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Hepatocyte-targeted delivery of *Sleeping Beauty* mediates efficient gene transfer *in vivo*

Research Article

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Abbreviations: *Sleeping Beauty* (*SB*); green fluorescent protein (GFP); partial hepatectomy (PH); asialoglycoprotein receptor (ASGR); inverted repeats/direct repeats (IR/DRs); chicken β-actin/rabbit globin intron (CAGGS); elongation factor (EF)-1α; human embryonic kidney (HEK293)

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Summary

Currently, most gene therapy studies utilize viral vectors that can potentially produce immunological and toxic side effects. To circumvent these limitations, we evaluated the efficiency of nonviral hepatocyte-targeted *in vivo* delivery of plasmids that mediate stable genomic integration of transgenes via the *Sleeping Beauty* (*SB*) transposon system. We constructed plasmids that express a reporter green fluorescent protein (GFP) transposon and the *SB* transposase, required for transgene insertion into genomic DNA, from either a single plasmid (*cis*) or two different plasmids (*trans*). The constructs were compacted to an average diameter of < 50 nm with lactosylated polyethyleneimine, a polycation, for targeting to the hepatocyte asialoglycoprotein receptor. Intravenous administration of the *cis* plasmid resulted in greater efficiency of transgene integration in mouse liver compared to transposase expression from a separate plasmid. Furthermore, by western blot analysis and fluorescence microscopy, delivery of the *cis* plasmid to rat livers resulted in transgene expression that persisted for months even after regeneration from partial hepatectomy. Southern blot analysis of the regenerated livers indicated that SB mediated genomic integration of the GFP transgene at random sites, and this correlated with disappearance of *SB* transposase. In conclusion, receptor-mediated targeted delivery of a transposon system capable of transgene integration and stable expression provides an attractive alternative to viral vectors for gene therapy to the liver.

I. Introduction

Recombinant viral vectors are the current mainstay of gene therapy for inherited metabolic disorders (Kay et al, 2001). However, clinical trials have achieved only modest success, in part, because of the limitations set by viral vectors. For example, adenovirus-based vectors do not integrate into host chromosomes (Harui et al, 1999) and their immunogenicity precludes repeated gene transfer. Furthermore, in contrast to the highly efficient gene transfer to livers of laboratory animals, clinical trials with adenovirus have produced low levels of transgene expression in human liver (Raper et al, 2002). Recombinant adeno-associated viral vectors also do not integrate efficiently in liver (Hillgenberg et al, 2001), resulting in progressive loss of the episomal DNA (Nakai et al, 2001; Ehrhardt and Kay, 2002). Moreover, the low level integration appears to occur preferentially into active genes and is associated with chromosomal deletions at the site (Nakai et al, 2003). Although oncoretroviral vectors integrate into the host genome, the process is very inefficient in non-replicating cells such as hepatocytes *in vivo* (Kalpana, 1999). Lentiviruses, which appear to partially overcome this (Pfeifer et al, 2001; Follenzi et al, 2002), are difficult to generate in quantities adequate for

human therapy. Moreover, despite removal of the viral genes, potential safety concerns persist. Thus, development of efficient non-viral methods for long-term gene transfer would be important for gene therapy.

Plasmid-based non-viral gene transfer has been attempted by direct injection into liver, with limited levels of transgene expression. A "hydrodynamic" method that relies on rapidly injecting plasmids in large volumes intravenously has been used to transfer nucleic acids to the livers of rodents (Zhang et al, 1999; Maruyama et al, 2002). An elegant alternative employs targeted delivery of nucleic acids to hepatocytes via the asialoglycoprotein receptor (ASGR) (Wu and Wu, 1988). Unfortunately, the delivery of naked plasmids to hepatocytes results in little or no integration of the transferred DNA into the host genome (Zhang et al, 1999; Maruyama et al, 2002). A potential solution to this problem arises from the discovery that the *Sleeping Beauty* (*SB*) transposon system developed from fish can mediate the transposition of DNA into chromosomes for a broad range of vertebrates, including humans (Ivics et al, 1997; Izsvák et al, 2000).

The *SB* transposon system functions by a cut-and-paste mechanism catalyzed by binding of the *SB* transposase to the inverted repeats/direct repeats (IR/DRs) of the transposons. It excises the transposon at the outside ends of the IR/DRs and inserts the element into a new TA dinucleotide site. The hydrodynamic delivery of two separate plasmids in mice, one expressing *SB* transposase and another comprising a transgene flanked by the IR/DRs, resulted in long-term gene expression in the liver even after partial hepatectomy (PH) (Yant et al, 2000, 2002; Montini et al, 2002). This gene transfer method reproducibly transduced up to 5% of hepatocytes. However, although useful for delivery of naked DNA in mice (Nakai et al, 2001; Yant et al, 2000, 2002; Montini et al, 2002), and rats (Maruyama et al, 2002), the rapid hydrodynamic delivery of large volumes may pose considerable restrictions for clinical use.

In this study, we determined the efficiency of transposition in liver using a single plasmid, containing both a transposon with a transgene and *SB* transposase, targeted for delivery to hepatocytes via the ASGR. Our results indicated that the SB complex efficiently delivered green fluorescent protein (GFP) genes *in vivo* to hepatocytes of mice and rats. Long-term gene expression occurred only in animals that received both the transposon and transposase. In addition, transposition was increased when the GFP transgene and *SB* were delivered in *cis*, rather than in *trans* as separate plasmids.

II. Materials and methods

A. Construction of transposon vectors

Two different GFP reporter transposons were constructed using either the elongation factor (EF)-1α promoter (Johnson and Krieg, 1994) (pT/GFP), or the hybrid CMV enhancer chicken β-actin/rabbit globin intron (CAGGS) promoter (Okabe et al, 1997) (pT2/GFP). pT/GFP was flanked by the original IR/DRs (Ivics et al, 1997) while pT2/GFP, constructed by cloning the *Eco*R V-*Sma* I coding sequence of pT/GFP into the *Eco*R I site of the CAGGS vector, was flanked by alternate IR/DRs (Cui et al, 2002). For the *cis SB* constructs, the 2 kb *SB*10 transgene was removed from pCMV*SB*10 using *Eco*R I and *Xba* I (Ivics et al, 1997) and inserted outside the IR/DRs at either the unique *Nar* I (pT/GFP//*SB*10) or *Xho* I site (pT2/GFP//*SB*10). The pT2/CAGGS//DsRed2 (pT2/DsRed2//*SB*10) construct contains the DsRed2 fluorescent protein gene (BD Biosciences Clonetech, Palo Alto, CA) driven by the CAGGS promoter and the same 2 kb CMV*SB*10 transgene inserted in the unique *Bsa*A I site. All plasmids were prepared using Qiagen™ (Valencia, CA) endotoxin free plasmid isolation kits according to standard protocols.

