

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*

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We systematically compared human factor IX gene expression from a variety of plasmids containing different cis-regulatory sequences after transfection into different hepatocyte cell lines, or *in vivo*, after their injection into the livers of mice. Although there was a 1.5- to 2.0-fold variation in gene expression from cultured cells, a 65-fold variation was observed in the *in vivo* studies. We found that a plasmid containing the apolipoprotein E locus control region (HCR), human α 1-antitrypsin (hAAT) promoter, hFIX minigene (hFIXmg) sequence including a portion of the first intron (intron A), 3'-untranslated region (3'-UTR), and a bovine growth hormone polyadenylation signal (bpA) produced the highest serum level of human factor IX, reaching 18 μ g/ml (normal = 5 μ g/ml) 1 day after injection. Although most of the plasmid DNAs resulted in transient gene expression, inclusion of an intron, a polyadenylation signal from either the 1.7-kb 3'-UTR or the 0.3-kb bpA, and the HCR resulted in persistent and therapeutic levels of hFIX gene expression, ranging from 0.5 to 2 μ g/ml (10 to 40% of normal) for 225 days (length of experiment). These data underscore the importance of cis sequences for enhancing *in vivo* hepatic gene expression and reemphasize the lack of correlation of gene expression in tissue culture and *in vivo* studies.

Key Words: liver gene therapy; nonviral and viral vectors; hemophilia.

INTRODUCTION

Hemophilia B is a bleeding disorder that results from a deficiency of factor IX in plasma. It is an X-linked disorder occurring in about 1/25,000 males (1). Different gene therapy techniques are being developed for treatment of hemophilia [reviewed in (2)]. Retroviral vectors have been used successfully to accomplish long-term expression of therapeutic genes, including factor IX, from the livers of normal and hemophilic animals (3–9). However, the levels of factor IX expression have been low (6). In contrast, recombinant adenoviral gene transfer results in therapeutic but transient expression of factor IX (10–14). Recently, adeno-associated viral vectors have successfully transferred therapeutic and persistent levels of factor IX in the livers of hemophilic mice and dogs (15–19). However, increasing transgene expression is desirable as

a way to decrease the amount of viral vector required to achieve a clinical effect. In addition, there are advantages to restricting vector-mediated gene expression from hepatocytes by using liver-specific promoters and enhancers, e.g., reducing the probability of inducing an immune response to the transgene (20). Thus, it is important to develop high expressing and regulated gene transfer vectors to achieve therapeutic levels of transgene expression in the liver for effective gene therapy of hemophilia.

As a first step toward producing better expression cassettes, various cis-acting regulatory sequences, such as different promoter–enhancer–HCR combinations, introns, and 3'-UTR, were considered for incorporation into retroviral expression plasmids. Traditionally, intron sequences have not been used in gene transfer vectors because of their large sizes and lack of dramatic enhancing function on gene expression in cultured cells. However, it has been shown that intron sequences can potentiate gene expression *in vivo* (21–23). In transgenic mice, inclusion of the full-length 6.2-kb or a truncated

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1.4-kb fragment of the first intron of the factor IX gene increased gene expression 40- to 200-fold (24). Recently, Kurachi *et al.* (25) showed that subregions of the first factor IX intron (intron A) inserted immediately upstream of the hFIX promoter exerted only marginal enhancing or even weakly negative regulatory activities on factor IX gene expression. However, minigene constructs containing further truncated first intron sequences (1.4 and 0.27 kb, respectively) and legitimate splicing sequences (donor, acceptor, and branch sites) increased factor IX gene expression 7- to 9-fold compared with constructs that lacked intronic sequences in cultured hepatoma cells. In addition, a synthetic 5' intron has been shown to augment factor VIII gene expression in cultured hepatic cells (26).

Similarly, the 3'-untranslated regions may also affect the stability of mRNAs. Five families with hemophilia B have been described in which a point mutation in the 1.4-kb 3'-UTR of the factor IX gene severely reduces the plasma factor IX concentration to less than 3% of the normal level (27, 28). In addition, a 653-bp deletion located in the 3'-UTR of the factor IX gene was found to be responsible for mild hemophilia B (29). Furthermore, in the context of a nonintegrating, episomal recombinant adenoviral vector, there are limited data suggesting that the intron and 3'-untranslated region may have synergistic effects on gene expression *in vivo*, increasing gene expression by 1000-fold (30). Recently it has been reported that although 3'-UTR had a negative regulatory effect on factor IX gene expression *in vitro*, it substantially increased the steady-state hFIX mRNA and gene expression and is involved in the age-related regulation of the factor IX gene *in vivo* (31). Taken together, these data suggest that the 3'-untranslated region may have an important role in the regulation of expression *in vivo*.

Limited studies have suggested that viral-mediated gene expression from the liver did not always correlate with that obtained from tissue culture studies (32, 33). It has been shown from retroviral gene transfer studies that although viral promoters such as the cytomegalovirus (CMV) promoter are quite active in primary hepatocytes *in vitro*, they are inactivated *in vivo* and confer little or no transgene expression in the livers of animals (5, 6, 33, 34). Furthermore, primary hepatocytes in culture have very different transcription rates and posttranscriptional regulation of endogenous genes compared with hepatocytes *in vivo* (35). Because there is no good cell culture model that closely simulates hepatic gene expression *in vivo*, it is suggested that the evaluation of the gene expression vectors must be established *in vivo*.

Recently, two different methods for plasmid-mediated gene delivery to mouse livers have been described. First, plasmid DNAs were delivered in hypertonic solution into the portal veins of mice whose hepatic veins were transiently occluded. The intraportal delivery of naked DNA is at least a thousand times more efficient than interstitial delivery into mouse liver (36). High levels of luciferase expression and β -galactosidase expres-

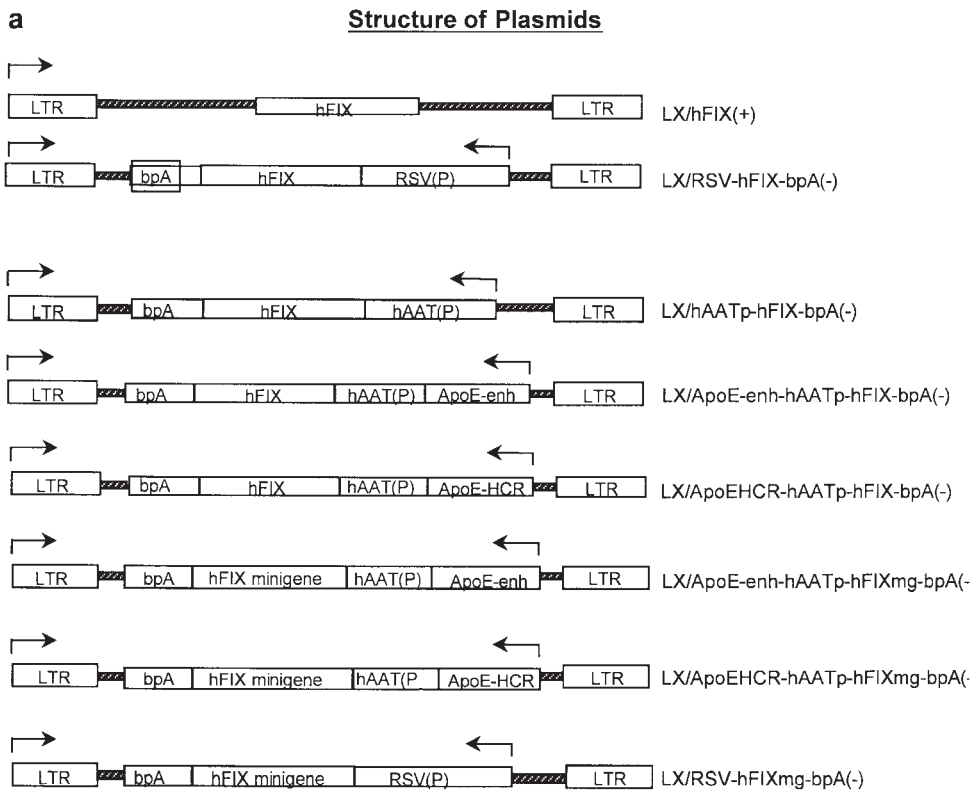
sion in 1% of the hepatocytes throughout the entire liver were obtained using 100 μ g of the plasmid. Second, a hydrodynamic transfection method using the rapid infusion of plasmids in aqueous solutions via the tail vein has been developed to achieve even higher levels (~40%) of transgene expression in the liver (37, 38). Although our original goal was to use this approach to screen for sequences that might enhance gene expression in viral vectors *in vivo*, the unexpected results related to prolonged gene expression allowed us to pursue specific cis DNA elements that may be useful in achieving long-term as well as therapeutic gene expression from nonviral vectors *in vivo*.

