

Optimization of Cis-Acting Elements for Gene Expression from Nonviral Vectors *In Vivo*

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ABSTRACT

While naked DNA gene transfer *in vivo* usually results in transient gene expression, in some cases long-term transgene expression can be achieved. Here we demonstrate that cis-acting DNA elements flanking the transgene expression cassette and components in the plasmid backbone can significantly influence expression levels from nonviral vectors. To demonstrate this, we administered our most robust human coagulation factor IX (hFIX) expression cassette placed in two different plasmid backbones, into the livers of mice, by hydrodynamic transfection. We found that placing the expression cassette within a minimal plasmid vector pHM5, a modified version of pUC19, resulted in 10 times higher serum hFIX expression levels (up to 20,000 ng/ml, 400% of normal hFIX serum levels), compared to a pBluescript backbone. To optimally increase expression levels from a nonviral vector, we added matrix attachment regions (MARs) as cis-acting DNA elements flanking the hFIX expression cassette. We detected five fold higher hFIX expression levels *in vivo* for up to 1-year posttransfection from a vector that contained the chicken MAR from the lysozyme locus. Together, the present work demonstrates that in addition to the transgene expression cassette, cis-acting DNA elements within and outside of the plasmid backbone need to be evaluated to achieve optimal expression levels in a nonviral gene therapy approach.

OVERVIEW SUMMARY

Nonviral gene therapy vectors encoding for the human coagulation factor IX (hFIX) with various cis-acting DNA elements were injected via high pressure tail vein injection into C57Bl/6 mice ($n = 5$ per group). After injection of the nonviral vector pHM5 in which the bacteriophage $\phi 1$ origin of replication and a portion of the *lacZ* gene is deleted from the bacterial DNA backbone, 10 times higher hFIX serum concentrations (up to 20,000 ng/ml) were obtained for the duration of the study (4 weeks). To increase transgene expression levels further, matrix attachment regions (MARs) as additional cis-acting elements were placed into the nonviral vector pHM5. We found that MARs can result in prolonged transgene expression levels from a circular nonviral vector *in vivo*. Taken together, these results suggest that cis-acting elements including the bacterial DNA backbone are essential in achieving optimal transgene expression levels from a nonviral vector *in vivo*.

INTRODUCTION

THERE HAVE BEEN VARIED RESULTS related to the degree of persistence of transgene expression after naked DNA gene transfer *in vivo* (Zhang *et al.*, 1999, 2000; Yant *et al.*, 2000; Chen *et al.*, 2001; Herweijer *et al.*, 2001; Miao *et al.*, 2001; Stoll *et al.*, 2001). Nonetheless, long-term correction of genetic diseases was demonstrated in studies in which episomal or integrating nonviral vectors were used (Miao *et al.*, 2000; Yant *et al.*, 2000; Chen *et al.*, 2001). The ability to maintain transgene expression in the absence of vector integration is important because it eliminates safety concerns such as insertional mutagenesis from random integration events. In the present study we engineered nonreplicating and nonintegrating vectors with stabilization elements and evaluated changes in expression levels *in vivo*.

While clinically relevant and efficient delivery methods for nonviral transduction are currently being developed, it is important to establish optimal expression cassettes for DNA trans-

fer *in vivo*. Some progress in determining factors involved in optimizing the nonviral vector system has been made. For nonviral liver gene therapy approaches, it has been demonstrated that liver specific enhancers and promoters increase transgene expression levels *in vivo* (Brooks *et al.*, 1991; Miao *et al.*, 2000). Furthermore, we have recently shown that the molecular conformation of the delivered DNA can significantly affect transgene expression levels. For example, when the DNA is linearized into two pieces prior to delivery, higher and more sustained transgene expression levels were obtained compared to an intact circular DNA (Chen *et al.*, 2001). Additionally, there is strong evidence that the bacterial sequences that exist in most nonviral gene therapy vectors influence transgene expression levels. It was demonstrated that a minicircle deleted for all bacterial sequences resulted in 13- to 50-fold higher reporter gene expression levels *in vitro* and *in vivo* than with an undeleted vector (Darquet *et al.*, 1997, 1999). It is well established that in eukaryotes, the presence of 5-methylcytosine dinucleotides -CG- is involved in the silencing of gene expression (Allamane *et al.*, 2000; Yew *et al.*, 2002). Furthermore it was demonstrated that deletion of CpG motifs in the nonviral DNA vector results in a reduced immunostimulatory response *in vivo* (Krieg, 1999; Yew *et al.*, 2000). However, the mechanism by which these dinucleotide motifs silence gene expression has not been definitively established.

Another group of cis-acting elements are scaffold or matrix attachment regions (MARs), which contain 100 to 1000 bp AT-rich sequences without defined consensus sequences. MARs have been shown to increase transgene expression levels *in vitro* and *in vivo* (McKnight *et al.*, 1992; Goyenechea *et al.*, 1997) and are known to provide insulation effects between transcription units and may function as origins of replication. Viral recombinant gene therapy vectors with and without MARs have been constructed (Agarwal *et al.*, 1998; Parks *et al.*, 1999; Dang *et al.*, 2000; Park and Kay, 2001; Ehrhardt and Kay, 2002). In the context of integrating vector systems (retroviruses and lentiviruses), MARs can either enhance or decrease transgene expression levels. In adenoviral episomal vector systems MARs seem to have no significant effect on either stability of the vector genomes or the transgene-derived expression levels. In this study, we examined MARs to determine if they had an effect on expression levels from episomal nonviral gene therapy vectors *in vivo*.

MATERIALS AND METHODS

Plasmid expression vectors

The plasmids pBS-ApoEHCR-hAATp-FIX-Int-bpA, pBS-ApoEHCR-hAATp-FIX-Int-3'UTR-bpA, and pHM5 were recently published (Mizuguchi and Kay, 1998, 1999; Miao *et al.*, 2000). The plasmid pBS-ApoEHCR-hAATp-FIX-Int-3'UTR-bpA contains, in addition to the plasmid pBS-ApoEHCR-hAATp-FIX-Int-bpA, the 3' untranslated region (3'UTR) of the human coagulation factor IX (hFIX) gene. Throughout the paper, the plasmids pBS-ApoEHCR-hAATp-FIX-Int-bpA and pBS-ApoEHCR-hAATp-FIX-Int-3'UTR-bpA are called pBS/hFIX and pBS/hFIXmg, respectively. The *Bam*HI site of pHM5 was changed to *Spe*I resulting in pHM5*Spe*I. The hFIX expression cas-

sette from pBS/hFIXmg was released by *Spe*I and cloned into the *Spe*I site of the plasmid pHM5*Spe*I resulting in pHM5/hFIXmg. The *Pst*I IgκMAR fragment from pPCRMAR (Ehrhardt and Kay, 2002) was cloned into the *Pst*I site of pHM5*Spe*I and the *Xba*I ChMAR fragment from pBS-2x(B-1-X1) (Phi-Van *et al.*, 1990) was ligated into the *Xba*I site of pHM5*Spe*I resulting in pHM5*Spe*I/IgκMAR, pHM5*Spe*I/ChMAR and pHM5*Spe*I/IgκMAR/ChMAR, respectively. The *Spe*I fragment from pBS/hFIX was cloned into the *Spe*I site of pHM5*Spe*I, pHM5*Spe*I/IgκMAR, pHM5*Spe*I/ChMAR, and pHM5*Spe*I/IgκMAR/ChMAR to generate pHM5/hFIX, pHM5/hFIX/IgκMAR, pHM5/hFIX/ChMAR and pHM5/hFIX/IgκMAR/ChMAR. To generate pHM5/RSV/hFIX the *Xho*I fragment from pBS/RSV/hFIX (Miao *et al.*, 2000), in which the hFIX cDNA is expressed under the control of the Rous sarcoma virus promoter (RSV), was ligated into the *Sal*I site of pHM5.

