Gene Expression in Lung and Liver After Intravenous Infusion of Polyethylenimine Complexes of *Sleeping Beauty* Transposons

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Abstract

Two methods of systemic gene delivery have been extensively explored, using the mouse as a model system: hydrodynamic delivery, wherein a DNA solution equivalent in volume to 10% of the mouse weight is injected intravenously in less than 10 sec, and condensation of DNA with polyethylenimine (PEI) for standard intravenous infusion. Our goal in this study was to evaluate quantitatively the kinetics of gene expression, using these two methods for delivery of *Sleeping Beauty* transposons. Transposons carrying a luciferase expression cassette were injected into mice either hydrodynamically or after condensation with PEI at a PEI nitrogen-to-DNA phosphate ratio of 7. Gene expression in the lungs and liver after hydrodynamic delivery resulted in exponential decay with a half-life of about 35–40 hr between days 1 and 14 postinjection. The decay kinetics of gene expression after PEI-mediated gene delivery were more complex; an initial decay rate of 6 hr was followed by a more gradual loss of activity. Consequently, the liver became the primary site of gene expression about 4 days after injection of PEI-DNA, and by 14 days expression in the liver was 10-fold higher than in the lung. Overall levels of gene expression 2 weeks postinjection were 100- to 1000-fold lower after PEI-mediated delivery compared with hydrodynamic injection. These results provide insight into the relative effectiveness and organ specificity of these two methods of nonviral gene delivery when coupled with the *Sleeping Beauty* transposon system.

Introduction

GENE TRANSFER TO THE LIVER has been studied intensively as a potential means of treating several human diseases, most notably hemophilia (Manno *et al.*, 2003, 2006; High, 2005) and other monogenic diseases such as ornithine transcarbamylase deficiency, phenylketonuria, and those that cause various forms of mucopolysaccharidosis (Cabrera-Salazar *et al.*, 2002; Ellinwood *et al.*, 2004; Hodges and Cheng, 2006; Sands and Davidson, 2006; Beck, 2007; Ponder and Haskins, 2007). Clinical trials for these diseases, as well as preclinical work in numerous animal models of human disease, have employed primarily viral vectors to mediate gene transfer into hepatocytes. Although viral vectors mediate a

high frequency of gene transfer, clinical outcomes in several gene therapy trials have served to highlight key problems in their therapeutic application. Immune/inflammatory responses to adenoviral vectors compromise their effectiveness and can result in adverse reactions, exemplified in the death of one patient (Raper *et al.*, 2003). Adeno-associated viral (AAV) vectors are not associated with acute inflammatory reactions, but the effectiveness of AAV-mediated gene therapy in a liver-directed clinical trial for hemophilia B was limited by a cellular immune response against AAV capsid protein, with subsequent cytotoxic effects against the transduced hepatocytes (Manno *et al.*, 2006). A further complication in the use of retroviral, lentiviral, and AAV vectors is their preference for integrating close to promoters and into transcriptional units,

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where they may have increased chances of causing adverse effects as a result of activation of oncogenes or their regulators (Schroder *et al.*, 2002; Nakai *et al.*, 2003; Wu *et al.*, 2003; Laufs *et al.*, 2004; Mitchell *et al.*, 2004; Bushman *et al.*, 2005; De Palma *et al.*, 2005; Ciuffi *et al.*, 2006; Donsante *et al.*, 2007; Hackett *et al.*, 2007).

An alternative method for gene therapy is to use DNA without associated proteins against which immune responses might be directed. There are two major problems with most methods of nonviral gene therapy. First, expression of the transgene from plasmids is most often brief because of lack of integration and because of cellular responses that extinguish their expression. Second, directing DNA molecules to a specific organ or cell type is difficult and inefficient. We have addressed the first problem by developing the *Sleeping Beauty* (SB) transposon system (Ivics *et al.*, 1997), which combines the advantages of viruses and naked DNA (Izsvak and Ivics, 2004; Essner *et al.*, 2005; Hackett *et al.*, 2005). The SB system has integrating activity for long-term gene expression without a preference for transcriptional units and lacks the problems associated with highly immunogenic viral proteins (Izsvak and Ivics, 2004; Hackett *et al.*, 2005). In mice SB transposons have been used to correct several genetic deficiencies including those for hemophilia B (Yant *et al.*, 2000, 2002) and hemophilia A (Ohlfest *et al.*, 2005b; Liu *et al.*, 2006; Kren *et al.*, 2009), tyrosinemia type I (Montini *et al.*, 2002), junctional epidermolysis bullosa (Ortiz *et al.*, 2003), diabetes (He *et al.*, 2004), Huntington disease (Chen *et al.*, 2005), and mucopolysaccharidosis (Aronovich *et al.*, 2007, 2009) as well as for treatment of a xenograft model for glioblastoma (Ohlfest *et al.*, 2004, 2005a). Current methods for delivery of plasmids to liver cells are about 1–10% as efficient as viral delivery (Yant *et al.*, 2000; Aronovich *et al.*, 2007; Jacobs *et al.*, 2008). However, if the therapeutic gene in an expression cassette contains a powerful promoter, a few cells can provide therapeutic levels of gene expression (Aronovich *et al.*, 2009). The major remaining obstacle to effective nonviral gene therapy with the SB system involves the scaling up of delivery into large animals.

