Gene Therapy (2004) 11, 856–864

www.nature.com/gt

RESEARCH ARTICLE

Silencing of episomal transgene expression by plasmid bacterial DNA elements in vivo

ZY Chen, CY He, L Meuse and MA Kay
Departments of Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA, USA

We previously demonstrated that sustainable enhanced levels of transgene products could be expressed from a bacterial DNA-free expression cassette either formed from a fragmented plasmid in mouse liver or delivered as a minicircle vector. This suggested that bacterial DNA sequences played a role in episomal transgene silencing. To further understand the silencing mechanism, we systematically altered the DNA components in both the expression cassette and the bacterial backbone, and compared the gene expression profiles from mice receiving different DNA forms. In nine vectors tested, animals that received the purified expression cassette alone always expressed persistently higher levels of transgene compared to 2fDNA groups. In contrast, animals that received linearized DNA by a single cut in the bacterial backbone had similar expression profiles to that of intact plasmid groups. All three linear DNAs formed large concatemers and small circles in mouse liver, while ccDNA remained intact. In all groups, the relative amount of vector DNA in liver remained similar. Together, these results further established that the DNA silencing effect was mediated by a covalent linkage of the expression cassette and the bacteria DNA elements.
Gene Therapy (2004) **11,** 856–864. doi:10.1038/sj.gt.3302231
Published online 18 March 2004

Keywords: *linearized DNA; expression cassette; human α-1 antitrypsin; human factor IX; bacterial backbone; transcriptional silencing*

Introduction

The short duration of transgene expression is an important obstacle to overcome before nonviral vectors become useful for a wide variety of gene therapy applications. Some progress has been made in understanding the mechanism underlining the loss of transgene expression, as well as in developing the methodology to prolong the production of the therapeutic gene product. It has been found that after transfection of liver, muscle and lung *in vivo*, the transgene product from the plasmid DNA is typically expressed for a short period of time, even though vector DNA is not lost.[1–6] These observations are consistent with the hypothesis that gene silencing, rather than loss of vector DNA, is the primary mechanism limiting long-term episomal transgene expression.[2,6,7] The immunogenic CpG dinucleotides in the bacterial backbone of plasmid, and the interaction between the vector DNA elements and a variety of cytokines have been suggested as playing key roles in episomal gene silencing.[6–10] At the same time, some prolongation of transgene expression from nonviral vectors has been achieved. For example, by using a composite promoter/enhancer in the context of a minigene, therapeutic levels of human factor IX (human FIX) from mouse liver were achieved for up to 18 months.[11] A high level of transgene expression was maintained for at least 8 weeks in the lung after delivery of a reporter gene attached to the human polyubiquitin C promoter.[2] In another case, Yew *et al*[12] used a hybrid promoter composed of cytomegalovirus (CMV) promoter and ubiquitin B promoter combined with the deletion of CpG dinucleotides in kanamycin resistance gene, and shortening of plasmid origin of replication in bacterial backbone, to express high and sustainable levels of human alpha galactosidase A from murine liver for up to 35 days. Using CMV or chimeric promoter, Liu *et al*[13] and Kramer *et al*[14] also achieved prolonged and high levels of transgene products from mouse liver. Nevertheless, the mechanism of the promoter inactivation remains poorly defined, and the development of vectors capable of expressing high level of transgene products so far remains as the result of occasional findings. A thorough understanding of the mechanism underlying the episomal vector silencing effect will be important for the successful development of versatile nonviral vectors that can be used to achieve persistent gene expression in different cell types.

Recently, we demonstrated that the transcriptional silencing phenomenon observed with plasmid delivery *in vivo* could be decreased or abolished by cleaving a closed circular plasmid into two DNA fragments before delivery into mice.[1] Persistent high levels of serum human α1-antitrypsin (human AAT) or human FIX, were obtained by cutting the expression plasmid to separate the expression cassette from the bacterial backbone prior to transfection of mouse liver, while the equivalent molar amount of ccDNA resulted in 10–100 times less gene expression. Analysis of the molecular structure of the injected linear vector DNA in mouse liver demonstrated

Correspondence: MA Kay, Departments of Pediatrics and Genetics, 300 Pasteur Dr. Rm G305, Stanford, CA 94305-5208, USA
Received 24 June 2003; accepted 20 November 2003; published online 18 March 2004

that the total amount of vector DNA was similar in both groups of mice, but linear DNA resulted in the formation of large random concatemers as well as smaller circles, while the ccDNA remained as intact circular structures. Accordingly, we have considered the possibility that the large concatemers might be responsible for the enhanced transgene expression, or the increase in transgene expression might be a consequence of the distance or dissociation of the expression cassette from the bacterial DNA, which might influence its inhibitory role in transgene expression.[1] Here, we present additional evidence that the bacterial backbone is involved in silencing the transgene of episomal vectors *in vivo*. We also demonstrate that a high and sustainable level of transgene expression can be achieved by simply excluding bacterial DNA and using a purified expression cassette. Taken together, our observations have laid down a foundation for further understanding the molecular mechanism responsible for the inhibitory effect of the bacterial DNA, and the development of nonviral vectors capable of expressing sustainable high level of transgene and suitable for use in human gene therapy.

Results

High level of transgene expression by infusion of the purified expression cassette

To begin to unravel the mechanisms whereby there is a discordant level of gene expression from the same DNA sequences delivered as linear fragments *versus* circular DNA molecules, we investigated two different possibilities: (1) the bacterial backbone played an inhibitory role in transgene expression, which was lost or weakened when separated from the expression cassette during concatemer or small circle formation, and/or (2) the structure of large concatemers was more favorable for transgene expression. We compared transgene expression from mice receiving a purified expression cassette to those receiving two-linear fragments consisting of the expression cassette and bacterial plasmid backbone. We reasoned that if bacterial DNA plays an inhibitory role in transgene expression, mice transfected with the purified expression cassette free of bacterial DNA would express higher levels of the transgene than mice receiving the expression cassette together with bacterial DNA. We infused mice with 20 μg of uncut circular plasmid pRSV.hAAT.bpA (ccpRHB), two-fragment linear DNA (2fRHB) or different doses of purified expression cassette (1fRHB), and compared serum human AAT levels at various time points (Figure 2a). Consistent with previous observations,[1] the uncut circular plasmid injected mice expressed a high level of serum human AAT that declined by more than 3 logs within 4 weeks after DNA infusion. In contrast, mice receiving equal molar amounts of 2f- or 1fRHB had 28- and 40-fold higher serum human AAT, respectively, over the same period. The serum human AAT levels were approximately proportional to the vector DNA doses in the 1fRHB groups. In all, 20 μg of 2fRHB contained 8 μg of the RSV.hAAT.bpA expression cassette. However, serum human AAT levels from the mice receiving the same molar amount of the expression cassette delivered as 2fRHB were 40–60% lower than that of mice receiving the expression cassette alone. These data suggested that a substantial proportion of the human AAT expression cassette was silenced by the bacterial backbone and support the hypothesis that bacterial DNA sequence has a negative effect on transgene expression.

