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Increased Maintenance and Persistence of Transgenes by Excision of Expression Cassettes from Plasmid Sequences *In Vivo*

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ABSTRACT

Persistence of transgene expression is a major limitation for nonvirus-mediated gene therapy approaches. We have suggested that covalent linkage of bacterial DNA to the expression cassette plays a critical role in transcriptional silencing of transgenes *in vivo*. To gain insight into the role of the covalent linkage of plasmid DNA to the expression cassette and transcriptional repression, and whether this silencing effect could be alleviated by altering the molecular structure of vector DNAs *in vivo*, we generated a scheme for converting routine plasmids into a purified expression cassette, free of bacterial DNA after gene transfer *in vivo*. To do this, the human α-1-antitrypsin (hAAT) and human clotting factor IX (*hfIX*) reporter genes were flanked by two I-*Sce*I endonuclease recognition sites, and coinjected together with a plasmid encoding the I-*Sce*I cDNA or a control plasmid into mouse liver. Two weeks after DNA administration, mice injected with the reporter gene alone or with the irrelevant control plasmid showed low serum levels of hAAT or hFIX, which remained low throughout the length of the experiment. However, animals that expressed I-*Sce*I had a 5- to 10-fold increase in serum hAAT or hFIX that persisted for at least 8 months (length of study). Expression of I-*Sce*I resulted in cleavage and excision of the expression cassettes from the plasmid backbone, forming mostly circles devoid of bacterial DNA sequences, as established by a battery of different Southern blot and polymerase chain reaction analyses in both C57BL/6 and *scid* treated mice. In contrast, only the input parental circular plasmid DNA band was detected in mice injected with the reporter gene alone, or an I-*Sce*I plasmid together with the hAAT reporter plasmid lacking the I-*Sce*I sites. Similar results were obtained when the Flp recombinase system was used to make mini-plasmids in mouse liver *in vivo*. This study presents further independent evidence that removing the covalent linkage between plasmid and transgene sequences leads to a marked increase in and persistence of transgene expression. Unraveling the mechanisms by which the covalent linkage of bacterial DNA to the expression cassette is connected to gene silencing is fundamental to establishing the mechanism of transcriptional regulation in mammalian systems and will be important for the development of versatile nonviral vectors that can be used to achieve persistent gene expression in different cell types.

OVERVIEW SUMMARY

The short duration and shutdown of transgene expression are important limitations to overcome for many potential clinical gene therapy applications. We have previously suggested that covalent linkage of bacterial DNA to the expression cassette may play an important role in transcriptional silencing. To overcome the negative effect of covalent linkage in gene repression we have generated a scheme for converting routine plasmids into a purified expression cassette, free of bacterial DNA after gene transfer *in vivo*. To do this, the human α-1-antitrypsin (hAAT) and human clotting factor IX (*hfIX*) reporter genes were flanked by two I-*Sce*I endonuclease recognition sites, and co-injected together with a plasmid encoding the I-*Sce*I cDNA into mouse liver. Our studies showed that I-*Sce*I-treated mice formed mostly small circles containing the expression cassette vector DNA, free of plasmid DNA sequences. The removal of the covalent linkage between plasmid and transgene sequences led to a marked increase in the level and persistence of transgene expression. These studies further establish the importance of removing the bacterial plasmid backbone from the expression cassette to achieve optimal transgene expression *in vivo*.

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INTRODUCTION

The ability to achieve reproducible, persistent, and therapeutic levels of gene expression *in vivo* by nonviral gene transfer approaches has been problematic. Episomal transgene expression in mice is silenced shortly after administration of exogenous DNA. Typically, a high level of transgene expression is detected shortly after DNA is delivered to target cells, but this expression is rapidly silenced within a few weeks, even though vector DNA remains in these cells (Wu and Wu, 1987; Zhang *et al*., 1999; Nishikawa *et al*., 2000; Yant *et al*., 2000; Chen *et al*., 2001; Niidome and Huang, 2002; Herweijer and Wolff, 2003). The short duration and the shutdown of transgene expression are important limitations to overcome for many potential clinical gene therapy applications (Wu and Wu, 1987; Zhang *et al*., 1999; Nishikawa *et al*., 2000; Yant *et al*., 2000; Niidome and Huang, 2002; Herweijer and Wolff, 2003).

Although improvement has been achieved in prolonging gene expression in several tissues (Wolff *et al*., 1992; Hartikka *et al*., 1996; Chen *et al*., 2001; Gill *et al*., 2001; Miao *et al*., 2001, 2003; Nicol *et al*., 2002), the mechanisms underlying gene silencing are complex and remain unclear. Understanding the mechanisms required for persistent gene expression will be important before clinical implementation. Because one of our major interests is to develop versatile nonviral vectors that can be used to achieve persistent gene expression in different cell types, we have focused on unraveling the processes responsible for gene silencing *in vivo*. In a previous study, we made progress in understanding these episomal transgene silencing mechanisms by determining how the molecular structure of the DNA vectors delivered into mouse liver affected the persistence of transgene expression *in vivo*. Thus, transgene expression from closed circular DNA (ccDNA) plasmids was lost soon after delivery, whereas linearized DNA containing only the expression cassette (obtained by cutting twice through the plasmid ccDNA) expressed high levels of transgene product that persisted for up to 9 months (Chen *et al*., 2001). Interestingly, this linear DNA formed large random concatemers as well as small circles, whereas the ccDNA remained intact in mouse liver. Accordingly, we hypothesized that either concatemer formation or removal of the expression cassette from the plasmid backbone was responsible for the enhanced gene expression (Chen *et al*., 2001). By altering the DNA components in both the expression cassette and the bacterial backbone before delivery into mice, we have demonstrated that the bacterial DNA silencing effect is independent of the sequence, and that covalent linkage of the bacterial and expression cassette DNA was required for transcriptional repression *in vivo* (Chen *et al*., 2004). In other words, a physical connection between the two DNA components was required for the silencing effect to occur (Chen *et al*., 2004). These observations were in accordance with previous studies showing increased gene expression when either polymerase chain reaction (PCR) products (Felgner and Liang, 1999; Sykes and Johnston, 1999; Hofman *et al*., 2001; Liang *et al*., 2002) or capped DNA (Zanta *et al*., 1999) was delivered into animals. Taken together, the data suggest that covalent linkage of bacterial DNA to the expression cassette may play an important role in transcriptional silencing of the transgene *in vivo*.

We took this observation for further study by attempting to establish whether silencing could be alleviated by generating *in vivo* expression cassettes free of bacterial DNA after plasmid vector delivery *in vivo*. To do this, human α-1-antitrypsin (hAAT) and human clotting factor IX (*hfIX*) expression cassettes were flanked by two I-*Sce*I meganuclease recognition sites and coinjected together with a plasmid encoding the I-*Sce*I cDNA or a control plasmid into mouse liver. I-*Sce*I is a rare-cutting and highly specific endonuclease that has been widely used in cells from many organisms (reviewed in Jasin, 1996) and in experiments ranging from DNA repair to genome analysis (Mahillon *et al*., 1998; Pipiras *et al*., 1998; Anglana and Bacchetti, 1999).