To produce linear DNA fragments for infusion into mouse liver, the plasmids were cut outside the expression cassette followed by a heat inactivation of the restriction endonucleases (schematically shown in Fig. 4A). The plasmid pBS/hFIX was cut with *Spe*I, pHM5/hFIX was cut with *Sal*I and *PI-Sce*I and the plasmids pHM5/hFIX/IgκMAR, pHM5/hFIX/ChMAR, and pHM5/hFIX/IgκMAR/ChMAR were cut with *I-Ceu*I and *PI-Sce*I.

Animal studies

C57Bl/6 mice (approximately 8 weeks old) were injected via high-pressure tail vein injection as previously described (Liu *et al.*, 1999; Zhang *et al.*, 1999). In order to inject the same molar amounts of the recombinant vectors in groups that were directly compared, 20 to 30 μg of vector DNA dependent of the size of the plasmid was infused into mouse liver in each experiment. To obtain serum samples blood was collected by retro-orbital technique at the predicted time points.

DNA analysis

For genomic DNA isolation the liver was removed, homogenized, and a 100-mg portion was used for DNA extraction as described previously (Kay, 1995; Peeters *et al.*, 1996).

For Southern blot analyses 20 μg of genomic DNA was digested with *Hind*III, run on a 0.8% agarose gel, and electrotransferred to a Hybond membrane (Amersham, Piscataway, NJ). The blots were hybridized with a [α -³²]dCTP-labeled cDNA hFIX probe (*Hind*III/*Eco*RI fragment from pAAVCM2), using a random priming kit (Stratagene, La Jolla, CA). To detect the total copy number per cell 1 year post-injection, a quantitative real-time polymerase chain reaction (PCR) was performed. The PCR product is located in the hFIX cDNA (nt 5032-nt 5800 from pBS-ApoEHCR(s)-hAATp-hFIXmg-bpA) and for the amplification a hFIX forward primer (5' AAGATGCCAAACCAGGTCAATT3') and a hFIX reverse primer (5' GATAGAGCCTCCACAGAATGCA3') were used.

Blood analysis

Mouse serum hFIX antigen levels were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (Kay, 1995). The normal human serum level was 5000 ng/ml. Serum glutamate pyruvate transaminase (SGPT) assays

were performed by using a diagnostic kit for colorimetric determination of SGPT (Sigma procedure No. 505-OP).

RESULTS

Optimization of the plasmid backbone for high hFIX expression levels in hepatocytes *in vivo*

Previously, we tested different hFIX expression cassettes *in vivo* and obtained the highest and most sustained transgene expression levels with an expression cassette that contained a por-

tion of the first intron of the hFIX gene and the 3' untranslated region region (3'UTR) driven by the α_1 -antitrypsin promoter, apolipoprotein E (Apo E) enhancer and hepatocyte control region (HCR) (Miao *et al.*, 2000). We placed this robust hFIX expression cassette into the two plasmid backbones pHM5 and pBS+/- resulting in pHM5/hFIXmg (Fig. 1A, I) and pBS/hFIXmg (Fig. 1A, II), respectively. The plasmid pHM5 is a modified version of the cloning vector pUC19 in which the bacteriophage f1 origin of replication and a portion of the *lacZ* gene were deleted. Thus, we called the plasmid pHM5 a minimal vector, because it only contains the two essential components required for plasmid amplification in bacteria. To directly compare

