Silencing of Episomal Transgene Expression in Liver by Plasmid Bacterial Backbone DNA Is Independent of CpG Methylation

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Minicircle DNA vectors devoid of plasmid bacterial backbone, (BB) DNAs, are transcriptionally persistent, whereas their parent plasmid counterparts are silenced in the liver. In this study we establish that circular plasmid BB provided *in trans* did not silence a transgene expression cassette *in vivo*, further confirming our previous conclusions that the covalent attachment of the plasmid BB to the expression cassette is required for DNA silencing. Given the high concentration of CpG dinucleotides in the plasmid BB, we investigated the role of DNA methylation on transgene silencing *in vivo*. The presence or absence of methylation in CpG motifs in routine plasmid BBs had no significant effect on transcriptional silencing. Furthermore, the removal of the CpG motifs from the BB did not ameliorate transcriptional silencing. Transgene silencing was partially inhibited when two tandem copies of the chicken cHS4 insulator at each end of a routine plasmid vector were used. These results are consistent with the idea that the transcriptional repression observed with plasmid DNA vectors in the liver is caused by formation of repressive heterochromatin on the plasmid DNA backbone, which then spreads and inactivates the transgene *in cis,* and that CpG content or methylation has little or no influence in the process.

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INTRODUCTION

One of the major obstacles in the application of non-viral vectors for human gene therapy is the short duration of transgene expression. Standard plasmid vectors composed of a transgene expression cassette and plasmid bacterial backbone (BB) DNA are able to express a high level of transgene product shortly after entering the cells, but the transgene product usually declines to very low or undetectable levels in a period of days even though the vector DNA is not lost.[1–6] In fact, only very rare constructs in certain circumstances are able to express significant levels of transgene product for a prolonged period of time.[7–10] There are a number of different factors (*e.g.*, transgene product, mouse strain, and promoter) that may explain some of the variations in inter- and intralaboratory experimental results. Nonetheless, this does not change the general finding that plasmid DNAs are commonly silenced when transfected into the liver. We have made some progress in understanding the mechanism of the silencing.[1–3] The episomal transgene is silenced when there is a covalent connection of plasmid BB DNA sequences to the expression cassette. Dissociation of the transgene expression cassette from the plasmid BB, achieved either by delivery of a linear expression cassette[1,3] or plasmid BB-free episomal transgene circles (*e.g.*, minicircles) *in vivo*,[1,3,11] results in more sustained and higher level expression of the transgene product. On the basis of these observations, we have earlier developed a øC31-based intramolecular recombination system that allows the plasmid BB to be excluded and degraded in bacteria, and the plasmid BB-free circular expression cassette, the minicircle, to be one-step purified from a bacterial culture using a commercially available affinity column.[12] This allows for robust production of DNA minicircle vectors that can be used for achieving high level and persistent transgene expression from the liver[2,3,12] and muscle (data not shown) of treated animals.

Although plasmid BB DNA sequences have been completely removed from the minicircle, we wanted to establish the mechanism underlying plasmid BB DNA-mediated silencing. Earlier studies have focused on the role of the highly enriched CpG dinucleotide motifs present in plasmid BB DNA.[3,5,13–16] It has been well documented that unmethylated CpG is inflammatory and can trigger innate immune responses,[17] resulting in the loss of cells harboring the vector DNA.[5,13–16] Interestingly, there are study results suggesting that DNA sequences, in addition to the CpG motifs in plasmid BB, contribute to the activation of cytokine expression when complexed with lipid.[16,18] Further studies demonstrated that the inflammatory responses depended upon the complexity of the lipid and plasmid DNA,[19] whereas lipid or plasmid DNA alone had little or no effect.[20] The inflammatory responses are believed to be elicited partially by toll-like receptor 9 resulting in cellular injury and loss of DNA.[21] In most circumstances, in the absence of lipid carriers, the loss of transgene expression is because of transcriptional silencing, with the transgene product becoming undetectable while the vector DNA concentration remains constant in cells.[1–6,15,16] Nonetheless,

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other reports provide evidence to suggest that the unmethylated CpG motifs in plasmid DNA may play a role in transcriptional silencing of the episomal transgene. For example, the reduction or depletion of CpG dinucleotides in plasmid DNAs result in a moderate enhancement of transgene expression.[5,6,15,22] In these studies, the methylation status of the vector DNA, once inside the cells, was not determined. It is believed that methylated CpG motifs may bind to specific nuclear proteins, such as MeCP2 and MEBs, resulting in the formation of repressive heterochromatin and consequent gene silencing.[23–25] In the absence of CpG methylation, however, the gene silencing mechanism is less well understood. In order to further elucidate the molecular mechanism underlying the transcriptional silencing, we conducted a series of experiments to study the effects of CpG content and methylation of plasmid BB, and of genetic insulators used for blocking the interaction between the CpG-rich plasmid BB and transgene expression cassette, on silencing of a transgene in mouse liver. In this report, we provide evidence to demonstrate that transgene silencing is independent of plasmid BB CpG methylation status, and that the application of genetic insulators and removal of CpG dinucleotides had only a minor effect on transcriptional silencing *in vivo*.