METHODS

Construction of plasmids. Factor IX expression cassettes were first constructed in Bluescript vector backbones (pBSKS). Plasmid pBS-hAAT-bpA (39) was digested with *EcoRI* to remove the hAAT cDNA and blunt ended. The human factor IX cDNA was cut out from pCMV-FIX (40) by *BamHI* and *KpnI*, blunt ended, and then cloned into the blunt-ended *EcoRI* site of pBS-bpA to yield pBS-FIX-bpA. Next, pBS-FIX-bpA was digested with *Sall* and blunt ended. The 408-bp hAAT promoter was obtained from digestion of pLTR-hAATp-FX (41) with *BglII* and *NotI*, and the apolipoprotein E (ApoE) enhancer-hAAT-promoter fragment was obtained by digestion of pLTR-ApoE-enh-hAATp-hAAT (9) with *BglII*. Both enhancer-promoter fragments were then blunt ended and inserted into the *Sall* blunt-ended site of pBS-FIX-bpA to generate pBS-hAATp-FIX-bpA and pBS-ApoE-enh-hAATp-FIX-bpA constructs. Plasmid pBS-hAATp-FIX-bpA was further digested with *KpnI* and ligated with a 711-bp *KpnI* site flanked fragment of ApoE-HCR amplified by PCR from plasmid pLIV7 (42) to generate pBS-ApoE-HCR-hAATp-FIX-bpA. The factor IX minigene (including a 1.4-kb factor IX intron A and a 1.7-kb 3'-UTR) was obtained from digestion of plasmid AVS17H9F.CM (30) by *SpeI* and *Clal* and blunt ended. pBS-FIX-bpA was digested with *XhoI* to release hFIX cDNA and blunt ended and then ligated with the factor IX minigene to give pBS-FIXmg-bpA. The FIXmg-bpA fragment was then cut out from pBS-FIXmg-bpA by *Clal* and *SpeI*. pBS-hAAT-FIX-bpA, pBS-ApoE-enh-hAAT-FIX-bpA, and pBS-ApoE-HCR-hAAT-FIX-bpA were digested with *SpeI* and *Clal* to give three fragments from each plasmid, pBS, FIX-bpA, and three different enhancer-promoter-HCR combinations. The digested plasmid, pBS, was then ligated with the FIXmg-bpA fragment and three different enhancer-promoter-HCR combinations to give three plasmids, respectively, pBS-hAAT-FIXmg-bpA, pBS-ApoE-enh-hAAT-FIXmg-bpA, and pBS-ApoE-HCR-hAAT-FIXmg-bpA. The ApoE-HCR fragments amplified by PCR were sequenced and all the constructs made were checked to ensure their correctness prior to their cloning into the expression plasmids.

The plasmid containing the retroviral vector backbone, pLX, was generated by deleting the neo gene from pLNCX (43). Plasmid pLX was then digested with *HpaI*. Various hFIX expression cassettes were cut out from the pBS constructs described above with *SpeI*, blunt ended, and cloned into the *HpaI* site of pLX to yield a series of LX constructs listed in Fig. 1a.

For characterizing the essential elements for persistent factor IX gene expression, more Bluescript constructs were made for comparison with all eight plasmids in animal experiments (Fig. 1b). Either bpA or factor IX 3'-UTR was deleted from plasmid pBS-ApoEHCR-hAATp-hFIXmg-bpA to make pBS-ApoEHCR-hAATp-hFIXmg or pBS-ApoEHCR-hAATp-hFIX-Int-bpA. Next, bpA was deleted from pBS-ApoEHCR-hAATp-hFIX-Int-bpA to yield pBS-ApoEHCR-hAATp-hFIX-Int. Plasmid pBS-hAATp-FIX-bpA was digested with *KpnI* and ligated with a shorter, 331-bp *KpnI* site flanked fragment of ApoE-HCR amplified by PCR from plasmid pLIV7 (42) to generate pBS-ApoEHCR(s)-hAATp-FIX-bpA. The ApoEHCR(s)-hAATp or 408-bp hAAT promoter fragment was ligated with pBS-bpA and hFIX-mg fragments purified as described earlier to yield pBS-ApoEHCR(s)-hAATp-hFIXmg-bpA or pBS-hAATp-hFIXmg-bpA. All plasmid DNAs were pre-



hFIX minigene = 1.4 kb hFIX cDNA + 1.4 kb truncated Intron A + 1.7 kb 3'-UTR



pared by alkaline lysis method and purified by Maxi-Prep kits from Qiagen. No protein or RNA was detectable in these preparations.

Cell culture and transfections. Mouse hepatoma cells (Hep1A) were cultured in Dulbecco's modified Eagle's medium supplemented with L-glutamine, antibiotics (penicillin and streptomycin), and 10% fetal calf serum. Human hepatoma cells (HepG2) were cultured in minimal Eagle's medium supplemented with L-glutamine, antibiotics, 1% nonessential amino acids, 1% sodium pyruvate, and 10% fetal calf serum. Nontransformed mouse hepatocyte cells (NMH) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's and Ham's F-12 medium supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenium, 0.1 mM dexamethasone, 0.1% gentamycin, 10 mM nicotinamide, and 20 ng/ml epidermal growth factor. All three cell lines were maintained in a 5% CO₂ atmosphere at 37°C.

Plasmid DNA (2 μ g) and pBS-RSV-hAAT-bpA (1 μ g) used as an internal control were cotransfected into cultured cells by Lipofectamine reagents (Life Technologies). Each transfection was repeated at least three times. Sixty hours after transfection, cell culture media were collected and analyzed for total human α 1-antitrypsin or factor IX antigen (11) by an ELISA using polyclonal antisera to α 1-antitrypsin or hFIX (44), respectively.