MATERIALS AND METHODS

Vector construction

Construction of the plasmids pBShAAT, containing the human α-1-antitrypsin (hAAT) cDNA under the control of the Rous sarcoma virus (RSV) long terminal repeat promoter, and pT-CM1, containing the human clotting factor IX cDNA (*hfIX*) under the control of a hybrid promoter/enhancer composed of the hAAT promoter and the hepatic locus control region (HCR) from the ApoE gene, was previously described (Chen *et al*., 2001). To prepare the pBSATI plasmid, a pBluescript KS+ (Stratagene, La Jolla, CA) containing the hAAT expression cassette flanked by two I-*Sce*I sites, we first generated an I-*Sce*I site at the *Xho*I site in pBluescript KS+, using the primers 5′-TCGATAGGGATAACAGGGTAAT-3′ and 5′-TCGAATTACCCTGTTATCCCTA-3′, resulting in an intermediate plasmid, pBS-I-*Sce*I. After I-*Sce*I digestion of the hAAT cDNA cassette from the pHN3-hAAT plasmid (a gift from H. Mizuguchi, Stanford, CA), the cassette was inserted into the I-*Sce*I site of the pBS-I-*Sce*I plasmid, to obtain the pBSATI plasmid. Schematic maps and molecular size of the various DNA species used in this study are shown in Fig. 1A and B. To obtain the pfrtFIXI plasmid, a pBluescript KS+ containing the *hfIX* expression cassette flanked by I-*Sce*I and Flp recognition target (*frt*) sequence sites, we generated an I-*Sce*I site at the *Xho*I site in the pBSfrt plasmid (a gift from S. Yant, Stanford, CA), using the primers described above, to obtain the intermediate plasmid pBSfrtI. We isolated the *hfIX* expression cassette from the pT-CM1 plasmid by *Sac*I/I-*Ceu*I digestion and inserted it into the pHN3-hAAT plasmid, replacing the hAAT expression cassette and resulting in the intermediate plasmid pHN3-hFIX. Finally, from there the *hfIX* cassette was released by cutting with I-*Sce*I and then transferred into the I-*Sce*I site of pBSfrtI, yielding plasmid pfrtFIXI. The CMV/I-*Sce*I plasmid was kindly provided by M. Calos (Stanford, CA). A schematic diagram of the expected DNA molecular forms after I-*Sce*I cleavage is shown in Fig. 1C.

DNA and RNA preparation

Plasmid DNA was amplified in DH10B cells (GIBCO-BRL; Invitrogen, Carlsbad, MD) and purified with endotoxin-free kits (Qiagen, Valencia, CA). All plasmid preparations were filtered through a 0.22-μm pore size syringe filter unit and were dialyzed against Tris–EDTA (TE) for 24 hr before *in vivo* admin-

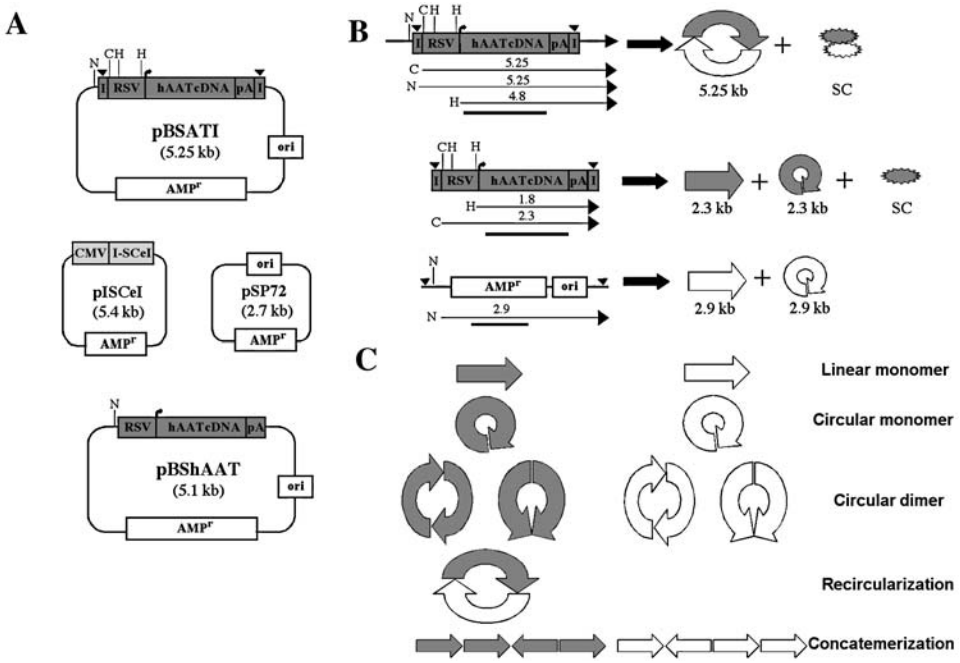

FIG. 1. Maps and restriction enzyme sites of input plasmids. (**A**) Maps and molecular size of the plasmids used for injection (I, I-*Sce*I cleavage site, also indicated by solid triangles; *ori*, plasmid origin of replication; AMP^r, ampicillin resistance gene; pA, bovine growth hormone polyadenylation signal; see text for plasmid abbreviations). (**B**) Diagrams of predicted forms and sizes (kb) of the DNA molecules obtained after digestion with restriction enzymes are shown with either the input parental plasmid (*top*), or in the linear expression cassette monomer (*middle*) and the bacterial backbone monomer (*bottom*). SC, circular supercoiled forms (these DNA species will migrate faster than their linear/relaxed circular forms). Thick bars indicate probes used in the Southern blot analyses. The letters on the left represent the enzyme used in the experiment: C, I-*Ceu*I; H, *Hin*dIII; N, *Not*I. (**C**) Schematic representation of the likely DNA forms after I-*Sce*I cleavage in pBSATI+I mice (gray arrows, expression cassette DNA forms; white arrows, bacterial backbone DNA forms).

istration. Total RNA was obtained from liver, using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. RNA samples (10 μg) were electrophoresed on a 1% agarose gel containing formaldehyde (2.2 mol/liter). Northern blots were hybridized to an [α-^{32}P]dCTP-labeled I-*Sce*I cDNA probe. The 18S signal was used to correct for loading inequalities.

Determination of transgene expression in cells

293T cells were obtained from the American Type Culture Collection (Manassas, VA), cultured in Dulbecco's modified Eagle's medium (DMEM) containing high glucose, 10% fetal bovine serum (FBS), and 1× penicillin–streptomycin, and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were plated on 6-cm-diameter dishes the day before cotransfection with 4 μg of pBShAAT and pBSATI together with 1 μg of either pCMVI-*Sce*I or pBluescript, using SuperFect (Qiagen). A "humanized" green fluorescent protein (gfp) expression plasmid, pTRUF2 (Zolotukhin *et al*., 1996), was separately transfected and cells were analyzed the next day by fluorescence microscopy to guarantee high and consistent transfection efficiencies. Cells were washed off the plates 3 days after transfection, and extrachromosomally replicated DNA was extracted by a modified Hirt protocol (Grimm *et al*., 1998) and subjected to Southern blot analyses as described below. All transfections were done in triplicate.

Animal studies

We obtained 6- to 8-week-old C57BL/6 mice and DNA protein kinase-deficient (DNA-PK$^-$) C57BL/6 *scid* mice from the Jackson Laboratory (Bar Harbor, ME). We delivered 20 μg of ccDNA expression cassette into C57BL/6 and C57BL/6 *scid* mice together with 1, 5, or 20 μg of pCMVI-*Sce*I and 5 μg of pCMVFlpe or pSP72 (stuffer plasmid DNA) to mouse liver by a hydrodynamics-based technique. The amount of I-*Sce*I- and Flp-expressing DNAs was determined on the basis of the molecular weight of the DNA molecule, such that mice would receive the same number of molecules in comparable groups. This technique was used because this is the most efficient way to deliver DNA into the hepatocytes of experimental animals *in vivo* (Hartikka *et al*., 1996; Zhang *et al*., 1999; Herweijer and Wolff, 2003). We collected mouse blood periodically by a retroorbital procedure and determined serum hAAT and hFIX by enzyme-linked immunosorbent assay (ELISA) as described previously (Chen *et al*., 2003). All animals were treated according to National Institutes of Health (Bethesda, MD) and Stanford University (Stanford, CA) animal care guidelines.

Southern blot analysis of vector DNA structures in mouse liver

Mice were killed 1 day or 8 months after DNA injection and liver DNA was prepared by a salting-out method, as previously described (Chen *et al*., 2001). For Southern blot analyses, 10 μg of DNA from mouse liver injected with the hAAT and FIX vectors was digested with several restriction enzymes in order to distinguish between the various molecular forms. The expected band sizes are depicted in Fig. 1B. The vector genome copy number standards (the number of vector genomes per diploid genome equivalent) were total liver DNA from a naive mouse mixed with the appropriate amount of control plasmid. DNA was then separated by ethidium bromide agarose gel electrophoresis and transferred to nitrocellulose. Membranes were hybridized to [α-^{32}P]dCTP-labeled probes corresponding to either 1.3-kb hAAT, 1.7-kb *hfIX*, or 0.6-kb pBluescript fragments and subjected to autoradiography and phosphoimager analysis. Band intensities were quantified with a G710 calibrated imaging densitometer (Bio-Rad, Hercules, CA).