Covalent linkage of bacterial DNA is responsible for transgene silencing

Infusion of the 2fRHB DNA vectors into mouse liver resulted in the formation of random concatemers.[1] To differentiate whether the concatemers or the covalent linkage of the bacterial and expression cassette sequences were responsible for the fall off in gene expression, we compared serum human AAT levels from *Xba*I-linearized DNA (LRHB) and 2fRHB DNA injected mice. *Xba*I cuts once through the bacterial sequence (Figure 1), resulting in a linear expression cassette covalently attached to the bacterial DNA. We infused each mouse with 40 μg LRHB, 2fRHB, uncut ccpRHB or an equal molar amount of 1fRHB. Interestingly, the serum human AAT level in the LRHB group was similar to that of the mice receiving the ccpRHB (Figure 2b). As demonstrated in the above experiments, mice receiving the 1fRHB expressed 40–150% more transgene than the 2fRHB group, which was 20- to 55-fold higher than the LRHB or ccpRHB groups, 3 weeks after DNA infusion. These observations further confirm that the bacterial DNA had an inhibitory effect on transgene expression, and a covalent attachment between these two DNA elements was required for the silencing effect to occur.

It was possible that the fall off in transgene expression in the mice receiving LRHB mimicked that of ccpRHB because the DNA recircularized and formed ccpRHB-like molecules in mouse liver. To answer this question, we set forth to determine the molecular structure of the vector DNA in mouse livers by Southern blot analysis. Mouse liver DNA was digested with either *Bgl*II, which did not cut the vector DNA but cut the mouse genome frequently, or *Hin*dIII, which cut vector DNA once through the expression cassette (Figure 1). In the blot of liver digested with the 0-cutter *Bgl*II and probed with radio-labeled human AAT cDNA, strong vector DNA bands over 23 kb in size were found in all of the mice receiving 1fRHB, LRHB or 2fRHB linear DNA (Figure 3a). This established that all three linear DNA species formed large concatemers in mouse livers. Multiple DNA bands of smaller sizes were also seen in all three linear DNA groups, indicating that DNA circles with different numbers of DNA fragments were formed. A 1.6 kb band, seen in the 0-cutter blot, was replaced by a 2.1 kb band in the one-cutter blot in 1fRHB and 2fRHB groups, but was absent in the other two groups, indicating that this 1.6 kb band represented the recircularized expression cassette monomer. Consistent with previous observations,[1] the one-cutter *Hin*dIII converted all of the vector DNA bands observed in the 0-cutter *Bgl*II blot of the 2fRHB group into a DNA ladder, ranging from 2.1 kb to up to 23 kb (Figure 3a). Apparently, each band was composed of one or two hAAT.bpA sequences, with variable numbers of bacterial DNA sequences, suggesting that the large concatemers were formed by random linking of the two DNA elements in mouse liver. In contrast, only two DNA bands were found in the LRHB and 1fRHB groups. The two vector DNA bands, about

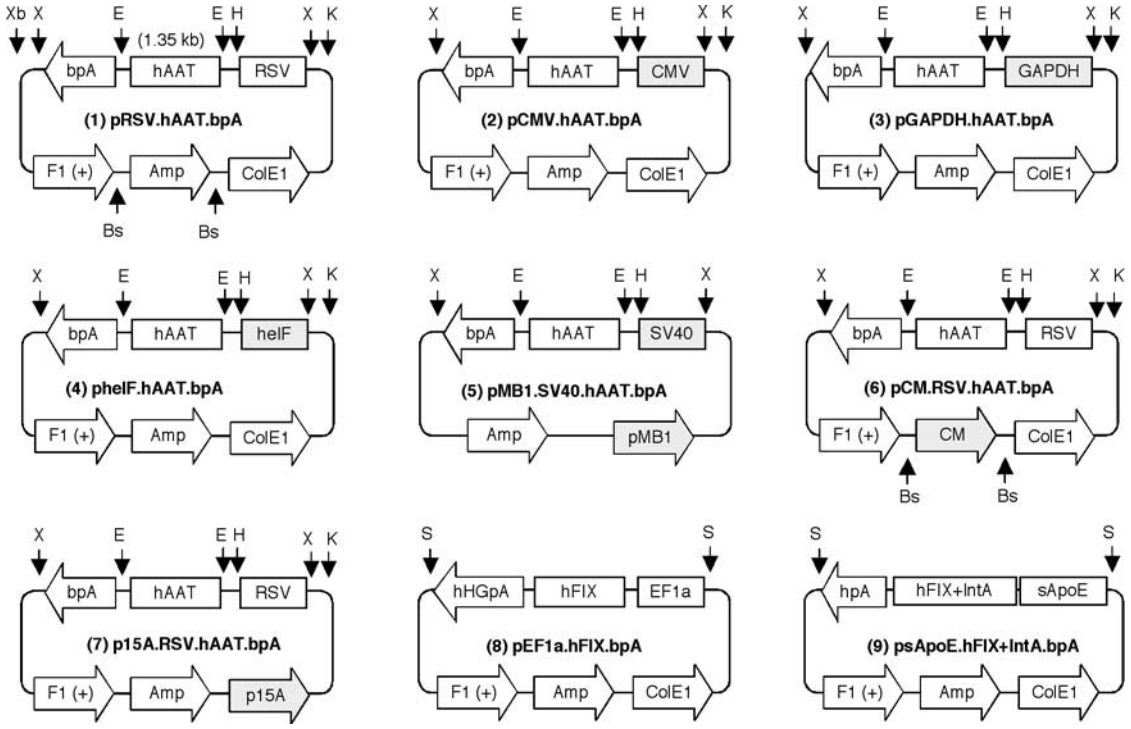

Figure 1 *Schematic illustration of nine DNA vectors expressing either human AAT or human FIX. Amp, ampicillin-resistant gene; CM, chloramphenicol-resistant gene; ColE1, 15A and MB1, plasmid origins of replication; F1(+), f1 phage origin of replication; abbreviation of restriction enzymes: Bs, BspH1; E, EcoR1; H, HindIII; S, SpeI; X, XhoI; Xb, XbaI. The DNA elements different from that in pRSV.hAAT.bpA are highlighted by using the filled symbols.*