Efficient delivery and expression of DNA are essential for nonviral gene therapy to become a feasible method of treating genetic disease. So far, the most effective method of nonviral gene transfer in mice is by the infusion of naked plasmid DNA under pressure, that is, hydrodynamic injection. The mechanism by which hydrodynamic delivery is achieved is poorly understood (Zhang *et al.*, 2004; Crespo *et al.*, 2005). Hydrodynamic injection into larger animals has also been achieved by direct infusion into selected, occluded organs such as the liver and muscle (Eastman *et al.*, 2002; Yoshino *et al.*, 2006; Aliño *et al.*, 2007; Fabre *et al.*, 2008; Sawyer *et al.*, 2008).

However, because of the concerns that hydrodynamic delivery may not be appropriate for humans, we have investigated alternatives to hydrodynamic delivery for *in vivo* delivery of SB transposons. In this paper we report on the ability of polyethylenimine (PEI) to mediate uptake of transposons into mouse tissues for long-term gene expression. In earlier work we and others have found that PEI could mediate delivery of the components of the SB transposon system to the lung for integration and long-term transgene expression (Belur *et al.*, 2003; L. Liu *et al.*, 2004) or to the liver when PEI was complexed with certain ligands

(Kren *et al.*, 2003). However, the kinetics of expression were not investigated in these studies. Here we evaluate quantitatively the kinetics of gene expression using these two methods for delivery of *Sleeping Beauty* transposons and show that over the course of 2 weeks, maximal expression after PEI-mediated delivery shifted from the lungs to the liver, where gene activity reached a steady state level that was considerably lower than that achieved by hydrodynamic delivery. These results have implications for the development of therapeutic nonviral gene therapies for targeting liver for gene delivery and expression in large animals.

Materials and Methods

Plasmids

Plasmid pT2/CAL//PGK-SB13 contains a T2 SB transposon (Geurts *et al.*, 2003) encoding the firefly luciferase gene (Luc) downstream of a β -actin/CMV hybrid promoter (CAGGS) and the SB13 hyperactive transposase regulated by a phosphoglycerate kinase (PGK) promoter (Carlson *et al.*, 2005). The DsRED-IRES-firefly luciferase transposon pKT2/dRIL and the SB11 transposase expression plasmid pKUb-SB11 have been described (Wilber *et al.*, 2005 and 2006, respectively). Further details and sequences of the constructs can be found at <http://biosci.cbs.umn.edu/labs/perry/> under Plasmid Info.

DNA/PEI complex formation

Linear 22-kDa PEI was obtained from two vendors: jetPEI from Polyplus-Transfection (Illkirch, France) and ExGen 500 from Fermentas (Glen Burnie, MD). DNA/PEI was complexed at a PEI nitrogen-to-DNA phosphate (N/P) charge ratio of 7, using either 50 μ g of pT2/mCAGGS-Luc/PGK-SB13 or 50 μ g of pKT2/dRIL with 25 μ g of pKUb-SB11. DNA/PEI complexes were prepared according to the manufacturers' instructions in 5% dextrose. Briefly, the DNA for each reaction was diluted to a total volume of 0.15 ml of 5% dextrose. Separately, the appropriate amount of linear PEI to achieve an N/P ratio of 7 was diluted to a total volume of 0.15 ml in 5% dextrose. The PEI and DNA solutions were then immediately vortexed in a 1.5-ml Eppendorf tube for 20 sec and centrifuged for about 2 sec. Supernatants containing the DNA/PEI complexes were incubated at room temperature for 15 min before injection into experimental animals.