B. Cell culture, transfection and cloning of transduced cells

To validate transposase expression, primary rat hepatocytes or HuH-7 cells (Bandyopadhyay et al, 1998) at ~ 40% confluent were transfected with 1 μg of the *cis* vector constructs as well as the initial pCMV*SB*10 plasmid using the same L-PEI amine (N):DNA phosphate (P) ratios as *in vivo*. Cells were harvested by scraping hepatocytes 48 h or HuH-7 cells 2 to 10 days after transfection. HEK293 cells seeded on a 10 cm^2 plate were transfected at ~ 60% confluence with 2 μg of the *cis* pT2/DsRed2 construct using Lipofectamine™(Invitrogen), After 72 h, the cells were transferred to a 75 cm^2 plate and grown to confluence. Subsequently, the cells were split 1:3 and passaged 4 times. Finally, ~ 100 cells from the fourth passage were plated on a 75 cm^2 plate. The positive clones were picked after a week using 8 mm cloning cylinders (Bellco Glass, Inc., Vineland, NJ) and cultured in DMEM with 10% fetal calf serum.

C. Electron microscopy

The size of the *cis* transposon:L-PEI complexes was determined by electron microscopy. The complexes in 5% dextrose were applied onto glow-discharged formvar-carbon coated 300 mesh grids (Polysciences Inc., Warrington, PA) for ~ 2 min. PEI complexes were negatively stained with aqueous 1% uranyl acetate and were visualized using a JEOL100-CX electron microscope.

D. *In vivo* administration

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Minnesota and Albert Einstein College of Medicine according to the *NIH Guidelines for Animal Care*. The plasmids were complexed using primary amine lactosylated 25 kDa branched PEI (L-25) (Aldrich, Milwaukee, WI) (Kren et al, 2002) and 10 kDa branched PEI (L-10) (Polysciences, Inc.) at a ratio of 1.5:1 (L-25:10) in 5% dextrose. The amine (N) to DNA phosphate (P) ratio was 6:1 (Bandyopadhyay et al, 1998). C57B16 *gus*$^{-/-}$mice (10 g) received a single tail vein injection of 400 μl containing 2.5 or 5 μg of pCMV*SB*10 and/or pT/GFP, or *cis* pT/GFP//*SB*10. Animals were sacrificed at 1, 2 and 8 weeks post-injection and liver tissue removed for analysis. For rats, the complexes were prepared identically except the concentration was increased to 100 μg/ml of transposons. The ~ 200 g Wistar rats received 500 μg/kg bw as a single bolus injection into the tail vein. Liver tissue was sampled at 1, 2 or 4 days by biopsy. PHs of 70% (Higgins and Anderson, 1931) were performed at 1, 2 or 3 weeks after injection. The animals were sacrificed at least 2 weeks post-PH and liver tissue removed for analysis.

E. Protein detection

Tissue for microscopic analysis was fixed in 4% paraformaldhyde in PBS, pH 7.4 at 4°C for 1 h prior to OCT.

Frozen sections of 6 μm were viewed using a Nikon, Diaphot (Melville, NY) fluorescent microscope or post-fixed for 10 min prior to examination with a BioRad MRC1000 Confocal Microscope (Hercules, CA). For western blot analysis, 100 to 150 μg protein/lane of a 10% (w/vol) homogenate of either tissue or cells in 0.25 M Tris acetate, pH 7.8, 0.25 M sucrose, 0.2 mM EDTA and complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) was separated by SDS 10% PAGE, and electrophoretically transferred to nitrocellulose membranes. GFP and SB were detected by ECL (Pierce Super Signal, Rockford, IL) with monoclonal anti-GFP (SC-9996; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-SB (Geurts et al, 2003), respectively (Kren et al, 1999).

F. Southern blot and PCR analysis

DNA for Southern blots and PCR was isolated from frozen tissue using DNAzol, High Pure PCR template kit (Roche Molecular Biochemicals) or a DNAeasy Tissue kit (Qiagen) according to the manufacturers' protocol. For plasmid copy number, samples were spiked with known amounts of pT/GFP prior to extraction of the DNA. For Southern blots, the samples were digested with *Afl* II or *Bsa* I and incubated with a ^{32}P-labeled 757 bp *Eco*R V *Sma* I GFP probe isolated from pT/GFP. To detect genomic transposition of GFP, DNA was isolated 4 weeks post-PH and digested with *Eco*R V or *Eco*R V and *Sma* I, prior to incubation with the ^{32}P-labeled probe. For PCR, primers F5 GGTGATGTTAATGGGCACA and B3 GGGATCTTTCGAAAGGGCA were used to amplify a 535 bp region of the GFP gene using 95°C 5 min (54°C 20 sec, 72°C 45 sec, 95°C 45 sec) x 40 cycles with Expand Hi-Fidelity polymerase (Roche Molecular Biochemicals). Under the same conditions, primers SBF GGACCACGCAGCCGTCATAC and SBR CCTGTTTCCTCCAGCATCTTCAC amplified a 136 bp region of the *SB* gene; and primers ApoBF CGTGGCTCCAGCATTCTA and ApoBR TCACCAGTCATTTCTGCCTTG were used to amplify a 72 bp region of the *apoB* gene. The PCR products were analyzed using 1% agarose gels and visualized using ethidium bromide staining and UV light. Quantitation was performed using NIH image 1.62; and statistical significance determined by two tailed unequal variance T-tests.

