delivery can bypass complications caused by low *in vivo* transfection efficiency and become a tool with clinical applications in humans.

The reconstructed *Sleeping Beauty* transposon is active in most vertebrate species [11] and transposes in HeLa cells about one order of magnitude more efficiently than the transposons Tc3, *Mos1,* and *Himar1* [5]. However, recent findings have shown that SB transposition can be even further improved by introducing genetic *cis* modifications within the terminal inverted repeats, resulting in elements that are up to fourfold more transposable than the original element [27,28], or by introducing a combination of amino acid changes in the protein [26]. Considering the manner in which *Sleeping Beauty* was reconstructed from defective elements [10], it is likely that a detailed genetic screen of transposase mutants may reveal *trans*-acting mutations that can further improve transposition. In support of this notion, hyperactive *Himar1* transposase mutants that are fivefold more active than the original transposase in HeLa cells have been described [5,29]. For SB-based vector systems, an optimized transposase does not only insert transposon DNA with increased efficiency but also ideally displays reduced overproduction inhibition. Although the molecular background for the inhibitory effects of transposase overexpression remains unclear, reduced transposition in mouse liver occurs through pathways causing loss of transposon donor plasmid (S. R. Yant, J. G. Mikkelsen, L. Meuse, Z. Huang, M. A. Kay, unpublished results), indicating that transposase-induced nonspecific plasmid degradation or cell growth defects may play a role. Further insight into these mechanisms may provide a new platform for isolating hyperactive transposase mutants which do not induce transposition inhibition, ultimately providing better gene-integrating tools for nonviral gene transfer including novel SB-based HITT vectors.

We show that SB HITT vectors facilitate long-term transgene expression in mouse liver and partially restore blood-clotting capacity in hemophilic mice. This single-plasmid technology holds great promise as a therapeutic tool and shares with other nonviral vector systems advantages in terms of safety and feasibility of large-scale manufacturing. However, as targeted and efficient *in vivo* delivery remains a limitation for some clinical applications of nonviral vector systems, incorporation of the transposon integration machinery in adenoviral vectors may be required for sufficient delivery of the SB vector components. We have previously shown that circular DNA carrying a transgene-tagged transposon and a CMV-transposase expression cassette, essentially like the SB HITT vectors, can be released from adenoviral DNA by Flp recombinase-based recombination. This DNA intermediate can serve as a combined source and substrate for DNA transposases [8]. In future investigations of such hybrid adeno-transposon vectors it will be important to elucidate

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**TABLE 2:** Origin and characteristics of promoter sequences

| Construct name | Promoter origin | Type | Length (bp) | GenBank accession No. (positions corresponding to amplified sequence) | Template for PCR amplification |
|---|---|---|---|---|---|
| pBS.RSV-hAAT | Rous sarcoma virus LTR | Viral | 401 | J02025 (157–550) | pBS.RSV.hAAT Ref. [30] |
| pBS.MoU3-hAAT | Moloney murine leukemia virus U3 region | Viral | 373 | AF033811 (7553–8000) | pLAPSN (Clontech) |
| pBS.CMV-hAAT | Human cytomegalovirus | Viral | 538 | X03922 (611–1148) | pCMV-SB Ref. [10] |
| pBS.EF1α-hAAT | Human elongation factor 1α gene | Cellular constitutive | 1112 | J04617 (450–1561) | pAAV.EF1α.hFIX Ref. [32] |
| pBS.Alb-hAAT | Mouse albumin gene | Liver-specific | 796 | J04738 (1258–2053) | pAT2-eG$_{3x}$ Ref. [33] |
| pBS.eG$_{3x}$Alb-hAAT[a] | Mouse albumin gene | Liver-specific | 959 | U04199 (527–555) | pAT2-eG$_{3x}$ Ref. [34] |
| pBS.TTR-hAAT | Mouse transthyretin gene | Liver-specific | 350 | M19524 (1–350) | Mouse genomic DNA[b] |
| pBS.DHFR-hAAT | Mouse dihydrofolate reductase gene | Cellular constitutive | 300 | J00382 (143–442) | Mouse genomic DNA[c] |
| pBS.PGK-hAAT | Mouse phosphoglycerate kinase gene | Cellular constitutive | 510 | M18735 (422–931) | pHR2.PGK.NLS.lacZ Ref. [35] |
| pBS.MTH-hAAT | Mouse metallothionein gene | Inducible | 262 | M11534 (1–262) | pHD.SB.Flp Ref. [8] |

[a]The eG$_{3x}$ insert upstream of the Alb promoter consists of three and a half direct repeats of a 29-bp hepatocyte nuclear factor 3 binding site derived from the albumin gene enhancer (GenBank Accession No. U04199). Compared to the PCR template, pAT2-eG3x, an internal *Kpn*I site located between the eG$_{3x}$ sequence and the Alb promoter, was removed to allow *Kpn*I–*Hind*III cloning of the complete element.
[b]Amplified TTR promoter sequence differs from listed GenBank entry sequence at positions 59, 106, and 107.
[c]Amplified DHFR promoter sequence differs from listed GenBank entry sequence at position 173.

whether the correlation between transposase promoter strength and transposition efficiency, described here for HITT-vector plasmids, applies also to circular DNA released from adenoviral vector platforms.