RESULTS

Plasmid BB did not silence the expression of the transgene *in trans*

We have previously established that the silencing effect of plasmid BB depends upon a covalent linkage to the transgene expression cassette.[1–3] Because bacterial plasmid DNA has a high concentration of CpG dinucleotides, we were interested in examining whether plasmid BB DNA, delivered with a eukaryotic expression cassette into the same cells *in trans*, can alter the expression of the transgene. In order to do this, we transfected each mouse liver with 2 μg of either intact (MC.RHB) or linearized minicircle (LMC.RHB) encoding the RSV.hAAT.bpA (RHB) expression cassette (**Figure 1a**), alone or in combination with a 13-fold molar excess (40 μg/mouse) of either circular (pBS) or linear pBlueScript (LpBS) DNA (**Figure 1b**). The relative transgene expression was monitored with periodic serum human α1-antitrypsin (hAAT) measurements. Consistent with earlier observations,[1,3] at the end of the 7-week experiment, a more than 100-fold lower level of serum hAAT was observed in mice infused with the standard plasmid pRSV.hAAT.bpA DNA (pRHB, **Figure 1c**), when compared with the mice receiving either MC.RHB or LMC.RHB alone. This was despite the fact that the molar amount of transgene DNA administered and subsequently quantified in pRHB livers was 8 times higher compared to the MC.RHB- or LMC.RHB-treated groups (**Figure 1d**). Consistent with our earlier results,[1,3] serum hAAT levels in mice receiving the LMC.RHB DNA plus LpBS were >4 times lower than those in mice receiving MC.RHB or LMC.RHB alone, thereby establishing that some plasmid BB-mediated transgene silencing did occur in these mice. By contrast, all the mice that received a mixture of MC.RHB DNA plus LpBS DNA, or LMC.RHB DNA plus pBS DNA, or MC.RHB DNA plus pBS DNA (**Figure 1e**) had a persistent and high level of transgene expression, similar to those in mice that had received either MC.RHB or LMC.RHB alone. We have earlier shown that linear

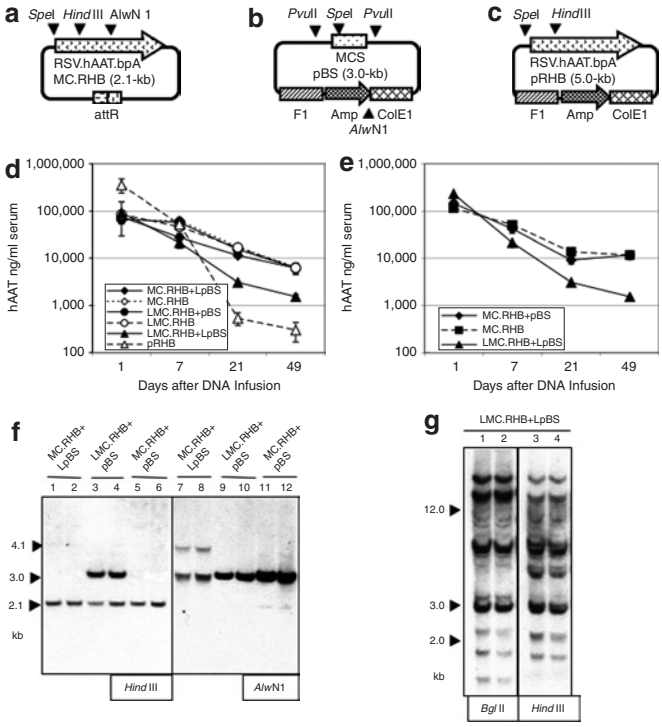

Figure 1 Transgene expression in mouse liver. (**a**) Structure of a minicircle, MC.RHB. MC, minicircle; R, Rous sarcoma long terminal repeat (RSV); H, human α1-antitrypsin (hAAT) gene; B, bovine growth factor polyadenylation signal (bpA); attR, recombination hybrid of bacterial attachment site attB and phage attachment site attP. (**b**) Structure of the plasmid bacterial backbone (BB), pBS. pBS, pBlueScript; MCS, multiple cloning sites; F1, F1 filamentous phage origin; Amp, Ampicilin resistant gene; ColE1, origin of plasmid DNA replication. (**c**) Scheme of plasmid pRHB. (**d**) Serum hAAT in mice infused with a mixture of circular and linear vector DNAs. Mice were injected with a mixture of the circular MC.RHB and linear pBS (MC.RHB+LpBS), or LMC.RHB and circular pBS (LMC.RHB+pBS), or the two linear vector DNAs (LMC.RHB+LpBS). The amounts of vector DNA used were: 2 μg of MC.RHB or LMC, 40 μg of pBS or LpBS, 40 μg of pRHB. (**e**) Serum hAAT in mice receiving two circular DNAs (MC.RHB+pBS). The amount of vector DNA was the same as in experiments described in **d**. (**f**) Molecular structure of vector DNAs in mouse liver. Liver DNA samples were taken from the mice described in **d** and **e** at the end of the 7-week experiment. Twenty micrograms of DNA was cut with either *Hin*dIII (**f**, lanes 1–6) or *Alw*N1 (**f**, lanes 7–12) before electrophoresis. The DNA bands were illustrated after hybridization with the 1.3-kilobase (kb) hAAT complementary DNA (cDNA) from pRHB (**f**, lanes 1–6) or the 2.5-kb *Pvu*II fragment of pBS (**f**, lanes 7–12) radiolabeled probe. (**g**) Large concatemer molecules formed by two linear vector DNAs. Twenty micrograms of DNA taken from the livers of the mice described in **d** at the end of the 7-week experiment were digested with a restriction endonuclease which either did not cut (zero cutter *Bgl*II) (**g**, lanes 1 and 2), or cut once in the vector (one cutter, *Hin*dIII) (**g**, lanes 3 and 4) before electrophoresis. All DNA bands were illustrated by the hAAT cDNA probe. $n = 5$ mice per group for all *in vivo* experiments.

DNAs form concatemers or monomeric circles in mouse liver through non-homologous end-joining[1,3] Therefore we predicted that the persistent expression observed in mice that received different mixtures of two different DNAs (**Figure 1e**) resulted from the lack of covalent connection between the transgene and plasmid BB, if both DNAs were circular or if one was circular and the other was linear.[1,3] In order to confirm this, we performed Southern blot assays to analyze the molecular structure of the