III. Results

A. Co-expression of the SB10 Transgene in cis

We constructed *cis* plasmids carrying both the transposon and *SB* transposase (**Figure 1**). Primary rat hepatocytes were transfected with the original pCMV*SB*10 plasmid (Ivics et al, 1997) (*SB* expression only), pT/GFP (no *SB*), or the *cis* plasmid, pT/GFP//*SB*10. Both *SB* constructs resulted in similar levels of transposase protein expression after 2 days (**Figure 2A**). We then transfected plasmids into HuH-7 cells to determine the duration of *SB* expression for pCMV*SB*10 and pT/GFP//*SB*10. There was no difference in the initial expression of *SB* after transfection of the two plasmids (**Figure 2B**), with peak levels being reached in 2 days. Interestingly, by day 5 a decrease in *SB* expression was observed in cells that received the *cis* construct relative to pCMV*SB*10 alone (**Figure 2B,** lanes 4, 5). At 10 days, *SB* expression was undetectable in both cases. By PCR analysis, the abundance of *SB* coding sequence in the *cis* pT/GFP//*SB*10 transfected cells showed an accelerated loss compared to pCMV*SB*10 (data not shown).

B. Transfection and integration of pT2/DsRed2//SB10 in HEK293 cells

To evaluate a second reporter construct in a different cell line, human embryonic kidney HEK293 cells were transfected with the *cis* transposon pT2/DsRed2//*SB10* to express both *SB* and the DsRed2 protein. After 48 h, ~ 30% of the cells expressed DsRed2 (**Figure 2C, A-C**). Following 4 passages, 6 DsRed2-positive clones were derived from single cells by dilutional cloning. The clones remained homogeneously positive for DsRed2, indicative of host genome integration (**Figure 2C, D-F**). For comparison, we transfected the HEK293 cells with a plasmid containing the DsRed2 transposon but without the *SB* transgene (pT2/DsRed2). This also resulted in the expression of DsRed2 in ~ 30% of the cells at 48 h. However, the transgene expression disappeared after 1 or 2 passages (data not shown).

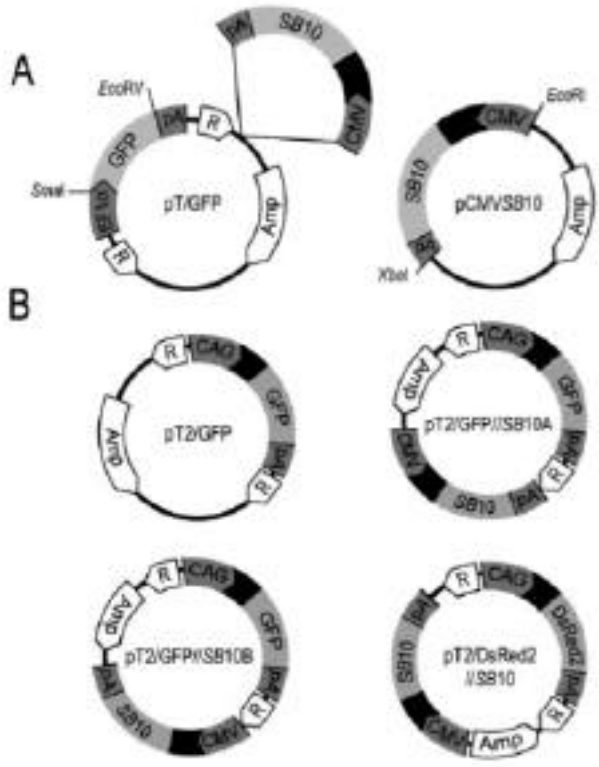

Figure 1 The *cis* and *trans Sleeping Beauty* vector systems. (**A**) To construct pT/GFP//*SB*10, CMV*SB*10 transposase (right) was inserted at a *Nar* I site outside the transposon EF-1α driven pT/GFP (left). (**B**) The CAGGS driven GFP (pT2/GFP) reporter transposon and *cis* counterpart pT2/GFP//*SB*10, and pT2/DsRed2//*SB*10 are shown. The location and orientation of the CMV driven transposase expression cassette are indicated relative to the reporter transgene as well as the direction of transcription (arrows). Amp, *bla* gene for plasmid selection; black regions, introns; EF-1α, elongation factor-1α enhancer/promoter; CMV, cytomegalovirus immediate-early gene promoter; p(A), polyadenylation signal; CAG, hybrid CMV enhancer, chicken β-actin/rabbit globin intron (CAGGS) promoter; R, IR/DRs.