ATP-dependent DNase- and PCR-based analyses

Ten micrograms of DNA from pBShAAT and pBSATI ± pCMVI-*Sce*I samples digested with *Not*I was heat inactivated and treated for 4 hr at 37°C with 40 U of plasmid-safe ATP-dependent DNase (Epicentre Biotechnologies, Madison, WI), a nuclease that selectively hydrolyzes linear double-stranded DNA (dsDNA) and shows low affinity for either closed circular supercoiled or nicked circular dsDNAs. After completion of the digestion, DNA was extracted with phenol–chloroform, ethanol precipitated, and analyzed by Southern blot, as described above.

PCR analyses were also performed for the detection of small circles. Specific oligonucleotide primers were chosen to yield a product uniquely in the presence of small circles, but not with either monomeric linear DNA or parental plasmid. We analyzed the DNA by PCR before and after the addition of ATP-dependent DNase, using oligonucleotide primers 5′ (5′-GAGGCCATGGATGCGATCGCTGCG-3′) and 3′ (5′-ATTGCGTCGCATCGACCCTGCGCC-3′). PCR products (expected size of 1.1 kb) were analyzed by ethidium bromide gel electrophoresis, gel purified, and subjected to direct DNA sequencing.

Statistical analysis

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

RESULTS

Generation of transgene expression cassette free of bacterial DNA by in vitro *expression of I-*Sce*I*

We cotransfected 293T cells with pBSATI (containing the hAAT expression cassette with the I-*Sce*I sites) together and pCMVI-*Sce*I (containing the I-*Sce*I expression cassette) to test our hypothesis that I-*Sce*I-mediated cleavage would result in the formation of a transgene expression cassette free of bacterial plasmid DNA. To ensure that either the I-*Sce*I endonuclease by itself or the presence of the recognition sites did not affect transgene expression, we did additional control studies in which we cotransfected 293T cells with a plasmid containing the hAAT expression cassette but lacking the I-*Sce*I sites (pBShAAT), together with the I-*Sce*I-expressing plasmid or stuffer DNA. Figure 1 presents the plasmid designation and molecular size of the various DNA species used in this study. After digesting DNA samples from transfected cells with *Nco*I (a restriction enzyme that does not cut within the plasmids) and hybridizing with the hAAT cDNA probe, we could detect two low molecular weight bands in the pBSATI plus I-*Sce*I (pBSATI+I)-transfected cells (about 1.4 and 2.3 kb), which probably corresponded to single supercoiled religated dsDNA monomers and minicircular/linear monomers of the expression cassette free of bacterial DNA, respectively (Fig. 2A).

In addition, when digesting with *Not*I, a restriction enzyme that cuts once outside the transgene, only the parental input plasmid DNA was detected in the control cells, which were transfected with the hAAT control plasmids mentioned above (Fig. 2B). In contrast, in the pBSATI+I cells we observed an additional DNA species, which corresponded to the generation of large concatemers and circular monomeric dsDNA (Fig. 2B). Furthermore, when rehybridizing the same Southern blot with a specific probe for the bacterial backbone, the parental plasmid containing the hAAT expression cassettes and/or circular monomers of the stuffer plasmid were also detected in the control cells (Fig. 2C). In the pBSATI+I-transfected cells, we detected an extra 2.9-kb band, suggesting the formation of linear and/or circular monomeric dsDNA pBluescript (the bacterial backbone of the original plasmid pBSATI), free of the transgene (Fig. 2C).

PCR analyses were also performed after treatment with ATP-dependent DNase to further confirm the presence of small circles containing the expression cassette free of bacterial DNA. This enzyme catalyzes the hydrolysis of linear DNA, and has an extremely low affinity for circular DNA. First, we digested liver DNA with *Not*I, in order to linearize the input parental DNAs but not any minicircular monomers generated in the pBSATI+I-cotransfected cells. Second, DNase treatment was used to remove the linear DNA but not the small DNA circles. Although no PCR-amplified products were observed in the control cells, we did detect a specific 1.1-kb PCR band in the pBSATI+I-treated cells before and after DNase treatment (Fig. 2D). All these results suggested that excision of the transgene and formation of purified expression cassette free of bacterial backbone were occurring *in vitro*.

*Mice coinjected with pBSATI and I-*Sce*I show increased transgene expression*

To determine whether the hepatic production of I-*Sce*I could affect the persistence of transgene expression from a plasmid containing I-*Sce*I recognition sites flanking the transgene expression cassette *in vivo*, we coadministered the plasmid pBSATI together with various concentrations of pCMVI-*Sce*I into mice. Whereas control mice co-injected with pBSATI together with a stuffer plasmid showed low levels of hAAT 2 weeks after vector administration, mice expressing I-*Sce*I from 1, 5, or 20 μg of plasmid had about 10 times higher levels of the transgene product throughout the length of the experiment (Fig. 3A). In addition, no differences were observed in the

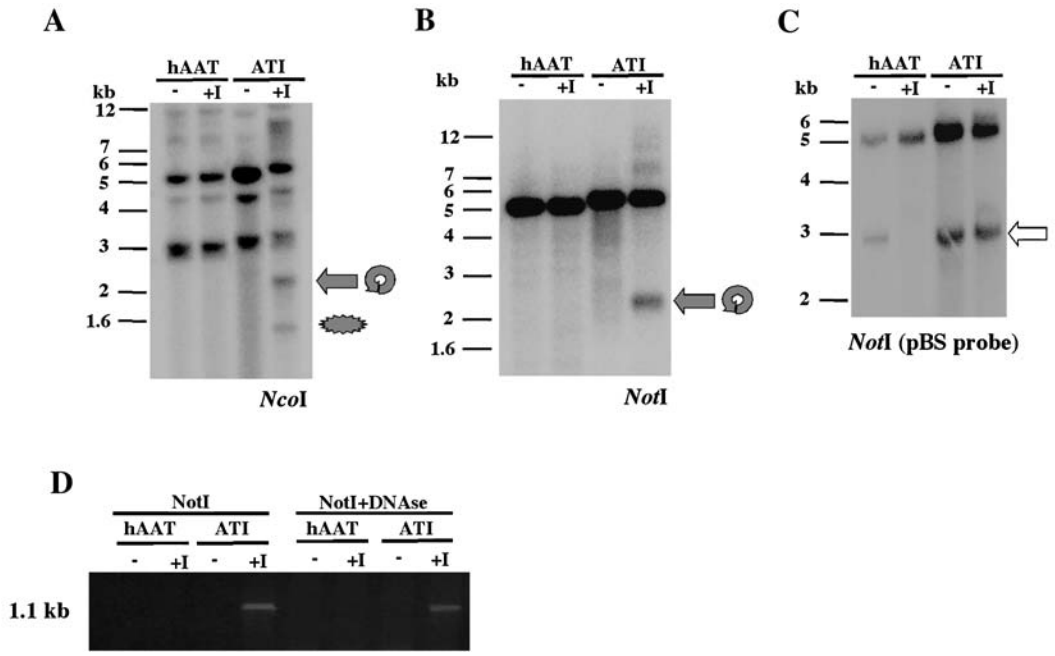

FIG. 2. I-*Sce*I expression in transfected 293T cells. Ten micrograms of Hirt DNA obtained from cells transfected with 4 μg of pBSATI and pBShAAT and 1 μg of either I-*Sce*I plasmid (+I) or pSP72 (−I) was digested with (**A**) *Nco*I and (**B**) *Not*I and probed with hAAT cDNA. A schematic with the proposed structures based on Fig. 1B and C is drawn to indicate the additional bands detected in the pBSATI+I mice (see text for explanation). (**C**) A Southern blot containing 10 μg of Hirt DNA digested with *Not*I and hybridized with hAAT cDNA was reprobed with a plasmid-specific probe, as indicated in Materials and Methods. (**D**) PCR analysis of Hirt DNA samples treated with ATP-dependent DNase from 293T cells transfected with the plasmids indicated above, as described in Materials and Methods.

serum levels of hepatic transaminase ALT between the experimental groups (data not shown), suggesting that the expression of I-*Sce*I in mice was not toxic. Moreover, 2 weeks after administration of the pBShAAT control plasmid together with the I-*Sce*I-expressing plasmid or stuffer DNA, mice showed low serum levels of hAAT, which remained low throughout the length of the experiment (Fig. 3B). These results suggested that expression of I-*Sce*I or the presence of the recognition sites alone did not affect transgene expression.