## Materials and Methods

***Plasmid construction.*** To generate a panel of hAAT-expression cassettes, a total of 10 PCR-amplified promoter sequences were inserted by standard cloning procedures into *Kpn*I–*Hind*III-digested pBS.RSV-hAAT [30] replacing the RSV promoter. Constructs, promoter origins, sources, lengths, and corresponding positions in GenBank entries are listed in Table 2. A construct without promoter, pBS.No-hAAT, was generated by end-polishing of *Kpn*I–*Hind*III-digested pBS.RSV-hAAT prior to religation. To generate *Sleeping Beauty* HITT vectors containing on single plasmids (i) a transposase expression cassette and (ii) a RSV-hAAT transposon, a 1262-bp PCR fragment containing the transposase coding sequence and the downstream SV40 poly(A) sequence was amplified from pCMV-SB [10] and inserted by standard techniques into the *Nde*I site (227 bp upstream of transposon) of pT/hAAT (previously described in [7]), generating pT/hAAT.PIL-SB (PIL-SB same orientation as RSV-hAAT) and pT/hAAT.PIL-SB.bid (opposite orientations of PIL-SB and RSV-hAAT). Introduction of a promoter insertion linker containing *Spe*I, *Not*I, and *Xba*I restriction sites in the upper primer allowed for insertion of CMV, Alb, TTR, DHFR, and MTH promoters into *Spe*I–*Xba*I-digested pT/hAAT.PIL-SB and of EF1α and PGK promoters into *Spe*I–*Not*I- and *Not*I–*Xba*I-digested pT/hAAT.PIL-SB, respectively. To generate bidirectional HITT vectors, we cloned CMV, Alb, PGK, and MTH promoters into pT/hAAT.PIL-SB.bid. pT/hAAT.CMV-SB.ds was generated by inserting the *Sca*I–*Xba*I-fragment from pT/hAAT into *Sca*I–*Ssp*I-digested pCMV-SB, creating a 606-bp plasmid linker sequence between T/hAAT and CMV-SB. To generate control vectors pT/hAAT.PGK-mSB and pT/hAAT-.MTH-mSB expressing a defective transposase, the mutant transposase sequence coding for a protein with a single amino acid substitution (D244A) in the catalytic domain was PCR-amplified from pCMV-mSB [7]. The mSB DNA fragment was connected to PCR fragments carrying the PGK and MTH promoters by overlap extension and PCR and the resulting fragment was cloned into *Nde*I-digested pT/hAAT. To determine HITT-vector integration frequencies in mouse liver, we cloned a *Kpn*I–*Xmn*I-flanked 6-kb β-galactosidase-encoding transposon (derived from pT/RSV-βgeo previously described by Yant *et al*. [7]) into *Kpn*I–*Xmn*I-digested pT/hAAT HITT vectors (CMV-, PGK-, MTH-, and PIL-SB promoter–transposase cassettes), replacing the hAAT transposon in these vectors. HITT vectors carrying a 5.4-kb transposon expressing EF1α promoter-directed hFIX (T/EF1α-hFIX) were created by inserting *Nde*I-digested transposase expression cassettes (PGK, MTH, Alb, and PIL) derived from pT/hAAT HITT vectors into *Nde*I-digested pT/EF1α-hFIX [7]. To generate pT/EF1α-hFIX-.CMV-SB and pT/EF1α-hFIX.PGK-mSB, a *Kpn*I–*Sal*I fragment containing T/EF1α-hFIX was cloned into *Kpn*I–*Sal*I-digested pT/hAAT.CMV-SB and pT/hAAT.PGK-mSB, respectively.

***Animal studies.*** Six-week-old C57Bl/6 and C57Bl/6-scid mice were obtained from The Jackson Laboratory. Animals were treated according to the NIH Guidelines for Animal Care and Guidelines of Stanford University. Plasmid DNA was injected essentially as previously described [19] in 0.85% saline (volumes 1.8 to 2.0 ml) into the tail vein over 5 to 8 s. For surgical partial hepatectomy and periodical retro-orbital blood sampling, mice were anesthetized with isoflurane (Abbott Laboratories, Abbott Park, IL). Partial hepatectomy was carried out as previously described [21]. Bleeding times of plasmid-treated hemophilic C57Bl/6 mice were determined by clipping a section of the tail (diameter about 1.5 mm) and measuring the time required for clotting to occur. Tails of all mice were subsequently cauterized to avoid possible bleeding complications. For *in vivo* induction of gene expression from the MTH promoter, animals were served drinking water supplemented with $ZnSO_4$ (25 mM final concentration).

***Blood work.*** Mouse serum levels of hAAT protein and hFIX were quantified by an ELISA as previously described [30,31]. Briefly, blood samples were

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spun for 5 min and the serum was serially diluted in appropriate buffer prior to incubation in ELISA plates coated with hAAT (Diasorin) or hFIX (Dako) primary antibody. After overnight incubation and subsequent washing, HRP-conjugated secondary hAAT (Research Diagnostics) or hFIX (Affinity Biologicals, Inc.) antibody was added, allowing for quantification using TMB (hAAT) or OPD (hFIX) substrate pellets (both Sigma) dissolved in citrate/phosphate buffer.

***β-Galactosidase expression in mouse liver.*** Mice treated with plasmid carrying cytoplasmic β-gal-encoding transposon (T/RSV-βgeo) were killed for liver isolation at day 81 after plasmid injection. Mouse liver tissues were frozen in OCT buffer on dry ice, sectioned (10 μm), and stained for β-gal expression using 5-bromo-4-chloro-3-indodyl β-D-galactoside. To determine the percentage of hepatocytes harboring an inserted T/RSV-βgeo transposon, a minimum of 2000 hepatocytes from random areas of three liver lobes from each mouse were counted and checked for β-gal-expression.

***Statistical analysis.*** Unpaired *t* tests were used to analyze differences between control and experimental groups and within selected experimental groups. $P < 0.05$ was considered statistically significant.

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