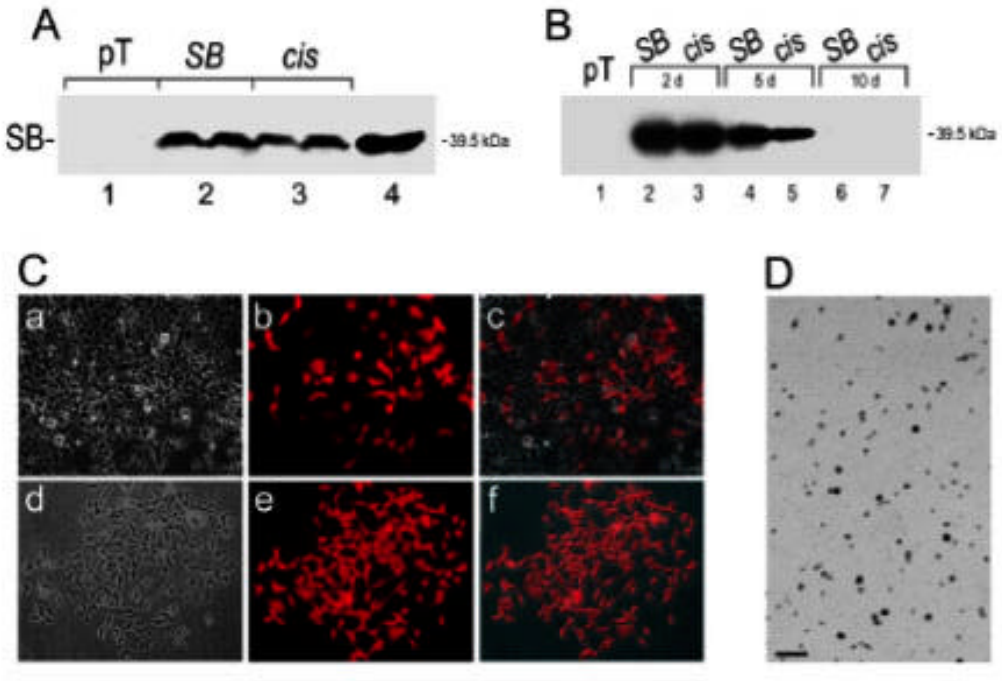

Figure 2 Characterization of the *cis SB*10 transposons. (**A**) Western blot analysis of *SB*10 transposase expression in primary rat hepatocytes. Hepatocytes were transiently transfected with pT/GFP (lane 1), pCMV*SB*10 (lanes 2) or pT/GFP//*SB*10 (lanes 3) for 48 h. Total protein from duplicate transfections was analyzed by immunoblot using anti-SB10 rabbit polyclonal antibodies. Transposase expression was detectable only in cells that received plasmids encoding *SB*10 (lanes 2,3). The transposase produced from transient expression co-migrated with purified SB10 protein (lane 4) at its predicted molecular size of 39.5 kDa (Geurts et al., 2003). (**B**) Western blot analysis of *SB*10 transposase expression in HuH-7 cells. HuH-7 cells were transiently transfected with pT/GFP (lane 1) pCMV*SB*10 (lanes 2,4,6) or pT/GFP//*SB*10 (lanes 3,5,7). Total protein from the cultures harvested at the indicated times was analyzed by immunoblot using anti-SB10 rabbit polyclonal antibodies. (**C**) Transposition of DsRed2 genes into HEK293 cells. Cells transiently transfected with pT2/DsRed2//*SB*10 were examined by (a) phase contrast and (b) fluorescence microscopy. (c) The overlay of (a) and (b) indicate that ~ 30% of the cells expressed DsRed2 fluorescent protein. Clonal isolation of DsRed2 positive cells following limiting dilution and expansion visualized by (d) phase contrast, (e) fluorescence and (f) both. Original magnification x 20. (**D**) Transmission electron microscopy of pT/GFP//*SB*10:L-PEI (lactosylated polyethyleneimine) complexes. A representative micrograph of negatively stained L-PEI:pT/GFP//*SB*10 complexes formed at a 1:6 (N:P) ratio in 5% dextrose showing their small size and monodisperse nature. Bar, 100 nm. N, PEI amine; P, DNA phosphate.

C. Size determination of the plasmid-vector complexes.

We determined the size of the transposon constructs complexed with branched L-PEI to insure that they were able to pass through the ~ 100 nm fenestrae into the Space of Disse (Hara et al, 1997). In 5% dextrose, the pT/GFP//*SB*10 construct formed monodisperse particles with an average diameter of ~ 50 nm at a 6:1 PEI amine to DNA phosphate ratio (**Figure 2D**).

D. Injection of GFP reporter transposons into mice and rats

Mice received a single tail vein injection of either pCMV*SB*10, pT/GFP, equal amounts of pCMV*SB*10 and pT/GFP (*trans*), or *cis* pT/GFP//*SB*10. Fluorescence microscopy of liver sections showed GFP expression in all the animals that received the GFP transposon after 1 week (**Figure 3, A-D**). In contrast, only those mice that also received the transposase in either *cis* or *trans* expressed GFP at 8 weeks (**Figure 3, E-H**).

Adult rats also received either pT/GFP or pT/GFP//*SB*10 complexed with L-PEI by a single tail vein injection. We then performed 70% PH 2 weeks post-injection to induce hepatocyte replication. Fluorescence microscopy of the removed tissue showed that 10-35% of the hepatocytes expressed GFP in rats that received either pT/GFP or *cis* pT/GFP//*SB*10 (**Figure 3, I, J, M, N**). After 3 weeks, the fully regenerated livers were harvested and analyzed by fluorescence microscopy. Rats that received the *cis* pT/GFP//*SB*10 showed GFP expression in single or in small clusters of hepatocytes at the same frequency as was initially observed (**Figure 3, O, P**). In contrast, < 1% of the hepatocytes remained positive after liver regeneration in rats that received pT/GFP (**Figure 3, K, L**).

GFP protein was detected by western blot analysis in mouse liver homogenates 1 and 2 weeks after injection of pT/GFP or *cis* pT/GFP//*SB*10 (**Figure 4A**). However, only mice that received *SB* in *trans* or *cis* continued to express GFP after 8 weeks. By 2 weeks post-injection, GFP expression was 28% and 8% greater in animals that had received *cis* and *trans* constructs, respectively, relative to pT/GFP alone. At 8 weeks, GFP expression in the *trans* group was ~ 2-fold less than that observed in the *cis* animals ($p < 0.05$).