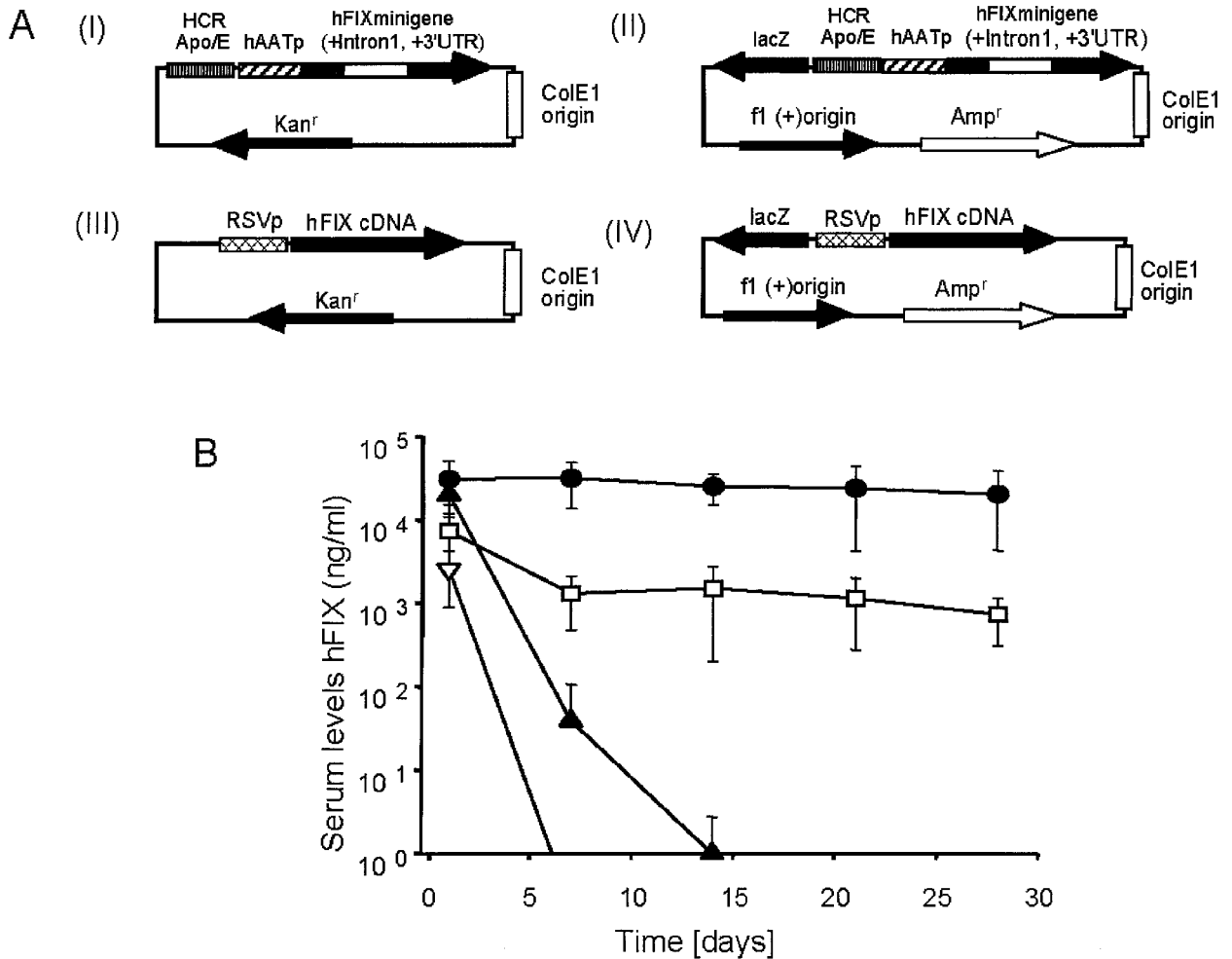


FIG. 1. Transgene expression levels from two different nonviral vectors in C57Bl/6 mice. **A:** The human coagulation FIX (hFIX) expression cassette contains the hFIX minigene with a portion of the first intron from the hFIX gene and the 3' untranslated region (3'UTR) driven by the human α_1 -antitrypsin promoter (hAATp) and the two liver-specific enhancers, apolipoprotein E (Apo E) and the hepatocyte control region (HCR). The less robust hFIX expression cassette contains the hFIX cDNA under the control of the Rous Sarcoma Virus (RSV) promoter. Both transgene expression cassettes were placed into the two bacterial backbones pBluescript (pBS, right panel) and the minimal vector pHM5 (left panel). The minimal vector pHM5 is a modified and deleted version of the plasmid pUC19 and contains the Kanamycin resistance gene and the bacterial origin of replication. The resulting plasmids were called pHM5/hFIXmg (I), pBS/hFIXmg (II), pHM5/RSV/hFIX (III) and pBS/RSV/hFIX (IV), respectively. **B:** Recombinant plasmids were infused into mouse liver by hydrodynamic delivery ($n = 5$ per group). Serum levels of hFIX after hepatic delivery of the recombinant plasmids pBS/hFIXmg = [□], pHM5/hFIXmg = [●], pBS/RSV/hFIX = [▽], and pHM5/RSV/hFIX = [▲]. Serum samples were periodically collected and hFIX serum concentrations were monitored by enzyme-linked immunosorbent assay (ELISA). Mean \pm standard deviation (SD) is shown.

the hFIX expression levels derived from pHM5/hFIXmg and pBS/hFIXmg *in vivo*, both plasmids were injected into C57Bl/6 mice by hydrodynamic infusion. Interestingly, the vector pHM5/hFIXmg resulted in higher hFIX expression levels, which were maintained during the following 4 weeks, whereas a 80% decline in hFIX serum concentrations was detected for pBS/hFIXmg (Fig. 1B). Four weeks post-injection, we detected 10-fold higher hFIX serum concentrations (up to 15,000 $\mu\text{g/ml}$) for pHM5/hFIXmg compared to pBS/hFIXmg- (1500 $\mu\text{g/ml}$) injected animals (Fig. 1B). No significant differences in liver enzyme (SGPT) levels were detected in mice treated with pBS/hFIXmg and pHM5/hFIXmg at 6 hr and 1 day after injection (Table 1). These findings demonstrate that the selection of the optimal plasmid backbone is important to obtain higher and sustained expression levels and suggested that the additional components in the pBS+/- backbone had a negative effect on transgene expression levels *in vivo*.

In a further study, we examined whether pHM5 could be used in combination with other transgene expression cassettes to obtain stabilized transgene expression levels *in vivo*. Thus, we cloned a less robust hFIX expression cassette into two vectors, pHM5 and pBS+/- in which the expression of the hFIX cDNA was driven by the RSV promoter. After infusion of the resulting plasmids, pHM5/RSV/hFIX and pBS/RSV/hFIX (Fig 1A, III and IV) into mouse liver, we found that transgene expression levels declined to undetectable levels by 6 days for pBS/RSV/hFIX, whereas pHM5/RSV/hFIX showed prolonged hFIX expression for up to 2 weeks (Fig. 1B). Two weeks post-injection, the transgene expression levels for pHM5/RSV/hFIX declined to undetectable levels. This suggested that perhaps the stabilization of transgene expression levels in the context of the vector pHM5 may be a general phenomenon.

In order to begin to unravel the molecular mechanism responsible for the differences in transgene expression levels *in vivo*, we determined vector genome copy numbers in transduced livers of treated mice. Southern blot analysis of liver genomic DNA 4 weeks post-injection revealed (Fig. 2) that there was variation in DNA copy number per cell among individual mice in each group. It is important to point out that the differences among the copy number per cell was less pronounced in the groups that received pHM5/hFIXmg and pBS/hFIXmg compared to the groups that received the plasmids, pHM5/RSV/hFIX and pBS/RSV/hFIX with the weaker expression cassette. Interestingly, and in contrast to the copy number per cell, the hFIX serum concentrations were similar in each analyzed indi-

vidual within a group. This result strongly suggests, that the negative effect of the plasmid backbone may occur at a transcriptional or posttranscriptional level, and is not simply the result of differential loss of vector genomes within transfected hepatocytes.

MARs as cis-acting DNA sequences in nonviral vectors influence transgene expression levels *in vivo*

In an attempt to assess the value of adding MARs to these plasmid vectors, we flanked the sequences around an expression cassette into our minimal vector pHM5 (Fig. 3A). In contrast to the hFIX expression cassette described in Figure 1A (I and II), we used a weaker hFIX expression cassette, allowing a simpler method to determine positive cis elements. We had previously shown that a hFIX transgene expression cassette with the inclusion of the 3'UTR resulted in higher hFIX expression levels compared the expression cassette without 3'UTR in the context of the plasmid backbone pBS (Miao *et al.*, 2000). To determine if this finding was also true for the plasmid backbone pHM5, we placed the two hFIX expression cassettes into the plasmid backbone pHM5. We obtained slightly higher hFIX serum concentrations for pHM5/hFIXmg that contained the 3'UTR compared to the vector pHM5/hFIX without 3'UTR (Fig. 3B). Taken together these data suggest that there may be a potential role of the 3'UTR in pHM5/hFIXmg. However, the use of a weaker expression cassette was beneficial for the following studies because we wanted to measure the relative transgene expression levels derived from nonviral vectors with and without MARs. The MARs used in this study were originally derived from the mouse immunoglobulin κ gene (Ig κ MAR) and the chicken lysozyme locus (ChMAR). After transfection into the liver, we found that the vector pHM5/hFIXs/ChMAR that contained the ChMAR resulted in sustained hFIX expression levels during the first 3 weeks (up to 30,000 ng/ml), whereas the expression levels derived from pBS/hFIXs, pHM5/hFIXs, and pHM5/hFIXs/Ig κ MAR, pHM5/hFIXs/ChMAR/Ig κ MAR decreased 5- to 10-fold (Fig. 3C). Interestingly, the vector pHM5/hFIXs/ChMAR resulted in the highest level of expression 1 day after injection and 1 year post-injection animals receiving this vector expressed 5 times higher levels of hFIX (up to 8000 ng/ml hFIX) compared to all other vectors used in this study (Fig. 3D). Overall, these results indicate that the ChMAR sequence, if contained in a nonviral vector, increases transgene expression levels *in vivo*.

