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Minicircle DNA Vectors Devoid of Bacterial DNA Result in Persistent and High-Level Transgene Expression *in Vivo*

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The loss of transgene expression has been a major obstacle to the development of nonviral vectors for the treatment of human diseases. We previously demonstrated that bacterial DNA linked to a mammalian expression cassette resulted in transcriptional silencing of the transgene *in vivo.* To confirm these studies and develop a means to produce a robust DNA vector that is not silenced *in vivo,* we developed a phage ϕC31 integrase-mediated intramolecular recombination technology to prepare minicircle vector DNA devoid of the bacterial backbone and then compared the transgene expression profile of the minicircle with different molecular forms of plasmid DNAs in mice. We demonstrate that minicircular DNAs devoid of bacterial sequences expressed 45- and 560-fold more serum human factor IX and α1-antitrypsin, respectively, compared to standard plasmid DNAs transfected into mouse liver. Our data suggest that minicircles are capable of expressing high and persistent levels of therapeutic products *in vivo* and have a great potential to serve as episomal vectors for the treatment of a wide variety of diseases.

Key Words: minicircle, ϕC31 integrase, linear DNA, expression cassette, bacterial backbone, transgene, human α1-antitrypsin, human factor IX, transcriptional silencing

INTRODUCTION

A barrier limiting the use of nonviral vectors for gene therapy is related to the short duration of transgene expression *in vivo.* Typically, a high level of transgene product is achieved shortly after DNA is delivered to target cells, but transgene expression falls to low levels within a few weeks even though vector DNA is retained in these cells [1–4]. Recently, we have made progress in understanding the mechanism of episomal transgene silencing. We observed that silencing of transgene expression was substantially alleviated by cutting closed circular DNA (ccDNA) twice through the bacterial DNA sequences before transfection into mouse livers [5]. We found that transgene expression was 10- to 100-fold higher in linear DNA- versus ccDNA-transfected mice. Interestingly, linear DNA formed large random concatemers as well as small circles, while the ccDNA remained intact in mouse livers. Accordingly, we hypothesized that covalent attachment of the bacterial backbone to the expression cassette plays an inhibitory role in transgene expression. The bacterial DNA silencing effect was further established in additional studies, in which mice receiving an equimolar mixture of linearized expression cassette and bacterial backbone resulted in a lower level of reporter transgene expression compared with mice receiving the expression cassette alone (Chen *et al.,* submitted for publication), suggesting that a proportion of the delivered expression cassette was silenced by the bacterial backbone. Finally, we demonstrated that linearized plasmid DNA containing an expression cassette covalently attached to the bacterial backbone resulted in an expression profile similar to that observed for uncut circular plasmids, and like other linear DNA molecules, large concatemers and circles were formed in mouse liver.

Taken together, the data suggest that covalent linkage of bacterial DNA to the expression cassette results in transcriptional silencing of the transgene *in vivo.* We hypothesized that recircularized expression cassette free of bacterial DNA sequences was an active vector form, responsible for persistent episomal expression of a transgene.

To test this hypothesis, we have developed a *Streptomyces* temperate phage integrase ϕC31-mediated site-specific intramolecular recombination technology to prepare DNA minicircles encoding expression cassettes excluding bacterial backbone and examined their expression profiles in mouse liver. Our data suggest that vector DNA devoid of bacterial sequences is not silenced and is capable of expressing persistently high levels of transgene *in vivo.* This type of vector will be useful as an episomal gene