vector DNAs in the livers of mice after the experiments described earlier in the text. In the blots using liver DNAs cut with *Hin*dIII (cuts once through the minicircle) and probed with the hAAT complementary DNA (cDNA), a 2.1-kilobase (kb) band corresponding to the full length of minicircle DNA was detected in the livers of mice that had received the intact MC.RHB in combination with either LpBS (**Figure 1f**, lanes 1 and 2) or intact pBS DNA (**Figure 1f**, lanes 5 and 6); an additional 3.3-kb band, the product of a tail-to-tail ligation, was found in animals that had received LMC.RHB DNA mixed with pBS DNA (**Figure 1f**, lanes 3 and 4). These band patterns suggest that there was random end-joining among the linear LMC.RHB molecules but no recombination between the circular DNA molecules or between the linear and circular molecules. Similarly, in the blot using the same group of liver DNA samples cut with AlwN1 (cuts once through both pBS and minicircle DNAs) and probed with the 2.5-kb *Pvu*II fragment of pBS (**Figure 1b**), one single 3.0-kb band representing the full length pBS DNA was observed in the mice that had received circular pBS DNA together with MC.RHB or LMC.RHB DNA (**Figure 1f**, lanes 9–12). One additional 4.2-kb band was observed in the liver DNA obtained from the mice that had received LpBS plus circular MC.RHB DNA (**Figure 1f**, lanes 7 and 8), suggesting random end-joining among the LpBS molecules. A third predicted 1.7-kb band was not observed in the LpBS group. Judging from the intensity of the 3-kb and 4.2-kb bands, we conclude that a substantial proportion of the LpBS molecules were self-ligated, while only a small proportion of them formed intermolecular end-joining products. This results in an intense 3.0-kb band, a weak 4.2-kb band, and an expected but non-visible 1.7-kb band. Similar to what has been shown previously,[1,3] in the blot using DNA from mice that had received two linear DNAs, cut with the zero cutter *Bgl*II (which does not cut in vector DNAs), and probed with the hAAT cDNA (**Figure 1g**, lanes 1 and 2), a DNA ladder composed of DNA bands from about 1.0-kb to larger than 12.0-kb was observed, suggesting that the random non-homologous end-joining amongst the two species of linear DNA molecules occurred in the livers of the mice. When the same DNA samples were cut with the one cutter *Hin*dIII and also probed with hAAT cDNA (**Figure 1g**, lanes 3 and 4), a somewhat different DNA ladder was detected. The 1.0-kb band seen in the *Bgl*II blot disappeared, the intensity of the largest band decreased, and several bands from 3.0- to ~8.0-kb were observed. These band patterns suggest that the two linear DNA species were randomly ligated to each other to form large concatemers and small DNA circles, as we demonstrated earlier.[1,3] The DNA ladders were slightly different from the earlier ones because in this study we used a 1:13 (instead of a 1:1) molar ratio of linear LMC.RHB to LpBS, resulting in the linkage of many more LpBS molecules as compared to LMC.RHB DNA. Taken together, these results further confirm that circular DNA remained intact, while linear DNAs were ligated to form molecules of different sizes in the livers of the mice. Furthermore, there was no recombination between linear and circular, or between circular and circular vector molecules. Finally, plasmid BB did not silence transgene expression in the absence of the formation of a covalent connection with the transgene, even if the plasmid BB and expression cassette coexisted in the same cell.

Silencing of transgene expression is independent of CpG methylation

We wanted to investigate whether transgene silencing is influenced by the methylation status of the CpG motifs present in the plasmid BB. For this purpose, we used a dam- and dcm-deficient bacterial strain, SCS110, to make pBS DNA devoid of methylation. After purification, some of the DNA was treated with M.Sss I so as to methylate all the cytosines in the CpG motifs (**Figure 2a**). We next compared the effect of methylated and unmethylated pBS on transgene expression *in vivo*. In view of the fact that linear DNAs concatemerize *in vivo*, and because we wanted to make DNA molecules containing an expression cassette attached to a bacterial plasmid backbone, we injected 40 μg (13 times molar excess) of the linear plasmid BB DNAs containing (LmepBS) or lacking (LpBS) CpG methylation, together with 2 μg of LMC.RHB. The serum transgene product, hAAT, was periodically evaluated by enzyme-linked immunosorbent assay. Interestingly, at the end of the 7-week experiment, both groups of mice that had been coinjected with the two linear DNAs expressed a 7-fold lower serum hAAT (**Figure 2b**) than the mice that had received LMC.RHB alone. Quantitative Southern blot analyses of vector DNA isolated from mouse livers confirmed that there was no significant difference in hAAT cDNA copy number per diploid genome among the three groups of animals (data not shown). Southern blot assays using the same DNA samples demonstrated a DNA ladder (data not shown) similar to that obtained using liver DNA from mice that had received two linear DNAs without premethylation (**Figure 1g**). This suggested that the repressed transgene expression seen in both the groups that had received two linear DNAs was caused by transcriptional silencing resulting from ligation of the linear transgene expression cassette to the linear plasmid BB, and that the silencing was not dependent on the CpG methylation status of the plasmid BB at the time of delivery.

Methylation status of plasmid BB in mouse liver is unchanged

After having established that the presence or absence of CpG methylation of plasmid BB at the time of DNA delivery did not affect transgene silencing, we were interested in understanding whether the methylation status of the DNA changes after *in vivo* transfection. For this purpose, 20 μg of total liver DNA from mice receiving different vector DNAs was digested with MrcMC. This endonuclease cuts through a pair of methylated cytosine residues in the sequences of (G/A)MeC(G/N), in one or both DNA strands. The 3.0-kb pBS and the 2.1-kb MC.RHB contain 430 and 210 potential McrBC sites, respectively, so that McrBC cutting should serve as a sensitive method to detect alterations in vector DNA cytosine methylation isolated from liver. In the first blot (**Figure 2c**), we used the hAAT cDNA probe to detect methylation of the CpG motifs in the RHB expression cassette (**Figure 1a**) in mouse liver. In a second blot (**Figure 2d**), we used the 2.5-kb *Pvu*II fragment of pBS (**Figure 1b**) as a probe to examine whether methylation or demethylation of CpG occurred in pBS DNA. Control samples contained 20 μg of naïve mouse liver DNA spiked with 0.25 ng of CpG-methylated (**Figure 2c,** lane 6 and **Figure 2d**, lane 8) or non-methylated (**Figure 2c**, lane 5 and **Figure 2d**, lane 7) pRHB DNA (1.5 copies per diploidy genome). Vector copy