DNA/PEI particle sizes and polydispersity

Particle sizes of DNA/PEI complexes (Table 1) were measured by dynamic light scattering, using a Brookhaven Instruments (Holtsville, NY) BI-200SM goniometer with an AT9000 digital autocorrelator equipped with an adjustable intensity Lexel model 95 ion laser (488 nm) (Cambridge Lasers Laboratories, Lexel Products Division, Fremont, CA). The correlation functions were calculated between delay times of 2 and $1 \times 10^5 \mu$ sec and were determined by the non-negative least-squares analysis present in the Brookhaven software for Windows. Measurements were taken in dust-free Kimble 10 $\times$ 75 borosilicate glass culture tubes (Kimble Chase, Vineland, NJ) in a temperature-controlled (room temperature) mineral oil bath with the detector set to 90° to the incident laser beam. Hydrodynamic diameters were

TABLE 1. PHYSICAL CHARACTERISTICS OF POLYETHYLENIMINE/DNA COMPLEXES

Sample	H ₂ O/ saline	Effective diameter (nm)	Polydispersity
jetPEI/DNA (N/P @ 7:1)	dH ₂ O Saline ^a	80 468	0.198 0.232
ExGen PEI/DNA (N/P @ 7:1)	dH ₂ O Saline ^a	69 334	0.187 0.217

Abbreviations: dH₂O, distilled H₂O; N/P, nitrogen-to-DNA phosphate ratio; PEI, polyethylenimine.

^aAggregates observed when samples diluted into saline.

calculated by assuming the particles were spherical, with the viscosity set to pure water. Concentrations of the complexes ranged from 10 to 20 μ g/ml in 5% dextrose. Particle size measurements were taken in triplicate and generally had a standard deviation of 5 nm or less (Steele, 2006).

Animals and DNA injections

Female C57BL/6 mice, 6–12 weeks old, were obtained from the National Cancer Institute (Frederick, MD) and housed under specific pathogen-free conditions and provided food and water *ad libitum*. DNA/PEI complexes were delivered via standard lateral tail vein injection. For hydrodynamic introduction of uncomplexed plasmid DNA, samples were diluted with lactated Ringer's solution (Baxter, Deerfield, IL) to a volume equal to 10% of each mouse's weight and injected through the lateral tail vein in less than 10 sec as previously described (Liu *et al.*, 1999; Zhang *et al.*, 1999; Bell *et al.*, 2007).

Bioluminescence imaging in vivo

Expression of luciferase in living mice was monitored by bioluminescence imaging, using the Xenogen IVIS imaging system and the Living Image program (Xenogen, Alameda, CA). Mice were anesthetized with a cocktail containing ketamine-HCl (8 mg/ml; Phoenix Scientific, St. Joseph, MO), acepromazine maleate (0.1 mg/ml; Phoenix Scientific), and butorphanol tartrate (0.01 mg/ml; Fort Dodge Animal Health, Overland Park, KS) as described earlier (Bell *et al.*, 2007). Five minutes later, 0.1 ml of D-luciferin (28.5 mg/ml; Xenogen) was injected intraperitoneally. Five minutes after injection of luciferin substrate, mice were imaged according to the manufacturer's instructions. Luciferase expression determined by bioluminescence imaging is expressed as photons emitted per second per square centimeter.

Luciferase assay

Animals were killed and prepared for tissue harvest by cardiac perfusion with normal saline. The lungs and liver were removed, snap frozen in liquid nitrogen, and stored at -80°C . Lungs and livers were homogenized, using a Power-Gen 125 homogenizer (Fisher Scientific, Pittsburgh, PA). Lung tissue was homogenized for 20 sec in 1 ml of cold lysis buffer and liver tissues for 30 sec in 6 ml of cold lysis buffer. Cell lysates were cleared by centrifugation at 14,000 rpm for

10 min at 4°C . Twenty microliters of supernatant was assayed for luciferase activity after mixing with 100 μ l of luciferin substrate (Promega, Madison, WI), using a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Luminescence was measured over 10 sec. Luciferase activity is expressed as relative light units (RLU) per total organ mass.

Quantitative polymerase chain reaction

DNA was isolated from the cell lysates by means of a standard phenol-chloroform protocol. We used the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal control for genomic DNA content and measured the PCR threshold cycle (C_T) numbers for excision products (EPs) and GAPDH genes, using the forward and reverse primers listed below (Aronovich *et al.*, 2007, 2009). All quantitative polymerase chain reactions (qPCRs) were run in duplicate. Reaction volumes of 25 μ l included 0.5 μ g of DNA, 2 $\times$ SYBR GreenER qPCR supermix universal (Invitrogen, Carlsbad, CA), and a 200 nM concentration each of the forward and reverse primers. The number of excision events per cell was calculated from the following equation: Excision events/cell = 2×2^{-n} , where n is the difference between the C_T values for GAPDH and EP. This equation assumes that there is a diploid number of GAPDH genes but only one transposition event. In the case of the lung after hydrodynamic injection, there was no C_T value because of insufficient delivery to this organ; hence, integration values are expressed as upper limits. All measurements are accurate to $\pm$ one cycle, which is equivalent to an uncertainty of a factor of 2 in EPs per cell.