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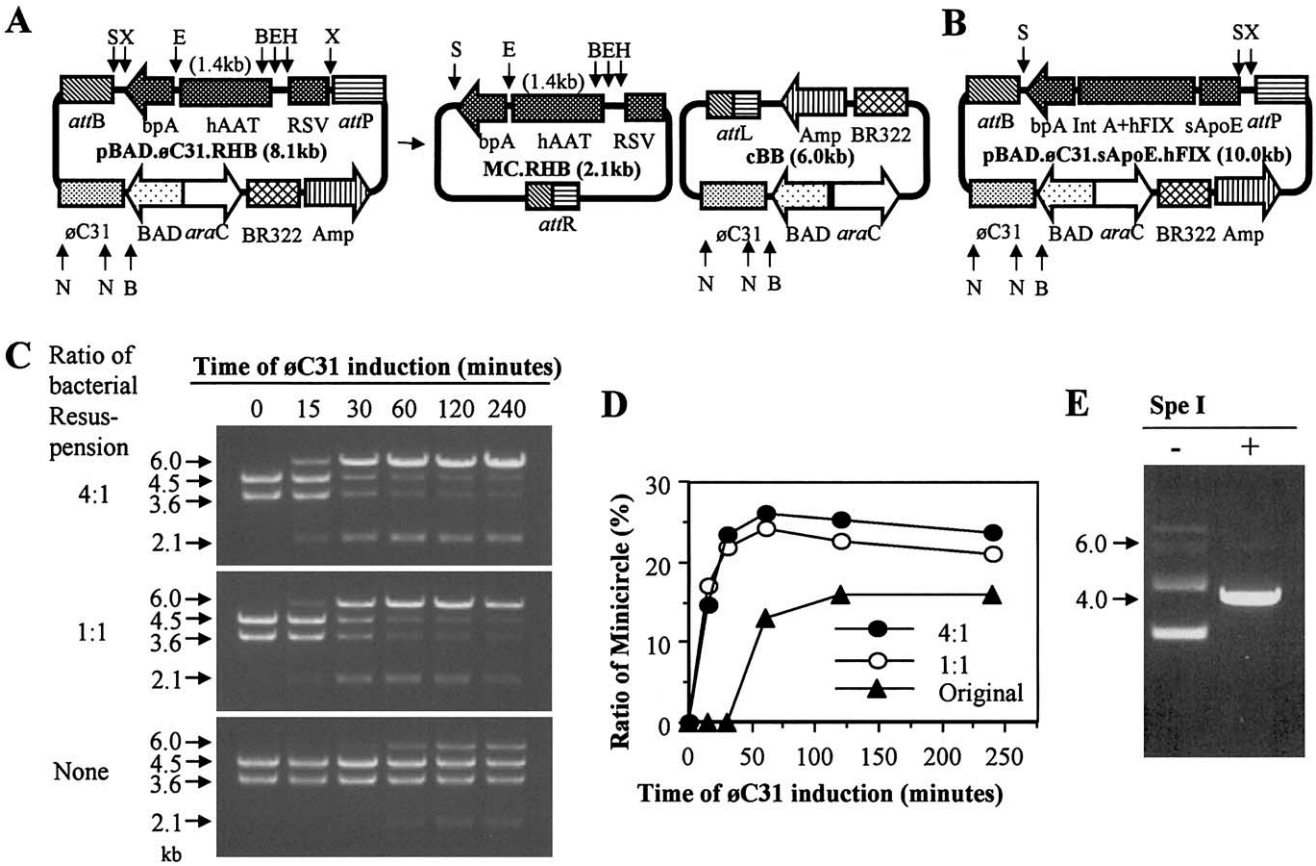

FIG. 1. ϕC31-mediated production of minicircle in *Escherichia coli.* (A) Flow chart of ϕC31 integrase-mediated intramolecular recombination of pBAD.ϕC31.RHB. The resulting products are shown as cBB and MC.RHB. RSV, Rous sarcoma virus long terminal repeat promoter; hAAT, human α1-antitrypsin; bpA, bovine growth factor polyadenylation signal; RHB, RSV.hAAT.bpA expression cassette; Amp, ampicillin resistance gene; BAD, *ara*BAD promoter; *ara*C, *ara*C repressor; *att*B, bacterial attachment site; *att*P, phage attachment site; *att*L, left hybrid sequence; *att*R, right hybrid sequence; BR322, pBR322 origin of DNA replication; MC, minicircle; cBB, circular bacterial backbone. Abbreviations of restriction sites: B, *Bam*HI; H, *Hin*dIII; N, *Nco*I; S, *Spe*I; and X, *Xho*I. (B) The vector pBAD.ϕC31.hFIX used for production of a minicircle expressing human factor IX (hFIX). sApoE, the artificial enhancer/promoter sApoE.HCR.hAAT [25]; Int A, intron A. (C) Kinetic analysis of L-(+)-arabinose-induced ϕC31-mediated formation of MC.RHB. The influence of different bacterial broth conditions on MC.RHB production was determined. Resuspension: 4:1 and 1:1 represent the volume of overnight bacterial growth versus volume of fresh LB broth containing 1% L-(+)-arabinose used to resuspend the bacteria. None: the inducer L-(+)-arabinose was added directly to the overnight bacterial growth medium at 1%. Plasmid DNA was isolated from the culture and purified. Each lane was loaded with 1 μg of *Bam*HI-digested DNA. The 2.1- and 6.0-kb bands represent the linear MC.RHB and cBB, respectively, while the 4.5- and 3.5-kb bands were derived from the unrecombined pBAD.ϕC31.RHB (A). (D) Determination of the time course of minicircle formation by quantification of DNA bands in the gel shown in (C). The values of minicircle are presented as the ratios of the 2.1-kb linear minicircle band compared to all four of the other bands in each lane. (E) Analysis of minicircle DNA. Each lane contains 1 μg of purified minicircle encoding RSV.hAAT.bpA with (+) or without (−) *Spe*I digestion. The 2.1-kb band represents the *Spe*I-cut minicircle and the 6.0-kb band the *Nco*I-linearized bacterial backbone.