Early in vivo *excision of transgene expression cassette from plasmid backbone by I-*Sce*I expression*

We hypothesized that I-*Sce*I-mediated cleavage would also result in the formation of a transgene expression cassette free of bacterial plasmid DNA *in vivo*. To confirm this, we analyzed liver DNA from mice 24 hr after injection with the various plasmids as described above. After digestion with *Not*I, and probing with the hAAT cDNA, all control mice showed a single DNA band corresponding to the input parental plasmid (Fig. 4A). In contrast, in the pBSATI+I group, we detected a high molecular weight band (>12 kb) and two different low molecular weight bands (about 1.4 and 2.3 kb) (Fig. 4A). Previously, we described the generation of large concatemers and circular monomeric DNA after injection of various combinations of linearized DNA (Chen *et al*., 2001, 2004). Accordingly, because *Not*I did not cut the expression cassette, the >12-kb band possibly corresponded to undigested large concatemers formed by fusion only of I-*Sce*I-derived transgenes. Indeed, another possibility is that once I-*Sce*I cuts the parental plasmid, large concatemers can be formed by fusion of random expression cassettes and plasmid molecules (they maintain the *Not*I site), which also could be responsible for the presence of high molecular weight bands (Fig. 4A). As described previously, the 1.4-kb band probably corresponded to single supercoiled religated dsDNA monomers, whereas the 2.3-kb band represented linear fragments of the expression cassette (Fig. 4A). Thus, the presence of these extra bands observed in the pBSATI+I mice also suggested the formation of large concatemers and linear and/or circular monomeric dsDNA relaxed/supercoiled forms of the transgene free of bacterial backbone *in vivo* (Fig. 4A).

When rehybridizing the same Southern blot with a probe specific for the bacterial backbone, the parental plasmid was also detected in all groups (Fig. 4B). In addition, in the control groups co-injected with pSP72 we detected a 2.7-kb band, corresponding to circular monomers derived from the stuffer plasmid (Fig. 4B). In the pBSATI+I-injected mice we detected an additional 2.9-kb band, suggesting the formation of linear and/or circular monomeric pBluescript (the bacterial backbone of the original plasmid pBSATI), free of the transgene. In the control group coinjected with pBShAAT+I, the 2.9-kb band was not detected (Fig. 4B). Taken together, these results indicated that the expression of I-*Sce*I was also able to specifically excise the expression cassette and generate DNA molecules containing the transgene without bacterial DNA *in vivo*. The molecular rearrangement appeared to be responsible for the ob-

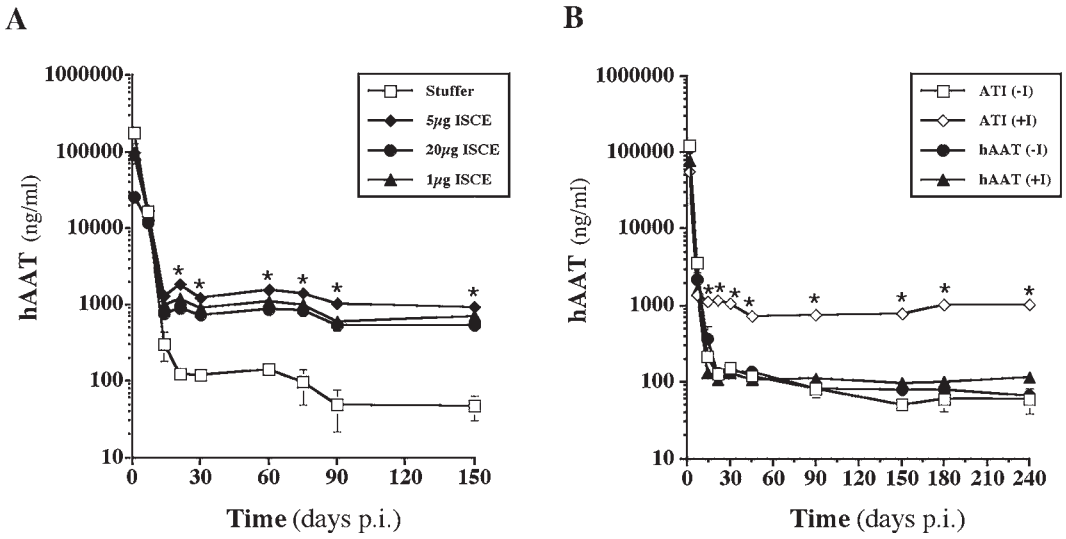

FIG. 3. Human α-1-antitrypsin expression in I-*Sce*I-injected mice. (**A**) Serum hAAT levels in mice injected with 1, 5, and 20 μg of I-*Sce*I plasmid or the stuffer plasmid pSP72. Data represent means ± SEM of six mice per group. $*p < 0.05$. (**B**) Serum hAAT production in mice injected with 20 μg of pBSATI and pBShAAT and 5 μg of either I-*Sce*I plasmid (+I) or pSP72 (−I) (n = eight mice per group). $*p < 0.05$.

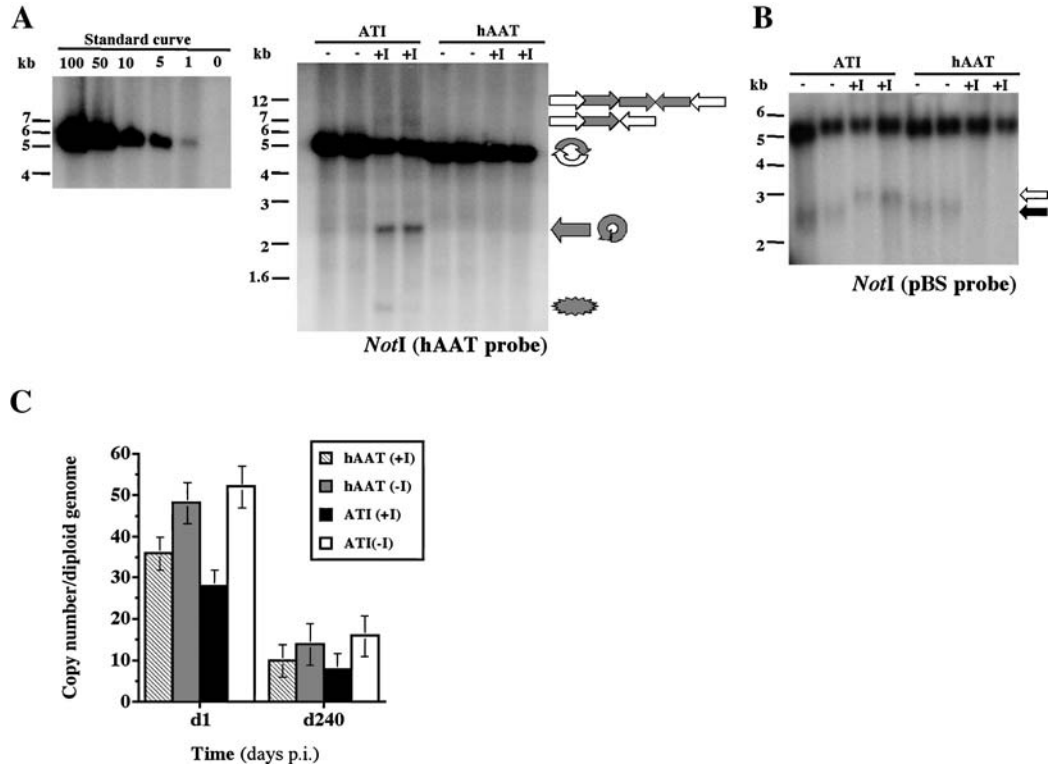

FIG. 4. Southern blot analysis of mouse liver DNA 24 hr after injection with hAAT, I-*Sce*I (+I), or pSP72 (−I) plasmids. (**A**) *Left*: *Not*I (cuts the input plasmid once) digestion for determination of plasmid copy number per diploid genome (0–100 are copy number standards; pBSATI was used for the standards.) *Right*: DNA molecular forms of plasmids. Liver DNA was digested with *Not*I. Topologies of proposed structures based on Fig. 1B and C are drawn to indicate the additional bands detected in pBSATI+I mice (see text for explanation). Each lane represents an individual mouse. hAAT cDNA was used as a probe. (**B**) Liver genomic DNA digested with *Not*I and hybridized with hAAT cDNA was reprobed with a plasmid-specific probe. Open and solid arrows indicate the presence of pBluescript and pSP72, respectively. (**C**) Vector copy number per diploid genome. Data represent means ± SEM of at least three mice per group.

served differences in transgene expression because no differences in the vector DNA copy number per diploid genome in the livers of these mice were found, excluding the possibility that the differences in transgene expression observed between the experimental groups resulted from different amounts of vector DNA (Fig. 4C).