To determine the plasmid copy number 1 year post-injection, liver genomic DNA was isolated and a quantitative real-time PCR assay was performed. A similar number of vector genome copies per hepatocyte for groups that received pBS/hFIXs, pHM5/hFIXs, pHM5/hFIXs/ChMAR, pHM5/hFIXs/ChMAR/Ig κ MAR, and pHM5/hFIXs/Ig κ MAR were detected (Fig. 3C). This finding confirmed that the differences in transgene expression levels were not caused by the loss of vector genomes within the hepatocytes.

Effects of cis-acting elements on transgene expression levels after infusion of linear DNA fragments into mouse liver

Previously, we established that injection of linear fragments but not circular plasmid DNA resulted in sustained transgene

TABLE 1. SERUM GLUTAMIC-PYRUVIC TRANSAMINASE (SGPT) LEVELS (ALANINE AMINOTRANSFERASE ACTIVITY [IU]) 6 AND 24 H POST-INJECTION IN MICE RECEIVING EITHER THE NONVIRAL VECTOR pBS/hFIXmg OR pHM5/hFIXmg

Time (hr)	Serum SGPT levels		
	Nonviral vector		
	pBS/hFIXmg, U/ml	pHM5/hFIXmg, U/ml	Normal mice U/ml
6	274 \pm 99	185 \pm 23	11.7 \pm 4.8
24	275 \pm 77	245 \pm 40	

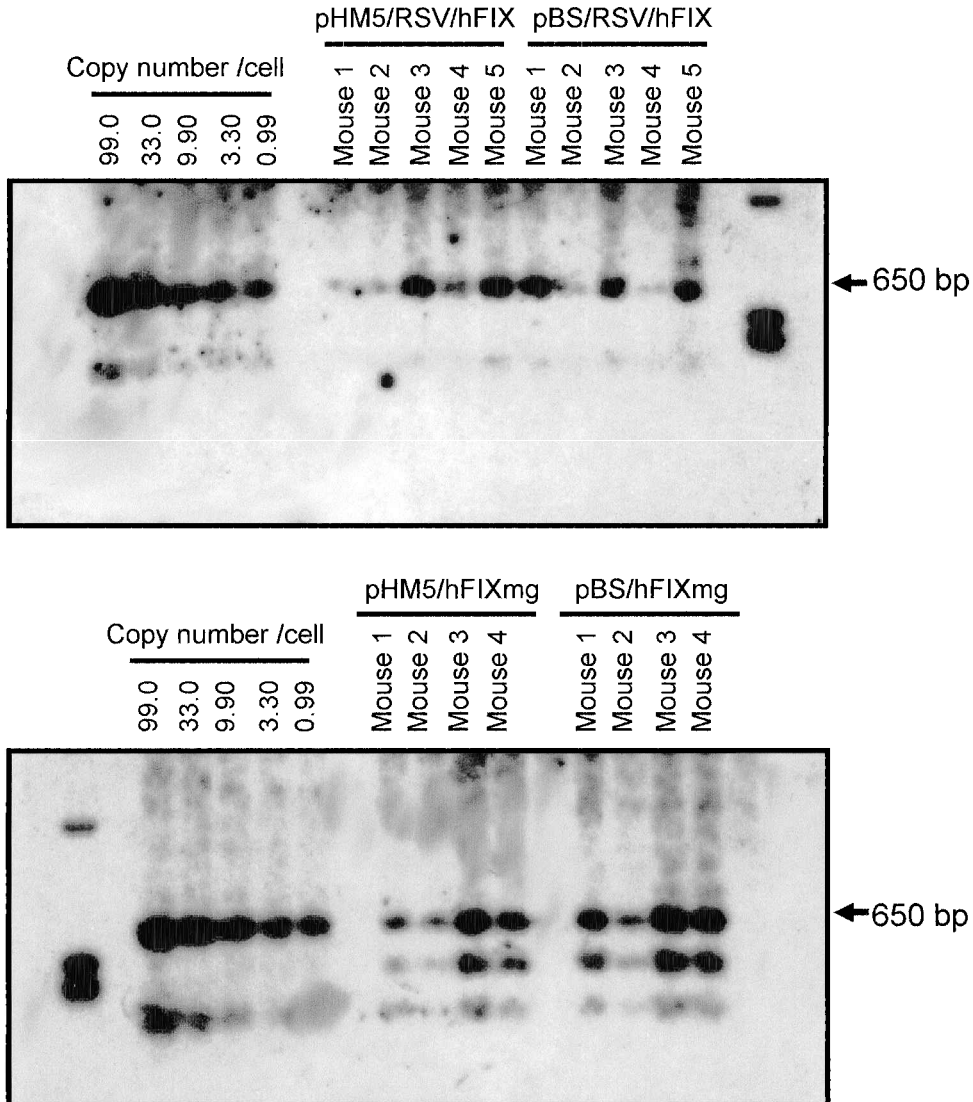
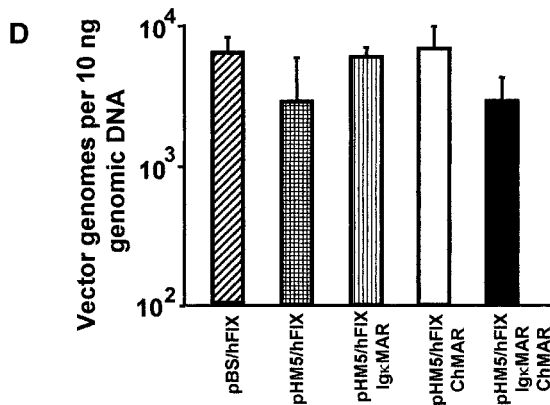
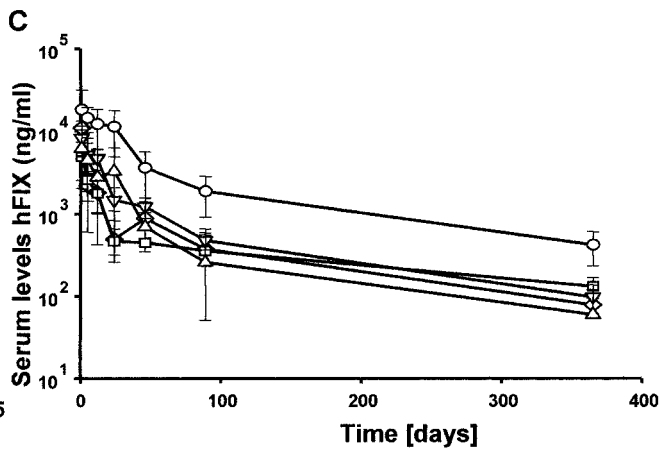
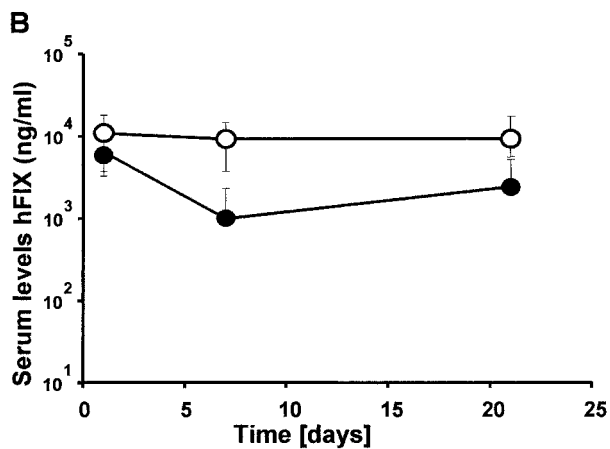
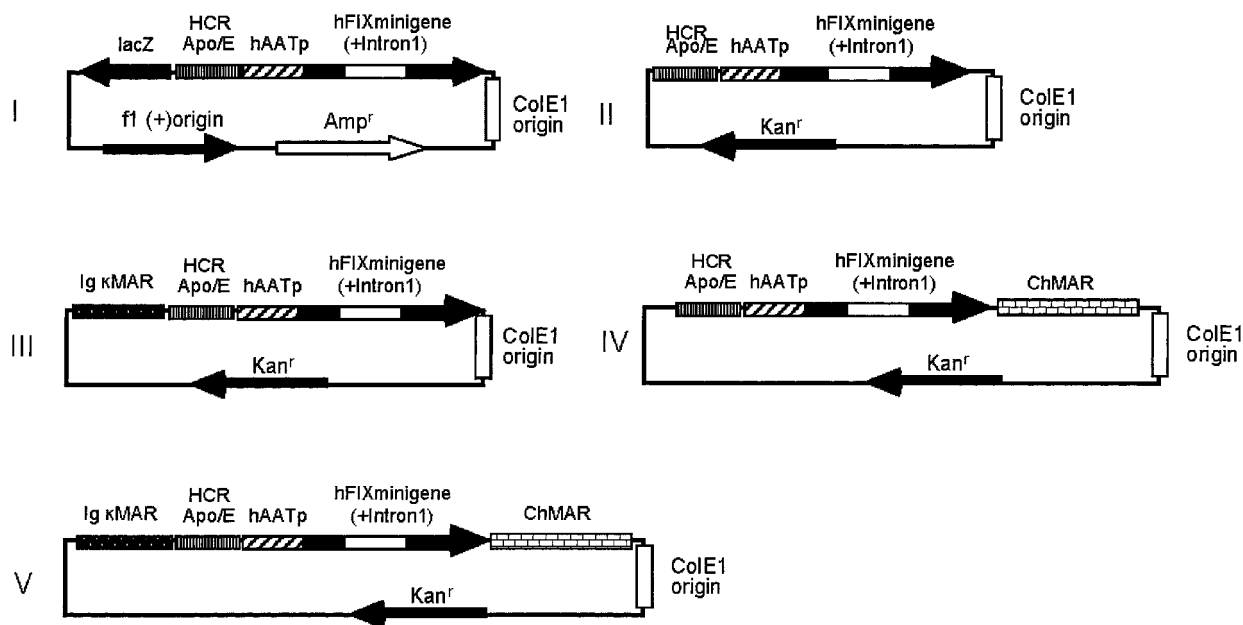