therapy vehicle for treatment of a wide variety of medical conditions. In addition, we have established a relatively simple method for producing minicircle DNA vectors for preclinical studies in animals.

RESULTS

Production of Minicircle DNA Vectors

We constructed the plasmids pBAD.ϕC31.RHB (Fig. 1A) and pBAD.ϕC31.hFIX (Fig. 1B) as precursors to the production of minicircle vectors expressing human α1-antitrypsin (human AAT) and human factor IX (human FIX), respectively. We used the plasmids to transform *Escherichia coli* Top 10, and the recombinase ϕC31 was produced after the addition of the inducer L-(+)-arabinose to the bacterial growth medium, to mediate the recombination between *att*B and *att*P. This resulted in two DNA circles, one consisting of the expression cassette with a 36-bp hybrid *att*R and one containing all other DNA elements with a 37-bp hybrid *att*L.

To determine the optimal conditions for induction of ϕC31 integrase-mediated recombination, we prepared an overnight culture (OD_{600} ~2.50) obtained from a single colony of transformed cells containing pBAD.ϕC31.RHB.

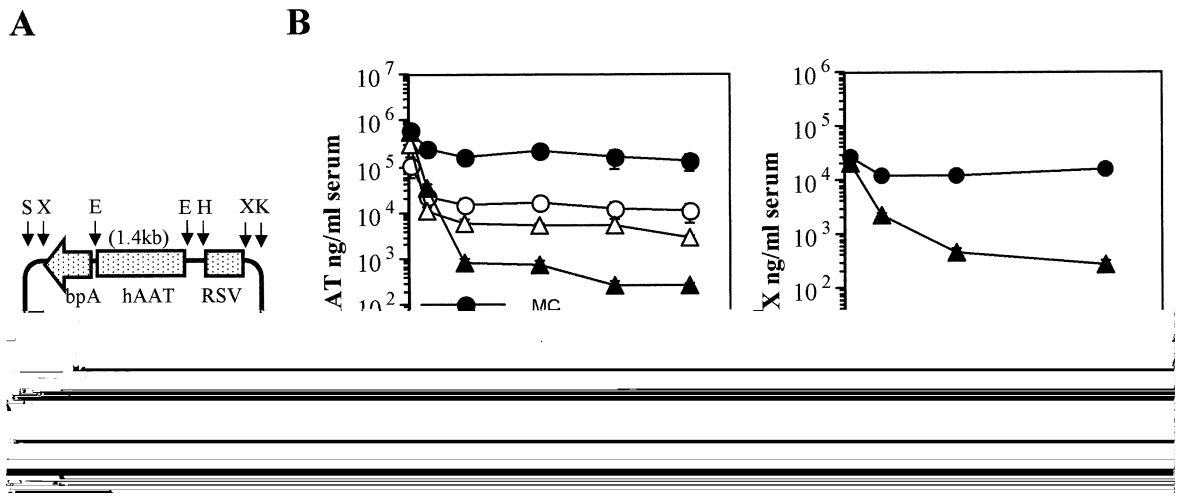

FIG. 2. Transgene expression profiles. (A) The pRSV.hAAT.bpA construct used for preparing the three different forms of DNA. (B) Serum human AAT and human FIX expression. Left: Serum human AAT from mice that received 20.0 μg of closed circular RHB (CC) or equivalent molar amounts of purified expression cassette (1f, 8.2 μg), two-fragment DNA (2f, 20.0 μg), or minicircle DNA (MC, 8.5 μg). Right: Serum human FIX from mice that received 40.0 μg of unrecombined plasmid pBAD.ϕC31.hFIX (▲) or equal molar amount of minicircle (16.2 μg, ●).