Long-term persistence of purified forms of transgene after DNA injection

Production of these extra DNA molecular forms in the liver of pBSATI+I mice occurred as early as 24 hr after DNA injection. Eight months after injection, these mice showed a markedly higher and more persistent level of transgene expression compared with the controls (Fig. 3A). Thus, we aimed to determine whether the extra DNA species free of bacterial backbone found in the livers of pBSATI+I mice was present at late time points after the initial injection. To do this, liver DNA analyses based on a combination of Southern blot and PCR were performed. When samples from the control mice (pBSATI-I) were digested with *Nco*I and probed with hAAT cDNA, we determined that the input plasmid DNA remained intact and migrated as single-copy or multicopy aggregates (Fig. 5A). These DNAs were converted to single full-length linear molecules after digestion with the one-cutter restriction enzymes *Not*I (Fig. 5B) and I-*Ceu*I (both cut in the hAAT expression cassette) (Fig. 5C), or the two-cutter restriction enzyme *Hin*dIII (*Hin*dIII cuts twice in the hAAT transgene) (Fig. 5D). These results were consistent with our previous results, supporting the notion that input plasmid DNAs remain long-term as episomal, circular monomers in the liver after vector administration (Chen *et al*., 2001, 2003, 2004). In contrast, in addition to the input plasmid DNA, *Nco*I-treated samples from pBSATI+I-injected mice also produced a high molecular weight band (>12 kb) and two low molecular weight bands (about 2.3 and 1.4 kb) that were not present in the DNA analyzed from the control group (Fig. 5A). Production of the high molecular weight band corresponded to the formation of concatemers *in vivo* from random linkage of the various DNA fragments generated by I-*Sce*I-mediated transgene cleavage and excision, and was similar to what was observed in previous studies in which linear DNA was transfected into mouse liver (Chen *et al*., 2001, 2003, 2004).

The 1.4- and the 2.3-kb bands observed with *Nco*I digestion in the pBSATI+I mice probably represented linear and/or supercoiled religated monomers and multiaggregates of the expression cassette free of bacterial DNA, because they were converted to a 2.3-kb band (size of the full-length linear expression cassette monomer) and a medium molecular weight band (about 4 kb), corresponding to the expected size of an aggregate of multiple dsDNA copies (Fig. 5C and D). In addition, similar to the input parental plasmid, these monomer/multiple copy aggregates of the expression cassette were maintained as intact

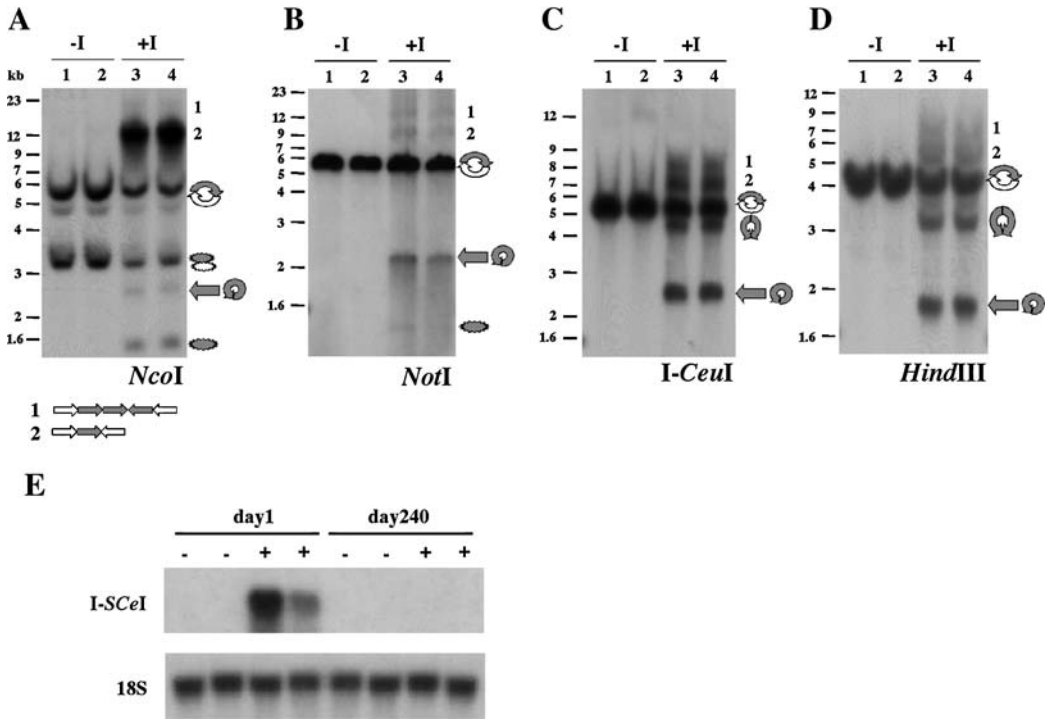

FIG. 5. Nucleic acid analysis of mouse liver DNA 24 hr after injection with pBSATI, I-*Sce*I (+I), or pSP72 (−I) plasmids. Liver DNA was digested with (**A**) *Nco*I, (**B**) *Not*I, (**C**) I-*Ceu*I, or (**D**) *Hin*dIII. Each lane represents an individual mouse. hAAT cDNA was used as a probe. Topologies of the proposed structures based on Fig. 1B and C are drawn to indicate the additional bands detected in pBSATI+I mice (see text for explanation). (**E**) Hepatic expression of I-*Sce*I. Total RNA was obtained from the liver of mice co-injected with pBSATI together with I-*Sce*I plasmid (+) or stuffer plasmid (−) on days 1 and 240 after the co-injection, as described in Material and Methods. A representative Northern blot hybridized with I-*Sce*I and 18S cDNA probes is shown. Each lane represents an individual mouse.

episomal circles in mouse liver for at least 8 months after the injection. Similar results were previously obtained when minicircle DNA vectors containing the expression cassette and devoid of bacterial plasmid DNA were injected into mice (Chen *et al*., 2003).

The expression of I-*Sce*I was specific to the pBSATI+I-injected mice, as determined by Northern blot to detect I-*Sce*I mRNA (Fig. 5E). I-*Sce*I expression was clearly observed on day 1 after injection, which paralleled the presence of the rearranged DNA molecular forms in the livers of these mice (Fig. 5A–D). Eight months after plasmid infusion, I-*Sce*I levels were undetectable (Fig. 5E) yet the pBSATI+I-treated mice still contained monomer/multiple copy aggregates of the expression cassette. Together this indicated that the formation of DNA species free of bacterial backbone found in the liver of the pBSATI+I mice occurred early after vector infusion and persisted long after I-*Sce*I expression was extinguished.