Primers for the excision qPCR assay of dRIL were as follows:

Forward: 5'-CCTGATTGCCCGACATTATC-3'
Reverse: 5'-GAGCGAGGAAGCGGAACAGA-3'

Primers for GAPDH were as follows:

GAPDH-F: 5'-TGTCTCCTGCGACTTCAACAGC-3'
GAPDH-R: 5'-TGTAGGCCATGAGGTCCACCAC-3'

We used a MasterCycler *realplex*² instrument (Eppendorf, Westbury, NY) with the following conditions: cycle 1, 50°C for 2:00 min; cycle 2, 95°C for 10:00 min, cycles 3–42, 95°C for 0:15 min, 60°C for 1:00 min. The melting profiles of every qPCR were examined to ensure that a single product of defined melting temperature was quantified in the PCRs.

Results

Transgene expression from SB transposons after PEI-mediated delivery

Our goal was to evaluate quantitatively two of the most commonly used methods of nonviral gene delivery, hydrodynamic injection and condensation of DNA with PEI, using these two methods for *in vivo* delivery of *Sleeping Beauty* transposons. PEI-complexed plasmids containing SB transposons can be effectively delivered to the lungs of mice to achieve long-term expression (Belur *et al.*, 2003; L. Liu *et al.*, 2004), but detailed analysis of the kinetics of gene expression in lung and liver was not done in these studies. Accordingly, we injected 50 μ g of PEI-complexed pT2/CAL//PGK-SB13,

a *cis* construct with a T2 hyperactive transposon containing a CAGGS-Luc expression cassette and, outside of the transposon but within the same plasmid, a PGK-SB13 hyperactive SB transposase expression cassette (Fig. 1A). We tested two different preparations of 22-kDa linear PEI from two vendors, jetPEI (Polyplus) and ExGen 500 (Fermentas), and complexed DNA at an *N/P* ratio of 7 (Fig. 2). Dynamic light scattering indicated that the particles were about 70–80 nm in size when suspended in 5% dextrose, but the same complexes in saline aggregated to 330–470 nm with greater polydispersity (Table 1). Thus, after injection, aggregation of the smaller particles in the solution may occur to an unknown extent as the dextrose solution mixes with isotonic blood.

Transgene expression from transposons delivered to the lung is less stable than in the liver after PEI-mediated delivery

We determined luciferase expression *in vivo* by bioluminescence imaging over 13 weeks after injection (Fig. 2). Animals imaged 1 day after injection of PEI/DNA complexes exhibited luciferase expression that was localized primarily to the region of the lungs (Fig. 2a). Surprisingly, after 1 week we observed that the primary image of luciferase expression had shifted from the lung to a region that was localized over

the abdomen, most likely the liver (Fig. 2b). Similar results were obtained for animals injected with 22-kDa jetPEI and ExGen 500 PEI from the two different vendors. Expression of firefly luciferase showed multiphasic decay kinetics with a rapid decrease in expression of about 50-fold (half-life, ca. 24 ± 2 hr; interval 1 in Fig. 2c) over the first week, followed by a more gradual loss in activity with a half-life of greater than 1000 hr (interval 2 in Fig. 2c). The result was a several hundred-fold loss after 2 months from initial peak activities that were recorded the first day postinjection (Fig. 2c).

Quantification of bioluminescence signals from multiple locations *in vivo* can be complicated by the masking of less intense signals by a comparatively higher signal emerging from a second site. Consequently, we carried out a detailed study to determine quantitatively the kinetics of the apparent shift of expression from lung to liver. In these experiments we also added mice that were injected hydrodynamically with the same transposon-containing plasmids in order to evaluate the overall effectiveness of the PEI-mediated delivery process in both lung and liver. In the second series of experiments we injected either a single *cis* plasmid that contained the entire transposon system on a single DNA molecule, pT2/CAL//PGK-SB13, or two plasmids, pKT2/dRIL and pKUb-SB11 (Fig. 1B), in which the transposon and transposase components are on separate DNA molecules (*trans* delivery). The *cis* and *trans* constructs were administered by standard tail vein injection of DNA/PEI conjugates and by hydrodynamic tail vein injection of naked plasmid DNA. The animals were assayed for gene expression 1, 4, 7, 10, and 14 days postinjection by whole-body *in vivo* bioluminescence imaging. Cohorts of four to six mice were killed to evaluate expression of dsRed fluorescence in various organs for those mice receiving the pKT2/dRIL construct, and by luminometer assay of tissue extracts for luciferase activity in mice that were injected with either the pKT2/dRIL or pT2/CAL//PGK-SB13 constructs. In agreement with the findings of others, transgene expression in organs other than the lung was about 100-fold lower on a per milligram of protein basis. Because the liver is the primary site of gene expression after hydrodynamic delivery and the lung is the primary site of delivery when using PEI-complexed DNA, in this work we focused on transgene expression as a function of time in these two organs.