We determined that the optimal incubation temperature was 32°C, with 1% inducer L-(+)-arabinose added to the bacterial culture (data not shown). However, we found that the recombination efficiency was poor when induction was carried out by adding L-(+)-arabinose to the overnight bacterial growth medium (Figs. 1C and 1D). The recombination efficiency was greatly enhanced by resuspending the bacteria in fresh LB broth before adding the L-(+)-arabinose. Moreover, we obtained a better yield of minicircle when the bacterial culture was resuspended in a 4:1 versus a 1:1 ratio of overnight growth culture to fresh LB medium (Figs. 1C and 1D). The optimal conditions for minicircle production include resuspending the overnight bacterial growth 4:1 in fresh LB broth containing 1% L-(+)-arabinose and incubating the bacteria at 32°C with shaking at 250 rpm for 60 to 120 min. We cannot exclude the possibility of some leaky expression from the arabinose-induced promoter that may have reduced the final minicircular DNA yield. Nonetheless, we monitored the formation of the minicircles over time by quantifying the four DNA bands in the agarose gel (Fig. 1C). Because the minicircle was about a quarter of the size of the parent vector pBDA.ϕC31.RHB, we estimated, according to the DNA quantification data (Fig. 1D), that under these culture conditions, the efficiency of recombination was greater than 97%. We purified recombined DNAs from bacterial growth using the Qiagen Plasmid DNA Kit and purified minicircles by standard gradient CsCl banding procedure [6] after linearizing the bacterial backbone circle with *Nco*I digestion. We removed ethidium bromide from the minicircle DNA using a butanol extraction procedure [6]. We obtained about 1 to 1.5 mg of recombined DNA before CsCl purification and 150 to 200 μg of purified supercoiled minicircle from 1000 ml of bacterial growth with minicircle-producing vector, pBAD.ϕC31.RHB or pBAD.ϕC31.hFIX. We found variable amounts of nonrecoverable minicircle DNA comigrating with linear bacterial DNA probably due to DNA nicking. We found minimal contamination of linearized bacterial backbone DNA, as represented by the 6.0-kb band, in the minicircle DNA preparation (Fig. 1E). Occasionally, a second CsCl banding was performed, removing this contaminant (data not shown).

Minicircle-Mediated Persistent and High Levels of Transgene Expression *in Vivo*

To determine if the human AAT-expressing minicircle was devoid of bacterial DNA silencing *in vivo,* we compared the expression profiles of this minicircular DNA with equal molar amounts of the uncut plasmid, pRSV.hAAT.bpA (Fig. 2A), a linear DNA mixture of expression cassette and bacterial backbone, or the purified linear expression cassette containing the same DNA sequence as the minicircle except for the 36-bp *att*R hybrid site after transfection into mouse liver.

Consistent with our previous observation (Chen *et al.,* submitted for publication), serum concentrations of human AAT obtained from mice injected with purified expression cassette were more than 3-fold higher than those of mice that received two-fragment DNA and 20- to 43-fold higher than those of ccDNA-injected mice (Fig. 2B, left) 3 weeks after DNA infusion. The mice receiving minicircle DNA produced 10- to 13-fold more serum human AAT than those receiving the purified expression cassette, which was 200- to 560-fold higher than that of the ccDNA group. Mice receiving ccDNA also expressed a high level

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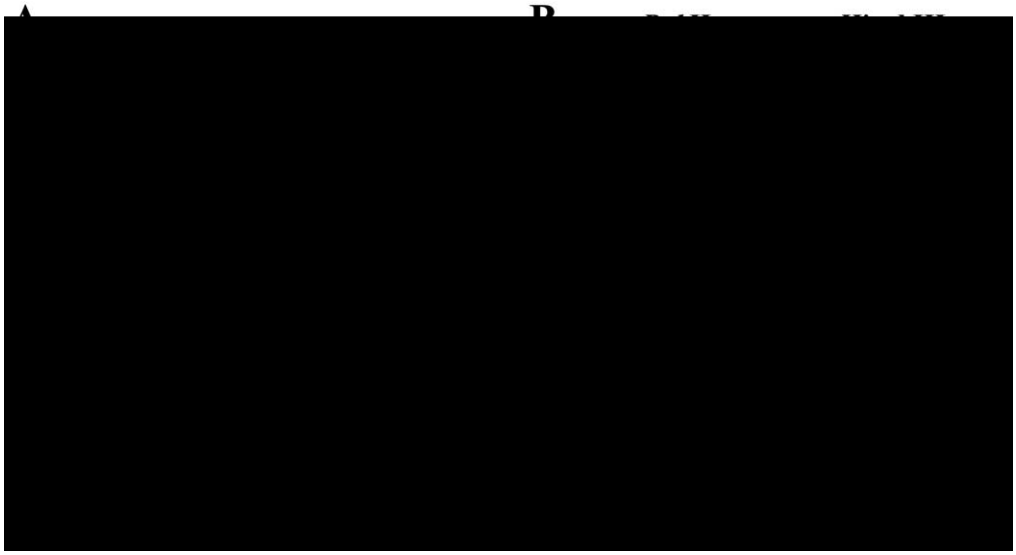