Direct injection of plasmids into portal vein of mice. C57Bl/6 mice were obtained from Jackson Laboratory and housed under SPF conditions. Animals were treated according to NIH Guidelines for Animal Care at the University of Washington and Stanford University. The method was similar to that described by Budker *et al.* (36) and resulted in ~1% transfected hepatocytes. The mice were injected with 100 μ g of the plasmid DNA in a hypertonic solution [containing 0.9% saline, 15% mannitol (Sigma), and 3.5 units/ml of heparin to prevent microvascular thrombosis] over 30 s through the portal vein. To temporarily occlude the hepatic veins during the injection, a 5-mm, Kleinert-Kutz microvessel clip (Edward Weck) was applied at the junction of the hepatic vein and vena cava. Mice were periodically bled by retroorbital technique. The human factor IX level in the mouse serum or plasma was determined as total hFIX antigen by an ELISA using polyclonal antisera to hFIX.

Direct injection of plasmids into tail vein of mice. C57Bl/6 mice (Taconic) were housed under SPF conditions at Stanford University and University of Washington. The method was similar to that described by Liu *et al.* (37) and Zhang *et al.* (38). Twenty micrograms of the respective plasmid for testing along with 5 μ g of a control plasmid (pBS-RSV-hAAT-bpA) in 2 ml 0.9% saline solution were injected into the tail vein of mice over 6–8 s. This method has been shown to transfect about 40% of the hepatocytes (45). The mice were bled periodically in order to evaluate hFIX or α 1-antitrypsin levels in the serum or plasma by ELISA as described above.

RESULTS

Design of Vector Constructions

To optimize production of human FIX in hepatocytes, gene expression was investigated by incorporating various combinations of cis-acting regulatory sequences including a tissue-specific promoter–enhancer, hepatic

locus control region, intron A, and 3'-untranslated region into retroviral-containing plasmid DNAs.

A simple retroviral backbone containing the human factor IX cDNA was first utilized. For hepatic gene transfer, a liver-specific promoter from the α 1-antitrypsin gene was added, with or without four copies of the ApoE enhancer (46). The α 1-antitrypsin promoter was selected because it is a strong liver-specific promoter (9), and its function is not inhibited by the LTR promoter in the retroviral vector (47). Furthermore, it was reported that by adding four copies of the strong 154-bp liver-specific ApoE enhancer (46) upstream of the hAAT promoter, expression of the α 1-antitrypsin gene was further increased more than 15-fold over the constructs having other promoters–enhancers (48).

If these sequences were to be used in integrating vectors, to overcome the possible silencing effect by the position of integration, we elected to incorporate a recently reported locus control region (LCR) for the liver-specific expression of the ApoE gene ApoE–HCR (49). This hepatic control region is located in the ApoE/CI/CII locus and localized to a 319-bp region for full functional LCR activities. This element confers copy-number-dependent, position-independent gene expression and was shown to exhibit 10-fold higher activity than the ApoE enhancer in a transgenic mouse model (42). Because it is not clear how a multimerized LCR would affect gene transcription *in vivo*, we used a single ApoE–HCR sequence in the plasmid expression cassettes. RSV-LTR and Moloney LTR promoters were used as controls.

Retroviral vector-containing plasmids were constructed to compare the strength of these cis elements systematically (Fig. 1a). We used the control viral LTR (LX/hFIX) and RSV-LTR (LX/RSV-hFIX-bpA(–)) promoters and then incorporated the liver-specific elements: the hAAT promoter alone, or with the addition of an ApoE enhancer, or ApoE–HCR [LX/hAAT-hFIX-bpA(–), LX/ApoE-enh-hAAT-hFIX-bpA(–), and LX/ApoE-HCR-hAAT-hFIX-bpA(–), Fig. 1a]. The expression cassettes with added internal promoters were inserted in an opposite transcriptional orientation relative to the LTR promoter to avoid promoter interference with the viral LTR promoter *in vivo* (50, 51) and for comparison with the constructs containing the incorporation of an intron and 3'-UTR. Additionally, an exogenous polyadenylation signal was added to the 3' end of the factor IX cDNA to ensure prop-

FIG. 1. (a) Retroviral constructs made with different combinations of cis-acting regulatory elements for evaluation of factor IX gene expression levels. (+) denotes that the expression cassettes were inserted in the natural orientation to the transcription directed by 5'-LTR; (–) denotes that the expression cassettes were inserted in the opposite orientation to the transcription directed by 5'-LTR; (P) indicates promoters; and (E) indicates enhancers. (b) Bluescript plasmids containing various cis DNA elements. Abbreviations used: LTR, long terminal repeats; hFIX, human factor IX cDNA (1.4 kb); hAAT, human α 1-antitrypsin promoter (408 bp); bpA, bovine growth hormone polyadenylation signal (265 bp); ApoE, four copies of the 154-bp ApoE enhancer sequence; ApoEHCR, hepatic locus control region from the ApoE gene locus (771 bp); ApoEHCR(s), a shorter fragment of the hepatic locus control region from the ApoE gene locus (331 bp); RSV, Rous sarcoma virus-LTR promoter (400 bp); hFIXmg, a human factor minigene containing factor IX cDNA (1.4 kb), 1.4 kb truncated intron A, and 1.7 kb 3'-UTR.

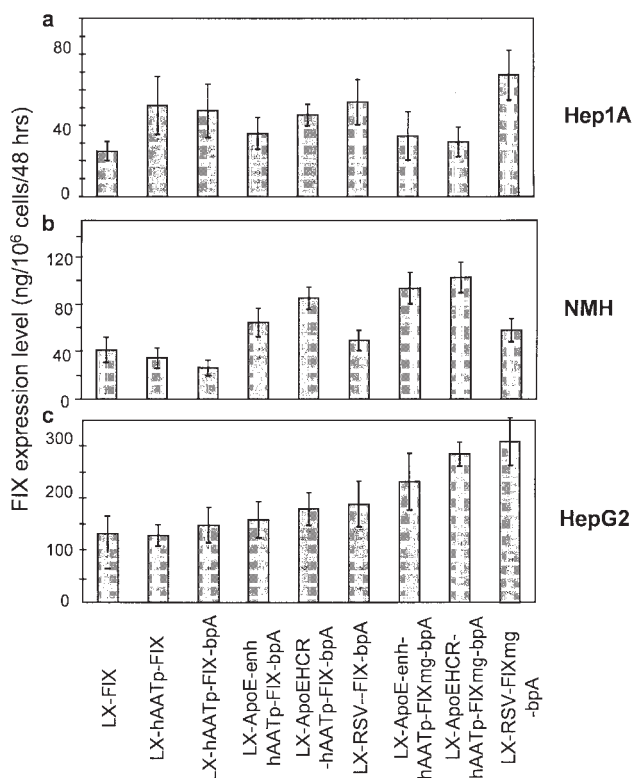


FIG. 2. Evaluation of promoter strength in the cell culture system. Different plasmid constructs were transiently transfected into three hepatic cell lines. Culture media were collected 2 days after transfection and analyzed for human factor IX and α 1-antitrypsin levels by ELISA. Each transfection was repeated at least three times. Factor IX expression levels were then normalized against α 1-antitrypsin expression levels produced by a control pBS-RSV-hAAT-bpA plasmid for transfection efficiencies in (a) Hep1A, (b) NMH, and (c) HepG2 cells.

er processing of the mRNA. The goal was to compare the relative strength of the Moloney LTR and an internal RSV-LTR with the different liver promoters discussed above.