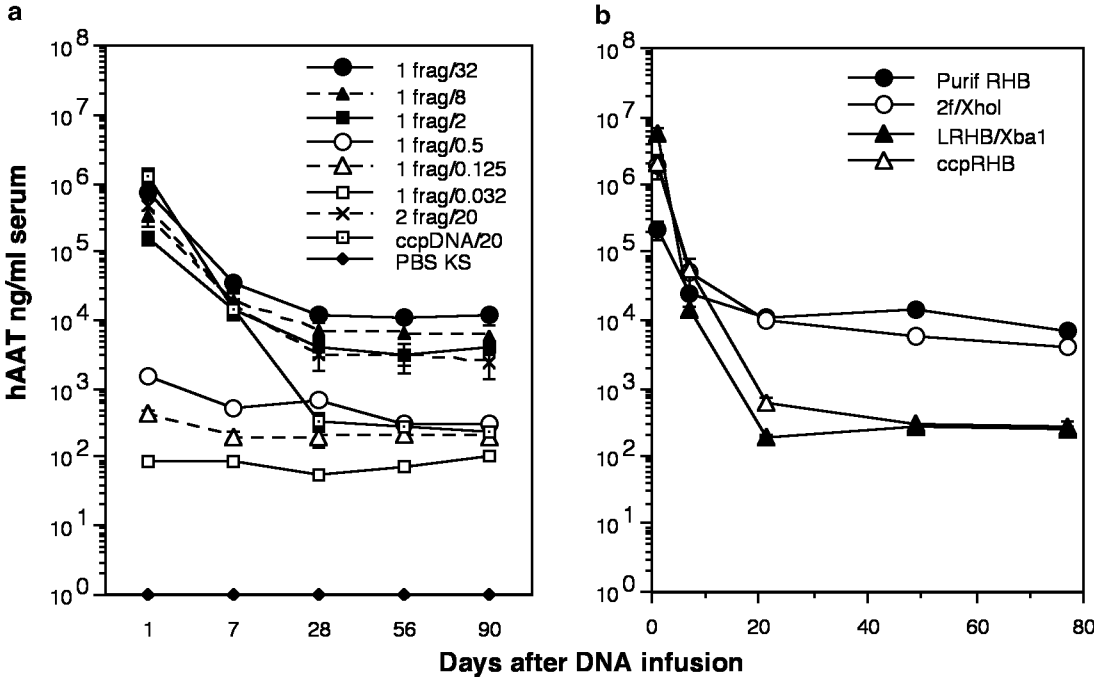

Figure 2 *Serum human AAT levels from mice injected with different doses of purified RSV.hAAT.bpA expression cassette (1fRHB), 2fRHB, LRHB or uncut closed circular DNA (ccpRHB). (a) Each mouse received 32 ng to 32 μg of 1fRHB, or 20 μg of 2fRHB or ccpRHB, which represents the same amount of expression cassette equal to 8 μg of 1fRHB (n = 5 mice per group). (b) Each mouse received 40 μg of 2fRHB, LRHB, ccpRHB or an equal molar amount of 1fRHB (16 μg of the pure RSV.hAAT.BpA fragment per mouse, n = 5 mice per group).*

3.4 and 5.0 kb in the LRHB samples, and 2.1 and 3.4 kb in the 1fRHB group, represent the *Hin*dIII cleavage products of head to tail, and tail to tail sequences in each group. When the blot was probed with a full-length expression cassette instead of human AAT cDNA, a third band of about 0.8 kb, and 6.4 kb in the 1fRHB and LRHB groups, respectively, was found (data not shown), indicating the existence of head to head junctions. These