To determine whether linear or single circular supercoiled religated monomers were generated in the liver of pBSATI+I mice, we used a specific ATP-dependent DNase. We first digested liver DNA with *Not*I, in order to linearize the input parental DNAs but not the circular monomers generated in the liver of pBSATI+I mice. Further DNase treatment resulted in the removal of the 5.2-kb band corresponding to the linear input parental plasmid DNA (pBSATI) in both control (−I) and I-*Sce*I (+I)-treated mice (Fig. 6A). In contrast, a 2.3-kb band was detected after DNase treatment in the pBSATI+I mice, suggesting that circular supercoiled DNA monomers were the major species produced *in vivo* (Fig. 6A). Similar to the *in vitro* studies, PCR analyses were also performed after treatment with the ATP-dependent DNase to further confirm the presence of circular monomers containing the expression cassette free of bacterial DNA generated in the liver of pBSATI+I-treated mice. While no amplification was observed in the control mice, we could detect only a 1.1-kb PCR band in the pBSATI+I-injected mice before and after DNase treatment (Fig. 6B), which was consistent with circle formation. Production of these small circles containing the expression cassette free of bacterial DNA was detected as early as day 1 after the injection, and then also 8 months thereafter (Fig. 6B). The formation of circular supercoiled dsDNA monomers was further confirmed by sequencing of the PCR band (data not shown). Taken together, all these results suggested that expression of I-*Sce*I led to the excision of the transgene from the parental bacterial backbone and to the subsequent formation of concatemers, as well as single supercoiled religated dsDNA monomers and multiple copy aggregates, of the expression cassette free of bacterial DNA *in vitro* and *in vivo*. These molecular forms were likely responsible for the long-term persistence of transgene expression in the I-*Sce*I-treated mice.

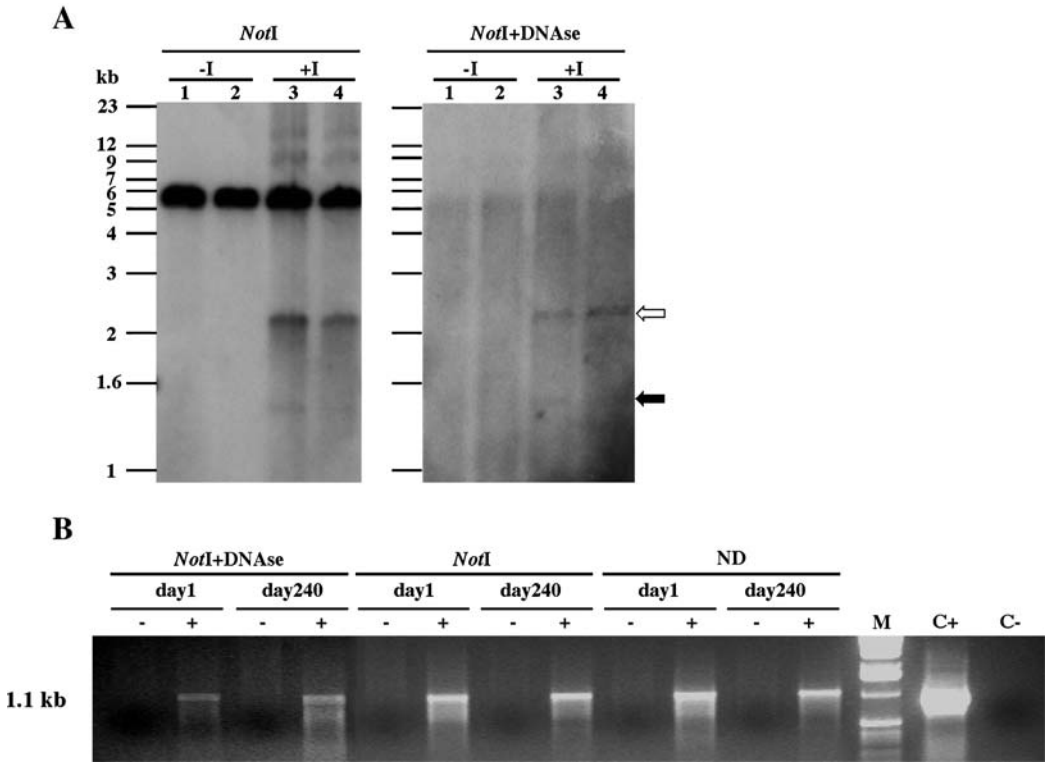

FIG. 6. Small circle production in pBSATI+I-injected mice. (**A**) Southern blot analysis. Liver DNA was digested with *Not*I (*left*) or with *Not*I and ATP-dependent DNase (*right*), as indicated in Materials and Methods. Each lane represents an individual mouse. hAAT cDNA was used as a probe. (**B**) PCR analysis of liver genomic DNA samples taken from mice on days 1 and 240 after co-injection of pBSATI plasmid together with I-*Sce*I (+) or stuffer plasmid (−), as indicated in Materials and Methods; the samples were digested with *Not*I, or digested with *Not*I plus ATP-dependent DNase, or not digested (ND). M, marker; C+, positive control; C–, negative control.

Circle formation of transgene expression cassette is independent of nonhomologous end-joining DNA repair mechanisms

To determine whether nonhomologous end-joining (NHEJ) DNA repair mechanisms were necessary for the formation of concatemers, single supercoiled religated dsDNA monomers, and multiple copy aggregates of the expression cassette free of bacterial DNA, we injected C57BL/6 severe combined immunodeficient (*scid*) mice with the pBSATI plasmid with or without I-*Sce*I. These mice are defective in the enzyme DNA-dependent protein kinase (DNA-PK), which makes them defective in DNA repair mechanisms. Like immunocompetent C57BL/6 mice, 2 weeks after administration of pBSATI together with the stuffer plasmid control mice showed low serum levels of hAAT, which remained low throughout the length of the experiment (Fig. 7A). In contrast, *scid* mice infused with pBSATI+I had a 5- to 10-fold higher level of serum hAAT (compared with the controls) that persisted for at least 7 months (length of the study) (Fig. 7A).

A Southern blot analysis was performed to identify the various DNA forms in the liver of pBSATI+I-injected mice. A single full-length linear band corresponding to the input plasmid DNA was detected in DNA from control mice (injected with pBSATI-I), after digestion with I-*Ceu*I or *Hin*dIII and probing with hAAT cDNA (Fig. 7B). In contrast, in addition to the input plasmid DNA, I-*Ceu*I- and *Hin*dIII-treated samples from pBSATI+I-injected *scid* mice produced additional DNA bands that were not present in the control group (Fig. 7B). These bands corresponded to small circles containing the expression cassette, free of bacterial DNA (momomers/dimers), similar to those observed in the pBSATI+I-injected wild-type mice described above. However, no large concatemer bands were detected in the pBSATI+I *scid* mice. This could be related to the preference for self-circularization of free DNA ends over concatemer formation, as described by Nakai and co-workers (2003b). When the vector DNA copy number per diploid genome was determined in the livers of these mice, we observed a lower vector DNA copy number in the livers of pBSATI+I *scid* mice compared with wild-type mice. Thus, greater amounts of transgene product were expressed from significantly less pBSATI plasmid, excluding the possibility that differences in transgene expression resulted from differences in the amount of vector DNA.