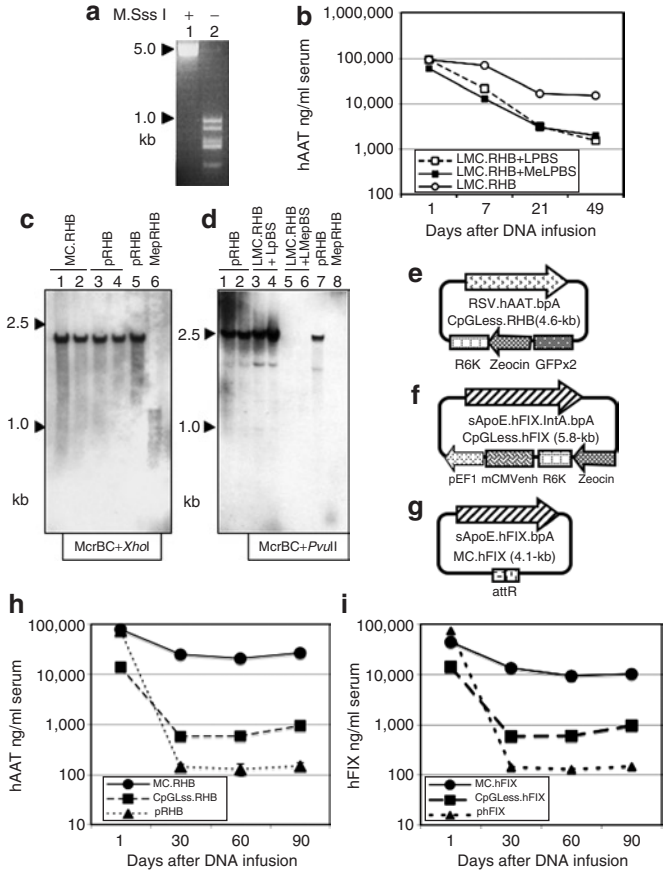

Figure 2 Effect of methylation of plasmid bacterial backbone (BB) CpG on transgene expression. (**a**) Determination of completeness of CpG methylation in pBS. pBS DNA with M.Sss 1 treatment (lane 1) or without the treatment (lane 2) was cut with *BstU*1, the restriction enzyme that cuts unmethylated CpG but not methylated CpG. M.Sss 1 is the CpG methylase capable of methylating all cytosine residues (C^5) in CpG motif. (**b**) The effect of CpG methylation status in the pBS backbone on production of serum human α1-antitrypsin (hAAT) by transgene RHB. Groups of mice were infused with a mixture of 2 μg of LMC.RHB and 40 μg of linear plasmid BB, with M.Sss I-mediated CpG methylation (meLpBS) or without (LpBS) before delivery. (**c**) Determination of CpG methylation status in the RHB expression cassette DNA in mouse liver. From the mice described in **b** and **Figure 1d**, that were killed at the end of the 49-day experiment period, 20 μg of liver DNA was cut with McrBC, an endonuclease cleaving DNA containing methylcytosine in one or both strands before electrophoresis, plus *Xho*I; the bands were illustrated using the 1.3-kilobase (kb) hAAT complementary DNA (cDNA) radiolabeled probe. pRHB (lane 5) and MepRHB (lane 6) were control samples containing 20 μg of naïve mouse liver DNA spiked with 0.25 ng of pRHB with M.Sss 1-mediated CpG methylation (MepRHB) or without (pRHB), respectively. (**d**) Determination of CpG methylation status in pBS in mouse liver. Mouse liver DNA samples were taken from the same experiments as described in **c**, but were cut with McrBC plus *Pvu*II and probed with the 2.5-kb *Pvu*II fragment derived from pBS. (**e**) Structure of plasmid CpGLess.RHB. CpGLess, CpG-free plasmid BB. R6K, a plasmid DNA replication origin; Zeocin, zeocin resistant gene; GFPx2, two copies of enhanced green fish protein cDNA.[26] (**f**) Structure of plasmid CpGLess. hFIX. pEF 1, human elongation factor 1α core promoter and 5′UTR containing a synthetic intron; mCMVenh, mouse cytomegalovirus enhancer. (**g**) Structure of minicircle MC.hFIX. (**h**) Serum hAAT levels in mice that had each received 20 μg of CpGLess.RHB or pRHB, or 8 μg of MC.RHB. (**i**) Serum human factor IX (hFIX) levels in mice each infused with 20 μg of CpGLess.hFIX or phFIX, or 11.5 μg of MC.hFIX. n = 5 mice per group for all *in vivo* experiments.

number determination by Southern blot revealed ~1.0 copies of the transgene RHB, or 3.0 copies of the transgene RHB, or 10.0 copies of pBS per diploidy genome in the livers of mice that had received 2 μg of MC.RHB, 40 μg of pBS, or 40 μg of pRHB DNA, respectively (data not shown). As illustrated in **Figures 2c** and **d**, there were no additional bands, nor any significant decrease in signal intensity of the vector DNA bands after digestion of liver DNAs from mice that had received any of the three unmethylated DNAs digested with *Xho*I and McrBC. By contrast, the entire pBS DNA signal disappeared when the same enzymes were used in the samples obtained from mice that had received a mixture of LMC.RHB and LmepBS DNAs (**Figure 2d**, lanes 5 and 6). Taken together, these findings lead to the conclusion that the CpG methylation status did not change after episomal DNA delivery into mouse liver, further establishing that the transcriptional activity of the transgene expression cassette was not influenced by the CpG methylation status of the plasmid BB.