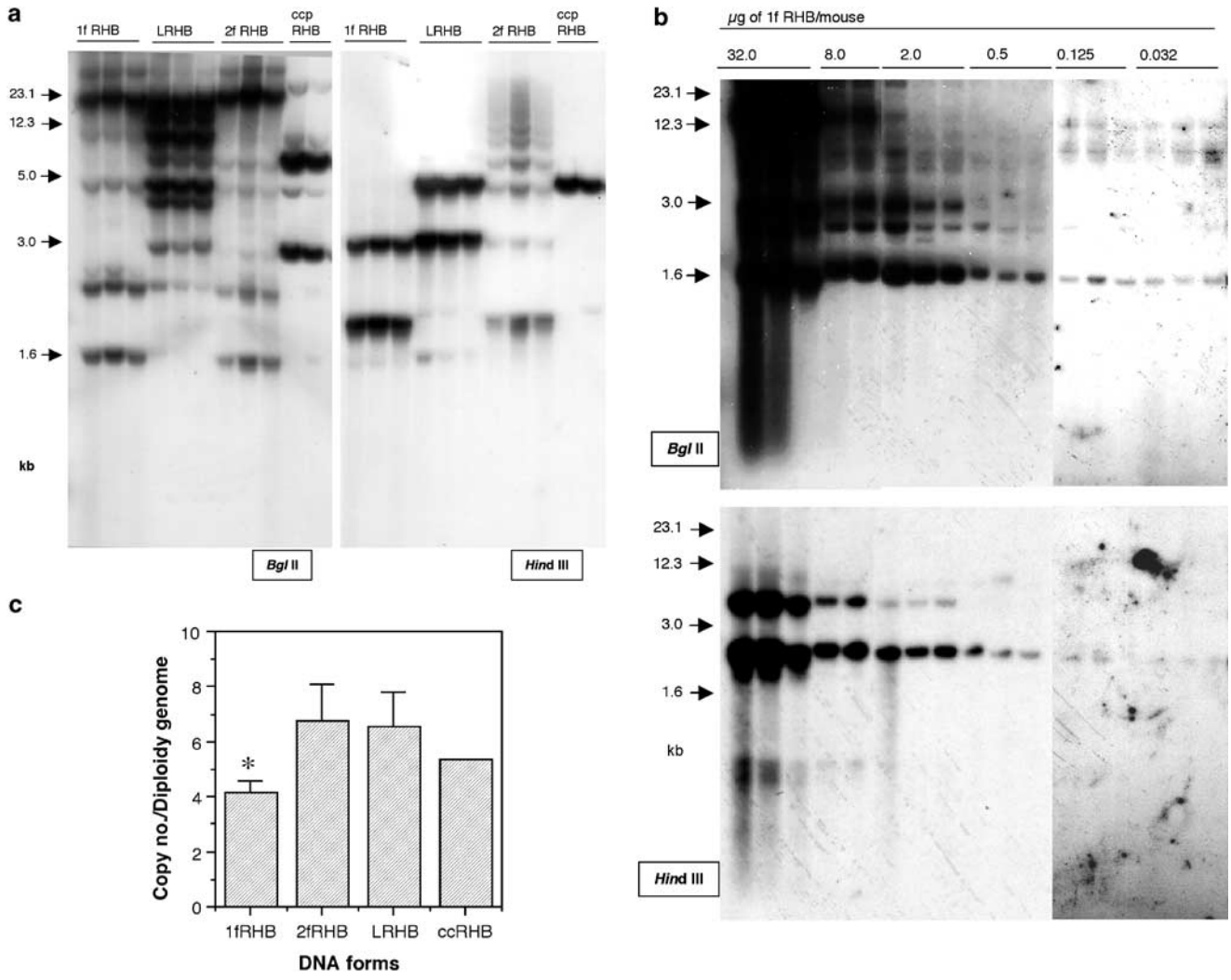

Figure 3 *Southern blot analysis of vector structure in mouse livers. (a) In all, 20 μg of liver DNA from mice receiving the four forms of DNA, as indicated in the legend of Figure 2b and killed 11 weeks post-DNA injection, was digested with either the 0-cutter Bgl II (left panel) or the one-cutter HindIII (right panel). The blot was probed with a radio labeled 1.35 kb EcoR1 fragment of hAAT cDNA as indicated in Figure 1. (b) The liver DNA was digested with BglII in the upper panel and with HindIII in the lower panel. The liver DNAs were from the same group of mice as indicated in the legend of Figure 2a and killed at week 11. All the experimental conditions were the same as described in (a). (c) Phosphoimager quantification of vector DNA in liver DNA samples used in (a). *Indicates the difference in the copy number per diploid genome is statistically significant as compared to 2fRHB or LRHB group at $P<0.01$, and <0.01.*

observations strongly suggest that linear DNAs link together randomly, probably via a nonhomology end joining mechanism,[15] to form large concatemers or smaller circles. As the LRHB formed large concatemers and expressed a 20–55 times lower level of serum human AAT, with a pattern closely similar to that of the ccpRHB group (Figure 2b), our observations strongly suggest that formation of large concatemers was not responsible for the high levels of transgene expression in 1fRHB- and 2fRHB-treated animals.

When the structure of vector DNA in the livers of mice receiving different amounts of 1fRHB were analyzed by Southern blot, a very strong vector DNA signal >23 kb was detected in mice receiving 32 μg of 1fRHB, while a much weaker signal was detected in 8 μg 1fRHB-treated animals. No signal was detected in mice receiving lower doses of linear DNA (Figure 3b). In the mice receiving less than 0.5 μg each of 1fRHB DNA, a single band was observed in *Bgl*II- (no cutter) or *Hin*dIII- (single cutter) digested DNA, indicating that the vector formed a circularized RSV.hAAT.bpA expression cassette monomer. These observations suggested that formation of large concatemers occurred only when the linear DNA concentration reached a threshold in the hepatocytes. These results are not unexpected, because at low DNA concentrations intramolecular ligations are likely favored over intermolecular ligations. Since low but stable levels of serum human AAT were detected in all mice receiving three low doses, 32–500 ng each, of 1fRHB (Figure 2a), this suggested that the circular expression cassette monomer was the transcriptionally active DNA form *in vivo*. This view was furthermore confirmed by the observations that minicircle vectors could express robust levels of transgene reporters persistently.[16]

To determine if a difference in the amount of vector DNA in mouse liver contributed to the variation in transgene expression between the different experimental groups, liver DNA from mice receiving four different forms of DNA, as indicated in the legends of Figures 2b and 3a, were used for the determination of vector DNA

copy number (Figure 3c). Consistent with our previous observation,[1] about four to seven copies of vector DNA per diploid genome were detected among the 2fRHB, LRHB and ccpRHB groups. However, significantly less vector was observed in 1fRHB compared to 2fRHB- and LHRB-treated mice ($P<0.01$). Since higher amounts of transgene product were expressed from significantly less 1fRHB DNA in mouse liver, these results exclude the possibility that differences in transgene expression resulted from the difference in the amount of vector DNA in mouse liver. Taken together, these results suggest that it is the distance or dissociation of the expression cassette from the bacterial backbone that results in high levels of transgene expression from RSV.hAAT.bpA, and the formation of large concatemers, *per se*, did not contribute to the maintenance of high levels of transgene expression.

Bacterial DNA inhibitory effect was promoter/enhancer independent

It is possible that the bacterial DNA sequences present in plasmid DNA do not universally inhibit transgene expression from all expression cassettes. To determine if other sequences might be refractory, we used four other promoter/enhancers, including two viral promoters, CMV and SV40, and two cellular promoters, heI4AF1 and glyceraldehyde 3-phosphate dehydrogenase promoter (GAPDH), in place of the RSV promoter. Purified expression cassette, two DNA fragments and uncut ccDNA were infused into groups of mice and serum human AAT levels were compared (Figure 4a). Additional promoters with the human FIX expression cassettes in vectors psApoE.hFIX + Int A.bpA and pEF1α.hFIX.hGHpA were also tested (Figure 4b). As expected, serum transgene protein levels were different with the various promoters. The serum human AAT levels, from the respective purified hAAT expression cassettes varied by up to more than 100-fold. Furthermore, uncut plasmids psApoE.hFIX + Int A.bpA, pGAPDH.hAAT.bpA and pCMV.hAAT.bpA expressed a low level of serum human FIX or human AAT throughout the 11- or 20-week time period, while the transgene expression from uncut pEF1α.hFIX.hGHpA and pheIF.hAAT.bpA plasmids declined to undetectable levels within 7 weeks. While some of these observations may reflect the differences in transgene expression activity from different promoter/enhancers, a general expression pattern was revealed from all six constructs. All six purified expression cassettes expressed a higher level of transgene product than their two DNA fragment counterparts, and all of the uncut circular plasmids expressed the lowest levels of transgene product shortly after DNA infusion. The serum transgene reporter from mice receiving one fragment DNA could be 2- to 3-log higher than that of the uncut plasmid DNA group (Figure 4a). Thus, our data demonstrate that the inhibitory effect of bacterial DNA sequence was reporter and promoter/enhancer independent.