Next the hFIX minigene composed of the hFIX cDNA, 1.4-kb truncated intron A, and 1.7-kb 3'-UTR were incorporated with different combinations of the promoter-enhancer sequences. These expression cassettes were inserted in the opposite transcriptional orientation to that of the retroviral LTR [LX/ApoE-enh-hAAT-hFIXmg-bpA(-), LX/ApoE-HCR-hAAT-hFIXmg-bpA(-), and LX/RSV-hFIXmg-bpA(-), Fig. 1a]. This was done to ensure that splicing of the factor IX intron and the polyadenylation signal in the 3'-UTR would not interfere with the production of full-length viral transcripts in packaging cell lines if these plasmids were to be used to make retroviral vectors.

In Vitro Testing of Gene Expression in Cell Culture Systems

These plasmids were first tested in three hepatic cell culture systems for the following two reasons. First, we

were able to confirm construct functionality prior to *in vivo* studies. Second, we wanted to establish a baseline for comparison and predictability with *in vivo* studies.

Two days after transfection in cell culture, the culture media were collected and analyzed by ELISA (summary in Table 1). Factor IX expression levels were normalized by the human α 1-antitrypsin levels (hAAT) expressed from a cotransfected control pBS-RSV-hAAT-bpA plasmid. In Hep1A cells, low-level factor IX expression was observed with all the constructs with 1- to 2.5-fold variance (Fig. 2a). Interestingly, the RSV-LTR promoter was just as active as the liver-specific enhancers-promoters. The presence of the intron and 3'-UTR region had little effect on gene expression. Next these plasmids were tested in a nontransformed hepatocyte (NMH) cell line derived from mice transgenic for transforming growth factor alpha (52), which may have properties more closely related to *in vivo* conditions. In general, a higher level of factor IX expression was achieved in the NMH cells than those obtained in Hep1A cells (Fig. 2b) with most of the tested plasmids. The factor IX level was about 2-fold higher with either the addition of an ApoE enhancer or ApoE-HCR compared with the hAAT promoter alone. Lastly, we tested these plasmids in HepG2 cells, which is a human hepatoma cell line. Since all the liver-specific regulatory cis sequences used were derived from human sequences, these elements may only be fully functional in human hepatocytes. As shown in Fig. 2c, the factor IX gene directed by hepatocyte-specific enhancers-promoters in the vector were expressed at higher levels than those in the other two mouse cell lines. However, the RSV-LTR promoter was also more active and had similar strength as the liver-specific enhancers-promoters in HepG2 cells. The inclusion of the hFIX intron and 3'-UTR in the vector increased the gene expression level by ~1.5-fold.

In Vivo Direct Plasmid Injection via Portal Vein

We next proceeded to study the expression of the plasmid DNAs *in vivo* by the direct injection of plasmids into the portal vein (36). One hundred micrograms of the respective retroviral plasmid in hypertonic solution was delivered intraportally into C57Bl/6 mice whose hepatic veins were transiently occluded. Half of the mice died during surgery or 1 day after surgery. All remaining mice survived up to 40 days (the duration of the experiment).

Two days after plasmid injection, collected serum samples were analyzed for hFIX protein by ELISA. As shown in Fig. 3a, animals infused with LX-FIX(+) without internal promoters gave a basal serum level of 5–10 ng/ml FIX. The addition of an internal hAAT promoter, ApoE-enh-hAAT promoter, ApoE-HCR-hAAT promoter, or RSV-LTR promoter, did not augment factor IX expression from constructs containing factor IX cDNA (LX-hAAT-hFIX-bpA, LX-ApoE-enh-hAAT-hFIX-bpA, LX-ApoE-HCR-hAAT-hFIX-bpA, and LX-RSV-hFIX-bpA). However, in mice injected with the construct containing the RSV-LTR promoter element and a human factor IX

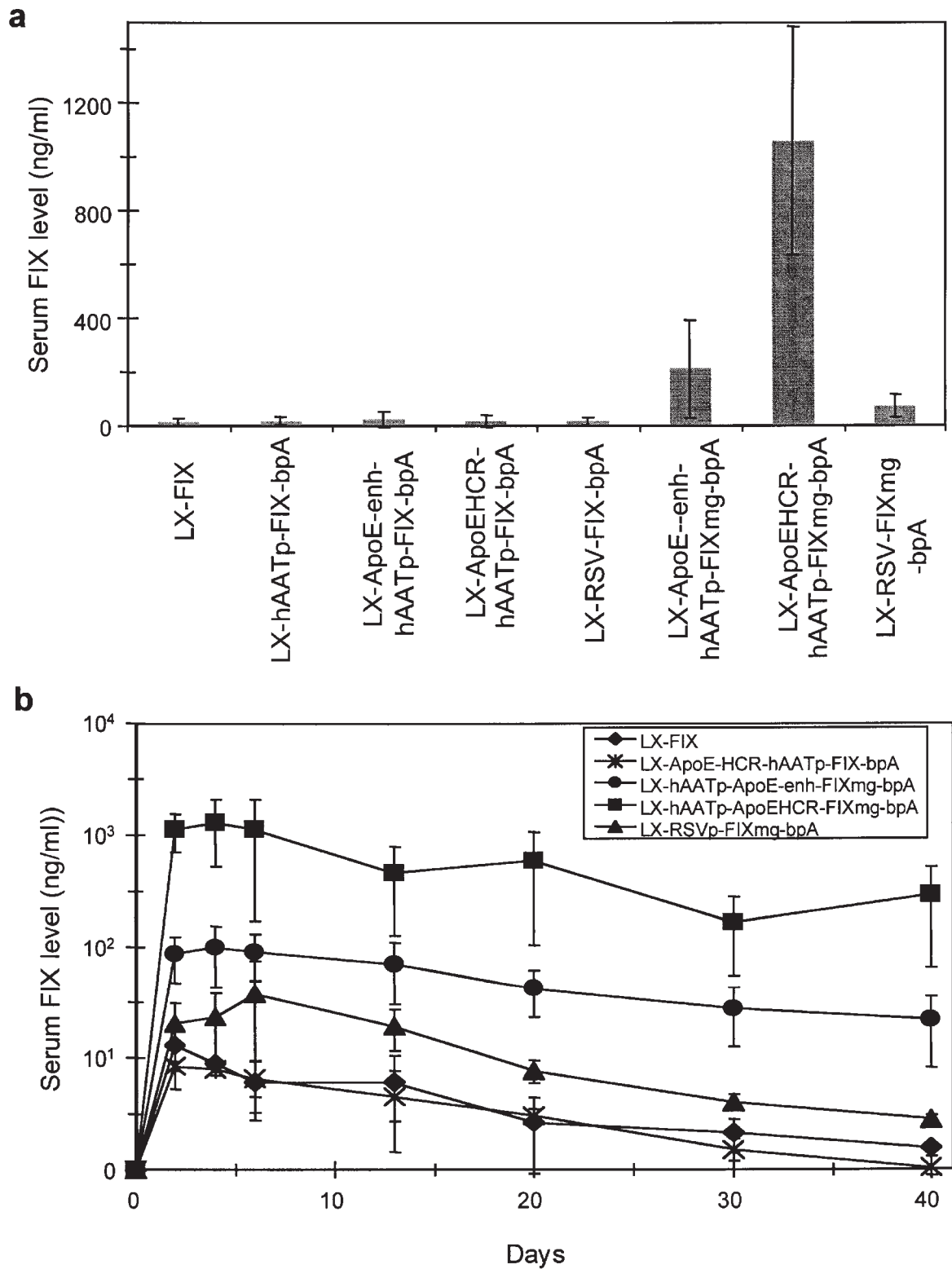


FIG. 3. (a) Factor IX gene expression after direct injection of plasmids into the portal vein of mice. One hundred micrograms of the respective plasmid was delivered intraportally into C57/BL6 mice ($n = 6$ per group; about half of the animals did not survive the procedure) whose hepatic vein was transiently occluded. Two days after infusion, the serum was analyzed for human factor IX levels by ELISA. (b) Factor IX serum levels were followed over time in five groups of mice described in (a). Different symbols represent expression levels from mice injected with different plasmids: diamonds, LX-FIX(+); asterisks, LX-ApoE-HCR-hAATp-FIX-bpA(-); circles, LX-ApoE-enh-hAATp-FIXmg-bpA(-); squares, LX-ApoE-HCR-hAATp-FIXmg-bpA(-); triangles, LX-RSVp-FIXmg-bpA(-).

