

Gene Insertion and Long-Term Expression in Lung Mediated by the *Sleeping Beauty* Transposon System

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Gene transfer to the lung could provide important new treatments for chronic and acquired lung diseases such as cystic fibrosis, α 1-antitrypsin deficiency, emphysema, and cancer. DNA-mediated gene transfer to the lung has been previously demonstrated, but anticipated effectiveness has been limited by low gene transfer efficiencies and by transient expression of the transgene. Here, we combine plasmid-based gene transfer with the integrating capacity of the nonviral *Sleeping Beauty* (SB) transposon vector system to mediate gene insertion and long-term gene expression in mouse lung. We observed transgene expression after 24 h in lungs of all animals injected with the luciferase transposon (pT/L), but expression for up to 3 months required codelivery of a plasmid encoding the *Sleeping Beauty* transposase. We also observed long-term expression in pT/L-injected animals transgenic for SB transposase. Transgene expression was localized to the alveolar region of the lung, with transfection including mainly type II pneumocytes. We used a linker-mediated PCR technique to recover transposon flanking sequences, demonstrating transposition of pT/L into mouse chromosomal DNA of the lung.

Key Words: *Sleeping Beauty*, transposon, gene therapy, lung, polyethyleneimine

INTRODUCTION

A significant obstacle to effective gene therapy is the lack of sustained gene expression required for treatment of chronic diseases. While the presence of newly introduced transgene sequences in somatic cells may be extended by incorporation of elements that support extrachromosomal maintenance, long-term transgene expression is more reliably accomplished by integration into the host cell genome [1,2]. Stable gene transfer in somatic tissues using retroviral vectors, which integrate into the host cell genome as a part of the viral replicative cycle, has been studied extensively. Adeno-associated virus vectors are also capable of mediating integration into host genomes by a mechanism that has not yet been fully characterized [3]. In contrast to viral transduction, integration following DNA-mediated gene transfer into somatic tissues usually relies on nonhomologous recombination and is extremely rare.

Transposons are mobile genetic elements that integrate into host cell genomes and are used extensively as tools for insertional mutagenesis [4,5]. In the simplest of

cases, a mobile element encodes a transposase protein that recognizes the ends of the transposon (indirect repeats, IRs), excises the transposon from its location in the genome, and reinserts it elsewhere into chromosomal DNA. The *Sleeping Beauty* transposase is an enzyme that was molecularly reconstructed from teleost fish sequences and is capable of mediating transposition in vertebrate cells [6]. The synthetic transposase gene encodes 340 amino acids and contains five regions that are highly conserved in all TcE transposases: a bipartite nuclear localization signal in the N-terminal portion of the transposase, a DNA binding domain, a glycine-rich motif in the center of the transposase, and three segments in the C-terminal domain that contain the DDE motif which catalyzes the transposition [6]. By introducing a therapeutic gene between transposon IRs and supplying the transposase function either on the same plasmid or on a separate plasmid, it is possible to use this transposon as a vector system for gene therapy.

Sleeping Beauty-mediated transposition has recently been reported for a wide variety of mammalian cell types *in vitro* [7] and has also been reported *in vivo* in liver [8]

and in the mouse germ line [9–12]. Here we describe experiments using *Sleeping Beauty* (SB) as a nonviral gene therapy vector that combines the advantages of plasmid-mediated delivery along with an integrative capacity that confers long-term transgene expression in the lung. Long-term (up to 3 months) expression of newly introduced sequences was achieved by codelivery or transgenic expression of an SB transposase gene and was associated with transposition into mouse chromosomal DNA. The SB transposon system, which provides a reliable means of long-term expression in the lung after plasmid-mediated gene delivery, is potentially applicable to genetic therapy of a variety of diseases affecting the lung.

RESULTS

Duration of Transgene Expression after Systemic Co-injection of Transgene with SB10 Transposase