Different bacterial DNA sequences had the same inhibitory effect

Five of the six constructs tested were based on the plasmid backbone pBS.KS, while the sixth construct, although containing different sequences, had the same ampicillin resistance gene and the pMB1 DNA origin of replication, which was highly homologous to ColE1 of pBS.KS. To determine if any of the bacterial sequences in common between the plasmids were responsible for the transcriptional inhibition, the DNA origin ColE1 was replaced with p15A from pACYC184, and the ampicillin resistance gene was replaced by the chloramphenicol (CM) resistance gene from pBC.KS. Transgene expression pattern from uncut plasmid DNA of these two constructs was compared with that from mice receiving either 2fRHB or uncut ccpRHB (Figure 4c). Both uncut circular pCM.RHB and p15A.RHB demonstrated a serum human AAT expression pattern similar to that of ccpRHB. These two experiments suggest that the negative effect of bacterial backbone was independent of a specific bacterial DNA sequence.

Discussion

In this study, we tested a total of nine constructs with seven different expression cassettes. All of the data indicate that covalent linkage of the bacterial DNA silenced the transgene expression cassette in episomal vectors in mouse livers. Consistent with our previous observation that minicircle DNA vectors expressed sustainable high levels of transgene products,[16] exclusion of bacterial DNA by using purified expression cassette resulted in up to 2- to 3-log higher levels of transgene expression as compared to their standard plasmid counterparts in mouse livers. In agreement with previous observations,[1] the enhanced transgene expression also occurred in two fragment (linear expression cassettes and bacterial plasmid backbone) DNA groups, as compared to intact circular plasmid groups.

Importantly, the difference in transgene expression activity was not due to the difference in vector DNA in mouse livers because, as we have demonstrated previously,[1,16] the absolute amount of vector DNA was similar regardless of which form of the vector was infused. Although linear DNA molecules can concatemerize *in vivo*, our data indicate that concatemerization of vector DNA, *per se*, did not support transgene expression as we speculated earlier.[1] Linearized DNA generated by one cut through the bacterial DNA also formed large concatemers and demonstrated a similar depressed transgene expression profile as the uncut plasmids. Our data also suggest that concatemerization was not necessary for sustained high levels of transgene expression. In the two fragment DNA groups, expression cassette circles free of bacterial DNAs were formed alongside large concatemers. In the light of our earlier observations of the robust transgene expression profiles of minicircle vectors,[16] it appears that it was the bacterial DNA-free recircularized expression cassettes alone or in combination with large concatemers with one or more expression cassette units separated from the bacterial backbone sequence that remained transcriptionally active. Taken together, our data further suggest that a covalent connection between the bacterial DNA and expression cassette was necessary for transgene silencing *in vivo*.

The molecular mechanism underlining the bacterial DNA silencing is not known at present. However, based on the data of the present study, as well as others, several