FIG. 2. Vector DNA analyses quantification of plasmid transfected liver DNA 4 weeks post-injection. To determine the copy number 4 weeks post-injection, a Southern blot analysis of vector-transfected liver samples was performed. Twenty micrograms of mouse liver genomic DNA was digested with *HincII* and *EcoRV* to release a 650 bp fragment, which is characteristic for all injected vectors. The genomic DNA was separated by gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were hybridized with a 1.6-kb human coagulation factor IX (hFIX) cDNA probe.

expression levels *in vivo* (Chen *et al.*, 2003). It was demonstrated that transfection of linear DNAs into mouse liver resulted in formation of large DNA concatemers and small circular DNA forms but it was unclear how these molecules affected transgene expression (Chen *et al.*, 2001). In an attempt to understand if the deletions of bacterial sequences in the vector pHM5 influence transgene expression levels derived from concatemers *in vivo*, we first compared hFIX expression levels in the context of circular and linear DNA constructs using the two different backbones, pHM5 and pBS+/- (Fig. 4A). Although for all groups, similar serum hFIX levels were detected 1 day post-injection (up to 30,000 ng/ml), we observed 10 times lower levels of gene expression in both groups receiving circular DNA (Fig. 4B). Ten weeks post-injection, the hFIX ex-

pression levels in the linear DNA injected groups remained similar. To determine the molecular status of the linear DNA fragments that were infused into mouse liver, Southern blot analyses were performed. We cut the genomic DNA with *PacI*, which did not cut the input DNA, and found that the linear DNA fragments were organized as DNA concatemers and small circular DNA, the later, likely representing recircularized expression cassettes (Fig. 5A, lanes 1 to 4). Furthermore *ClaI* digestion, which cut the input DNA once demonstrated the presence of head-to-head, tail-to-tail and head-to-tail concatemers (Fig. 5B, lanes 6 and 7). Altogether these results demonstrate that deletions in pHM5 had no effect on transgene expression levels from linear DNAs *in vivo*. This suggests that the small circular DNA molecules were primarily responsible for high transgene levels.

A



We next compared circular and linear DNA sequences that contained the hFIX expression cassette and MAR elements. To produce linear DNA fragments, the backbone was released from the vector in such a way that the MAR sequences would still flank the hFIX expression cassette (schematically shown in Fig. 4A). We found that the ChMAR resulted in similar transgene expression levels for both groups that received linear or circular DNA constructs (Fig. 4C), and only slightly lower hFIX expression levels for the linear DNA versus circular DNA sequences either containing the Ig κ MAR or a combination of the ChMAR and the Ig κ MAR (Fig. 4D). In fact, our results indicate that MAR DNA sequences had a negative effect on transgene expression levels from linear DNA. Serum concentrations of hFIX derived from linear DNA without MARs were about 3-fold higher if directly compared to linear DNAs with MARs (Fig. 4B, 4C, and 4D). Southern blot analysis revealed that less circular DNA molecules were present in groups that received two pieces of linear DNA with MARs compared to groups without MARs (Fig. 5A, lanes 6 to 10). It remains to be determined if differential amounts of concatamers or circular DNAs were formed *in vivo* as a result of using different restriction enzymes (Fig. 4A, *I-CeuI* and *PI-SceI*) for cutting and linearizing the DNA prior to injection. Together, these results suggest that MARs have the ability to prolong transgene expression levels derived from circular but not linear nonviral vectors *in vivo*.