Kinetics of transgene expression from PEI-complexed transposons are different from those after hydrodynamic injection

Hydrodynamic injection of either 25 μ g of plasmid pT2/mCAGGS-Luc/PGK-SB13 (*cis* construct) or 25 μ g of transposon pKT2/dRIL plus 12.5 μ g of transposase plasmid pKUb-SB11 (*trans* constructs) resulted in luciferase expression levels, determined by bioluminescence imaging, between 10^9 and 10^{10} photons/sec 1 day postdelivery and fell about 1000-fold over the first 2 weeks (Fig. 3a). These decay kinetics are consistent with our earlier observations indicating that therapeutic benefit for correction of genetic deficiencies had been achieved (Ohlfest *et al.*, 2005b; Aronovich *et al.*, 2007; Bell *et al.*, 2007). Hydrodynamic delivery directs more than 99% of the expressing plasmids to the liver, as determined on the basis of luciferase expression 24–48 hr postinjection (Fig. 3b). Luciferase activities in lung and liver from groups

FIG. 1. Transposon and transposase-encoding plasmids. The plasmids were delivered in a (A) *cis* or (B) *trans* configuration. (A) pT2/CAL//PGK-SB13 contains a firefly luciferase transposon and also encodes SB13 *Sleeping Beauty* transposase on the same plasmid but outside of the transposon. (B) *Top*: pKT2/dRIL transposon includes genes that encode dsRed2 and firefly luciferase regulated by the CAGGS promoter. *Bottom*: pKUb-SB11 encodes *Sleeping Beauty* transposase transcriptionally regulated by the human ubiquitin C promoter.

FIG. 2. *In vivo* imaging of luciferase expression after PEI-mediated delivery. Luciferase gene expression was measured after injection of 50 μ g of pT2/CAL//PGK-SB13 (Fig. 1A) complexed at an *N/P* ratio of 7 with either of two brands of 22-kDa PEI: jetPEI (Polyplus) or ExGen 500 (Fermentas). Five mice were injected with each DNA/PEI complex and monitored for luciferase expression by bioluminescence imaging over 3 months. (a and b) Sample images from mice 1 and 7 days postinjection are shown. The pink lines represent the upper borders of the diaphragm and rib cage, determined by visual inspection, in the treated mice. The signal intensities are given as photons per second and the exposure times are given in seconds (in parentheses underneath each panel). (c) The complex decay kinetics of gene expression in both cohorts of mice over 13 weeks; the lines are least-squares best fits to the data divided into two phases (1 and 2) identified by the bars at the top of the panel. Color images available online at www.liebertonline.com/hum.

of mice killed at various times postinjection were determined to obtain a quantitative evaluation of gene expression in these two organs. As shown in Fig. 3c and d, there was no consistent significant difference between injections of the *cis* (solid lines) and *trans* (dashed lines) constructs by whole-body imaging in either liver or lung over the 2-week period of the test. We also noted that levels of expression in the liver were consistently more than 1000-fold higher than in the lung (Fig. 3d), which resulted in the whole-body images reflecting expression from the liver. On quantitative analyses we found that the rates of decay were essentially the same for all groups over the entire 14-day duration of the experiment. The half-lives of luciferase activity are plotted as black dotted lines in each panel (Fig. 3c and d). The half-life was 36 ± 7 hr for transgene expression from whole-animal imaging, 37 ± 5 hr for expression in liver extracts, and 36 ± 1 hr for expression in lung extracts. Overall, the kinetics of activity loss for the whole animal essentially paralleled that of the liver, as expected because expression in the liver was consistently much higher than in the lung.

As shown in Fig. 4A, luminescence was localized in the lungs of all animals 1 day after injection of DNA complexed with PEI. Luciferase expression was $4\text{--}5 \times 10^7$ photons/

sec/cm^2 for the *trans* and *cis* constructs. However, when the mice were imaged 4 days after injection, the site of expression detected by bioluminescence imaging had changed from the lung to the liver, and the level of expression was reduced by about 100-fold both in the liver and lung, to $3\text{--}5 \times 10^5$ photons/sec/cm². Fourteen days after injection, luciferase expression was seen by bioluminescence imaging only in the liver, similar to that shown in Fig. 2a. As with the hydrodynamic injections, there was no statistically significant difference between animals injected with the *cis* (Fig. 4B, green solid line) and the *trans* (Fig. 4B, green dotted line) constructs. Gene expression as determined by whole-body imaging declined by about 500-fold over 2 weeks (Fig. 4B) at rates that varied over time. However, when we examined gene expression from homogenized lung and liver tissues isolated separately, the decay of expression was substantially different in the two organs. For both the *cis* and *trans* orientation transposons, we observed that luciferase activity in lung dropped nearly 10,000-fold over 2 weeks compared with that in the liver, where the reduction in gene expression was only about 100-fold after 2 weeks. Half-lives of transgene expression (indicated as black dotted lines in Fig. 4C) were calculated either for days 1–4 and days 10–14 or, in the