FIG. 3. Southern blot analysis of vector DNA in mouse livers. Liver DNA from mice treated as indicated in the legend of Fig. 2B, left. (A) Quantification of vector DNA in mouse livers. 20 μg of liver DNA was digested with *Eco*RI to release the 1.4-kb human AAT cDNA (Figs. 1A and 2A) and quantified by PhosphoImager. (B) Molecular structure of vector DNA in mouse livers. 20 μg of liver DNA was digested with *Bgl*II (does not cut in the vector) or *Hin*dIII (cuts once in the vector, Fig. 2A), and vector expression cassette DNA bands were visualized after hybridization with a radiolabeled human AAT cDNA probe.

of serum human AAT initially, but the serum reporter level dropped by 710 times in the first 3 weeks and continued to decrease afterward. Our data clearly demonstrate that the minicircle was the most efficient vector form and could express persistent and high levels of transgene product.

To demonstrate the potential for therapeutic efficacy, we compared the human FIX-expressing minicircle to the corresponding unrecombined plasmid (Fig. 1B). Animals infused with this minicircle expressed a high level of serum human FIX, which stabilized at about 12 μg human FIX per milliliter of serum (more than twice normal) for up to 7 weeks (length of experiment; Fig. 2B, right). High levels of serum human FIX were produced in mice receiving the unrecombined plasmid 1 day after DNA infusion, but the serum therapeutic protein dropped more than 45-fold in 3 weeks and continued to decrease afterward.

Transgene Expression Levels and Quantity of Vector DNA in Mouse Liver

Although in previous studies, we found no difference in the amount of vector DNA after infusion of ccDNA or linear DNA [5], we wanted to establish if the same was true for minicircle-injected mice. We determined liver vector DNA copy number in mice receiving different forms of the human AAT vector DNA 15 weeks after injection (Fig. 2B, left). We detected about 13 to 20 copies of vector DNA per diploid mouse genome in each group, with only small differences among groups (Fig. 3A). Consistent with previous observations, our data indicate that the difference in serum human AAT levels was not due to variations in the amount of vector DNA in mouse liver.

Previously, we have demonstrated that circular plasmids remained as intact circles in mouse liver [5] (Chen *et al.*, submitted for publication). To establish if minicircle DNA behaved like other circular plasmids in mouse liver, we analyzed the molecular structure of vector DNA by Southern blot. With *Bgl*II digestion, which does not cut in the vector, we found multiple bands, ranging from 1.6 to about 23 kb, in the liver DNA samples from the mice infused with minicircle DNA. These bands were converted into a single-length 2.1-kb monomer by *Hin*dIII digestion, which cuts once in the vector (Fig. 3B), suggesting that the multiple bands in the *Bgl*II blot represented a single supercoiled DNA molecule or multiple copy aggregates of the minicircle. Thus, similar to a circular plasmid, the minicircle DNA was maintained as an intact episomal circle in mouse liver.

Consistent with our previous observations, animals infused with the two-fragment linearized DNAs formed large concatemers, as represented by the 23-kb bands, which were converted into a DNA ladder by *Hin*dIII digestion, suggesting a random linkage of the two DNA fragments in mouse liver [5]. In addition, small circles were also observed in the *Bgl*II-digested blot (Fig. 3B). The 1.6-kb DNA band in the *Bgl*II blot probably represented a supercoiled religated expression cassette because it was converted to a 2.1-kb band of a full-length linear expression cassette monomer by *Hin*dIII digestion. All the DNA bands seen in the *Bgl*II blot of the one-fragment linear DNA-injected group were converted by *Hin*dIII digestion into a 2.1- and a 3.4-kb band, representing the head-to-tail and tail-to-tail junctions, respectively. The 0.8-kb fragments resulting from the head-to-head end-joining were not expected to be detected with the human AAT cDNA probe (Fig. 2A). The 1.6-kb bands in the *Bgl*II blot, like the one seen in the two fragment linear group, most likely represented the recircularized expression cassette mono-

mer and was converted to the 2.1-kb linear monomer by *Hin*dIII digestion.