Elimination of CpG dinucleotides from plasmid BB does not eliminate transgene silencing

In order to evaluate whether the CpG motifs were involved in a non-methylation dependent manner in transgene silencing, we injected mice with CpG-repleted transgene expression cassettes contained within CpGLess plasmid BB, CpGLss.RHB (**Figure 2e**) and CpGLess.hFIX (**Figure 2f**), and compared the transgene product fall off over time in these mice with the values in those that had received an equimolar amount of the same expression cassette in a comparable minicircle (**Figure 2g**) or routine plasmid. In order to make the sizes of all plasmid BBs comparable, we composed the two CpGLess plasmid BBs by including either two copies of green fluorescent protein cDNA[26] or the elongation factor 1α promoter plus mouse cytomegalovirus (mCMV) enhancer DNA sequences to R6K-Zeocin backbone. We found that the levels of transgene products in mice infused with CpGLess plasmids dropped 6- to 23-fold in 4 weeks after DNA infusion, and remained 10- to 28-fold lower than in the animals in the minicircle groups (**Figure 2h** and **i**; **Table 1**). At the end of the 90-day experiment, the expression in these mice remained fourfold to sixfold higher than the expression in mice in the regular plasmid groups. This pattern was similar to that reported in studies by others.[5,22] In order to rule out the possibility that the silencing was caused by a selective loss of vector DNA, we compared the copy numbers of vector DNAs in mouse liver by quantitative real-time polymerase chain reaction (PCR). The DNA amounts were summarized as relative copies to host genomic DNA: hAAT (pRHB = 65 ± 17; MC.RHB = 32 ± 16; and CpGLess.RHV = 61 ± 16 relative copies) and the human factor IX (hFIX) (phFIX = 27 ± 5; MC.hFIX = 15 ± 4; and CpGLess.hFIX = 24 ± 4). The vector DNA copy numbers were comparable between the livers of mice that had received either one of the CpGLess plasmids or corresponding standard plasmids, and remained higher than those in the animals that had received minicircle DNA. These results establish that the difference in transgene expression demonstrated was not the result of a differential loss of vector DNA. These results show that, even in the absence of CpG motifs in the BB, the vector was silenced, albeit to a slightly lesser degree than in the case of routine plasmids. We cannot rule out the possibility that the CpG did have a slight effect on silencing but, in

Table 1 Effect of CpG-reduction and genetic insulator cHS4 on stability of transgene expression

Experiment[a]	Group	Serum hAAT or hFIX level (Mean±SE ng/ml serum) Day 1	Day 90	Ratio of hAAT or hFIX level between day 90 and 1 (%)	Relative hAAT or hFIX level on day 90 (fold)[b]
1	CpGLess.RHB	14,000 ± 2,500	960 ± 85	6.8	6.4
	MC.RHB	80,000 ± 5,500	27,000 ± 1,500	33.8	180.0
	pRHB	75,000 ± 4,200	150 ± 25	0.2	1.0
2	CpGLess.hFIX	31,000 ± 2,000	960 ± 200	3.1	3.5
	MC.hFIX	44,000 ± 7,500	10,100 ± 1,500	23.0	37.4
	phFIX	16,000 ± 2,000	270 ± 50	1.7	1.0
3	Pinsul.RHB	584,884 ± 30,747	2,503 ± 441	0.4	9.1
	1 Frag RHB	108,910 ± 51,540	11,632 ± 4,711	10.7	42.5
	pRHB	563,037 ± 95,218	274 ± 38	0.05	1.0

Abbreviations: hAAT, human α1-antitrypsin hFIX, human factor IX.
[a]Results of the experiments 1 and 2 were from the experiments described in legends of **Figure 2h** and **i**, respectively, and that of the experiment 3 from **Figure 3b**.
[b]As compared to that of the regular plasmid group in each experiment.

general, the silencing was not ameliorated by the absence of CpG motifs in the bacterial DNA backbone.

The effect of the genetic insulator, cHS4, on plasmid BB silencing

While this study as well as our earlier ones have firmly established that the covalent linkage of plasmid BB to the transgene expression cassette results in transcriptional silencing, we wanted to determine whether plasmid BB silencing could be overcome by a genetic insulator used to flank the expression cassette. In the eukaryotic genome, a variety of genetic insulators serve to prevent heterochromatin from spreading to silence the genes in active chromatin *in cis*. For this purpose, we placed two tandem units of the 5′-end of chicken β-globin locus cHS4, a well documented genetic insulator,[27] to each end of the RHB expression cassette in plasmid pRHB (pInsul.RHB, **Figure 3a**) and compared its hAAT expression pattern with the parent plasmid pRHB (**Figure 1c**) and the purified linear RHB expression cassette (1 Frag RHB) prepared from the standard plasmid.[3] Interestingly, at the end of the 11-week experiment, inclusion of cHS4 resulted in a ninefold increase in serum hAAT as compared to pRHB lacking the genetic insulator, although the transgene product in this group remained more than fivefold lower than that of the purified expression cassette group (**Figure 3b**; **Table 1**). Vector DNA quantification showed that the livers of the three groups of mice contained comparable copy numbers of hAAT cDNA per diploidy genome (data not shown), thereby suggesting that the difference in serum hAAT level was not due to the difference in the amounts of vector DNA among the mouse livers, and that cHS4 was capable of only partially overcoming plasmid-mediated BB transcriptional silencing *in vivo*.

Plasmid BB silenced transgene expression independent of host-adaptive immune system

Unmethylated CpG-rich bacterial DNA is highly immunogenic and is widely used as a vaccine adjuvant.[28] In order to rule out the role of the antigen-dependent immune response in plasmid BB-mediated transcriptional silencing, we injected 8.5 μg of MC.RHB, either alone or together with an equal molar amount of circular pBS DNA (11.5 μg per mouse), or an equal molar amount