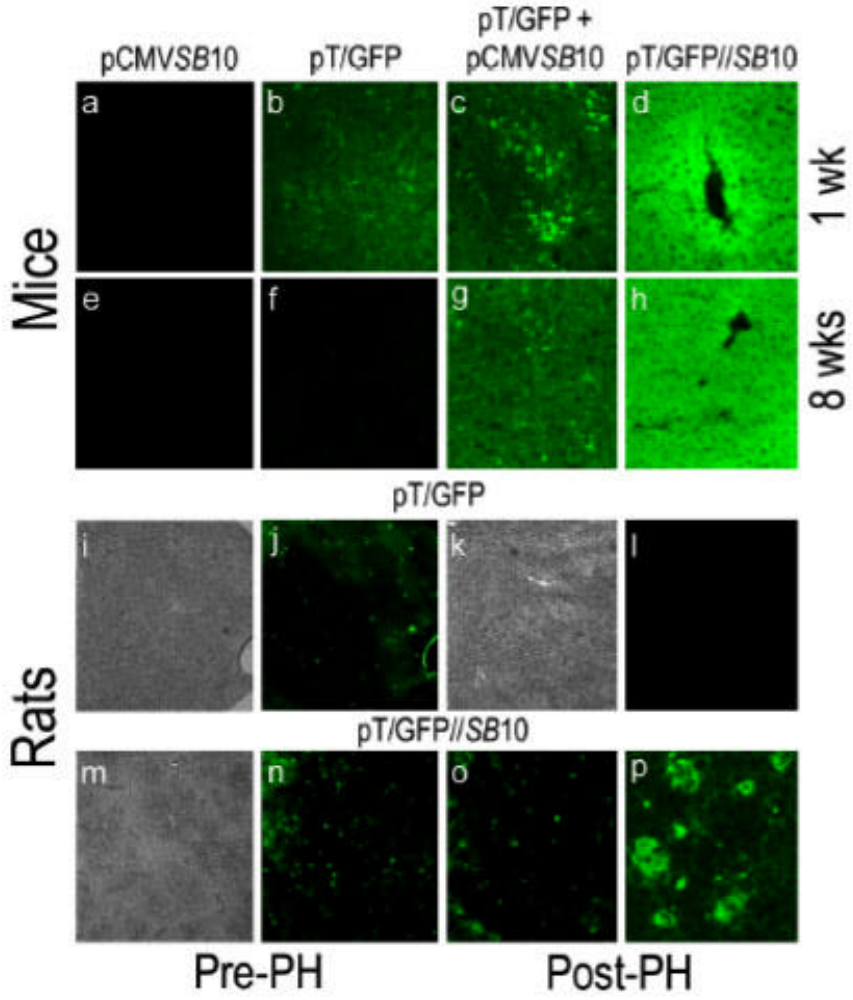

Figure 3 Expression of GFP fluorescent protein in rodent liver. Representative sections of liver tissue isolated 1 (a-d) or 8 (e-h) weeks after tail vein injection of 5 μg of (a,e) pCMV*SB*10, (b,f) pT/GFP, (c,g) 5 μg each of pT/GFP and pCMV*SB*10, or (d,h) 5 μg of pT/GFP//*SB*10 complexed with L-PEI. Original magnification x 20. Representative sections of resected rat liver 2 weeks post-injection (i,j,m,n) and regenerated liver 3 weeks post-PH (k,l,o,p) from animals injected with pT/GFP (i-l) or pT/GFP//*SB*10 (m-p) in complex with L-PEI; (i,k,m) phase contrast. Original magnification i-o x 4; p x 40.

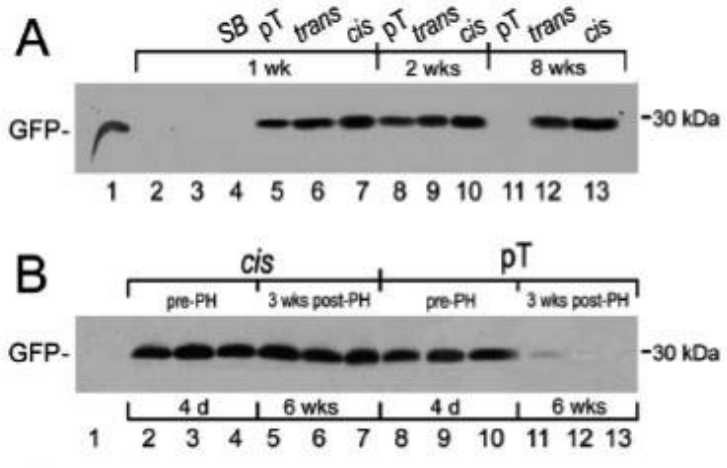

Figure 4 Immunoblot analysis of GFP protein expression. (A) Total protein was isolated from mouse livers 1 week (2-7), 2 weeks (8-10) and 8 weeks (11-13) after tail vein injection with 5% dextrose (lane 2), L-PEI only (lane 3), 5_g of L-PEI complexed pCMV*SB*10 (lane 4), pT/GFP (lanes 5,8,11), 5 μg each of pT/GFP and pCMV*SB*10 (lanes 6,9,12) or 5 μg of pT/GFP//*SB*10 (lanes 7,10,12). Immunopositive 30 kDa GFP (lane 1) was identified using a monoclonal anti-GFP antibody. (B) Immunoblot analysis of using anti-GFP monoclonal antibody of total protein isolated from rat liver 4 days after injection of 100 μg of L-PEI:pT/GFP//*SB*10 (lanes 2-4) or L-PEI:pTGFP (lanes 8-10). Six weeks after injection and 3 weeks post-PH, GFP expression in the regenerated livers of the same animals showed substantial GFP expression only in rats that had received pT/GFP//*SB*10 (lanes 5-7) rather than the transposon only (lanes 11-13). A non-reactive protein and alternate plasmid vector was used as control (lane 1).

In rats, the GFP levels observed 4 days after injection with GFP transposon alone or the *cis* construct were similar (**Figure 4B**). Only the animals that received *cis* pT/GFP//*SB*10 expressed high levels of GFP after PH, and those were unchanged from the original livers (lanes 2-7).

E. Integration into the host genome

We extracted total DNA from mouse livers harvested 1 or 8 weeks after injection. Ampicillin-resistant colonies from transformed electrocompetent *E. coli* were recovered with DNA isolated from the 1 week samples, but not after 8 weeks (data not shown). Southern blot analysis showed that non-integrated plasmids were present at less than a single copy per cell after 1 week (**Figure 5A**), and no free plasmid was detectable at 8 weeks. In mice treated with pT/GFP alone, PCR amplification of the GFP coding region showed the persistence of a small but detectable amount of the transgene at 8 weeks (**Figure 5B,** lane 2), suggesting that spontaneous integration of plasmids occurred at a very low level, as previously reported (Montini et al, 2002; Yant et al, 2000; 2002). Livers from control mice that received pCMV*SB*10 alone showed no GFP amplicons (lane 7). In contrast, mice that received the *SB*-transgene either in *cis* or *trans* showed persistent GFP coding sequences by PCR. Samples with *cis* constructs generated ~ 45% ($p < 0.05$) more amplicons (lanes 4,6) than those with pT/GFP plus pCMV*SB*10 in *trans* (lanes 3,5), and correlated with GFP expression by confocal microscopy and western blot analysis. Semiquantitative PCR using *apoB* as a genomic control indicated that gene transfer efficiency of the *cis* construct at 8 weeks was ~ 1 copy per genome (**Figure 5C,** lane 5). Delivery in *trans* resulted in significantly lower ($p < 0.05$) GFP copy number (lane 4).

We also examined the persistence of the *SB* coding region by semiquantitative PCR in mice that received the transposase either alone (**Figure 5D**, lanes 1,4), in *cis* (lanes 3,6) or in *trans* (lanes 2,5). No difference in *SB* amplicon levels between groups was observed 1 week post-injection (lanes 1-3). However, by 2 weeks a greater decrease in SB coding sequences was seen in animals that had received the *cis* construct (lane 6), compared with those that had received pCMV*SB*10 alone or in *trans* (lanes 4,5).