DISCUSSION

The potential advantages of nonviral gene therapy vectors have attracted much research interest in the past decade but technical limitations must still be overcome. In this study, the importance of cis-acting DNA elements for vector stability, and optimization of bacterial DNA sequences and cis-acting DNA elements in nonviral vectors for sustained expression levels *in vivo* are significant advancements toward the effective use of these vectors in gene therapy.

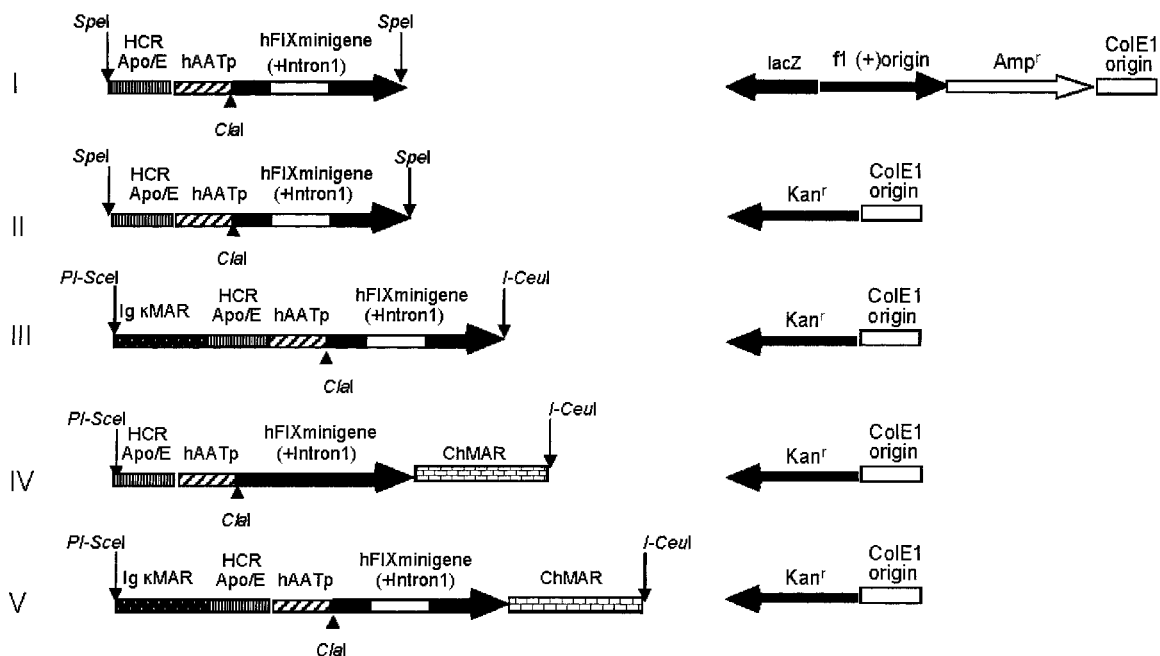
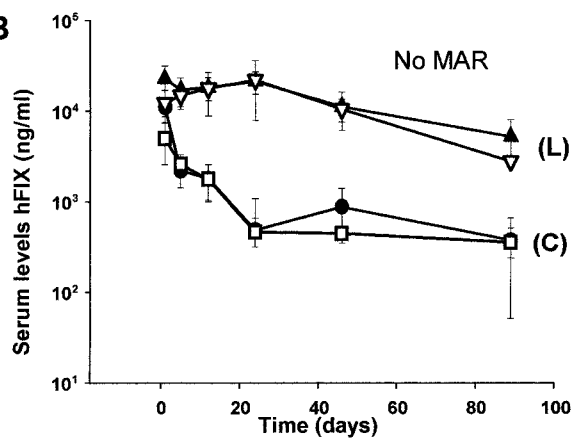
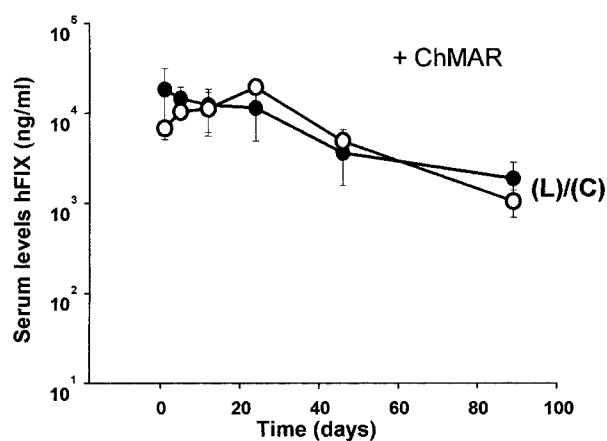
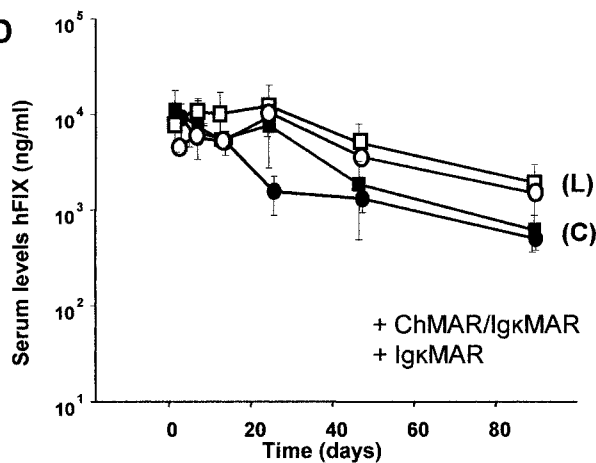
Although a number of cis-acting structures have been identified that can stabilize exogenous DNA, optimization of these elements will be beneficial. It was demonstrated that under certain conditions bacterial sequences can cause an inflammatory response in eukaryotes. A major factor in this inflammatory response is existence of large CpG islands in bacterial DNA (Yew *et al.*, 2000, 2002; McLachlan *et al.*, 2000). While removal of the CpG islands improved the persistence of transgene expres-

sion *in vivo*, our recent data suggests that the falloff in gene expression observed with bacterial DNA in the liver does not appear to be immune mediated (Chen *et al.*, 2003). Regardless of the mechanism, approaches were developed to circumvent this problem by removing bacterial sequences from the nonviral gene therapy vector. One method involved the generation of minicircle vectors consisting solely of the transgene expression elements (Darquet *et al.*, 1997, 1999). However high-scale production of pure minicircles was limited because the production is strongly dependent on tightly controlled expression of recombinases in bacteria. Other current approaches have experienced similar obstacles. To avoid these difficulties we reasoned that the individual elements in bacterial backbones that influence transgene expression can be identified through a systematic approach. Using a well-characterized vector expressing a reporter gene *in vivo*, we deleted single elements in the bacterial backbone to create a series of minimal vectors and thus were able to identify the regional elements involved in achieving stable transgene expression levels *in vivo*.