Intravenous injection of DNA–PEI (polyethylenimine) complexes has been shown to be an effective means of delivering DNA to the lung [13]. To optimize our conditions for PEI-mediated delivery of SB transposons to the lung, we complexed various doses of luciferase transposon DNA (pT/L) (Fig. 1A) with PEI at varying nitrogen-in-PEI to phosphate-in-DNA (N/P) ratios and then injected them into the lateral tail vein of C57BL/6 mice. We killed the animals after 24 h, harvested the lungs, and prepared homogenates for luciferase assay. Based on the results from these studies (data not shown), we settled on 100 μ g of total DNA at an N/P ratio of 7 as optimal for survival and gene expression. To determine the effect of transposase codelivery on the duration of gene expression, we complexed 50 μ g of pT/L with PEI at an N/P ratio of 7 and injected it intravenously into mice along with 0, 2.5, 25, or 40 μ g of pCMVSB10 DNA. We sacrificed the mice at 24 h, 2 weeks, and 2 months and assayed whole lung extracts for luciferase expression. There was a high level of luciferase expression observed at 24 h postinjection in all groups (Fig. 1B). After 2 weeks, luciferase expression in the control group (pT/L only) was reduced to near background, while expression in the test groups (pT/L + varying amounts of pCMVSB10) was slightly but not significantly higher than in the control group. At 2 months postinjection, expression in the control group remained at near-background levels, while in the groups that were co-injected with 2.5, 25, and 40 μ g of pCMVSB10 DNA, luciferase expression levels were 10-, 100-, and 80-fold higher, respectively, than in the control group. These levels represented 1, 30, and 20% of the luciferase expression level observed at 24 h. These data demonstrate that codelivery of SB transposase-encoding DNA with the luciferase transposon thus conferred substantial stability to expression levels achieved by intravenous injection of PEI–DNA complexes.

We also analyzed transgene expression levels in the liver and heart at various time points (data not shown). At

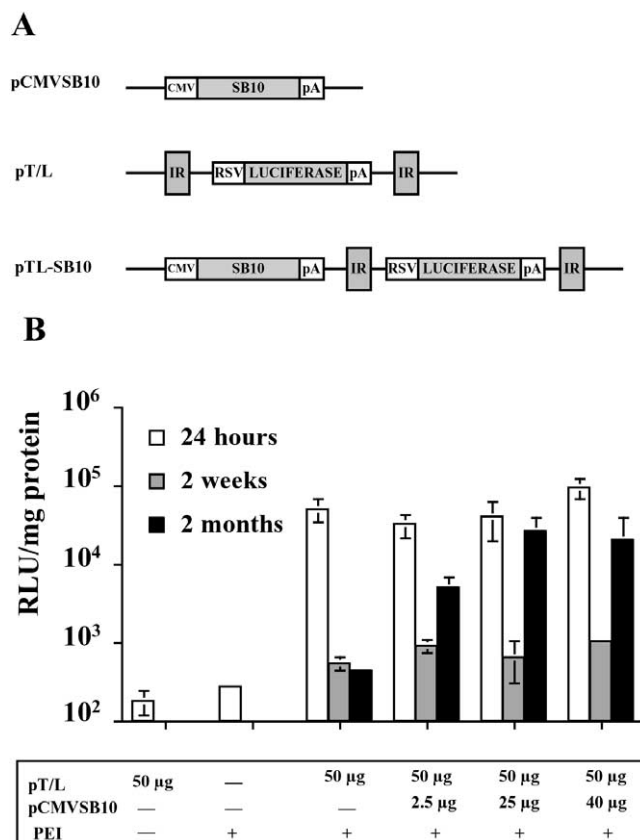


FIG. 1. Long-term expression of luciferase in mouse lungs mediated by the *Sleeping Beauty* transposon system. (A) Vectors used for *Sleeping Beauty*-mediated transposition in lung. RSV, Rous sarcoma virus promoter; CMV, cytomegalovirus promoter; pA, simian virus 40 polyadenylation signal; IR, transposon inverted repeat. (B) Luciferase levels in lung at various times after injection of pT/L with or without pCMVSB10. Plasmid pT/L (50 μ g) was injected intravenously with 0, 2.5, 25, and 40 μ g of pCMVSB10 at an N/P ratio of 7. Mice were sacrificed at various time points and whole lung extracts were assayed for luciferase activity. $N = 5$ for all experimental groups at each time point, except for the group injected with pT/L alone, $N = 4$ at 24 h.

24 h postinjection gene expression in both liver and heart in all groups was two orders of magnitude lower than in the lung. At 2 weeks and 3 months postinjection, there was no detectable gene expression in the liver in any of the experimental groups. There was no gene expression at 2 weeks postinjection in the heart, while at 3 months postinjection, gene expression was about 10-fold above background in SB transgenic animals injected with pT/L and 6-fold higher than background in nontransgenic animals co-injected with pT/L and pCMVSB10. Gene expression in the other groups was near background levels.