We then examined the loss of the plasmid and persistence of the GFP transgene in regenerating rat liver post-PH by PCR amplification. There was considerable loss of the plasmid vectors in the first week (**Figure 6A**, lanes 2-4) but the GFP coding sequence persisted in the genomic DNA of animals that received *cis* pT2/GFP//*SB*10 (lanes 5,7-9). Those rats that were given pT2/GFP alone retained no detectable GFP DNA by 3 weeks post-PH (lane 6). The data also suggested that the position and orientation of the *SB*10 expression cassette might influence the efficiency of transposition. Lower transgene levels were observed in animals that received the *cis* constructs in which transcription of the *SB*10 and GFP genes were in opposite directions (lane 5).

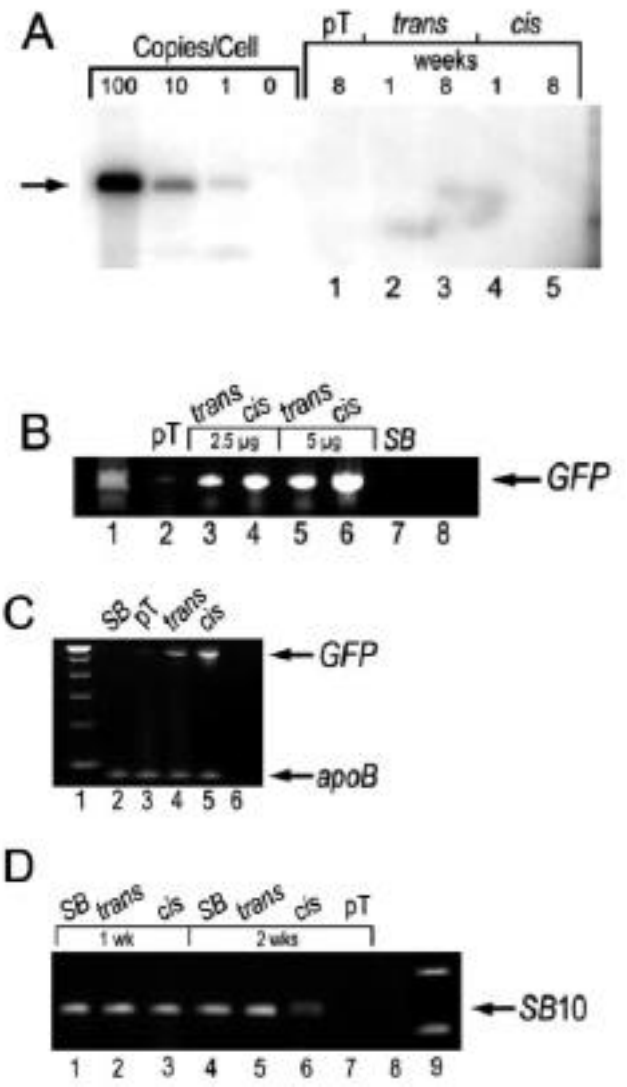

Figure 5 Analysis of the GFP coding sequence in mouse genomic DNA. (**A**) Southern blot detection of plasmid persistence in total DNA isolated from mouse livers 1 or 8 weeks after injection with L-PEI complexed with pT/GFP (lane 1); pT/GFP and pCMV*SB*10 (lanes 2,3), or pT/GFP//*SB*10 (lanes 4,5). The predicted plasmid size is indicated at left (arrow). (**B**) PCR amplification was used to detect GFP sequence in mouse DNA isolated 8 weeks post-injection of 5 µg of pT/GFP (lane 2), 2.5 µg (lane 3) or 5 _g (lane 5) each of pT/GFP and pCMV*SB*10, or 2.5 µg (lane 4) or 5 µg (lane 6) of pT/GFP//*SB*10; *SB*10 control liver (lane 7), and no DNA (lane 8). A 500 bp DNA standard (lane 1) and the predicted 535 bp amplification product are indicated (arrow). (**C**) Semiquantiative PCR was used to determine the efficiency of GFP transfer 8 weeks post-injection of either 5 µg pCMV*SB*10 (lane 2), pT/GFP (lane 3), 5 µg each of pT/GFP and pCMV*SB*10 (lane 4), or 5 µg of pT/GFP//*SB*10 (lane 5); and no DNA (lane 6). The 535 bp *GFP* and 72 bp *apoB* amplicons are indicated at right (arrows). DNA 100 to 600 bp ladder (lane 1). (**D**) PCR analysis was used to detect *SB*10 gene in mouse DNA isolated 1 week (lanes 1-3) or 2 weeks (lanes 4-7) post-injection of 5 µg of pCMV*SB*10 (lanes 1,4), 5 µg each of pT/GFP and pCMV*SB*10 (lanes 2,5), 5 µg of pT/GFP//*SB*10 (lanes 3,6) or pT/GFP (lane 7); and no DNA (lane 8). The 136 bp amplification product is indicated at right (arrow). DNA standards of 100 and 200 bp (lane 9).

We also investigated the persistence of pT/GFP and *cis* pT/GFP//*SB10* in rats. By Southern blot analysis, there was a significant loss of plasmids by 1 week (**Figure 6B**). Interestingly, the loss of plasmid appeared to be more rapid in the animals that received *cis* constructs suggesting that excision of the transposon might accelerate vector degradation. We did additional Southern blot analysis using a GFP probe to compare the transgene abundance in rats that received either pT/GFP or pT/GFP//*SB*10. At 4 days post-injection, GFP was undetectable in the high molecular weight region in DNA samples from rats that received pT/GFP (**Figure 6C**, lanes 1-3). In contrast, pT/GFP//*SB*10 delivery showed high molecular weight reactivity, consistent with integration of the transgene (lanes 4-6). As expected, DNA from regenerated livers of pT/GFP rats at 6 weeks did not contain detectable GFP sequence (lanes 7-9). In rats that received the *cis* construct, GFP transgene levels remained essentially unchanged from the original samples (lanes 10-12). Southern analysis of DNA extracted from regenerated livers of rats that had received *cis* pT/GFP//*SB*10 using a GFP probe showed that the transgene was detectable only in the high molecular weight DNA band (**Figure 6D**, lane 3). When digested with *Eco*R V, hybridization with the GFP probe generated a smear consistent with a large number of different integration sites (lane 4). This finding also excluded the presence of concatemers of episomal linearized plasmid DNA (Chen et al, 2001) or the integration of plasmid concatemers, both of which would have generated more distinct bands. The GFP sequence was released from the integrated transposon after digestion with both *Eco*R V and *Sma* I (lane 5).