FIG. 3. *In vivo* imaging and quantitative measurement of luciferase expression after hydrodynamic delivery. Either the *cis* or *trans* configurations of the luciferase expression transposons (Fig. 1) were administered by hydrodynamic tail vein injection. Twenty mice were injected for each group and organs from four mice were harvested for analysis on each of the indicated days postdelivery. (a) Sample bioluminescence images after *trans* (left) or *cis* (right) injections; the photon emissions and exposure times are given below the images, as in Fig. 2. The pink lines represent the upper borders of the diaphragm and rib cage, determined by visual inspection in the treated mice. (b) Luciferase expression 24–48 hr postinjection; more than 99% of the expressing plasmids are directed to the liver. (c) Graphical representation of luciferase expression over 2 weeks for both the *cis* (solid lines) and *trans* (dashed lines) configurations of transposon DNA. The standard errors are shown for each group of mice and the dotted black line shows the best fit for exponential decay. (d) Quantitative measurements of luciferase expression in isolated homogenized liver and lung samples harvested from mice at the indicated times. Luciferase expression in isolated organs after injection of *cis* (solid lines) and *trans* (dashed lines) configurations of transposon DNA is shown, and the dotted black line shows the best fit for exponential decay. There were no significant differences between expression from the *trans* constructs and the *cis* constructs.

case of liver, for the entire period. Whereas the decay in transgene expression in the liver was essentially constant with a half-life of 39 ± 3 hr, in the lung the initial half-life of Luc activity was an astonishing 6 ± 1 hr that leveled off to about 90 hr between 10 and 14 days after injection of the PEI/DNA complexes. The drop in gene expression in the lung was about 100-fold faster than in the liver over the first week (about 2000-fold in lung, compared with 20-fold in liver). As a result, whereas lung was 10-fold more active than liver in transgene expression 1 day after delivery, by 2 weeks the liver exhibited 10 times more activity than the lung, with a cross-over occurring at about 4 days postinjection. Thus, the rapid initial rate of loss in gene expression detected by whole-body bioluminescence imaging is due primarily to a steep decline in the lung and thereafter in the liver.

Transposition rates vary in the lung, depending on the method of plasmid delivery

We have previously shown that transposition is required to sustain long-term gene expression (Belur *et al.*, 2003; Aronovich *et al.*, 2007). Hence, the rapid loss of activity in the

lung suggests either that there was little transposition occurring in this tissue after PEI-mediated delivery or that cells taking up the plasmids were subsequently lost. Accordingly, we employed real-time qPCR to quantify the number of excision products (EPs, which are plasmids that have lost transposons) as a measure of transposition activity (G. Liu *et al.*, 2004; Aronovich *et al.*, 2007, 2009). The number of excision events was determined for just the pKT2/dRIL plasmid because expression from this construct matched that from the pT2/CAL//PGK-SB13 construct whether they were delivered hydrodynamically or with PEI. We found that excision events were detected in the liver after either hydrodynamic or PEI-mediated delivery but in the lung only after PEI-mediated delivery (Table 2). Our inability to detect transposition events in the lung after hydrodynamic delivery was due to the low level of plasmid delivery to this organ (Fig. 3b and d). We averaged the results from three different days for each of the four conditions and assumed two GAPDH genes per cell to anchor the number of excision events per cell. Our calculations indicate that transposition occurred in about 0.2% of liver cells and in less than 1 in 1 million lung cells after hydrodynamic injection compared with about