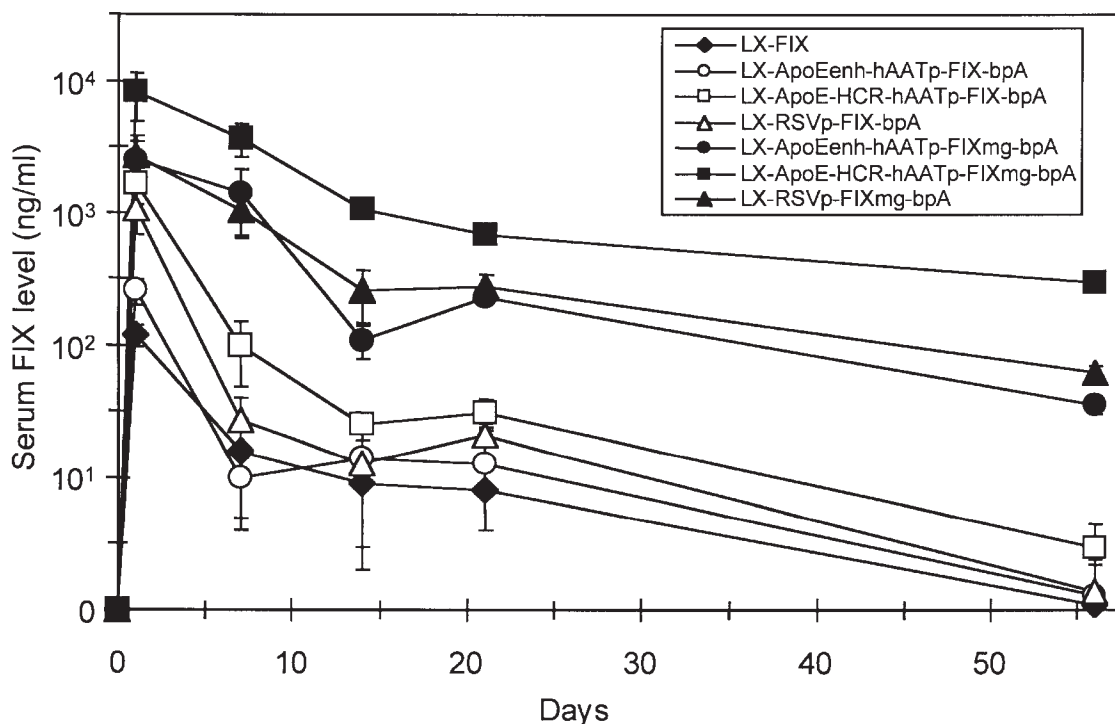


FIG. 4. Periodic plasma human factor IX levels after direct injection of the plasmids into the tail vein of mice. Twenty micrograms of the respective retroviral plasmid along with 5 μ g of a control pBS-RSV-hAAT-bpA plasmid in 2 ml 0.9% saline solution was injected into the tail vein of C57/BL6 mice ($n = 6$ per group) over 6–8 s. Plasma was analyzed for human factor IX and α 1-antitrypsin levels by ELISA at different time points. Human factor IX levels were then normalized against α 1-antitrypsin expression levels for plasmid delivery efficiencies. Different symbols represent expression levels from mice injected with different plasmids: filled diamonds, LX-FIX(+); open circles, LX-ApoE-enh-hAAT-FIX-bpA(-); open squares, LX-ApoE-HCR-FIX-bpA(-); open triangles, LX-RSV-FIX-bpA(-); filled circles, LX-ApoE-hAAT-FIXmg-bpA(-); filled squares, LX-ApoEHCR-hAAT-FIXmg-bpA(-); filled triangles, LX-RSV-FIXmg-bpA(-).

minigene sequence (LX-RSV-FIXmg-bpA), factor IX levels were 3-fold higher than those obtained from constructs containing hFIX cDNA. In mice injected with the construct containing the 154-bp ApoE enhancer element and hAAT promoter in combination with a hFIX minigene sequence (LX-ApoE-enh-hAAT-FIXmg-bpA), factor IX levels were about 10-fold higher (50–180 ng/ml) than those obtained from constructs with hFIX cDNA. Most interestingly, when the ApoE-HCR element and hAAT promoter were used in combination with the FIX minigene sequence (LX-ApoE-HCR-hAAT-FIXmg-bpA), factor IX levels were ~67-fold higher (0.7–1.5 μ g/ml) than the basal level obtained from animals receiving the LX-FIX construct. This level of factor IX, ~30% of the normal plasma factor IX concentration (5 μ g/ml), would be curative for individuals with hemophilia B.

To test how long plasmid-mediated gene expression persisted in the mice, serum samples were periodically analyzed for hFIX. In mice injected with constructs containing factor IX cDNA, factor IX levels dropped to an undetectable level 3 to 7 days post vector infusion. In mice injected with constructs containing the human factor IX minigene, factor IX levels were initially higher and persisted longer, yet the protein levels fell slowly over

time (Fig. 3b). Factor IX concentrations in mice injected with construct LX-ApoE-HCR-hAAT-FIXmg-bpA persisted at high levels for a week and slowly decreased to 200 ng/ml at 40 days post vector administration. The decline in gene expression was not unexpected because of the episomal nature of the plasmid in cells.

Direct Plasmid Injection via Tail Vein

Due to the labor-intensive nature of the surgical procedures for direct plasmid injection via portal vein as described above, another *in vivo* gene transfer method developed recently was used for testing the expression of these constructs in the liver. Twenty micrograms of the plasmid to be tested and 5 μ g of a control pBS-RSV-hAAT-bpA plasmid were injected into the tail vein of mice. The control plasmid was used to normalize for variations in plasmid injection procedures. Factor IX levels were highest 1 day after injection, but fell to lower or undetectable levels over time (Fig. 4). Although the absolute levels of factor IX gene expression were higher in tail versus portal vein infusions, the relative ratios of expression obtained from each construct relative to the basal expression level from the LX-FIX construct were comparable with those in the portal vein experiments (Table 1). The

expression level from the highest producing animals was achieved with injection of construct LX-ApoE-HCR-hAAT-hFIXmg-bpA (6 to 18 $\mu\text{g/ml}$). This level was 69-fold higher than that of the LX-FIX construct, similar to that observed from the portal vein experiments. Taken together, the results obtained from *in vivo* studies were strikingly different from those obtained from the cell culture experiments. Interestingly, at 8 weeks of age, mice injected with constructs containing an intron and 3'-UTR sequences still produced FIX, whereas mice infused with the other plasmids did not.