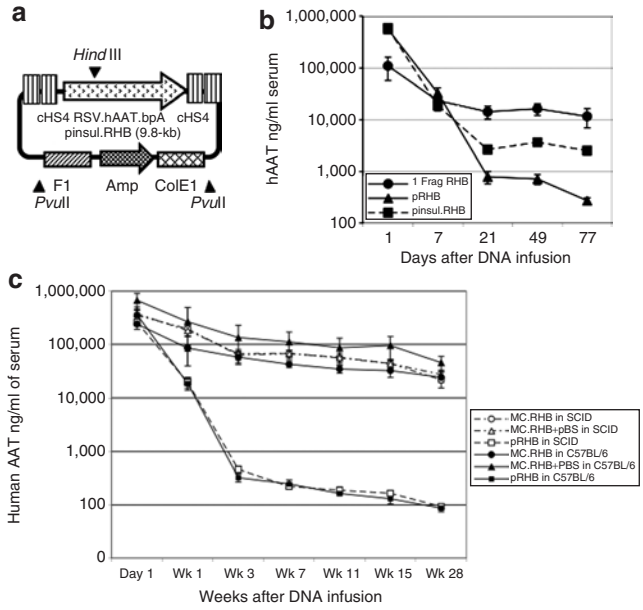

Figure 3 Effect of genetic insulator and host-adaptive immune system on transgene expression. (**a**) Structure of plasmid pInsul.RHB. Insul, genetic insulator; cHS4, the genetic insulator of the DNase I-hypersensitive site 5′ of the chicken β-globin locus;[27] 1 Frag RHB, a purified linear RHB expression cassette prepared from pRHB.[3] (**b**) Effect of insertion of genetic insulator on human α1-antitrypsin (hAAT) transgene expression. Groups of mice were infused with equal molar amounts of vector DNAs equaling 40 μg of pInsul.RHB. n = 5 mice were used per group. (**c**) Effect of host-adaptive immune system on transgene expression. 8.5 μg of MC.RHB with or without 11.5 μg of pBS were infused into livers of immune-competent as well as immune-deficient C57BL/6 severe combined immunodeficiency mice, using the standard hydrodynamic procedure.[48,49] Additional mice of either strain were administered equal molar amounts of pRHB (20 μg) to serve as controls. n = 9 mice were used for each group.

(20 μg) of pRHB, into the livers of immune-competent C57BL/6 and immune-deficient severe combined immunodeficiency mice. Interestingly, the same high level of serum hAAT was expressed from both strains of mice that received MC.RHB with or without coinjection of pBS, while the same silencing pattern (approximately 500-fold lower serum concentrations of hAAT) was observed in

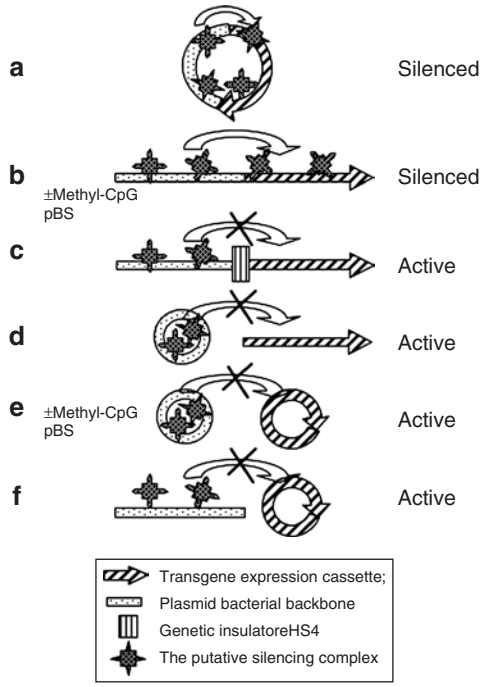

Figure 4 Summary of plasmid transcriptional activity in mouse liver and model for silencing. (**a**) Transgene in a regular plasmid bacterial backbone (BB) was inactive in mouse liver; the putative silencing complexes were initially formed over the plasmid BB; they then spread to repress the transgene in *cis* (**Figure 1d**)[1,3]; (**b**) transcriptional silencing occurred when a linear expression cassette covalently connected to a linear plasmid BB (produced by a restriction digestion cutting once in circular plasmid BB[1,3]) or formed from the two linear DNA elements that can ligate after injection into the mouse liver.[1,3] ±Methyl-CpG, the CpG motifs of the plasmid BB, may or may not be methylated (**Figure 2b**); (**c**) Alleviation of BB silencing of transgene expression by flanking the transgene expression cassette with two tandem copies of the genetic insulator cHS4[27] (**Figure 3a** and **b**); the transgene remained active when (**d**) a circular plasmid BB and a linear transgene expression cassette were co-delivered to mouse liver (**Figure 1d**); or when (**e**) circular forms of both plasmid BB and transgene were infused together (**Figure 1e**); or when (**f**) a linear plasmid BB and a circular transgene were co-delivered (**Figure 1d**).

both pRHB groups (Figure 3c) even though the hAAT vector copy number per diploid genome was similar between groups (data not shown). Our results agree with the observation of Gill *et al.*[4] that the adaptive immune system is not involved in plasmid BB-mediated transcriptional silencing of episomal transgene expression.

DISCUSSION

The results from this study are consistent with our earlier observation[1,3] that transgene silencing requires a covalent linkage of the plasmid BB to the eukaryotic expression cassette. However, this study further establishes that the silencing motif is not active *in trans*. Importantly, we establish that the transcriptional persistence or silencing is independent of the cytosine methylation status of CpG dinucleotides in the plasmid BB DNA, and that methylation status does not change once the vector is delivered into mouse liver. Furthermore, we demonstrated that elimination of CpG in the plasmid BB does not overcome the observed transcriptional silencing. A summary of plasmid transgene activity with different DNA constructs is provided in Figure 4.