*Increased expression of therapeutic gene by use of I-*Sce*I and Flp endonucleases*

Flp recombinase binds to its recognition target sites (called *frt* sites), and, via homologous recombination, generates small intact dsDNA circular monomers (Ahmad and Golic, 1996; Golic *et al*., 1997). Because I-*Sce*I-mediated excision of an expression cassette from the input plasmid generated both

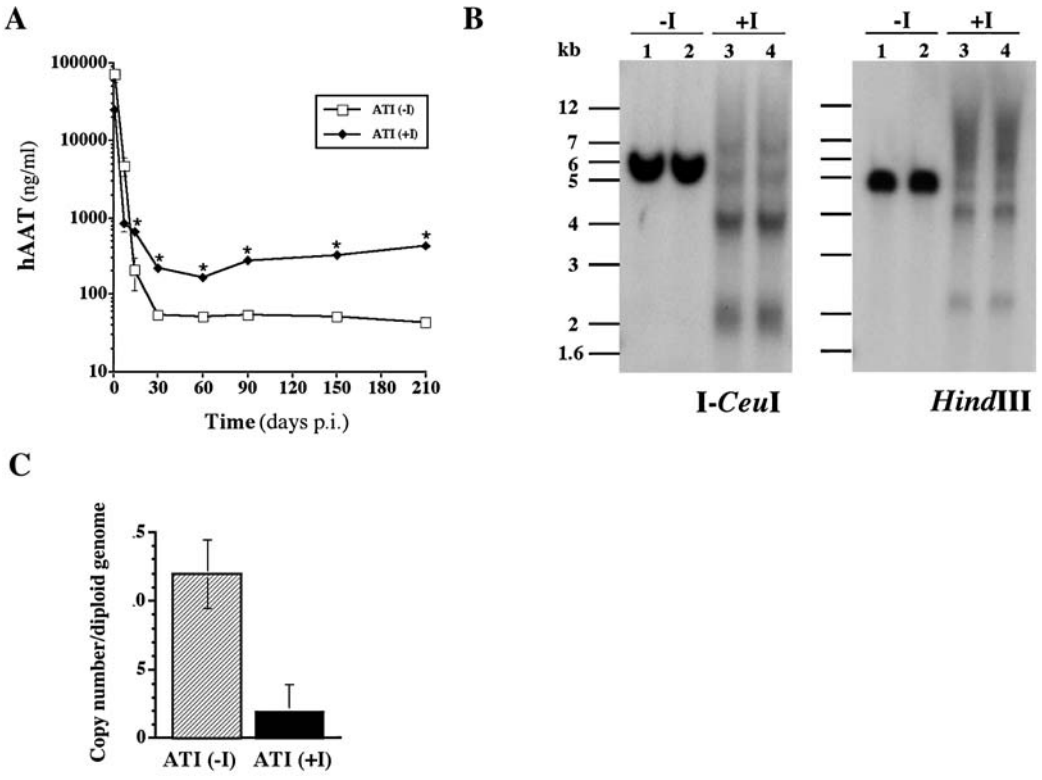

FIG. 7. I-*Sce*I expression in pBSATI+I-injected *scid* mice. (**A**) Serum hAAT production in C57BL/6 *scid* mice injected with 20 μg of pBSATI and pBShAAT and 5 μg of either I-*Sce*I plasmid (+I) or pSP72 (−I) ($n = 8$ mice per group). *$p < 0.05$. (**B**) Liver DNA Southern blot analysis. DNA samples were digested with I-*Ceu*I (*left*) or *Hin*dIII (*right*). Each lane represents an individual mouse. hAAT cDNA was used as a probe. (**C**) Vector copy number per diploid genome in pBSATI-injected *scid* mice. Data represent means ± SEM of at least three mice per group.

concatemers and small circular monomers containing the transgene free of bacterial DNA, we wanted to determine whether the effects of I-*Sce*I cleavage and Flp-mediated recombination were similar *in vivo*. We generated a human FIX expression cassette (pfrtIFIXI) flanked by two I-*Sce*I sites and two *frt* sites, and then co-injected them into mice, with the I-*Sce*I- or Flp-encoding plasmids (Fig. 8A). Control mice showed low serum levels of hFIX 1 week after administration of the pfrtIFIXI plasmid, and throughout the length of the experiment (Fig. 8B). In contrast, animals that were co-infused with the plasmid containing the *hfIX* gene flanked by I-*Sce*I and *frt* sites, and a plasmid expressing I-*Sce*I (pfrtIFIXI+I) or Flp (pfrtIFIXI+F), showed 5 to 15 times higher serum hFIX levels compared with the controls for at least 8 months (length of the study) (Fig. 8B). Importantly, the levels of hFIX observed in the pfrtIFIXI+I mice were within the therapeutic range (>0.5 μg/ml).

To determine whether I-*Sce*I or Flp expression had led to the conversion of the pfrtIFIXI plasmid into a transgene expression cassette free of bacterial DNA, we analyzed liver DNA from these mice. When digested with *Sac*I (a restriction enzyme that cuts once in the middle of the transgene) and probed with *hfIX* cDNA, all control mice showed a single 8.3-kb band corresponding to the input parental plasmid (Fig. 8C). In contrast, in liver of the pfrtIFIX+I and pfrtIFIXI+F groups, we also detected a 5.3-kb band, indicating the formation of dsDNA circular monomers containing the expression cassette, free of bacterial DNA (Fig. 8C). Furthermore, we could exclude the possibility that differences in transgene expression resulted from different amounts of vector DNA, because the plasmid copy number per diploid genome in the livers was similar in all three experimental groups (Fig. 8D).

DISCUSSION

The data presented here demonstrate that I-*Sce*I-directed release of an expression cassette from plasmid DNAs *in vivo* leads to the formation of concatemers and small circles containing the expression cassette vector DNA, free of plasmid bacterial DNA sequences, and results in an increase in transgene expression. This further corroborates our previous findings that bacterial DNA covalently attached to the expression cassette had an inhibitory effect on transgene expression. Moreover, we had shown that transcriptional repression was diminished when bacterial DNA became physically distanced or dissociated from the transgene, resulting in increased persistence of gene expression *in vivo*. We had also described that the inhibitory ef-

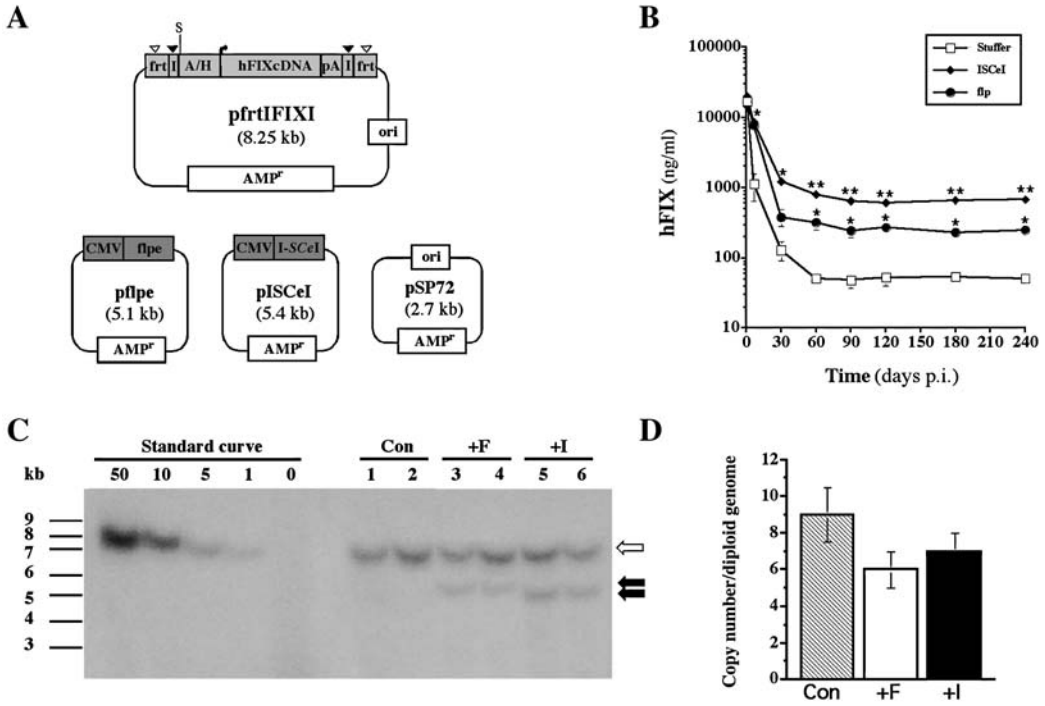

FIG. 8. Human FIX expression in I-*Sce*I- and Flp-injected mice. (**A**) Maps and molecular size of the plasmids used for injection (I, I-*Sce*I cleavage site, also indicated by solid triangles; *frt*, Flp recognition target sequence, also indicated by open triangles; *ori*, plasmid origin of replication; AMP^r, ampicillin resistance gene; pA, bovine growth hormone polyadenylation signal; see text for plasmid abbreviations). (**B**) Serum hFIX production in mice injected with 20 μg of pfrtIFIXI and 5 μg of I-*Sce*I- or Flp-expressing plasmids, or pSP72 ($n = 8$ mice per group). *$p < 0.05$, **$p < 0.01$. (**C**) Liver Southern blot analysis. DNA samples were isolated from pfrtIFIXI-injected mice, digested with *Sac*I, electrophoretically separated, and hybridized with the *hFIX* cDNA probe (Con, mice injected with stuffer plasmid; +I, mice injected with I-*Sce*I plasmid; +F, mice injected with Flp plasmid). pfrtIFIXI plasmid DNA was used for the 0–50 copy number standards. Open and solid arrows indicate the presence of input parental DNA and dsDNA circular monomers, respectively. (**D**) Vector copy number per diploid genome of pfrtIFIXI-injected mice (n = at least four mice per group).

fect of the plasmid bacterial backbone was sequence independent (Chen *et al*., 2003, 2004).