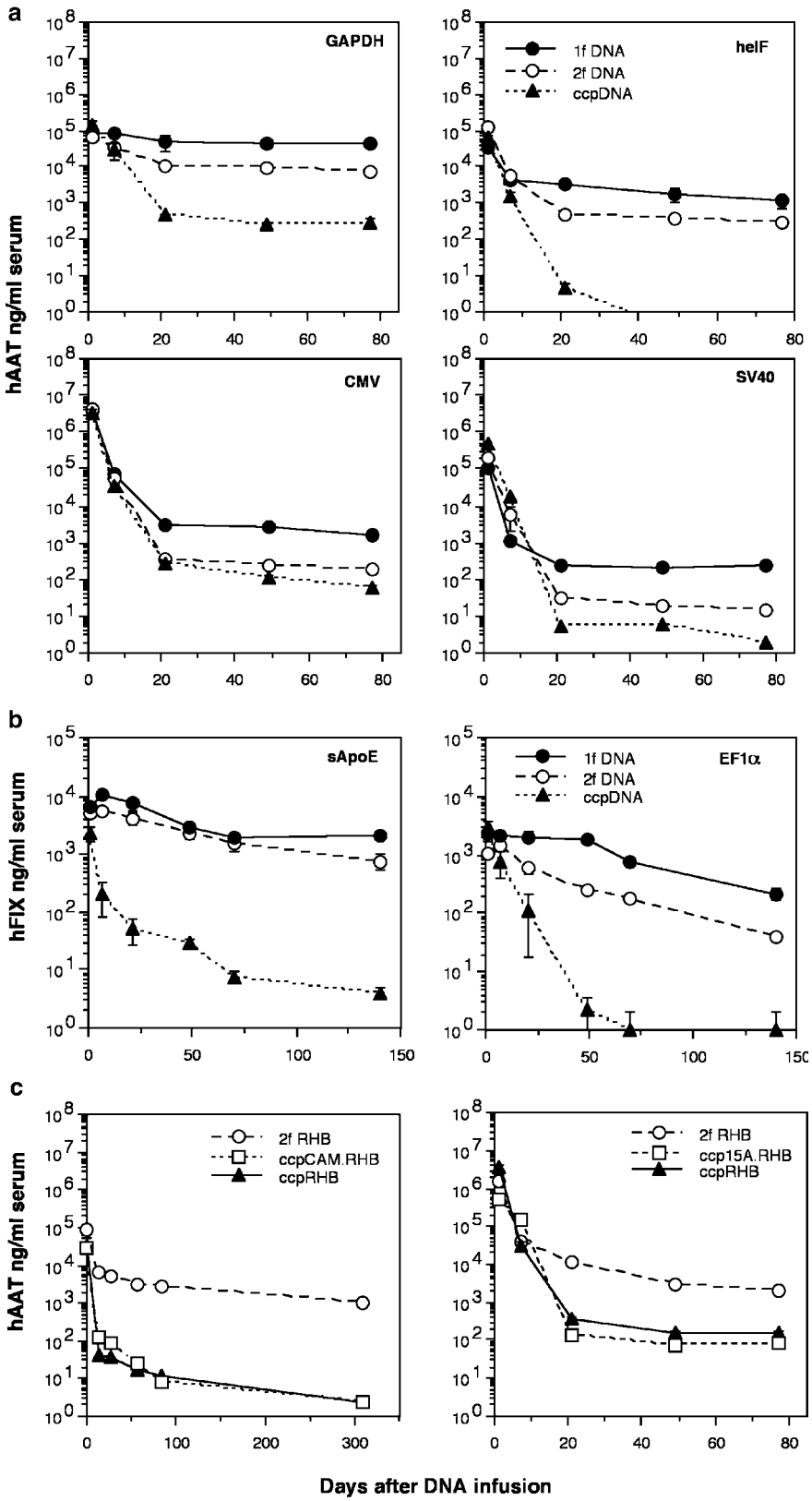

Figure 4 *Serum human AAT or human FIX from mice receiving one of the three forms of vectors (1fDNA, 2f DNA or ccDNA) with different DNA components. (a) Serum human AAT from mice receiving 40 μg of 2fDNA, ccDNA or an equal molar amount of purified expression cassette (1fDNA) from pGAPDH-, pheIF-, pCMV- or pSV.hAAT.bpA. (b) Serum human FIX levels from mice receiving 20 μg of two-fragment DNA (2fDNA), uncut ccDNA or an equal molar amount of purified expression cassette (1fDNA), from either p.EF1α.hFIX.bpA (EF1α) or psApoE.HCR.hAATp.hFIX + Int A.bpA (sApoE). (c) Serum human AAT from mice receiving 20 μg of 2fRHB, or ccpRHB derived from vectors containing the p15A (replacing the ColE1 DNA), or CM resistance gene (replacing the ampicillin resistance gene) (n = 5 mice per group).*

possibilities are worthy of serious consideration. One prominent functional feature common to all the bacterial backbones, for example, is that they are transcriptionally inactive in mammalian cells, although the eukaryotic expression cassettes in *cis*- are active. Functionally, these transcriptionally inert DNA sequences are similar to the heterochromatin in eukaryotic cells and we speculate that they may also share a similar chromatin structure.[17] Differences in many molecules composing the heterochromatin and euchromatin have been defined. For example, nonacetylated histones 3 and 4 bind DNA tightly and are the features of heterochromatin, while acetylation of these two histones will loosen their binding to DNA, allowing the formation of more open euchromatin.[17] The machinery responsible for the maintenance of chromatin status has also been studied in depth. It has been demonstrated that components of the transcription complex have acetylase activities, which are responsible for the addition of acetyl group to histones 3 and 4.[18] Since the bacterial DNA is not transcribed and lacks such a mechanism to maintain the acetylation status of histones 3 and 4, the chromatin in this region may be more condensed. If this is the case, it may be possible to unravel the mechanism of the inhibitory effect exhibited by the bacterial sequences.

It has been well documented that there are insulators between euchromatin and heterochromatin, and deletion of these insulators will result in the spreading of the heterochromatin into the neighboring euchromatin, and consequently the silencing of the genes.[19] As no known insulator was included in all the constructs tested in this study, heterochromatin of the bacterial backbone, if formed, could spread freely into the expression cassette, resulting in the silencing of the uncut circular plasmid. In contrast, in a scenario such as when the bacterial DNA and eukaryotic expression cassette were transfected as two molecules, heterochromatin spreading and transgene silencing would be limited to those molecules that remained physically connected with the bacterial sequence. Thus, a substantial number of the expression cassettes, for example, those that formed small circles free of bacterial DNA, and/or were distanced from bacterial DNA in large concatemers, could maintain a more open euchromatin status and a higher rate of transcription activity. Kass *et al*[20] have demonstrated the spreading of a 'repressive nucleoprotein structure' in the center of a methylation site into a neighboring promoter, resulting in the silencing of a plasmid injected into *Xenopus* oocytes.

The influence of cytosine methylation status in the vector DNA on the transgene expression is worthy of careful consideration. The frequency of CpG dinucleotides is much higher in bacterial DNA than in vertebrate DNA.[10] Consistent with this hypothesis, in the plasmid pRSV.hAAT.bpA used in this study, the frequency of CpG dinucleotides in the ampicillin resistance gene-ColE1 origin region (6.4 CpG per 100 bases) is 2.6 times of that in the RSV.hAAT.bpA expression cassette (2.8 CpG per 100 bases). A similar higher frequency of CpG was also seen in the CM resistance gene and the p15A DNA origin (4.9, and 5.8 per 100 bases, respectively). In this study, the CpG dinucleotides in plasmid DNA derived from the bacterial strain (XL1-blue F′ of Stratagene) were unmethylated; however, we cannot rule out methylation, which might occur after the DNA was introduced into the liver. Nonetheless, we demonstrated that the inhibitory effect of the bacterial backbone was DNA sequence independent. All three of the bacterial backbone elements, including the F1 phage origin of replication, plasmid origin of replication and antibiotic resistant gene, were systemically tested by either being deleted or placed in different combinations. All four plasmids demonstrated the same depressed transgene expression profile. This DNA sequence-independent inhibitory phenomenon suggests a common mechanism unrelated to specific DNA sequence, and the high frequency of unmethylated CpG dinucleotide common to all bacterial DNA elements is a logical candidate.

It has been well established that DNA carrying multiple unmethylated CpG motifs is highly immunogenic,[21] and has been used as an adjuvant for DNA vaccines against cancers and other diseases.[22] Unmethylated CpG motifs when complexed to cationic lipids could elicit acute inflammatory reactions, resulting in the activation of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), and necrosis-, apoptosis-mediated DNA-transduced cell death, and consequently the loss of transgene expression *in vivo*.[8,9,23–25] Inflammatory reactions stimulated by unmethylated CpG motifs are an unwanted side effect for vectors used to express therapeutic proteins. However, data from this and other studies were not consistent with this as a major mechanism of bacterial DNA inhibitory effect, especially when vector DNA was delivered without cationic lipids. In this and previous studies,[1,16] we have repeatedly demonstrated that 2fDNA always expressed a much higher level of transgene products than standard plasmids, although they shared exactly the same DNA sequence, and the same amount of vector DNA persisted in mouse liver long after administration and in no cases was there a selective loss of cells harboring the bacterial DNA. Thus, the preferential loss of vector DNA-containing cells is not a likely mechanism for the loss of transgene expression. In addition, a cytotoxic response was not observed after systemic delivery of either oligonucleotides containing potent immunogenic unmethylated CpG motifs,[9] or plasmid DNA[8,26] alone. Furthermore, a similar transient transgene expression profile was observed in both immune-competent and -deficient mice,[2,27] excluding the possibility of the involvement of a cytotoxic T-cell-mediated mechanism. Finally, several other studies[2,6,7] have provided evidence suggesting that it is the promoter silencing, not the vector DNA loss, that is responsible for the loss of transgene expression.