Duration of Transgene Expression after Delivery of Transposon and Transposase Functions on the Same Plasmid

Preliminary experiments carried out *in vitro* in Huh7 cells using a plasmid containing a neomycin-resistance trans-

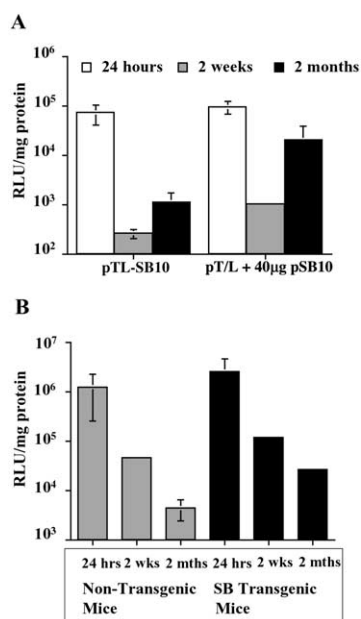


FIG. 2. Long-term expression of luciferase in mouse lungs. (A) Expression of luciferase in animals injected with transposon and transposase functions on the same plasmid (pTL-SB10). Plasmid pTL-SB10 was complexed with L-PEI at an N/P ratio of 7 and injected intravenously. Mice were sacrificed at various times and whole lung extracts assayed for luciferase activity. $N = 5$ for all experimental groups at each time point. (B) Detection of luciferase activity in lungs of SB transgenic and nontransgenic animals. $N = 3$ for all experimental groups at each time point, except for nontransgenic mice, $N = 2$ at 2 weeks. Injections and assays were performed as described under Materials and Methods.

poson demonstrated that when the Neo transposon and SB transposase functions were provided on separate plasmids (delivery *in trans*), the number of G418-resistant colonies obtained was an order of magnitude less than when both transposon and transposase functions were provided on the same plasmid (delivery *in cis*) [29]. We therefore prepared a plasmid that contains both a luciferase transposon and an SB transposase gene. This *cis* plasmid, pTL-SB10, is identical to the plasmid pT/L except for the addition of the CMV-SB10 transcription unit. We injected 50 µg of pTL-SB10 complexed with PEI at an N/P ratio of 7 into C57BL/6 mice through the tail vein. We sacrificed the animals at 24 h, 2 weeks, and 2 months postinjection and assayed whole lung extracts for luciferase expression (Fig. 2A). Luciferase activities observed at 24 h postinjection were similar to the levels observed in pT/L-injected animals, while expression levels at 2 weeks were not distinguishable from background. As observed in animals injected with pT/L + pCMVSB10, pTL-SB10-injected animals exhibited higher levels of luciferase expression at 2 months than at 2 weeks postinjection. However, these long-term expression levels were over an order of magnitude lower than the levels that were observed when the transposon and transposase components were delivered on separate plasmids.

Gene Delivery and Expression in the Lungs of Transgenic Mice Expressing *Sleeping Beauty* Transposase

We injected mice transgenic for *Sleeping Beauty* transposase as well as normal, nontransgenic control mice intravenously with 50 µg of pT/L alone, to determine whether the transposase component of the SB system could be provided endogenously. We sacrificed the mice at 24 h, 2 weeks, and 3 months postinjection and assayed whole lung extracts for luciferase expression. Transient expression of luciferase was similar in both transgenic and nontransgenic groups. However, at 3 months postinjection, the levels of expression in the control group were close to background, while those in the transgenic group were an order of magnitude higher (Fig. 2B). The long-term expression levels observed in transgenic mice were similar to those observed in normal mice co-injected with both transposon and transposase plasmids. These results demonstrate the importance and ability of the SB system to confer long-term transgene expression in the lung, whether delivered in plasmid form or expressed endogenously in tissues.