To distinguish between spontaneous integration and SB-mediated transposition of the GFP sequences, we extracted total DNA from post-PH regenerated livers of rats that had received *cis* pT/GFP//*SB*10. PCR was performed using two sets of equal size amplimers of (a) both sense and antisense primers corresponding to the coding region of GFP; and (b) a sense primer corresponding to the plasmid sequence immediately 5' to the 5' DR and an antisense primer corresponding to the GFP coding region.

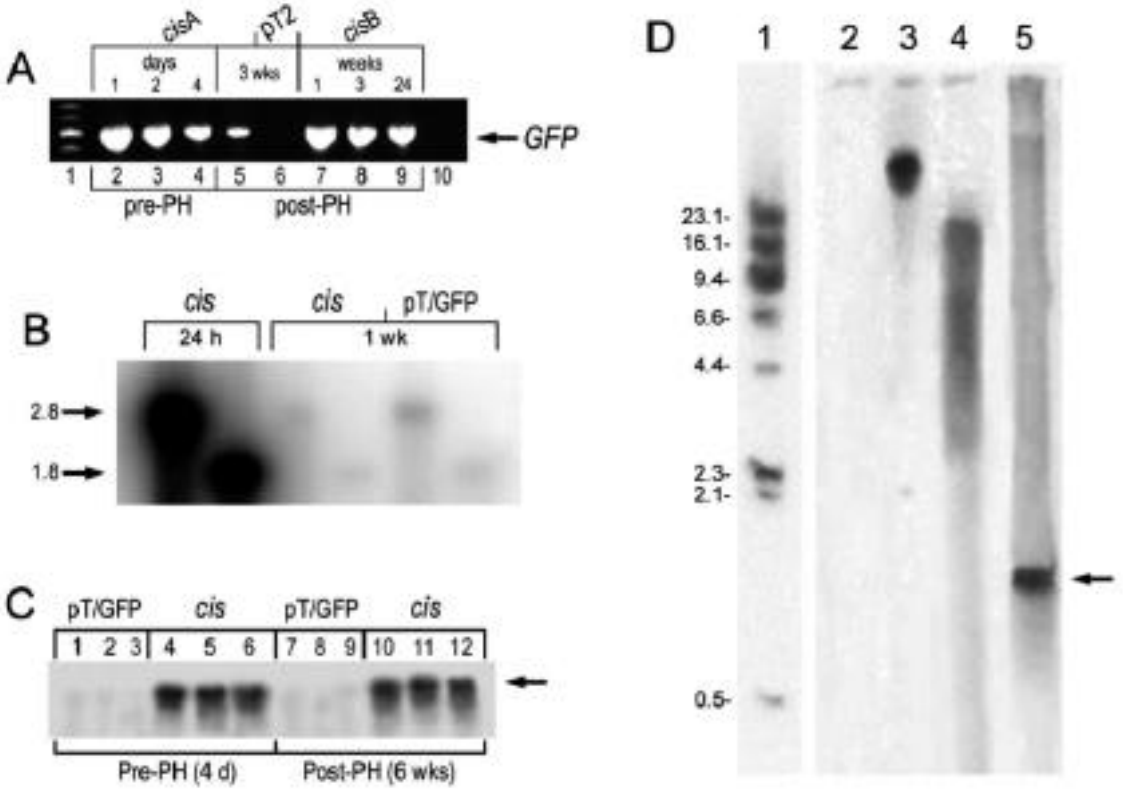

Figure 6 GFP coding sequence analysis in rat genomic DNA. (**A**) PCR amplification of GFP transgene in rat liver pre- and post-PH. Animals received either pT2/GFP (lane 6), pT2/GFP//*SB*10A (lanes 2-5), or pT2/GFP//*SB*10B (lanes 7-9) and liver tissue was removed for DNA isolation and PCR amplification of GFP. Standard DNA ladder (lane 1); DNA from livers 24, 48 and 96 h, respectively, post-injection of pT2/GFP//*SB*10A (lanes 2-4); DNA from regenerated liver 3 weeks post-PH (lanes 5,6,8); DNA isolated 1 week (lane 7) or 6 months (lane 9) post-PH, after injection of pT2/GFP//*SB*10B; control rat liver DNA (lane 10). The 535 bp GFP amplicon is indicated at right (arrow). (**B**) Southern blot analysis of plasmid disappearance in rat liver. At 24 h and 1 week after tail vein injection the transposon plasmid:L-PEI complexes, liver tissue was removed and total DNA isolated. The predicted size of the plasmid bands after *Afl* II (2.8 kb) or *Bsa* I (1.8 kb) digestion is indicted at left (arrows). (**C**) Southern blot analysis of the integrated GFP transgene (arrow) from genomic DNA of rat livers after PH. Liver lobes were resected 4 days after injection from 6 different rats that received only pT/GFP (lanes 1-3) or pT/GFP//*SB*10 (lanes 4-6), and 6 weeks post-PH for pT/GFP (lanes 7-9) or pT/GFP//*SB*10 (lanes 10-12). (**D**) Representative Southern blot ($n \geq 3$ for each of the groups) of DNA isolated from regenerated livers of rats that received *cis* pT/GFP//*SB*10. DNA standards (lane 1); undigested DNA from liver treated with an alternate plasmid vector (lane 2); the *cis* construct in a regenerated liver (lane 3). DNA from the regenerated liver was digested with *Eco*R V alone (lane 4) or both *Eco*R V and *Sma* I (lane 5) to release the GFP coding sequence (arrow).

An ampicon of predicted size was obtained when both primers corresponded to the GFP coding region, but no amplification product was observed when the sense primer was upstream to the direct repeat (data not shown). The result indicated that spontaneous integration of the plasmid was rare or absent, strongly suggesting that the integration had occurred at the transposon DR.