After hepatic infusion, the minimal vector pHM5 resulted in 10 times higher hFIX expression levels *in vivo* compared to the identical hFIX expression placed into the plasmid backbone pBS. The minimal vector pHM5 only contains the kanamycin resistance gene and the ColE1 origin of replication, whereas pBS contained the phage f1(-) origin of replication and a portion of the *lacZ* gene as additional DNA sequences. We suggest that the additional DNA sequences in the pBS backbone might have a negative effect on the transgene expression levels and speculate that the vector pHM5 simply contains a decreased number of CpG islands and/or less bacterial DNA sequences that may affect the vector conformation *in vivo* (Chen *et al.*, 2003). As a result, the additional sequences in the pBS backbone change the conformation of the plasmid in such way that the promoter sequences are not easily accessible to transcription factors. Further studies may elucidate the mechanisms resulting in the negative effect of the pBS backbone.

We then attempted to identify systematically any deletions in the pHM5 backbone that stabilize expression from the transgene expression cassettes. For better signal sensitivity, we placed a less robust hFIX expression cassette into our minimal vector in which the hFIX expression cassette was driven by the RSV promoter. Consistent to what we observed with a robust hFIX expression cassette, we found that the hFIX expression levels were higher for pHM5 than for pBS (Fig. 1B). However, we would like to point out that the differences in hFIX ex-

FIG. 3. Matrix attachment regions (MARs) as cis-acting elements in circular plasmids effect the *in vivo* performance from nonviral vectors. **A:** Circular recombinant human coagulation factor IX (hFIX)-expressing plasmids that contain no MAR in either the backbone pBS (I, pBS/hFIX) or pHM5 (II, pHM5/hFIX), the Ig κ MAR (III, pHM5/hFIX/Ig κ MAR), the chicken MAR (IV, pHM5/hFIX/ChMAR) or two MARs (V, pHM5/hFIX/ChMAR/Ig κ MAR). The hFIX expression cassette contains the hFIX cDNA with a portion of the first intron from the hFIX gene driven by the human α_1 -antitrypsin promoter (hAATp) and enhanced by the liver specific enhancers, apolipoprotein E (Apo E) and hepatocyte control region (HCR) but lacks the 3' untranslated region (3'UTR). **B:** *In vivo* comparison of two hFIX expression cassettes either with or without the 3'UTR. The plasmids were infused into mouse liver and the hFIX serum concentrations were measured by enzyme-linked immunosorbent assay (ELISA). (○) = pHM5/hFIXmg, (●) = pHM5/hFIXmg. **C:** After infusion into mouse liver of C57Bl/6 mice ($n = 5$ per group) using high-pressure plasmid injection, serum hFIX concentrations were measured periodically by ELISA. (◇) = pBS/hFIX, (□) = pHM5/hFIX, (▽) = pHM5/hFIX/Ig κ MAR, (△) = pHM5/hFIX/ChMAR/Ig κ MAR. Mean \pm standard deviation (SD) is shown. **D:** Quantification of vector DNA in plasmid transfected liver DNA. The nonviral vector genome copy number per 10 ng liver genomic DNA 1 year post-injection was determined by a quantitative real-time polymerase chain reaction (PCR).

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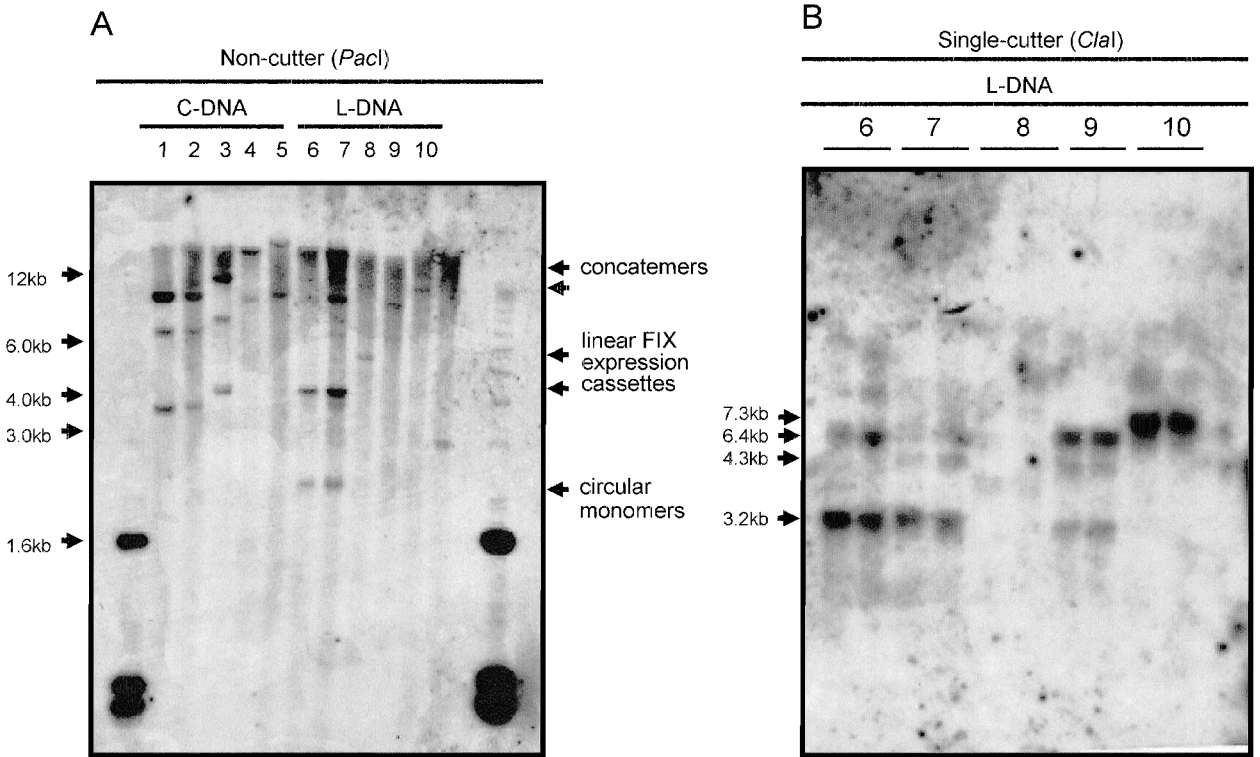


FIG. 5. Analyses of genomic liver DNA 10 weeks post-injection. Circular plasmids and linear fragments either with or without matrix attachment regions (MARs) (Figs. 3A and 4A) were infused into mouse liver, and 10 weeks post-injection a Southern blot was performed. Twenty micrograms of genomic liver DNA from two mice per group was isolated and analyzed with the restriction enzyme nucleases *PacI* (A), which does not cut the input DNA and *ClaI* (B), which did cut the input DNA once (Fig. 4A). The presence of head-to-head, tail-to-tail, and head-to-tail concatemers in (B) is marked by different arrows. After electrophoresis, the DNA was transferred to a nitrocellulose membrane and hybridized with a 1.6-kb human coagulation factor IX (hFIX) cDNA probe. Lane 1 = (C)-pBS/hFIX, lane 2 = (C)-pHM5/hFIX, lane 3 = (C)-pHM5/hFIX/ChMAR, lane 4 = (C)-pHM5/hFIX/IgκMAR, lane 5 = (C)-pHM5/hFIX/ChMAR/IgκMAR, lane 6 = (L)-pBS/hFIX, lane 7 = (L)-pHM5/hFIX, lane 8 = (L)-pHM5/hFIX/ChMAR, lane 9 = (L)-pHM5/hFIX/IgκMAR, lane 10 = (L)-pHM5/hFIX/ChMAR/IgκMAR.