Our results may seem to be in disagreement with those of Yew *et al.*[6,15,22] and Hodges *et al.*[5] who have demonstrated that the removal of CpG from the BB results in an enhancement of the transgene expression in liver. However, it should be pointed out that the CpG-dependent diminution of transgene expression in the lung observed after systemic delivery[5,6,15,22] was caused by the loss of the cells harboring the transgene, resulting from activation of the toll-like receptor responses triggered by the lipid–DNA complex containing CpG.[14,17,18,22] In our studies, we eliminated this particular variable because we never used lipid formulations in minicircle studies. Yew *et al.*[6,15,22] and Hodges *et al.*[5] used non-lipid liver transfection with synthetic vectors in which most of the CpG sequences were removed from the plasmid BB as well as from the CMV promoter that drove the transgene expression. The CMV enhancer–promoter used for inducing expression is notorious for its tendency to be silenced in the liver, even when delivered within a viral vector.[29,30] Furthermore, many transcriptional regulator binding sites in promoters, including the Sp1/Sp3 sites and the *cis* repression sequences[31] in the human CMV promoter, contain one or multiple CpG dinucleotides; removal of these CpG motifs can dramatically alter the promoter activity.[16] Yew and colleagues raise the possibility that the sustained transgene expression seen in the constructs with CpG-deleted CMV promoter may have resulted from accidentally mutating its repressor binding sites during CpG removal.[15] In addition to reducing CpG motifs, the CpG-deleted plasmids contained a shortened plasmid DNA replication origin and kanamycin resistance gene, resulting in a shorter (33% less) plasmid BB than that in the controls.[32] We have observed that the transgene silencing effect was more pronounced with larger plasmid vectors than in smaller ones containing similar expression cassettes (data not shown). Taken together, the more dramatic enhancement in transgene expression observed with CpG-deleted plasmids by Hodge *et al.*[5] and Yew *et al.*[6,15,22] was not the effect of CpG removal alone, but a combination of multiple factors.[15,16] In this study, we used the two CpGLess plasmid backbones that were comparable in size to the control plasmids. We also used the same CpG-containing expression cassette in all three constructs because of our concern that the removal of sequences within the promoter region might affect its transcriptional activity. Nonetheless, the vectors lacking in CpG motifs in plasmid BB had little effect in enhancing transgene expression.

Cytosine methylation in CpG motifs and the pathway of methyl-CpG-mediated silencing of gene expression in the genome and integrated vector DNA have been well studied. Heterochromatin formation and gene silencing are believed to begin with the association of the methyl-CpG motif with methyl-CpG DNA binding proteins, *i.e.*, MeCP2, MCB1 to 4, and Kaiso,[25] followed by recruitment of additional proteins, *e.g.*, the transcriptional cosuppressors Ski and N-CoR[33] and the histone deacetylase Sin3A,[24,34] formation of heterochromatin, and gene inactivation. In fact, some viral vectors that undergo transgene silencing may do so through such a mechanism. For instance, the episomal adenovirus vector[35] and the integrated lentivirus vector[36] undergo DNA methylation within or near the promoter. This may explain the differences between our results and those of Yew *et al.*[6,15,22] and Hodges *et al.*[5] (referred to earlier), where silencing of a CMV driven expression cassette was maximized with DNA methylation,

and transcription was enhanced when CpG motifs were removed from the bacterial plasmid as well as from the CMV promoter sequences. In addition, there have been scenarios wherein CpG motifs may be involved in silencing in the absence of methylation. Methyl-CpG binding proteins such as MeCP2 and MBD1 are able to bind to non-methyl-CpG motifs (albeit with a lower affinity as compared to the binding of methyl-CpG motifs) and to trigger the formation of repressive heterochromatin.[37] However, in our studies, the CpGLess plasmid DNAs resulted in slightly less silencing than what we obtained with routine plasmids. At this time, we cannot conclude whether this slight enhancement of transgene persistence was somehow related to the lack of CpG dinucleotides. Nonetheless, CpG motifs and DNA methylation were not the primary factors responsible for DNA silencing observed with our episomal plasmids *in vivo*.

The requirement of covalent attachment of the plasmid BB to the transgene expression cassette for silencing to occur leads to our current hypothesis that the silencing complex is first formed over the plasmid BB and then spreads out to silence the transgene *in cis*. The putative silencing complex may simply be heterochromatin, which spreads out to repress the expression of the neighboring transgene as per the heterochromatin propagation model proposed by Grewal *et al*.[38] and Maison *et al*.[39] It has been suggested that the deacetylation and methylation of histone 3 lysine 9 will facilitate the binding of silencing factors such as Swi6/HP1 or Sir3. These will subsequently provide an interface for interaction with histone-modification enzymes deacetylases and methyltransferases to modify the adjacent histones, and thereby create another binding site for the silencing factors. The sequential rounds of modification and binding then result in the stepwise bidirectional spreading of the silencing heterochromatin. The observation that the genetic insulator cHS4 was able to partially alleviate plasmid BB-mediated transgene silencing further strengthens our hypothesis, because the 5′ DNase hypersensitive site of the chicken β-globin locus has been demonstrated to prevent heterochromatin from silencing a chromosomal gene in other circumstances.[27] Our data suggest that, as in the context of a host genome, cHS4 played a role, albeit partially, in protecting the transgene from being silenced.

We also favor the hypothesis that silencing is dictated by chromatin structure rather than by a specific DNA sequence. Our hypothesis is consistent with the observations of Dalle and colleagues[26] who demonstrated that expression of human β-globin transgenes regulated by the locus control region was repressed by an 812-bp CpG-free green fluorescent protein coding sequence in transgenic mice, thereby suggesting the existence of one or more silencing pathways that recognize non-mammalian DNA sequences in the absence of CpG dinucleotides. There is evidence to suggest that binding of transcriptional factors and transcriptional activity are critically important for decondensation of chromatin and maintenance of an open chromatin structure.[40] It has been shown, for example, that the binding of Sp1 to its sites in mouse adenine phosphoribosyltransferase gene promoter prevents methylation of its CpG island,[41] and blocks epigenetic gene inactivation.[42] Furthermore, many transcriptional factors, such as nuclear factor-κB and interferon regulatory factors, upon binding to their recognition sites in the promoter/enhancer, will recruit chromatin remodeling complexes, such as SWI/SNF and histone acetylases and methyltransferases, to modify histone tails, including histone 3 lysine 9 acetylation and histone 3 lysine 4 methylation. The end result is decondensation of the chromatin.[40] The modified histone tails will become the docking sites for recruiting TFIID to initiate messenger RNA synthesis. The transcription of DNA by RNA polymerase through a gene is coupled to mechanisms for propagation of chromatin breakdown.[43] Some very actively transcribed genes contain only a few nucleosomes, and this highlights the importance of the transcriptional complex in destabilizing chromatin structure.[44] Neither the plasmid BB nor the CpG-free green fluorescent protein DNA contains a mammalian promoter and corresponding transcriptional factor binding sites and, like numerous transposon sequences in mammalian genome, lack any transcriptional activity. It is conceivable that, having once entered the mammalian cell, these vector DNAs, like numerous "desert DNA" transposon sequences in mammalian genomes, will serve as templates for the formation of heterochromatin, which can then propagate and spread out to silence the transgene *in cis* as described in the heterochromatin propagation model,[38–40,45,46] unless there is a genetic insulator to block the repressive heterochromatin from spreading, as we have shown by using cHS4 in this study. Consistent with this hypothesis, recent studies using chromatin immunoprecipitation assays by our group[47] and others[48] suggest that heterochromatin is first formed in the plasmid BB DNA, and then spreads out to silence the transgene *in cis*. By contrast, a minicircle DNA encoding solely the mammalian expression cassette free of plasmid BB DNA maintains a euchromatin pattern accompanied by a stable and high level of transgene expression in cultured cells[48] as well as in mouse liver. Interestingly, these specific histone marks take a period of a week or more to accumulate, and this possibly explains the observed time course over which silencing occurs.