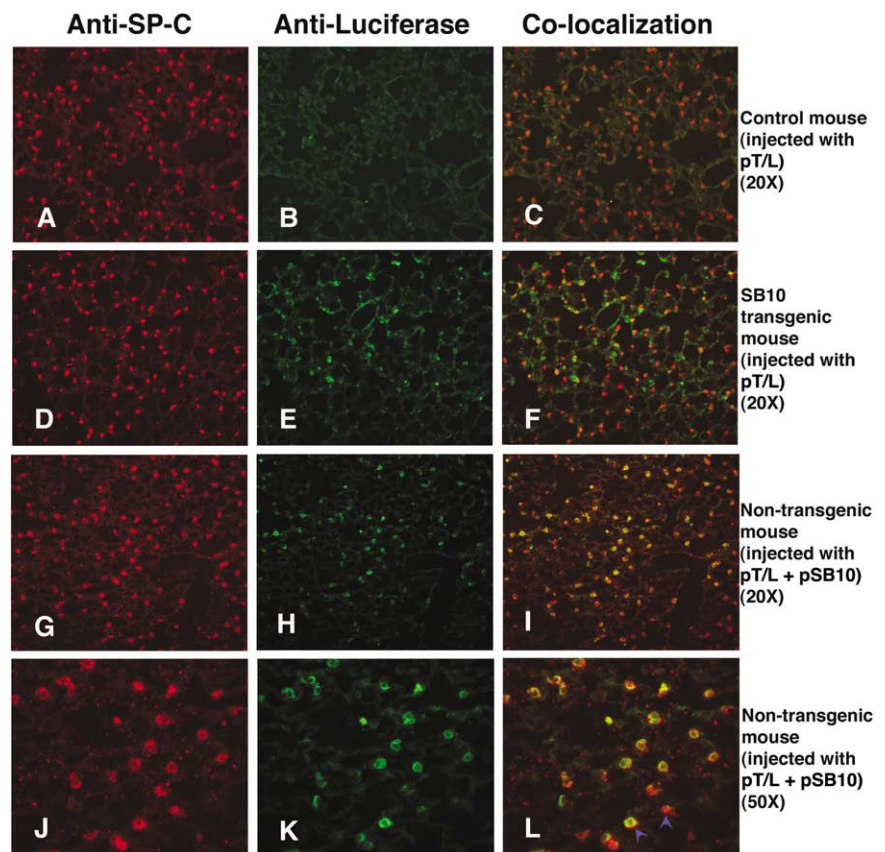
Localization of Transgene Expression in Lung Tissue

To assess the distribution of gene expression in the lung, we visualized luciferase protein by immunohistochemistry in frozen lung sections prepared from the control group (i.e., pT/L only), from SB transgenic mice injected with pT/L, and from a co-injected group (i.e., pT/L + 25 µg pCMVSB10) at 24 h and 3 months postinjection. Luciferase-positive cells were widely distributed in the respiratory zone, with no staining found in the conducting airways (Figs. 3A–3L). We carried out double staining with an antibody against surfactant protein C, which is expressed in type II pneumocytes, to confirm the cellular target of the polyplexes. In the control group (injected with pT/L alone), we saw no luciferase-positive cells at 3 months postinjection. Cells expressing luciferase were seen in both the transgenic SB group and the co-injected group. In both these groups, approximately 2–3% of total cells expressed luciferase at 3 months postinjection. Fifteen to 18% of the cells expressing SP-C also expressed luciferase. These results correlated well with the gene expression levels observed in the luciferase assays of these two groups at 2 and 3 months postinjection, respectively.

Molecular Evidence for Transposition into Mouse Chromosomal DNA

The *Tc1/mariner* family of transposons, including the SB transposon, requires for integration a TA dinucleotide that is usually duplicated upon insertion. We molecularly cloned several transposon insertion junctions to verify integration of the transgene into the host chromosome by transposition. We isolated flanking sequences using a splinkerette-mediated PCR strategy [9]. We digested genomic DNA extracted from lungs with either *Sau3AI* or

FIG. 3. Luciferase expression pattern in the alveolar region of the lung at 3 months postinjection. Animals were sacrificed at 3 months postinjection and lungs were fixed and sectioned as described under Materials and Methods. Sections from control animals (pT/L alone; A, B, C), SB transgenic animals injected with pT/L (D, E, F), and nontransgenic animals injected with pT/L + pCMVSB10 (G, H, I) were stained with anti-luciferase antibody (A, D, G) or anti-SP-C antibody (B, E, H). The anti-luciferase and anti-SP-C images were merged to visualize cells expressing both luciferase and SP-C (C, F, I). All images were at 20 \times original magnification, except for images J, K, and L, which are 50 \times magnifications of images G, H, and I, respectively. Left and right arrows in L indicate type II cells expressing both SP-C and luciferase and SP-C alone, respectively. Nuclei were counterstained with DAPI for total cell quantification.