DISCUSSION

We demonstrate that minicircle DNA vectors devoid of bacterial DNA sequences can be produced by using the recombinase ϕC31-mediated recombination in *E. coli* in quantities that are sufficient for systemic administration into animals. The product is a unique circular expression cassette monomer that was used to establish a more robust alternative nonviral vector expression cassette that could be used for gene therapy. Moreover, it allowed us to establish further our hypothesis that covalent linkage of a bacterial plasmid DNA sequence to a eukaryotic expression cassette results in transcriptional silencing (Chen *et al.,* submitted for publication). The recombinase ϕC31 used in this study is derived from *Streptomyces* temperate phage. It belongs to the resolvase/invertases family, and its intramolecular recombination activity has been characterized thoroughly by Thorpe *et al.* [7,8]. Groth and colleagues [9] have determined the minimal functional sizes of the *att*B and *att*P sites as 34 and 39 bp, respectively. In our study, the ϕC31 was placed under the control of *ara*BAD. The recombination between the *att*B and the *att*P flanking a transgene expression cassette downstream of this recombinase went almost to completion within 2 h when the induction conditions were optimized. Minicircle DNA vectors have also been produced from other recombinases (e.g., λ integrase and *Cre* recombinase) by intramolecular recombination. Darquet *et al.* [10,11] and Bigger *et al.* [12] have prepared minicircle DNA vectors excluding bacterial DNA via λ integrase and *Cre* recombinase, respectively. Production of minicircle DNA using ϕC31 integrase is in theory more efficient compared to other recombinases such as *Cre* recombinase because the former recombinase mediates a unidirectional reaction. Consequently, the intramolecular recombination can proceed to completion, resulting in higher yields of a unique population of circular expression cassette monomers. In contrast, *Cre* mediates a bidirectional reaction, resulting in lower yields of minicircular DNA [12]. Moreover, the product of the bidirectional reaction is a mix of circular molecules composed of a different number of repeated sequences [12], making it difficult to produce pure monomers. The λ recombinase can also mediate a unidirectional reaction in the absence of the *Xis* protein [13]. However, small amounts of dimer were always seen in the preparation of minicircle DNA using λ integrase in the absence of *Xis* protein in the studies of Darquet *et al.* [10,11], and the mechanism underlying the presence of this contamination is not completely understood.

This study establishes that minicircles can express high and persistent levels of transgene products in mouse liver. Delivered via the hydrodynamic procedure [14,15], minicircles expressed 45- and 560-fold more serum human FIX and human AAT than their parent unrecombined plasmids in mouse liver 3 weeks after transgene delivery. Importantly, and similar to our previous results [5] (Chen *et al.,* submitted for publication), the difference in gene expression was not related to differences in the amount of vector DNA in mouse liver. Compared to the linear purified expression cassette, minicircle DNA expressed more than 10-fold higher levels of serum human AAT, suggesting that the minicircle was an optimal episomal vector form for transgene expression, possibly because of its circular configuration. Alternatively, substantial amounts of linear expression cassette might be inactivated via the partial loss of promoter and/or polyadenylation DNA sequences during the nonhomology-end-joining process [16,17].

There is some variation in the degree of silencing when different bacterial backbones and expression cassettes are used in any particular combination. The common property resulting in silencing is the physical linkage of the two DNA elements, whether the DNA is delivered in this manner or the linkage occurs in the liver (e.g., linear DNA delivery) [5] (Chen *et al.,* submitted for publication). While linear DNA is more robust than intact plasmids, more studies are required to establish the relative differences in gene expression when different plasmid backbones and expression cassettes are used in combination. Nonetheless, based on the data in this and our previous studies [5] (Chen *et al.,* submitted for publication), the absence of bacterial DNA covalent linkages to the expression cassette results in more robust and persistence gene expression *in vivo*. There are two additional advantages of minicircle vs linear DNA vectors that should be considered: (1) Purified linear vectors are more labor intensive for production and (2) linear DNA molecules may be less safe because they have detectable and higher levels of integration into liver host chromosomal DNA compared to circular DNA plasmids [18,19].

Other groups have provided limited data suggesting that minicircle DNA vectors are more robust in other tissues/cell types. Darquet *et al.* [11], for example, demonstrated that minicircles expressed 13 to 50 times more transgene product when injected into mouse skeletal muscle or human head and neck carcinoma grafts in nude mice, compared to an equal amount of the parent plasmid. Gene expression was followed for only 1 week and the mechanism (e.g., transcriptional versus DNA persistence) for the enhanced expression for this short period was not determined.