FIG. 4. *In vivo* imaging and quantitative measurement of luciferase expression after PEI-mediated delivery. Either the *cis* or *trans* configurations of the luciferase expression transposons (Fig. 1) were administered by standard tail vein injection after complexation with PEI. Thirty-seven mice were injected with the *cis* configuration and 32 mice were injected with the *trans* configuration and organs from four mice were harvested for analysis on each of the indicated days postdelivery. (A) Sample bioluminescence images after injections of the *trans* constructs (left) or the *cis* construct (right). The photon emissions and exposure times are given below the images, as in Fig. 2. The pink lines represent the upper borders of the diaphragm and rib cage, determined by visual inspection, in the treated mice. (B) Graphical representation of luciferase expression over 2 weeks for both the *cis* and *trans* configurations of transposon DNA. The standard errors are shown for each group of mice and the dotted black lines that are broken into two time intervals, days 1–4 and days 7–14, show the best fits for exponential decay. (C) Quantitative measurements of luciferase expression in isolated liver and lung samples harvested from mice killed at the indicated times after delivery of the *cis* and *trans* configurations of transposon DNA. The best fits for exponential decay are shown by the dotted black lines; for liver the decay is over the entire 2 weeks, with decay kinetics determined for two time intervals, days 1–4 and days 7–14, for lung. There were no significant differences in expression between the *trans* versus the *cis* constructs.

TABLE 2. QUANTITATIVE PCR ANALYSIS OF SLEEPING BEAUTY TRANSPOSITION AFTER PLASMID DELIVERY^a

Tissue	Harvest	Hydrodynamic injection				PEI-mediated delivery			
		C _T				C _T			
		EPs	GAPDH	ΔC _T	EPs/cell	EPs	GAPDH	ΔC _T	EPs/cell
Liver	Day 1	34	20	14	1/16,000	31	19	12	1/4,100
	Day 4	31	22	9	1/510	30	21	11	1/510
	Day 7	32	23	9	1/510	34	21	13	1/8,200
	Average ^b	32	22	10	1/1,000	32	20	12	1/4,100
Lung	Day 1	>40	18	>22	<1/17×10 ⁶	30	18	12	1/4,100
	Day 4	>40	20	>20	<1/4×10 ⁶	30	17	13	1/8,200
	Day 7	>40	16	>24	<1/67×10 ⁶	33	17	16	1/66,000
	Average ^b	>40	18	>12	<1/17×10 ⁶	31	17	14	1/16,000

Abbreviations: C_T, PCR threshold cycle; EPs, excision products; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
^aThe average C_T values for each of the PCR reactions is shown. There were four mice per sample and each set was run in duplicate; values are ± 1 cycle.
^bAverage values are rounded to two places.

0.04% of liver cells and 0.01% of lung cells after PEI-mediated delivery. These results suggest that the rate of transposition in the liver was slightly higher than in the lung after PEI-mediated delivery, which is consistent with the higher level of expression in the liver 2 weeks after PEI-mediated delivery compared with the lung (Fig. 4C). The higher level of transposition per cell in the liver compared with the lung explains the temporal shift of luciferase expression from the lung to the liver after PEI-mediated delivery compared with hydrodynamic delivery.

Discussion

Efficient delivery of DNA into cells of specific tissues is the major challenge for nonviral gene therapy. Our results show that condensation of DNA with PEI results in rapid uptake and expression of transgenic DNA in both lung and liver after intravenous injection. However, gene expression in the lung was lost at an astonishing rate, with a half-life of about 6 hr. Consequently, although 1 day postinjection transgene expression in the lung was 10-fold higher than in the liver, within 4–7 days most transgene expression was observed in the liver, where it was lost at a much slower rate ($T_{1/2}$ = 35–40 hr). This shift was evident in the bioluminescence images (Fig. 2a and Fig. 4A) of expression in living mice as well as in extracts of harvested organs (Fig. 4C). Because there are about 5-fold more cells in the liver than in the lung, the overall shift in levels of expression from lung to liver is even greater, about 500-fold over 6 days.

The 6-hr half-life of luciferase gene expression in the lung could be due to several factors, including the following: (1) the instability of plasmids, which in the cytoplasm (where they are not transcribed but could be a reservoir for delayed entry into the nucleus) may last for only a couple of hours (Lechardeur *et al.*, 1999); (2) the duration of transcription of the transgene, which varies depending on the promoter and stability of the DNA; (3) the half-life of the mRNA, which is about 1 hr in cultured cells for luciferase mRNA (Chrzanowska-Lightowlers and Lightowlers, 2001); and (4) the half-life of the protein, which for luciferase is about 1–4 hr (Carlsen *et al.*, 2002; Baggett *et al.*, 2004), similar to that for the widely used reporter β -galactosidase (about 3–4 hr in the lung; Scherpereel *et al.*, 2001). These available numbers for the stability of luciferase mRNA and protein suggest that the duration of luciferase expression correlates directly with transcription of the luciferase reporter gene. Hence, the half-life of 6 hr in the lung compared with the 36- to 40-hr half-life in the liver indicates that either (1) PEI-mediated delivery to the lung results in almost immediate breakdown of 99% of the plasmids taken up into the cells in the lung; (2) there is rapid down-regulation of the CAGGS promoter; or (3) there is PEI-mediated toxicity in cells. PEI toxicity in the lung, perhaps due to aggregation of the PEI/DNA complexes in the microvasculature, has been previously reported (Moghimi *et al.*, 2005; Sharma *et al.*, 2005). PEI alone or complexed with DNA induces a substantial increase in the number of CD11b-positive cells deposited on the surface of the lung endothelium as soon as 30 min postinjection, peaking at 3 hr postinjection (Chollet *et al.*, 2002). The initial burst of gene expression seen at 24 hr may occur in cells that do not survive over the next 3 days, when we took our second set of measurements. This is apparently not the case in the liver, where the decay is nearly