*Nla*III to characterize the left or right flanking sequences, respectively. We cloned and sequenced PCR products and aligned the sequences with the transposon plasmid (pT/L) to identify new sequences flanking the IRs. Of the 12 clones isolated, 10 clones contained novel sequences, of which 2 clones contained sufficient sequence (5 bp) to be certain that integration had not occurred into either transposon or transposase plasmids, i.e., most likely into chromosomal DNA, even though the sequences were too short to identify on mouse chromosomes. The other 8 clones, although not derived from pT/L, did not contain sufficient sequence to ascertain that transposition had not occurred into transposon or transposase plasmids. The remaining two clones contained sufficient flanking sequences to be identified on mouse chromosomes. The original plasmid sequence flanking the transposon was missing, and instead the transposon was flanked by a TA dinucleotide and entirely new sequence. We mapped these new junction sequences to the whole mouse genome database (Celera Genomics, Inc.). Clone 1, obtained from a co-injected mouse (pT/L + pCMVSB10), showed 100% homology to a region in mouse chromosome 1 at location 156,606,527–157,106,527 Mb, while clone 2 (from a transgenic mouse injected with pT/L) also exhibited 100% homology to a location in mouse chro-

mosome 1, but at location 182,029,898–182,529,897 (Fig. 4). *Sleeping Beauty* transposase, expressed either from a co-injected plasmid or endogenously from a preexisting transgene, thus mediated transposition from the pT/L plasmid into mouse chromosomal DNA in lung tissue. These results provide molecular evidence for *Sleeping Beauty*-mediated transposition in the lungs of injected animals.

DISCUSSION

Therapeutic benefits for gene therapy of chronic diseases require long-term expression of a transgene. As a target

	IR Sequence	New Sequence
pTL	AAGGCTGAAGTTGAC	ATCCCATGGCTCGAGGTTTAAGC
Clone 1	AAGGCTGAAGTTGAC	ATCGAGTCGTGGAACGAAAAGGGGAC
Clone 2	AAGGCTGAAGTTGAC	ATTTTGAGAAAAGAAAACAAGAAAGT

FIG. 4. Molecular analysis of transposition. Flanking sequences of transposon insert sites. Clone 1, sequence from a mouse co-injected with both pT/L and pCMVSB10; Clone 2, sequence from an SB10-transgenic mouse. Transposon sequences and flanking TA dinucleotides are aligned with mouse chromosomal sequences extending to the right.

organ for gene transfer, the lung is accessible either directly through the airway or through the vascular system, with different cell types being transfected depending on the route of administration [14]. Targeting of airway epithelium has been studied extensively for CFTR gene therapy, in which gene transfer has been achieved using several different vector systems administered by intranasal, intratracheal, and endobronchial routes of delivery [15,16]. Introduction of genes to the lung via the vascular system targets the endothelial and epithelial cells of the lung. The primary limitation of nonviral gene delivery in the lung has been the short duration of gene expression achieved.

Transposons are useful gene therapy vectors because in the presence of a transposase they bring about integration of transgene sequences into the host chromosome [5]. Here we have used a nonviral, transposon-based vector system to deliver genes to the lung and have demonstrated sustained expression of the transgene upon co-introduction of the *Sleeping Beauty* transposase. Animals injected with the transposon plasmid + PEI alone exhibited no transgene expression at either 2 or 3 months postinjection, whereas animals that received transposon plasmid + varying amounts of SB transposase plasmid + PEI continued to express the transgene at levels that ranged from 10- to 100-fold higher than control. Introduction of the transposon plasmid into transgenic SB mice yielded transgene levels at 3 months postinjection that were similar to levels obtained by co-injection of transposon and transposase on separate plasmids, thus demonstrating that transposase function can be provided endogenously, as well as proving that transposition can occur in a different mouse strain. Supplying both transposon and transposase functions on the same plasmid was not as efficient as providing the two functions on different plasmids, since long-term expression was just 2-fold higher than background in the former case.