The results above suggested that some of the cis DNA elements may have been responsible for differences in the persistence in gene expression. Systematic studies were undertaken in an attempt to determine which elements were responsible for this finding. To avoid the influence from retroviral LTR sequences, the highest expressing cassette, ApoEHCR-hAATp-hFIXmg-bpA, was cloned into a Bluescript vector and 20 μg of the resulting plasmid in 2 ml of saline solution was injected into the tail vein of six mice. An average of 10 $\mu\text{g/ml}$ (two times the normal human level) was achieved 1 day after injection that slowly decreased to lower levels (Fig. 5a). However, the serum concentrations stabilized at 7–8 weeks after injection in the range from 0.5 to 2 $\mu\text{g/ml}$ and were maintained over 30 weeks (duration of the experiments). This concentration of hFIX was in the therapeutic range for treating hemophilia B. To begin to systematically delineate the critical element(s) required for persistent transgene expression, seven additional plasmids with a Bluescript vector backbone were constructed (Fig. 1b) and injected into seven groups of mice ($n = 6/\text{group}$). As shown in Fig. 5b, plasmids containing no intron sequences (pBS-hAATp-hFIX-bpA, pBS-ApoEHCR-hAATp-hFIX-bpA) or a polyadenylation signal (pBS-ApoEHCR-hAATp-hFIX-Int) resulted in low to undetectable levels and transient gene expression. On the other hand, plasmids containing the combination of ApoE-HCR and hAAT promoter, an intron, and

polyadenylation signal(s) whether from 1.7-kb 3'-UTR (pBS-ApoEHCR-hAATp-hFIXmg) or 0.3-kb bpA (pBS-ApoEHCR-hAATp-hFIX-Int-bpA) or both (pBS-ApoEHCR-hAATp-hFIXmg-bpA) produced persistent, therapeutic levels (0.5–2 $\mu\text{g/ml}$) of hFIX. The plasmids containing a 3'-UTR gave ~2-fold higher expression than those containing bpA alone. Furthermore, plasmid pBS-hAATp-hFIXmg-bpA without ApoE-HCR sequences produced a lower level of initial gene expression, and the level became undetectable 4-1/2 weeks after plasmid injection, indicating that ApoE-HCR was an essential element for persistent hFIX gene expression. Similar results were obtained when the 711-bp ApoE-HCR was replaced with a shorter 331-bp ApoE-HCR in plasmid pBS-ApoEHCR(s)-hAATp-hFIXmg-bpA. This demonstrates that the 331-bp ApoE-HCR fragment containing full functional LCR activities is sufficient for augmenting hFIX gene expression, consistent with results from transgenic mice experiments (42). Taken together, these data led us to conclude that the inclusion of an intron and one polyadenylation signal increased and prolonged transgene expression in the context of a plasmid after direct injection and in combination with a locus control region (ApoE-HCR) resulted in persistent as well as therapeutic levels of hFIX gene expression.

DISCUSSION

In this study, we designed plasmid DNA vectors that, when delivered to the liver, resulted in both persistent and therapeutic concentrations of plasma factor IX. The best-expressing plasmid tested in the *in vivo* study, LX-ApoE-HCR-hAAT-FIXmg-bpA, produced a maximal factor IX level of 18 $\mu\text{g/ml}$ 1 day after direct plasmid infusion. This level of factor IX expression is 3.6-fold higher than the normal plasma factor IX concentration (5 $\mu\text{g/ml}$) and therefore in great excess of a curative level needed for treating hemophilia. Even over long periods of time, hFIX serum levels remained therapeutic at levels that

TABLE 1
Comparison of Factor IX Expression Levels from Constructs Obtained from *in Vivo* and *in Vitro* Experiments

Constructs	<i>In vitro</i>			<i>In vivo</i>	
	Hep1A	NMH	HepG2	Portal vein	Tail vein
LX-FIX	1.0 \pm 0.21	1.0 \pm 0.26	1.0 \pm 0.26	1.0 \pm 0.75	1.0 \pm 0.18
LX-hAAT-FIX	2.0 \pm 0.64	0.8 \pm 0.20	1.0 \pm 0.16	—	—
LX-hAAT-FIX-bpA	1.9 \pm 0.59	0.6 \pm 0.16	1.1 \pm 0.26	1.3 \pm 0.91	—
LX-ApoE-hAAT-FIX-bpA	1.4 \pm 0.35	1.6 \pm 0.29	1.2 \pm 0.27	1.6 \pm 1.84	2.2 \pm 0.47
LX-ApoE-HCR-hAAT-FIX-bpA	1.8 \pm 0.24	2.1 \pm 0.24	1.4 \pm 0.24	1.2 \pm 1.38	14.0 \pm 4.42
LX-RSV-FIX-bpA	2.1 \pm 0.49	1.2 \pm 0.21	1.4 \pm 0.34	1.2 \pm 0.92	8.8 \pm 3.00
LX-ApoE-hAAT-FIXmg-bpA	1.3 \pm 0.54	2.3 \pm 0.32	1.8 \pm 0.42	13.2 \pm 11.4	21.3 \pm 8.62
LX-ApoE-HCR-hAAT-FIXmg-bpA	1.2 \pm 0.32	2.5 \pm 0.32	2.2 \pm 0.18	66.2 \pm 26.6	68.9 \pm 27.5
LX-RSV-FIXmg-bpA	2.7 \pm 0.55	1.4 \pm 0.24	2.3 \pm 0.35	4.6 \pm 2.63	22.8 \pm 8.25