Since the transcriptional silencing effect is overcome by using minicircular DNA, transgene expression will not be lost except during cell division [20] or cell death. It has been hypothesized that when plasmid DNA is delivered within lipid–DNA complexes, a loss of transgene expression occurs due to an immune response against unmethylated CpG dinucleotides present in bacterial DNA

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[21,22]. We have previously demonstrated (Chen *et al.,* submitted for publication), and confirmed in this study, that an immune response is not likely responsible for the loss of gene expression because there was no selective loss of vector DNA harboring bacterial sequences. It has been well documented that plasmids can undergo nucleation [23] and persist in an episomal status for months or years, not only in liver [5], but also in the heart [24] and skeletal muscle [4]. It is reasonable to expect that persistence of transgene expression from minicircle DNA can also be achieved from these and other organs with a low cell turnover rate.

EXPERIMENTAL PROTOCOL

Vector construction. To prepare the human AAT minicircle-producing construct pBAD.ϕC31.RHB (Fig. 1A), we amplified ϕC31 integrase from the plasmid pCMV.ϕC31 [9], using the primers 5′-CCGTCCATGGACACGTACGCGGGTGCT and 5′-ATGCGCGAGCTCGGTGTCTCGCTACGCCGCTAC and inserted the PCR product into *Nco*I and *Sac*I sites of pBAD/*Myc*-His (Invitrogen, Carlsbad, CA), resulting in an intermediate plasmid, pBAD.ϕC31. We composed *att*B and *att*P using the corresponding DNA oligonucleotides [9] and inserted them into the *Spe*I and *Kpn*I sites, respectively, to flank the human AAT expression cassette of the plasmid pRSV.hAAT.bpA (Fig. 2A). The *att*B and *att*P binding sites-flanked expression cassette was inserted into *Sac*I and *Kpn*I sites of pBAD.ϕC31, resulting in pBAD.ϕC31.RHB. We prepared the vector pBAD.ϕC31.sApoE.hFIX (Fig. 1B) to produce minicircle expressing human FIX by inserting the expression cassette, derived from pBS.sApoE.HCR.hAAT.hFIX+IntA.bpA [5,25,26], into the *Spe*I site of pBAD.ϕC31.RHB after the expression cassette was removed by *Xho*I digestion.

Production of minicircles. We used minicircle-producing vectors to transform *E. coli* Top 10 (Invitrogen). The bacteria were grown using a New Brunswick Scientific incubator (Model C24, Edison, NJ). We obtained L-(+)-arabinose from Sigma Chemical Co. (St. Louis, MO). We quantified the DNA bands after agarose electrophoresis using Quant One from Bio-Rad Laboratories (Hercules, CA). We prepared purified expression cassette and two-fragment DNA from plasmid pRSV.hAAT.bpA as described previously [5] (Chen *et al.,* submitted for publication). We dialyzed all the DNA preparations against TE overnight before delivery to animals.

Determination of transgene expression in mice. We obtained 6- to 8-week-old C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME). We delivered DNA to mouse livers using a hydrodynamic technique [14,15]. We collected mouse blood periodically using a retro-orbital procedure and determined serum human AAT and human FIX by ELISA as described earlier [27]. All animals were treated under the NIH and Stanford University Animal Care Guidelines.

Southern blot analysis of vector DNA structure in mouse livers. We prepared liver DNA using a salt-out procedure. Twenty micrograms of liver DNA from mice receiving one of the four forms of vector DNA expressing human AAT, as described in the legend for Fig. 2B, left, was digested with *Bgl*II, which did not cut the vector, or *Hin*dIII, which cut once through the expression cassette. We separated the digested DNAs by electrophoresis in a 0.8% agarose gel and blotted them onto a nitrocellulose membrane. Vector DNA was detected after hybridization with a [^{32}P]dCTP-labeled 1.4-kb human AAT cDNA probe (Fig. 2A) and autoradiography or phosphoimagery.