Members of the *mariner/Tc1* family of transposable elements including SB have been shown to be active in a wide variety of species, including bacteria. *Sleeping Beauty* has been shown to transpose in mouse embryonic stem cells [17], the one-cell mouse embryo [12], and mouse somatic tissue [8] as well as in the mouse germ line [11]. In comparison to *Tc1*, *Tc3*, *Himar1*, and *Mos1* transposon elements SB has been demonstrated to be most efficient in mediating transposition in cultured cells from a variety of vertebrate species [11]. Efficiency of transposition varies substantially among cell lines, suggesting a possibility for the involvement of host-specific factors *in vitro* [4,7,18,30]. This transfer between species does not mean that the activity of these elements is unregulated. Indeed, regulation of *mariner* elements is complex. When a *mariner* element transposes into a new site, it must limit the damage that it inflicts [19]. Activity of both *Mos1* and *Himar1* elements (members of the *Tc1/mariner* family) is dependent on the concentrations of transposase present

and is decreased at higher concentrations of transposase, a phenomenon termed overexpression inhibition [20]. In contrast to *mariner* transposons, it has been demonstrated that in HeLa cells, SB-mediated transposition increases proportionally with the amount of transposase present in cells [7]. However, the dependence of transposition on host factors could explain our finding that under the tested conditions *in vivo*, delivery of the transposase gene in the *cis* configuration provided a less optimal level of transposase that is either more or less than that achieved in the *trans* configuration.

We chose linear PEI (L-PEI) as a DNA-complexing agent, based on its highly efficient gene delivery to the lung [21,22]. Every third atom of linear PEI is an amino nitrogen that can be protonated, thus conferring on it a unique buffering capacity that allows escape of the DNA from lysosomes upon transfection and subsequent endocytosis of the PEI-DNA complex. The alveolar region, including the pneumocytes, is the primary site of gene delivery after intravenous administration of L-PEI-DNA complexes, perhaps due to increased permeability of the lung vasculature. This increased permeability could be the result of L-PEI-mediated toxicity, regarding which there are limited *in vivo* data. There are several reports addressing this issue, demonstrating that administration of L-PEI-DNA complexes leads to severe inflammatory responses even at 24 h, characterized by a high frequency of infiltrating inflammatory cells in the lung [23,24]. The severity of the inflammatory response is related directly to the degree of positive charge on the complexes. We used an N/P ratio of 7, in which the complexes were slightly electropositive. In all the co-injected experimental groups, luciferase activity was reduced at 2 weeks postinjection, while expression levels were increased at 2 months postinjection. This is presumably due to loss of transiently transfected cells through cell turnover and PEI toxicity, with the rebound of expression at 2 months resulting from transposed gene sequences in cells replicating in response to the lung injury.

A major limitation of plasmid-based gene transfer in general has been the limited duration of gene expression. After either intravenous administration or gene delivery via the airway, transgene expression is for the most part transient, subsiding 3–7 days after injection [14,23]. Loss of vector DNA from the tissue, transcriptional inactivation of the viral promoter, or an inflammatory response to the DNA or gene product are all potential causes for the observed loss of gene expression [25,26]. There have been two recent reports demonstrating extended DNA-mediated expression in the lung. Plasmid-based gene expression has been demonstrated out to nearly 3 months postinjection using plasmid-cationic lipid complexes with the extended duration of expression attributed to decreased CpG content of the DNA in combination with the use of a hybrid CMV-ubiquitin B promoter, to regulate expression of the transgene (β -galactosidase) [27]. Gill

et al. also reported extended transgene expression (luciferase) regulated by the ubiquitin C promoter, in which expression in airway cells was detected out to 6 months postinjection [28]. In both studies, however, the level of transgene expression was relatively low and, in the former case, was insufficient to provide a therapeutic benefit.

Use of an integrating-plasmid-based system such as *Sleeping Beauty* may provide a higher, more long-lasting level of gene expression than an episomal system. We showed that *Sleeping Beauty* transposons were inserted at TA dinucleotides into the mouse genome, thereby enabling sustained transgene expression in the lung. Approximate efficiency of gene transfer was 2–3% based on immunohistochemical staining. To our knowledge, this is the first demonstration of sustained expression in the lung due to transposition-mediated gene delivery using a plasmid-based vector. *Sleeping Beauty's* ability to mediate stable integration can be used as a means to optimize conditions for gene delivery, for transposition, and for regulation of long-term expression in the lung. The SB transposon system may potentially be used as an effective vector for the treatment of a variety of pulmonary diseases that require long-term expression of a missing or defective protein such as cystic fibrosis or α 1-antitrypsin deficiency.