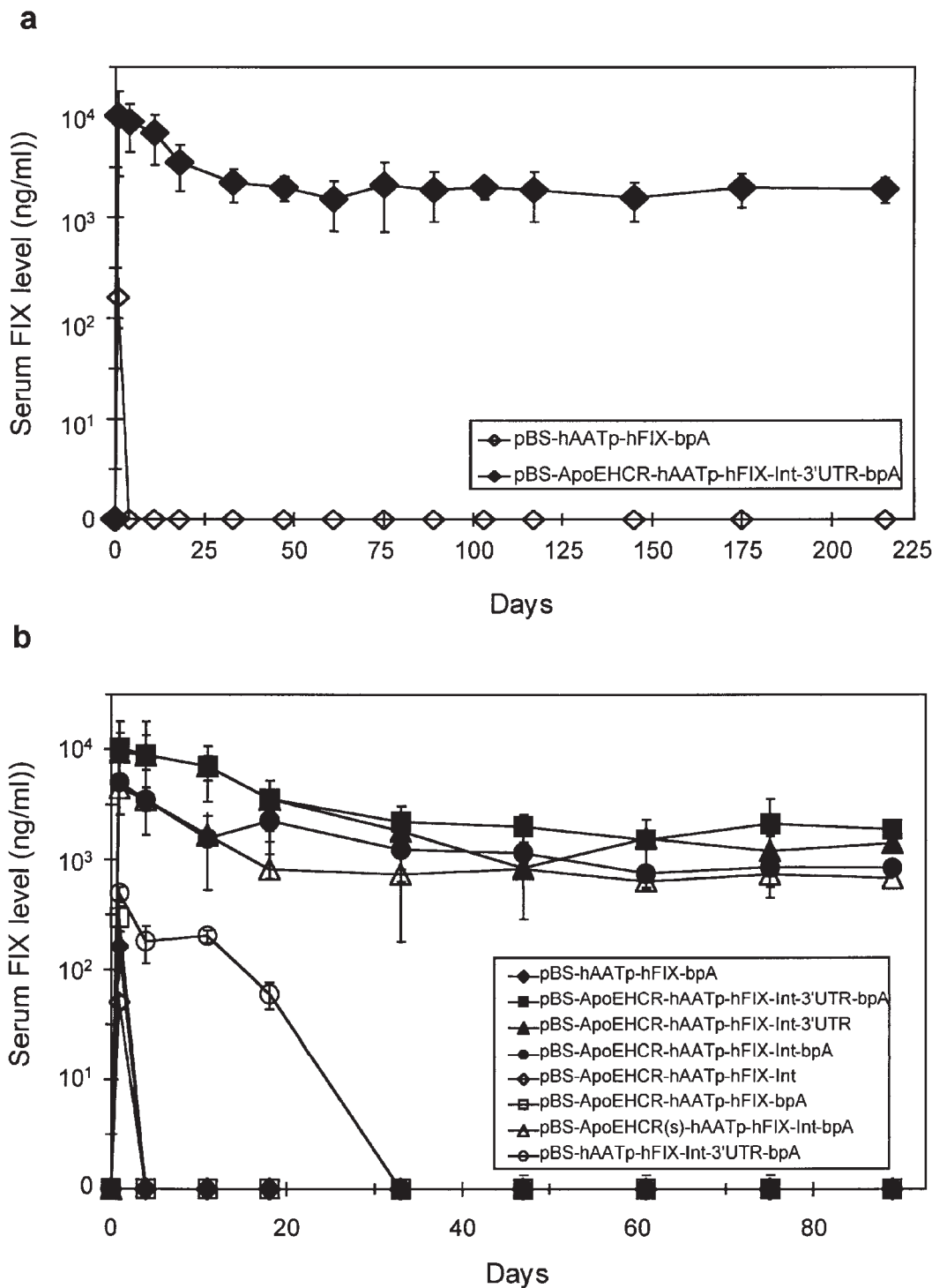


FIG. 5. Human factor IX gene expression levels after direct injection of various Bluescript plasmids into the tail vein of mice. Twenty micrograms of the respective Bluescript in 2 ml 0.9% saline solution was injected into the tail vein of C57Bl/6 mice ($n = 6$ per group) over 6–8 s. Serum or plasma was analyzed for human factor IX levels by ELISA at different time points. (a) Long-term gene expression of human factor IX levels was monitored after direct injection of the Bluescript plasmids pBS-ApoE-HCR-hAATp-hFIX-Int-3'UTR-bpA (filled diamonds) and pBS-hAATp-hFIX-bpA (open diamonds). (b) Delineation of essential elements required for persistent hFIX gene expression by testing different plasmids in mice experiments. Different symbols represent expression levels from mice injected with different plasmids: filled diamonds, pBS-hAATp-hFIX-bpA; filled squares, pBS-ApoEHCR-hAATp-hFIX-Int-3'UTR-bpA; filled triangles, pBS-ApoEHCR-hAATp-hFIX-Int-3'UTR; filled circles, pBS-ApoEHCR-hAATp-hFIX-Int-bpA; open diamonds, pBS-ApoEHCR-hAATp-hFIX-Int; open squares, pBS-ApoEHCR-hAATp-hFIX-bpA; open triangles, pBS-ApoEHCR(s)-hAATp-hFIX-Int-bpA; open circles, pBS-hAATp-hFIX-Int-3'UTR-bpA.

were 10 to 40% of normal. The mechanism for persistent gene expression from some of the plasmid DNAs is currently unknown. The ApoE-HCR contains a matrix attachment region (MAR) as well as liver-specific enhancer elements, which may augment gene expression in the plasmid. The MAR and intronic sequences together may interact with the nuclear matrix and may be primarily responsible for the persistence of gene expression from the plasmid DNA.

Intron A and the 3'-UTR were tested independently to assess their ability to enhance factor IX gene expression. It was previously speculated that the augmented gene expression with the inclusion of the first intron of factor IX in transgenic studies was not due to specific enhancer elements present in intron A, but rather to the increased precursor mRNA stability mediated by its splicing sequences (25). It may be important and even necessary to include at least one intron in gene therapy vectors so that the resulting transcripts can be more efficiently assembled into spliceosome complexes and better protected in the nucleus from random degradation. Similarly, the mechanism by which the 3'-UTR can enhance factor IX expression is currently unknown. From deletion analyses, it was demonstrated that although only one polyadenylation signal either from hFIX 3'-UTR or bpA was essential for high-level gene expression, the hFIX 3'-UTR enhanced hFIX gene expression approximately twofold more than bpA alone. This could be due to specific functional sequences in the 3'-UTR region. There may be enhancer elements contained in the 3'-UTR region to potentiate the transcription of the gene, regions that are needed to stabilize the mRNA, or elements required for posttranscriptional or posttranslational regulation. Additional studies that quantitate different sized mRNA species produced by constructs with and without the 3'-UTRs may help resolve some of these issues. It is interesting to note that recently a polymorphism found in 3'-UTR of the gene coding for another coagulation protein, prothrombin, significantly affected the level of prothrombin gene expression. A genetic variant due to a G to A transition at nucleotide 20210 is common and associated with elevated plasma prothrombin levels and an almost threefold increased risk of venous thrombosis (53, 54). Whether the 3'-UTRs of prothrombin and factor IX share similar function in controlling expression of respective genes needs to be further investigated.

Our study establishes the importance of studying expression cassettes *in vivo* before implementation into gene therapy applications. An additional application will be to determine if the same relative enhancement in gene expression is observed when these cis DNA elements are placed within integrating DNA vectors such as retroviruses or rAAV. The presence of a locus control region may help overcome the chromosomal positional effects that may influence transcription when the vector DNA is integrated. Furthermore, because enhancers can potentiate gene transcription in a chromosome position-

dependent manner, by combining multimerized liver-specific transcription factor binding sites such as HNF4, HNF3, HNF1, or C/EBP with the hepatic locus control region and a liver-specific promoter, higher levels of gene expression may be obtained. Some of these enhancer sites, when placed upstream of several promoters in a retroviral vector encoding hAAT, had marginal enhancing or even inhibitory effect on gene expression (47). This was attributed to the interaction of retroviral LTR with internal enhancer promoters and to individual protein-binding sites, which may interact not only with enhancing factors but also with inhibitory factors. However, these enhancer sites have not been tested *in vivo*. Further studies are required to determine if a correlation exists between gene expression from an integrating and plasmid based vector system *in vivo*.

This study clearly demonstrates that an intron and 3'-UTR sequences are important for optimizing *in vivo* hFIX gene expression. Furthermore, in combination with an hepatic locus control region and a liver-specific promoter, persistent and long-term, therapeutic level gene expression can be obtained after direct plasmid injection. It will be of interest to determine if the cis sequences described here can function in a similar manner with heterologous cDNAs. It is also of importance to investigate the mechanism leading to persistent gene expression, which should aid in developing a nonviral gene transfer strategy for human factor IX deficiency.