An alternative noncytodestructive immunologic mechanism explaining the transgene silencing effect was proposed by Qin *et al*,[6] who demonstrated that IFN-γ and TNF-α could selectively inhibit several viral promoters delivered in adenoviral, retroviral or plasmid vectors *in vitro* and *in vivo*, while the constitutive cellular promoter β-actin was less affected. It was also suggested that the cytokines exert an inhibitory effect at multiple levels including a direct effect at the transcriptional level by turning off the viral promoter.[10] It is well known that cytokines, including IFN-γ and TNF-α, are a part of the innate immune system playing a key role in host antiviral activities. It has been suggested that the unmethylated CpG motifs can bind to a specific

intracellular receptor and activate the nuclear factor-κB (NF-κB), which is a transcriptional factor responsible for the induction of many cytokines.[10,28] The role of CpG dinucleotide-cytokine signaling in transgene silencing appears to occur in other gene transfer studies where an enhancement of transgene expression was demonstrated by the administration of the anti-inflammatory agent dexamethasone,[9] and partial deletion of CpG motifs in plasmid bacterial backbone.[7] In the present study, all three viral and four mammalian enhancer/promoters examined were silenced in mouse liver when delivered either as intact circular plasmid or as linearized DNA generated by one cut through bacterial backbone, whereas the enhanced transgene expression resulted when dissociation between the two DNA elements occurred, in either two fragment DNA, purified expression cassette, or minicircle group.[16] Furthermore, our results indicate that the silenced promoters (those that connected with bacterial backbone) and the active promoters (those that form DNA circle free of bacterial DNA) could coexist in the same cell in two fragment DNA group, indicating that a promoter remained active, as long as it was not covalently connected with bacterial backbone. Taken together, our result suggest that the silencing is not the consequence of a direct inhibition, but more likely resulted from an event which first occurred at the bacterial DNA sequences, and then spread to the downstream promoter, resulting in its inactivation, a phenomenon as described by Kass *et al*,[20] and discussed earlier.

Another CpG motif-related mechanism was raised by Hong *et al*,[29] who provided preliminary evidence to suggest that CpG dinucleotides in episomal vector DNA could undergo *de novo* methylation in mammalian cells, and suggested that this was a mechanism of episomal transgene silencing. In eukaryotic cells, C5 methylation of the CpG is one major mechanism of the regulation of gene expression in chromosomes.[30,31] Methylated CpG dinucleotides can become the targets of a group of cellular proteins, including CPM1 and CPM2, whose binding would result in the condensation of DNA, and consequently, the silencing of genes.[32] If this was the case, the problematic CpG clusters located within the bacterial DNA sequence could trigger a nucleosome condensation process,[33,34] which could then spread to the vicinity of the transgene, resulting in its silencing. It remains possible that this *in vivo* CpG methylation is a consequence of cytokine activation and a mechanism of intracellular defense that evolved over time to silence pathogenic organisms.

Materials and methods

Vector construction

Construction of the plasmids pRSV.hAAT.bpA (Figure 1) expressing human AAT driven by RSV promoter, and psApoE.hFIX + Int A.bpA expressing the human FIX under the control of a hybrid promoter/enhancer composed of the human AAT promoter and the hepatic locus control region (HCR) from the ApoE gene, were described previously.[1,35] Human CMV immediate-early gene promoter derived from pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), human glyceraldehyde 3′-phosphate dehydrogenase promoter (GAPDH) and eukaryotic initiation factor 4A1 promoter (heIF) from pDRIVE-hGAPDH and pDRIVE-heIF4A1 (InvivoGene, San Diego, CA, USA), respectively, were used to replace the RSV promoter in pRSV.hAAT.bpA, resulting in plasmids pCMV.hAAT.bpA, pGAPDH.hAAT.bpA and pheIF.-hAAT.bpA. These promoters were amplified from individual plasmids using PCR primers embedding a *Hin*dIII or *Kpn*I site, allowing them to replace the RSV promoter in pRSV.hAAT.bpA. The hAAT.bpA fragment was used to replace the EM7.Zeocin.SV40 poly-A fragment in the pSV40/Zeo2 (Invitrogen, Carlsbad, CA, USA), resulting in pMB1.SV40.hAAT. The pCM.RSV.hAAT.bpA was constructed by replacing the *Bsp*H1–*Bsp*H1 fragment encoding the Ampicillin resistance gene in pRSV.hAAT.bpA with the *Bsp*H1–*Bsp*H1 fragment encoding the CM resistance gene from pBC.KS(+) (Stratagene, La Jolla, CA, USA). PCR product of the DNA origin p15A using pACYC184 (New England Biolabs, Beverly, MA, USA) as template was used to replace the ColE1 in pRSV.hAAT.bpA, resulting in p15A.RSV.hAAT.bpA. The plasmid pEF1α.h-FIX.hGHpA expressing the human FIX under the control of the human elongation factor 1α promoter (EF1α), was derived from pT.EF1α.hFIX[36] by inserting a pair of DNA oligonucleotides encoding an *Spe*I site into its *Not*I site downstream of the human growth hormone polyadenylation signal (hGHpA), allowing the EF1α.hFIX.hGHpA expression cassette to be released by *Spe*I digestion and relocated into the *Spe*I site of pBS.KS.II (Stratagene). All replacement of promoter/enhancers, antibiotic resistance genes, the DNA origin or the expression cassette was confirmed by DNA sequencing.

DNA preparation

All plasmid DNA were prepared using Qiagen (Valencia, CA, USA) endotoxin-free kits. The two-fragment DNA (2fDNA) form of pEF1α.hFIX.hGHpA (Figure 1) and psApoE.hFIX + Int A.bpA were prepared by digestion of the plasmids with *Spe*I, which cut twice through the bacterial DNA to release the expression cassettes. The 2fDNA from all other plasmids were prepared by digestion of individual plasmid with *Xho*I. After gel electrophoresis confirmation of completeness of the digestion, the reactions were extracted once with phenol:choroform:amyl alcohol (25:24:1), twice with chloroform:amyl alcohol (24:1), and the DNA was recovered by ethanol precipitation. To prepare the purified expression cassette, the two DNA fragments resulted from restriction digestion were separated by gel electrophoresis, the desired expression cassettes were cut out and the DNA was electroeluted from the gel and recovered by ethanol precipitation. All DNAs were dialyzed against TE (pH 8.0) for at least 24 h before delivery to mice.