pression levels in the context of the two plasmid backbones, pHM5 and pBS seem to vary among the different groups and are dependent on the DNA sequences contained in the transgene expression cassette. The differences in transgene expression levels between the two plasmids pHM5/hFIX and pBS/hFIX (Fig. 3C) were smaller than for the plasmids pHM5/hFIXmg and pBS/hFIXmg (Fig. 1B). This indicates that the 3'UTR may have a role in mRNA stability, contain enhancer elements for enhanced transgene expression levels, and/or synergistic effect between the 3'UTR and other components of the recombinant vector. There are data suggesting that the 3'UTR

and the intron may have a synergistic effect which would result in increased transgene expression levels *in vivo*. However, further studies are required to investigate the potential role of the 3'UTR in plasmid pHM5/hFIXmg. Furthermore, we observed a variation in DNA copy number per cell among the different mice that were analyzed in each group (Fig. 2). It is important to point out that the differences among the copy number per cell is less pronounced in the groups which received pHM5/hFIXmg and pBS/hFIXmg compared to the groups that received the plasmids pHM5/RSV/hFIX and pBS/RSV/hFIX with the weaker expression cassette. Interestingly, and in con-

FIG. 4. The effect of matrix attachment regions (MARs) as cis-acting elements on human coagulation factor IX (hFIX) expression levels from linear DNA fragments. **A:** The plasmid backbone without MARs was released by restriction enzyme nucleases and the linear fragments including the backbone were injected via high-pressure tail vein injection into C57Bl/6 mice ($n = 5$ per group). Serum samples were collected periodically and hFIX serum concentrations were measured by enzyme-linked immunosorbent assay (ELISA). Shown are hFIX expression levels derived from linear (L) and circular (C) DNA without MARs **B:** (∇) = (L)-pBS/hFIX, (□) = (C)-pBS/hFIX, (▲) = (L)-pHM5/hFIX, (●) = (C)-pHM5/hFIX; with the ChMAR (C) (○) = (L)-pHM5/hFIX/ChMAR, (●) = (C)-pHM5/hFIX/ChMAR; with both the IgκMAR and the ChMAR or with the IκMAR. **D:** (□) = (L)-pHM5/hFIX/ChMAR/IgκMAR, (●) = (C)-pHM5/hFIX/ChMAR/IgκMAR, (○) = (L)-pHM5/hFIX/IgκMAR, (■) = (C)-pHM5/hFIX/IgκMAR. Serum concentrations of hFIX were measured at the predicted time points. Mean ± standard deviation (SD) is shown.

trast to the copy number per cell, the hFIX serum concentrations were similar in each analyzed individual within a group. We speculate that only a portion of the recombinant DNA infused into mouse liver transfects hepatocyte nuclei and potentiates transgene expression dependent on the components within and outside of the transgene expression cassette. After hydrodynamic transfection some DNA molecules may transfect non parenchymal cells in the liver and/or may not make it to nucleus. These molecules may be transcriptionally inactive and the duration of these nonviral vectors in other cells types and/or subcellular local may vary dependent on the components in the nonviral vector. Taken together this provides support that the stabilization of transgene expression in the context of pHM5 may be at least in part expression cassette independent consistent with recent studies from our laboratory (Chen *et al.*, 2003). Previous comparisons of viral and tissue-specific promoters revealed differences in temporal expression patterns *in vivo*. Viral promoters are found to initially express at high levels during the first days post-injection and display a greater fall in expression later, whereas tissue-specific promoters result in more stable expression levels (Miao *et al.*, 2000; Herweijer *et al.*, 2001). The downregulation of viral promoters in eukaryotes was also described in the context of transgenic mice (Lee *et al.*, 1998), retroviral vectors (Kay *et al.*, 1993), and adenoviral vectors (De Geest *et al.*, 2001). In the present study the same effect was observed for the viral RSV promoter versus the liver-specific hAAT promoter. Four weeks post-injection the hFIX serum levels decreased to undetectable levels for the RSV promoter containing constructs but not the hAAT promoter even though the copy number per cell was similar for both constructs.

MARs have been shown to influence transgene expression levels derived from various viral vectors *in vivo* (Parks *et al.*, 1999; Park and Kay, 2001; Ehrhardt and Kay, 2002). In the present study, we demonstrate that MARs influence expression levels from nonviral vectors *in vivo* as well. We found that the ChMAR in circular plasmids results in about five times higher hFIX expression levels 1 year post-injection compared to other plasmids with other MARs or without MARs. We observed that the inclusion of the ChMAR, the Ig κ MAR or a combination of the ChMAR and the Ig κ MAR resulted in similar elevated and sustained hFIX expression levels for both, linear and circular DNA sequences. This suggests that MARs have a positive effect on expression levels from circular nonviral vectors. A reasonable explanation draws upon recent evidence that has shown that MARs act as insulators to block the negative effect of the backbone in the context of integrating systems (Wang *et al.*, 1995; Villemure *et al.*, 2001). More importantly some MARs were shown to have a high unwinding potential (Bode *et al.*, 2000) and directly influence transcription (Stief *et al.*, 1989; Nickerson *et al.*, 1995). Thus, one explanation for higher transgene expression levels derived the plasmid pHM5/hFIX/ChMAR (Fig. 3B) might be that the ChMAR causes the DNA to unwind and thus makes it more accessible for transcription factors. In addition we found that in the context of linear DNA, MARs have a negative effect on transgene expression levels (Fig. 4B, 4C–4E). We observed 3-fold higher hFIX serum concentrations in groups that received linear DNA without MARs than in groups that received linear DNA with MARs. Previously it was shown that in retroviral vectors MARs have an enhancing or inhibitory effect on transgene expression levels de-

pendent on the orientation of the MAR (Agarwal *et al.*, 1998). Agarwal *et al.* (1998) could measure an enhancing effect on the transgene expression cassette if the MAR was placed in the forward orientation but not if the MAR was placed in the reverse orientation relative to the transgene expression cassette. Genomic DNA analyses of our liver samples transduced with linear DNA revealed that large concatemers were formed *in vivo* (Fig. 5A). As previously demonstrated by Chen *et al.* (2001) these large DNA molecules consist of random head-to-head, head-to-tail, and tail-to-tail concatemers derived from the linear input DNA (Fig. 5B). Thus, the MAR used in our study is present in both forward and reverse orientation, which could explain the negative effect on the transgene expression levels. In fact, we detected less small circular DNA and more large DNA molecules in mice that received two fragments of linear DNA with MARs (Fig. 5A).

Altogether, the data in this study advance the progress toward prolonged, sustained transgene expression levels from nonviral vectors *in vivo* and clearly demonstrates that cis-acting DNA sequences and bacterial sequences need to be studied carefully for a successful nonviral gene therapy approach.

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