MATERIALS AND METHODS

Plasmid construction. The luciferase transposon (pT/L) was constructed by cloning the RSV promoter from plasmid pBluescript/AAT (Dr. Mark Kay, Stanford University) into plasmid pGL3C (Promega, Madison, WI) using *HindIII* and *XhoI*. The resulting plasmid contained the luciferase transgene under transcriptional control of the RSV promoter. This luciferase expression cassette was excised with *SmaI* and *BamHI* and inserted between the *EcoRV* and the *BglII* sites into plasmid pT/HB [9] to yield plasmid pT/L. For construction of plasmid pTL-SB10, the CMVSB10 expression cassette was excised from pCMVSB10 using *Sall* and *EcoRI* and cloned into *Sall* and *EcoRI* sites in pBluescript KS(+) (Stratagene, La Jolla, CA) to generate pBlueSB10. The luciferase transposon cassette was then cloned as a *KpnI* fragment into the *KpnI* site of pBlueSB10 to yield pTL-SB10.

Animals and PEI-DNA complex administration. C57BL/6 and FVB/N mice ages 8–10 weeks were purchased from The Jackson Laboratory (Bar Harbor, ME) and provided food and water ad libitum. SB transposase transgenic mice were established on an FVB/N background as described by Dupuy *et al.* [10]. Linear 22-kDa PEI (ExGen 500; MBI Fermentas, Hanover, MD) at 0.1 M was used for the *in vivo* studies. DNA-PEI complexes were prepared in 5% dextrose as per the manufacturer's instructions. Briefly, 50 μ g of luciferase transposon plasmid was mixed with 0, 2.5, 25, or 40 μ g of pCMVSB10 DNA. The total amount of DNA was kept constant at 100 μ g with appropriate amounts of pBluescript. One hundred micrograms of plasmid was diluted in 0.25 ml of 5% glucose, and linear PEI at a charge ratio of 7 [charge ratio is expressed as PEI nitrogen (N) to DNA phosphate (P)] was diluted in 0.25 ml of 5% glucose. The PEI was added to the DNA, vortexed immediately, and centrifuged briefly. DNA-PEI complexes were allowed to form at room temperature for 10 min before injecting mice through the tail vein. For *in vivo* experiments with SB transgenic mice, the linear 22-kDa form of PEI from Polyplus (Illkirch Cedex, France) was used.

Luciferase assay. Animals were sacrificed at indicated time points and perfused with 0.9% saline. The lungs were removed, washed in ice-cold 0.15 M NaCl, and homogenized for 20 s in 0.2 ml of ice-cold lysis buffer

(Promega) using a PowerGen 125 homogenizer (Fisher Scientific, Pittsburgh, PA). The cell lysate was centrifuged at 16,000g at 4°C for 5 min. Fifty microliters of the supernatant was assayed for luciferase activity in a Berthold Lumat LB 9507 luminometer. Upon injection of 100 μ l of luciferase substrate, luminescence emitted over 10 s was measured. Total protein concentration in the tissue samples was assayed using the Bradford DC protein assay kit (Bio-Rad, Hercules, CA). Luciferase activity is expressed as relative light units (RLU)/mg protein. In standardization experiments, 10⁵ RLU was equivalent to 3 ng of purified firefly (*Photinus pyralis*) luciferase (Roche Applied Sciences, Indianapolis, IN).

Immunohistochemistry. Animals were sacrificed at indicated time points and perfused for 10 min with saline, followed by perfusion for 10 min with 4% paraformaldehyde in PBS. Lungs were removed and further fixed in 4% paraformaldehyde at 4°C for 4 h, followed by immersion in 10 and 20% sucrose for 2–3 h and overnight, respectively, at 4°C. Lungs were then snap frozen in Tissue Tek Optimal Cutting Temperature compound (Sakura Finetek, Inc., Torrance, CA) and stored at –70°C until sectioned. Five- to six-micrometer sections were stained with goat anti-luciferase (Promega) and rabbit anti-human surfactant protein C (Chemicon, Temecula, CA) polyclonal primary antibodies followed by donkey anti-goat-Cy2 and donkey anti-rabbit-Cy3 (The Jackson Laboratory) secondary antibodies, respectively. Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride, and specimens were examined by fluorescence microscopy at the University of Minnesota Biological Image Processing Laboratory.

Transposon insert site flanking sequence analysis. A splinkerette PCR technique was utilized to recover sequences flanking transposon inserts on either the 5' or the 3' side as previously described [9]. PCR products consistent with a flanking sequence different from that of plasmid pT/L were gel purified, cloned into pCR2.1-Topo (Promega), and sequenced at the Advanced Genetics Analysis Center of the University of Minnesota.

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