On the basis of the observations arising from our studies and the current understanding of the chromatin regulation of gene expression, it is reasonable to hypothesize that, in addition to plasmid BB, any DNA sequences, including those from mammals, that lack the DNA sequences for binding the regulator factors to maintain an active transcription will form heterochromatin in mammalian cells. If this is true, we would expect that mammalian transcriptional expression cassettes that are not transcriptionally active in liver and covalently attached to liver-active expression cassettes would result in silencing. Further studies are underway to test this hypothesis.

MATERIALS AND METHODS

DNA vector construction. Preparation of the standard plasmids [pRHB (**Figure 1b**) and phFIX] and minicircles [MC.RHB (**Figure 1a**) and MC.hFIX (**Figure 2g**)] have been described earlier.[1–3,12] The plasmid pBS (**Figure 1c**) was purchased from Strategene (La Jolla, CA). LMC.RHB and LpBS were generated by digesting MC.RHB and pBS with *Spe*I followed by a standard phenol–chloroform extraction procedure and ethanol precipitation. In order to make the CpGLess plasmid encoding expression cassette RHB (CpGLess.RHB, **Figure 2e**), the βGloMAR and INFβ/S/MAR sequences were removed from the CpG-free plasmid pCpG-mcs of InvivoGen (San Diego, CA), and the RHB expression cassette was inserted into the *Nhe*I–*Spe*I sites; two tandem copies of 0.8-kb each of CpG-free enhanced green fluorescent protein cDNA, obtained from Dalle

and colleagues,[26] were inserted into the intermediate downstream of RHB. For constructing the CpGLess.hFIX (**Figure 2f**), the sApoE.HCR.hATT. hFIX+Intron A.bpA expression cassette[2,7] was inserted into the same site as the RHB in the above pCpG-mcs plasmid, after removing the two MAR sequences. CpG-methylated plasmids were prepared by incubating pBS and pRHB with CpG methylase M.Sss I (New England Biolabs, Beverly, MA) followed by a standard phenol–chloroform extraction–purification procedure. The completeness of the CpG methylation in pBS was verified by digestion of methylated and unmethylated pBS with *Bst*U1 (**Figure 2a**). For constructing the plasmid with the RHB expression cassette flanked with two tandem copies of the 1.2-kb chicken β-globin insulator cHS4 (GenBank accession no. U78755; pInsul.RHB, **Figure 3a**) in pBS, the *Xho*I-restriction fragment of RHB expression cassette in the pRHB was relocated to a modified pBS vector carrying a *Xho*I site flanked on either side by a *Bgl*II site. The RHB expression cassette prepared by *Bgl*II digestion of the intermediate plasmid was inserted into the *Bam*H1 site of pJC13-1[27] vector with two tandem units of the insulator in each side. The *Nde*I–*Sal*I fragment containing the RHB expression cassette together with two tandem units of cHS4 from the intermediate construct was relocated to another modified pBS vector prepared by *Nde*I+*Sal*I digestion. All DNA was dialyzed against TE at 4°C overnight before delivery to the animals.

Determination of* in vivo *transgene expression. Female C57BL/6 mice, 7- to 8-weeks of age, purchased from Jackson Laboratories (Bar Harbor, ME), were infused through their tail veins with 2-μg of MC.RHB (**Figure 1a**), 40 μg of pBS (**Figure 1b**), a combination of both vector DNAs, or 40 μg of pRHB (**Figure 1c**) in 1.8 ml of saline using the hydrodynamic technique.[49,50] For the hFIX study, 11.5 μg of MC.hFIX (**Figure 2g**), or an equimolar amount of CpGLess.hFIX (**Figure 2f**) or regular plasmid phFIX (20 μg each) was delivered to each mouse. Blood was collected periodically from the mice using the retro-orbital procedure. Serum hAAT and hFIX levels were determined by enzyme-linked immunosorbent assay. The animals were treated according to the National Institutes of Health and Stanford University guidelines.

Southern blot analysis of DNA vector structure in mouse liver. Total liver DNA was prepared using a salt-out protocol.[1] The vector copy number was determined using a PhosphaImage procedure.[1] For determining the CpG methylation status of vector DNA, 20 μg of liver DNA was digested with McrBC together with *Xho*I or *Pvu*II before electrophoresis, and the 1.3-kb hAAT cDNA or the 2.5-kb *Pvu*II fragment of pBS labeled with digoxiginin was used as a probe. The DNA band was illustrated using the chemoillustration protocol of Kirkegaard & Perry Laboratories (Gaithersburg, MA).

Determination of transgene copy numbers. Mouse liver genomic DNA was prepared 49–196 days after DNA injection using a salt-out protocol as described earlier,[1] and used as a template to determine the vector DNA copy number by quantification Southern blot or real-time PCR. Real-time PCR products were produced from 100 ng of template DNA with primers complementary to mouse hFIX or hAAT cDNA; their amounts were expressed as values relative to the endogenous glyceraldehyde 3-phosphate dehydrogenase gene. Reactions were carried out in accordance with the manufacturer's instructions and analyzed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with a SYBR Green PCR Kit from Applied Biosystems. Quantitative values were obtained from the threshold cycle (C_t) number that indicates exponential amplification of the PCR product.

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