Animal studies

Each dose of DNA, ranging from 32 ng to 80 μg per mouse, was dissolved in 1.8 ml of saline and infused into mouse liver using the hydrodynamic protocol of Liu *et al*[13] and Zhan *et al*.[37] C57L/6 mice (5–8 weeks old) from Taconi Farms, Inc. (Germantown, NY, USA) were used. Mice were bled periodically using a retro-orbital technique. Serum human FIX and human AAT were quantified by ELISA as described previously.[36]

Southern blot analysis

Mouse liver DNA was prepared using a salt-out protocol. In all, 20 μg of liver DNA from mice receiving different forms of DNA derived from pRSV.hAAT.bpA was digested with *Bgl*II, which did not cut the vector, or *Hin*dIII, which cut once through the expression cassette, resolved by gel electrophoresis, and probed with the radio-labeled 1.35 kb hAAT cDNA fragment from pRSV.hAAT.bpA (Figure 1). The radioactive vector DNA bands were quantified by phosphoimager analysis as described previously.[1]

Acknowledgements

We are extremely grateful to Theresa Storm for critical reading of this paper. This work was supported by NIH HL-64274.

References

1 Chen ZY *et al*. Linear DNAs concatemerize *in vivo* and result in sustained transgene expression in mouse liver. *Mol Ther* 2001; **3**: 403–410.
2 Gill DR *et al*. Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. *Gene Therapy* 2001; **8**: 1539–1546.
3 Hartikka J *et al*. An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum Gene Ther* 1996; **7**: 1205–1217.
4 Nicol F *et al*. Poly-L-glutamate, an anionic polymer, enhances transgene expression for plasmids delivered by intramuscular injection with *in vivo* electroporation. *Gene Therapy* 2002; **9**: 1351–1358.
5 Wolff JA *et al*. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum Mol Genet* 1992; **1**: 363–369.
6 Qin L *et al*. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum Gene Ther* 1997; **8**: 2019–2029.
7 Yew NS *et al*. High and sustained transgene expression *in vivo* from plasmid vectors containing a hybrid ubiquitin promoter. *Mol Ther* 2001; **4**: 75–82.
8 Li S *et al*. Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. *Am J Physiol* 1999; **276**: L796–804.
9 Tan Y, Li S, Pitt BR, Huang L. The inhibitory role of CpG immunostimulatory motifs in cationic lipid vector-mediated transgene expression *in vivo*. *Hum Gene Ther* 1999; **10**: 2153–2161.
10 Paillard F. CpG: the double-edged sword. *Hum Gene Ther* 1999; **10**: 2089–2090.
11 Miao CH, Thompson AR, Loeb K, Ye X. Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer *in vivo*. *Mol Ther* 2001; **3**: 947–957.
12 Yew NS *et al*. CpG-depleted plasmid DNA vectors with enhanced safety and long-term gene expression *in vivo*. *Mol Ther* 2002; **5**: 731–738.
13 Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Therapy* 1999; **6**: 1258–1266.
14 Kramer MG *et al*. *In vitro* and *in vivo* comparative study of chimeric liver-specific promoters. *Mol Ther* 2003; **7**: 375–385.
15 Critchlow SE, Jackson SP. DNA end-joining: from yeast to man. *Trends Biochem Sci* 1998; **23**: 394–398.
16 Chen ZY, He CY, Ehrhrdt A, Kay MA. Minicircle DNA vectors devoid of bacterial DNA result in persistent and high level transgene expression *in vivo*. *Mol Ther* 2003; **8**: 495–500.
17 Wolffe AP. Histone modification and transcriptional competence. In: Driel RV, Otte AP (eds) *Nuclear Organization, Chromatin Structure, and Gene Expression*. Oxford University Press: Oxford, New York, Tokyo, 1997, pp 40–57.
18 Utley RT *et al*. Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. *Nature* 1998; **394**: 498–502.
19 Noma K, Allis CD, Grewal SI. Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science* 2001; **293**: 1150–1155.
20 Kass SU, Landsberger N, Wolffe AP. DNA methylation directs a time-dependent repression of transcription initiation. *Curr Biol* 1997; **7**: 157–165.
21 Krieg AM *et al*. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995; **374**: 546–549.
22 Krieg AM *et al*. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. *Proc Natl Acad Sci USA* 1998; **95**: 12631–12636.
23 Schwartz DA *et al*. CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract. *J Clin Invest* 1997; **100**: 68–73.
24 Yew NS *et al*. Contribution of plasmid DNA to inflammation in the lung after administration of cationic lipid:pDNA complexes. *Hum Gene Ther* 1999; **10**: 223–234.
25 Freimark BD *et al*. Cationic lipids enhance cytokine and cell influx levels in the lung following administration of plasmid: cationic lipid complexes. *J Immunol* 1998; **160**: 4580–4586.
26 Scheule RK *et al*. Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Hum Gene Ther* 1997; **8**: 689–707.
27 Huang CY *et al*. Enhancements in gene expression by the choice of plasmid DNA formulations containing neutral polymeric excipients. *J Pharmacol Sci* 2002; **91**: 1371–1381.
28 Hemmi H *et al*. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**: 740–745.
29 Hong K, Sherley J, Lauffenburger DA. Methylation of episomal plasmids as a barrier to transient gene expression via a synthetic delivery vector. *Biomol Eng* 2001; **18**: 185–192.
30 Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. *Science* 1975; **187**: 226–232.
31 Sager R, Kitchin R. Selective silencing of eukaryotic DNA. *Science* 1975; **189**: 426–433.
32 Bird AP, Wolffe AP. Methylation-induced repression – belts, braces, and chromatin. *Cell* 1999; **99**: 451–454.
33 Schubeler D *et al*. Genomic targeting of methylated DNA: influence of methylation on transcription, replication, chromatin structure, and histone acetylation. *Mol Cell Biol* 2000; **20**: 9103–9112.
34 Mummaneni P, Walker KA, Bishop PL, Turker MS. Epigenetic gene inactivation induced by a *cis*-acting methylation center. *J Biol Chem* 1995; **270**: 788–792.
35 Miao CH *et al*. 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* 2000; **1**: 522–532.
36 Yant SR *et al*. Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat Genet* 2000; **25**: 35–41.
37 Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* 1999; **10**: 1735–1737.