IV. Discussion

We have shown that a single *cis* transposon plasmid that expresses *SB* and carries a transgene can effectively promote long-term gene expression in the liver of mice and rats. Using hepatocyte-targeted *in vivo* delivery, the *cis* transposon system was almost 2-fold more efficient by PCR and western blot analysis than the *SB* transposase delivered in *trans*. This finding is in contrast to a recent study using hydrodynamic delivery of a *cis SB* transposon in a murine model of tyrosinemia type I (Montini et al, 2002). The authors reported reduced transposition using the *cis* construct, and concluded that this had resulted from overproduction of the transposase in mouse liver (Yant et al, 2000, 2002; Montini et al, 2002). Therefore, we compared the efficiency of the *cis* and the *trans* systems by using equal amounts of the transposase and transposon plasmid vectors when co-delivering the two plasmids in *trans*. In fact, both systems produced similar levels of transposase protein immediately after transfection, suggesting that inhibition by transposase overproduction would be equivalent. In contrast, other mouse studies (Yant et al, 2000, 2002; Montini et al, 2002) used a 1 to 25 ratio of transposase construct to transposon. Interestingly, the optimal transposon/transposase ratio appears to be influenced by the amount of transposon (Geurts et al, 2003). The lower doses were optimal at a 1:3 transposon to transposase construct ratio, while increasing the transposon levels 5-fold decreased the optimal ratio to 1:0.2. Thus, the reduced amounts of transposon in our study may, in part, account for the observed increased transposition in *cis*. Additionally, the more rapid loss of the *cis* construct may have reduced levels of transposase, thereby increasing transposition. By targeted delivery, the *cis* plasmid showed greater efficacy for GFP transposition in mice. A potential advantage of using the *cis* system is that the transposition can result in self-destruction of the *cis* plasmid, eliminating the possibility of repeated transposition from the effect of any persisting *SB* expression.

In the present study, the DNA delivery system provides a potentially useful application to human trials. In previous studies, naked plasmids were delivered to the liver by rapid high volume intravenous administration that causes transient congestive heart failure and hepatic stasis, thereby enhancing DNA uptake by liver cells (Budker et al, 2000). This method, although useful in animal studies, is unlikely to find clinical application. In contrast, we achieved targeted delivery of DNA to the liver via hepatocyte-specific ASGR-mediated endocytosis (Wu and Wu, 1998; Wu et al, 2002). The fate of plasmid DNA delivered to the murine liver by the hydrodynamic method differs dramatically from that with PEI (Oh et al, 2001). Using the branched 25 kDa polycation, 50% of the plasmid initially delivered to the liver was still present up to 10 days after transfer. In contrast, 50% of the naked plasmid DNA delivered to the liver was lost within 15 min of administration, and there was no detectable plasmid at 3 days. Although delivery of branched PEI to the lung has been associated with a systemic immune response (Regnstrom et al, 2003), branched PEI-DNA complexes showed no significant liver toxicity (Oh et al, 2001). Moreover, it efficiently transfects quiescent cells such as hepatocytes (Pollard et al, 1998), protects the DNA from nuclease degradation (Boussif et al, 1995), and promotes efficient endosomal disruption (Behr, 1997). L-PEI may also enhance nuclear uptake of DNA by binding to a lectin-like protein with galactose specificity in the nuclear pore complex (Klink et al, 2001). Finally, in contrast to cationic lipids, PEI does not appear to inhibit transgene expression (Pollard et al, 1998). Safety, efficacy and hepatocyte-specificity of this endocytosis-based DNA delivery system makes it attractive for potential use in gene therapy.

This study is the first report of transposon-mediated gene transfer in a mammal other than the mouse. In the rat liver, a single dose of the *cis* transposon, or pT/GFP alone resulted in stable transgene expression, although significantly less homogeneous than in mice. For the *cis* plasmid, the expression observed in the intact liver was similar to that after regeneration post-70% PH. In contrast to the results reported with transposon delivery to the mouse liver using adenovectors, PH in the rats markedly reduced the transgene content and expression in the animals that received pT/GFP alone (Yant et al, 2002). One explanation for this finding is the different regenerative response of hepatocytes post-PH in the two species (Fausto, 2000; Higgins and Anderson, 1931). Also, there is increased persistence of the adenoviral vectors relative to plasmids during cell cycling *in vivo* (Ehrhardt et al, 2003). Finally, the method of DNA delivery may have contributed to the observed differences.

The finding of small clusters of cells expressing GFP after PH in the *cis* transposon rats suggested that the integration event preceded hepatocyte replication. Integration of the transgene was confirmed by Southern blot analysis and like the mouse studies (Dupuy et al, 2001; Fischer et al, 2001; Horie et al, 2001; Yant et al, 2000, 2002) *SB*-mediated gene transfer in rats also occurred randomly within the genome. Interestingly, on average a single copy of the transposon was observed per diploid liver genome when clonal selection for transgene expression occurs *in vivo* (Montini et al, 2002), yet single copies of a transposon were not associated with transgene expression in other *in vivo* mouse systems (Dupuy et al, 2002; Horie et al, 2001). Thus, the observed transgene expression may underestimate the overall transposition frequency. Expression of randomly inserted transgenes can be variable because of the known positional effects (Ivics et al, 1997; Izsvák et al, 2000; Yant et al, 2000, 2002; Dupuy et al, 2001, 2002; Horie et al, 2001; Montini et al, 2002). Insertion of insulator sequences flanking the transgene carried by transposons might abrogate the positional variation of transgene expression and time-

related gene silencing (Pikaart et al, 1998). The efficiency of transposition by the *cis* transposon will most likely be increased using different promoters to regulate transposase expression (Mikkelsen et al, 2003).

In summary, our data indicate that by using a receptor-mediated DNA delivery system and equivalent initial levels of transposase expression, the *cis* delivery of transposons is more efficient than *trans* for transgene integration into the liver. Furthermore, we have demonstrated that the combination of a *cis* construct and a nonviral DNA delivery system could achieve stable transgene expression at levels required to potentially treat many inherited metabolic disorders of the liver. *SB* promises to play an important role in the gene therapy of human genetic diseases.

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