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REFERENCES

1. Nicol, F., *et al.* (2002). Poly-L-glutamate, an anionic polymer, enhances transgene expression for plasmids delivered by intramuscular injection with in vivo electroporation. *Gene Ther.* **9:** 1351–1358.
2. Gill, D. R., *et al.* (2001). Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. *Gene Ther.* **8:** 1539–1546.
3. Hartikka, J., *et al.* (1996). An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum. Gene Ther.* **7:** 1205–1217.
4. Wolff, J. A., Ludtke, J. J., Acsadi, G., Williams, P., and Jani, A. (1992). Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genet.* **1:** 363–369.
5. Chen, Z. Y., Yant, S. R., He, C. Y., Meuse, L., Shen, S., and Kay, M. A. (2001). Linear DNAs concatemerize *in vivo* and result in sustained transgene expression in mouse liver. *Mol. Ther.* **3:** 403–410.
6. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7. Thorpe, H. M., and Smith, M. C. (1998). In vitro site-specific integration of bacteriophage DNA catalyzed by a recombinase of the resolvase/invertase family. *Proc. Natl. Acad. Sci. USA* **95:** 5505–5510.
8. Thorpe, H. M., Wilson, S. E., and Smith, M. C. (2000). Control of directionality in the site-specific recombination system of the Streptomyces phage phiC31. *Mol. Microbiol.* **38:** 232–241.
9. Groth, A. C., Olivares, E. C., Thyagarajan, B., and Calos, M. P. (2000). A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. USA* **97:** 5995–6000.
10. Darquet, A. M., Cameron, B., Wils, P., Scherman, D., and Crouzet, J. (1997). A new DNA vehicle for nonviral gene delivery: Supercoiled minicircle. *Gene Ther.* **4:** 1341–1349.
11. Darquet, A. M., *et al.* (1999). Minicircle: An improved DNA molecule for in vitro and in vivo gene transfer. *Gene Ther.* **6:** 209–18.
12. Bigger, B. W., Tolmachov, O., Collombet, J. M., Fragkos, M., Palaszewski, I., and Coutelle, C. (2001). An araC-controlled bacterial Cre expression system to produce DNA minicircle vectors for nuclear and mitochondrial gene therapy. *J. Biol. Chem.* **276:** 23018–23027.
13. Kim, S., and Landy, A. (1992). Lambda Int protein bridges between higher order complexes at two distant chromosomal foci *att*L and *att*R. *Science* **256:** 198–203.
14. Liu, F., Song, Y., and Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6:** 1258–1266.
15. Zhang, G., Budker, V., and Wolff, J. A. (1999). High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* **10:** 1735–1737.
16. Wilson, J. H., Berget, P. B., and Pipas, J. M. (1982). Somatic cells efficiently join unrelated DNA segments end-to-end. *Mol. Cell. Biol.* **2:** 1258–1269.
17. Wu, X., Wilson, T. E., and Lieber, M. R. (1999). A role for FEN-1 in nonhomologous DNA end joining: The order of strand annealing and nucleolytic processing events. *Proc. Natl. Acad. Sci. USA* **96:** 1303–1308.
18. Nakai, H., *et al.* (2003). Helper-independent and AAV-ITR-independent chromosomal integration of double-stranded linear DNA vectors in mice. *Mol. Ther.* **7:** 101–111.
19. Nakai, H., Fuess, S., Storm, T. A., Meuse, L. A., and Kay, M. A. (2003). Free DNA ends are essential for concatemerization of synthetic double-strand adeno-associated virus vector genomes transfected into mouse hepatocytes in vivo. *Mol. Ther.* **7:** 112–121.
20. Ludtke, J. J., Sebestyen, M. G., and Wolff, J. A. (2002). The effect of cell division on the cellular dynamics of microinjected DNA and dextran. *Mol. Ther.* **5:** 579–588.
21. Li, S., *et al.* (1999). Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. *Am. J. Physiol.* **276:** L796–804.
22. Yew, N. S., *et al.* (2000). Reduced inflammatory response to plasmid DNA vectors by elimination and inhibition of immunostimulatory CpG motifs. *Mol. Ther.* **1:** 255–262.
23. Kass, S. U., Landsberger, N., and Wolffe, A. P. (1997). DNA methylation directs a time-dependent repression of transcription initiation. *Curr. Biol.* **7:** 157–165.
24. Gal, D., Weir, L., Leclerc, G., Pickering, J. G., Hogan, J., and Isner, J. M. (1993). Direct myocardial transfection in two animal models. *Evaluation of parameters affecting gene expression and percutaneous gene delivery. Lab. Invest.* **68:** 18–25.
25. Miao, C. H., *et al.* (2000). Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression *in vivo* but not in vitro. *Mol. Ther.* **1:** 522–532.
26. Miao, C. H., Thompson, A. R., Loeb, K., and Ye, X. (2001). Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer in vivo. *Mol. Ther.* **3:** 947–957.
27. Yant, S. R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z., and Kay, M. A. (2000). Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat. Genet.* **25:** 35–41.