constant over the first 2 weeks after delivery. The similar decay kinetics in both liver and lung over the first 2 weeks after hydrodynamic delivery (Fig. 3d), which are about the same as that for the liver after PEI-mediated delivery (Fig. 4C), strongly support the interpretation of PEI-mediated cellular toxicity specifically in the lung. Declining gene expression in the liver after either hydrodynamic or PEI-mediated delivery most likely is the result of degradation of unintegrated plasmids during the period immediately after infusion. The subsequent slower loss of expression is most probably due to epigenetic silencing of integrated or unintegrated plasmids, coupled with slow cellular turnover and loss of unintegrated plasmids. This interpretation is consistent with what we find for expression of the *Sleeping Beauty* gene after hydrodynamic delivery (Bell *et al.*, 2006).

PEI-mediated toxicity in the lung is supported by the results from our quantification of excision products (Table 2). Normally, excision products are stable in the liver (Bell *et al.*, 2006; Aronovich *et al.*, 2007, 2009); but, as can be seen in the lower right quadrant of Table 2, over 7 days the number of excision products in the lung decreases more than 10-fold on a per-cell basis, which could be the result of cell death in those cells that took up PEI/DNA complexes. After 2–4 weeks, we observed that luciferase expression stabilized in both lung and liver when transposons were delivered with a source of transposase, which is consistent with expression from transgenes that have transposed into chromatin in the lungs (Belur *et al.*, 2003; L. Liu *et al.*, 2004) as well as in the liver (Yant *et al.*, 2000; Ohlfest *et al.*, 2005b; Aronovich *et al.*, 2007). Although targeting of the liver with ligands has been reported previously (Kren *et al.*, 2003), in our studies using linear or branched, 25- or 70-kDa PEI, we have found in general that when ligands are added to either primary or tertiary amine groups on PEI and other complexing agents, luciferase activities are almost always 10- to 100-fold lower than without the ligands (data not shown), which may be due to aggregates that are too large to enter cells. Indeed, aggregation of PEI/DNA complexes has been known to severely undermine their utility for gene delivery into mammalian cells (Ogris *et al.*, 1999; Sharma *et al.*, 2005), thus compromising conditions for injection that are critical for success (Zou *et al.*, 2000). Hence there has been an intense effort to shield these complexes with other polymers such as polyethylene glycol (PEG) with or without further modifications. However, in general these methods have resulted in gene delivery that has been 100- to 1000-fold lower than that achieved by hydrodynamic injection (Fig. 3c vs. Fig. 4b; Chen *et al.*, 2007).

In terms of the overall level of expression achieved, we note that the levels of luciferase activity 2 weeks postinfusion were 100- to 1000-fold lower after PEI-mediated delivery compared with hydrodynamic injection under the conditions we employed. On the basis of our experience and that of others who have successfully used hydrodynamic injection to treat murine models of human genetic disease (Yant *et al.*, 2000; Ohlfest *et al.*, 2005b; Aronovich *et al.*, 2007, 2009), it is clear that this procedure is the method of choice for effective *in vivo* introduction of SB transposons if not all naked DNAs, into hepatic cells. The challenge now for nonviral gene therapy is to develop effective hydrodynamic methods of gene delivery in larger animals as a staging ground for ultimate application to human disease.

Acknowledgments

The authors thank John R. Ohlfest, Elena L. Aronovich, and Joel L. Frandsen for sharing plasmid constructs, for assistance with hydrodynamic injections, and for helpful discussions. This project was supported by grants P01 HD32652 and R44 HL072539 from the National Institutes of Health to the University of Minnesota and Discovery Genomics, respectively, and by a Developmental Grant in Drug Design, 882-1010 from the Medicinal Chemistry Department (Robert Vince Fund) of the University of Minnesota.

Author Disclosure Statement

P.B.H. and R.S.M. have financial interests in Discovery Genomics, Inc., which is investigating the potential of SB transposons as a vector for human gene therapy.

No competing financial interests exist for the other authors.

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Received for publication July 22, 2009;
accepted after revision September 16, 2009.

Published online: December 30, 2009.