I-*Sce*I is one of the most specific homing endonucleases because of its long recognition site (>18 bp); it has not been reported to cleave in either the human or mouse genome (Jasin, 1996). Thus, one of the advantages of using I-*Sce*I is that its low frequency of cleavage at ectopic sites minimizes any deleterious effects on cell viability (Thierry *et al*., 1991; Jasin, 1996). Accordingly, we did not detect any hepatotoxicity in mice injected with the gene for this endonuclease, and the size of the DNA fragments generated by I-*Sce*I cleavage only in plasmids that contained the recognition sites indicated that the cleavage was site specific.

The presence of free DNA ends (in linear DNA fragments) is essential for efficient concatemerization and the *in vivo* production of small circles free of bacterial DNA in hepatocytes (Nakai *et al*., 2003a). In our study, I-*Sce*I expression resulted in the formation of concatemers and small circles as early as 24 hr after DNA administration, which persisted extrachromosomally for at least 8 months thereafter. The production of linear DNA by I-*Sce*I (which generates free DNA ends) likely induces the nonhomologous end joining (NHEJ) enzymatic system, which is responsible for DNA double strand break (DSB) repair in mammalian cells (Labhart, 1999). NHEJ is a complex process believed to be the major mechanism involved in ligating DNA ends in mammalian cells (Labhart, 1999). The free dsDNA ends generated by I-*Sce*I could serve as a DSB signal and a single unrepaired DSB can be lethal to cells (Bennett *et al*., 1993). Thus it is critical that I-*Sce*I-treated mice remove free vector ends not only by degradation, but also by activating the NHEJ pathway and ligating free DNA ends both inter- and intramolecularly. The I-*Sce*I homing endonuclease has also been shown to enhance gene targeting *in vitro* by increasing the recombination frequency (Donoho *et al*., 1998; Moure *et al*., 2003). Thus, in addition to NHEJ mechanisms, recombination events could also be involved in the generation of inter- and intramolecular repair of free DNA ends. This might also be an advantage for gene therapy approaches, because integration of vector sequences into the genome has the risk of insertional mutagenesis (Hacein-Bey-Abina *et al*., 2003). To date, although we cannot exclude the possibility that some low level of linear DNA integration occurred *in vivo*, our results indicate that neither the concatemers nor the dsDNA circular monomers observed here were substrates for efficient chromosome integration in hepatocytes *in vivo*. These results were consistent with previous observations showing that linear DNA-based vectors can efficiently concatemerize *in vivo* and produce small circles that remain extrachromosomal and active for several months (Chen *et al*., 2001). Similarly, injection minicircles devoid of bacterial backbone can express high and persistent levels of transgene products in liver (Chen *et al*., 2003). Furthermore, I-*Sce*I expression resulted in a higher and longer level of expression than that noted by previous studies when either PCR products (Sykes and Johnston, 1999; Hofman *et al*., 2001; Liang *et al*., 2002) or capped DNA (Zanta *et al*., 1999) was delivered into animals.

DNA-PKcs (the catalytic subunit of DNA-dependent protein kinase) is a pivotal component of the NHEJ repair pathway, and lack of normal DNA-PKcs catalytic activity results in the SCID phenotype (Bosma *et al*., 1983). Nakai and co-workers previously determined the presence of a hierarchical organization for processing free DNA end removal/ligation (Nakai *et al*., 2003b), and showed that self-circularization is the preferred pathway over concatemerization, although the former has a limited capacity to remove free vector ends (Nakai *et al*., 2003b). Indeed, concatemerization occurred only when vector genomes were present in excess or beyond the capacity of self-circularization (Nakai *et al*., 2002). These effects were observed in both wild-type and *scid* mice, suggesting the preferential use of the circularization pathway over concatemerization even when DNA-PKcs is deficient. Similarly, when *scid* mice were treated with I-*Sce*I, small DNA circles containing expression cassettes free of bacterial DNA were formed to the same extent as in wild-type (repair-competent) C57BL/6 mice, corroborating the presence of a DNA-PKcs repair-independent circularization pathway. In contrast, whereas concatemer formation was detected in wild-type mice, we did not detect large concatemers in the livers of I-*Sce*I-injected *scid* mice, most probably because of the production of a nonsaturating concentration of double-stranded linear molecules of the transgene, which led mainly to circularization rather than concatemerization of the transgene sequences free of bacterial DNA. The production of these small circles free of bacterial DNA led to the increase in gene expression in both wild-type and *scid* mice. Therefore, this independent study further confirms the hypothesis that the covalent linkage of bacterial backbone to the expression cassette plays a critical role in transcriptional transgene silencing *in vivo*.

Although in mammalian cells the mechanisms underlying gene silencing are complex and remain unclear, transcriptional gene silencing is most often related to hypermethylation of the promoter region and a closed chromatin conformation associated with specific histone modifications (Rice and Allis, 2001; Richards and Elgin, 2002; Grewal and Moazed, 2003; Rice *et al*., 2003). Large-scale silencing of genes is mediated by the packing of DNA in highly condensed heterochromatin structures involving hypermethylation of DNA at cytosines in defined cytosine–guanine sequence (CpG) sites and histone H3 methylation at Lys-9 (Tariq *et al*., 2003). As demonstrated by Kass and co-workers, a methylation center can result in chromatin condensation that can spread to a downstream promoter and result in silencing of the gene (Kass *et al*., 1997). Furthermore, the frequency of CpG dinucleotides is much higher in bacterial DNA than in vertebrate DNA (Paillard, 1999). Together with our results reported here, this leads us to speculate that bacterial DNA sequences are more prone to methylation by endogenous DNA methylases than eukaryotic DNA, leading to a higher extent of methylation and, hence, silencing.

Chromatin structure and chromatin remodeling are also critical determinants of cellular DNA methylation patterns in mammalian cells and play a key role as a mediator between DNA methylation and transcriptional silencing of genes (Rice and Allis, 2001; Richards and Elgin, 2002; Grewal and Moazed, 2003; Rice *et al*., 2003). One prominent functional feature common to all the bacterial backbones is that they are transcriptionally inactive in mammalian cells, although the eukaryotic expression cassettes *in cis* are active (Chen *et al*., 2004). In addition to the possible effect of hypermethylation in bacterial DNA silencing, this suggests that bacterial sequences may assume a chromatin structure similar to heterochromatin in eukaryotic cells (Wolffe, 1997; Chen *et al*., 2004). Accordingly, we hy-

pothesize that concatemers and small circles free of bacterial DNA are more likely to be in a transcriptionally active chromatin conformation than the parental input circular plasmid. However, the mechanisms that result in changes in the methylation status and in the chromatin remodeling/structure are somehow related to the covalent linkage of bacterial DNA to the expression cassette. Experiments to further explore the mechanisms of gene silencing are currently ongoing.

In summary, we have generated a scheme for converting routine plasmids into a purified expression cassette, free of bacterial DNA after gene transfer *in vivo*. Removal of the transgene expression cassette from the bacterial backbone must be considered to further optimize the production of nonviral vectors for gene therapy. Furthermore, understanding the mechanisms by which changes in chromatin remodeling/structure are connected to the covalent linkage of bacterial DNA to the expression cassette and transcriptional gene silencing is fundamental to unravel the mechanism of transcriptional regulation in mammalian systems. Ultimately this will assist us in the development of new tools that specifically reactivate the silenced transgenes of interest.

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