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REFERENCES

- Brownlee, G. G. (1987). The molecular pathology of haemophilia B. Fourth Wellcome Trust lecture. *Biochem. Soc. Trans.* 15: 1-8.
- High, K. A. (1999). Gene therapy for disorders of hemostasis. *American Society of Hematology, 41st Annual Meeting, Education Program*, p. 438.
- Hatzoglou, M., Lamers, W., Bosch, F., Wynshaw-Boris, A., Clapp, D. W., and Hanson, R. W. (1990). Hepatic gene transfer in animals using retroviruses containing the promoter from the gene for phosphoenolpyruvate carboxykinase. *J. Biol. Chem.* 265: 17285-17293.
- Cardoso, J. E., Branchereau, S., Jeyaraj, P. R., Houssin, D., Danos, O., and Heard, J. M. (1993). *In situ* retrovirus-mediated gene transfer into dog liver. *Hum. Gene Ther.* 4: 411-418.
- Kay, M. A., Li, Q., Liu, T. J., Leland, F., Toman, C., Finegold, M., and Woo, S. L. (1992). Hepatic gene therapy: Persistent expression of human alpha 1-antitrypsin in mice after direct gene delivery *in vivo*. *Hum. Gene Ther.* 3: 641-647.
- Kay, M. A., Rothenberg, S., Landen, C. N., Bellinger, D. A., Leland, F., Toman, C., Finegold, M., Thompson, A. R., Read, M. S., Brinkhous, K. M., et al. (1993). *In vivo* gene therapy of hemophilia B: Sustained partial correction in factor IX-deficient dogs. *Science* 262: 117-119.
- Rettinger, S. D., Kennedy, S. C., Wu, X., Saylor, R. L., Hafenrichter, D. G., Flye, M. W., and Ponder, K. P. (1994). Liver-directed gene therapy: Quantitative evaluation of promoter elements by using *in vivo* retroviral transduction. *Proc. Natl. Acad. Sci USA* 91: 1460-1464.
- Kolodka, T. M., Finegold, M., Kay, M. A., and Woo, S. L. (1993). Hepatic gene therapy: Efficient retroviral-mediated gene transfer into rat hepatocytes *in vivo* [published erratum appears in *Somat. Cell. Mol. Genet.* 20(3):251, 1994]. *Somat. Cell. Mol. Genet.* 19: 491-497.
- Hafenrichter, D. G., Wu, X., Rettinger, S. D., Kennedy, S. C., Flye, M. W., and Ponder, K. P. (1994). Quantitative evaluation of liver-specific promoters from retroviral vectors after *in vivo* transduction of hepatocytes. *Blood* 84: 3394-3404.

- ¹⁰Smith, T. A., Mehaffey, M. G., Kayda, D. B., Saunders, J. M., Yei, S., Trapnell, B. C., McClelland, A., and Kaleko, M. (1993). Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. *Nat. Genet.* 5: 397–402.
- ¹¹Kay, M. A., Landen, C. N., Rothenberg, S. R., Taylor, L. A., Leland, F., Wiehle, S., Fang, B., Bellinger, D., Finegold, M., Thompson, A. R., et al. (1994). *In vivo* hepatic gene therapy: Complete albeit transient correction of factor IX deficiency in hemophilia B dogs. *Proc. Natl. Acad. Sci. USA* 91: 2353–2357.
- ¹²Walter, J., You, Q., Hagstrom, J. N., Sands, M., and High, K. A. (1996). Successful expression of human factor IX following repeat administration of adenoviral vector in mice. *Proc. Natl. Acad. Sci. USA* 93: 3056–3061.
- ¹³Eisensmith, R. C., and Woo, S. L. (1997). Viral vector-mediated gene therapy for hemophilia B. *Thromb. Haemost.* 78: 24–30.
- ¹⁴Dai, Y., Schwarz, E. M., Gu, D., Zhang, W. W., Sarvetnick, N., and Verma, I. M. (1995). Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: Tolerization of factor IX and vector antigens allows for long-term expression. *Proc. Natl. Acad. Sci. USA* 92: 1401–1405.
- ¹⁵Snyder, R. O., Miao, C. H., Patijn, G. A., Spratt, K., Danos, O., Nagy, D., Gown, A. M., Winther, B., Meuse, L., Cohen, L. K., Thompson, A. R., and Kay, M. A. (1997). Persistent and therapeutic concentrations of human factor IX in mice after hepatic gene transfer of recombinant AAV vectors. *Nat. Genet.* 16: 270–276.
- ¹⁶Nakai, H., Herzog, R. W., Hagstrom, J. N., Walter, J., Kung, S. H., Yang, E. Y., Tai, S. J., Iwaki, Y., Kurtzman, G. J., Fisher, K. J., Colosi, P., Couto, L. B., and High, K. A. (1998). Adeno-associated viral vector-mediated gene transfer of human blood coagulation factor IX into mouse liver. *Blood* 91: 4600–4607.
- ¹⁷Snyder, R. O., Miao, C., Meuse, L., Tubbs, J., Donahue, B. A., Lin, H. F., Stafford, D. W., Patel, S., Thompson, A. R., Nichols, T., Read, M. S., Bellinger, D. A., Brinkhous, K. M., and Kay, M. A. (1999). Correction of hemophilia B in canine and murine models using recombinant adeno-associated viral vectors [see comments]. *Nat. Med.* 5: 64–70.
- ¹⁸Wang, L., Takabe, K., Bidlingmaier, S. M., Ill, C. R., and Verma, I. M. (1999). Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. *Proc. Natl. Acad. Sci. USA* 96: 3906–3910.
- ¹⁹Wang, L., Nichols, T. C., Read, M. S., Bellinger, D. A., and Verma, I. M. (2000). Sustained expression of therapeutic level of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. *Mol. Ther.* 1: 154–158.
- ²⁰Pastore, L., Morral, N., Zhou, H., Garcia, R., Parks, R. J., Kochanek, S., Graham, F. L., Lee, B., and Beaudet, A. L. (1999). Use of a liver-specific promoter reduces immune response to the transgene in adenoviral vectors. *Hum. Gene Ther.* 10: 1773–1781.
- ²¹Brinster, R. L., Allen, J. M., Behringer, R. R., Gelinas, R. E., and Palmiter, R. D. (1988). Introns increase transcriptional efficiency in transgenic mice. *Proc. Natl. Acad. Sci. USA* 85: 836–840.
- ²²Palmiter, R. D., Sandgren, E. P., Avarbock, M. R., Allen, D. D., and Brinster, R. L. (1991). Heterologous introns can enhance expression of transgenes in mice. *Proc. Natl. Acad. Sci. USA* 88: 478–482.
- ²³Liu, K., Sandgren, E. P., Palmiter, R. D., and Stein, A. (1995). Rat growth hormone gene introns stimulate nucleosome alignment *in vitro* and in transgenic mice. *Proc. Natl. Acad. Sci. USA* 92: 7724–7728.
- ²⁴Jallat, S., Perraud, F., Dalemans, W., Bolland, A., Dieterle, A., Faure, T., Meulien, P., and Pavirani, A. (1990). Characterization of recombinant human factor IX expressed in transgenic mice and in derived trans-immortalized hepatic cell lines. *EMBO J.* 9: 3295–3301.
- ²⁵Kurachi, S., Hitomi, Y., Furukawa, M., and Kurachi, K. (1995). Role of intron I in expression of the human factor IX gene. *J. Biol. Chem.* 270: 5276–5281.
- ²⁶Ill, C. R., Yang, C. Q., Bielingmaier, S. M., Gonzales, J. N., Burns, D. S., Bartholomew, R. M., and Scuderi, P. (1997). Optimization of the human factor VIII complementary DNA expression plasmid for gene therapy of hemophilia A. *Blood Coagul. Fibrinolysis* 8(Suppl. 2): S23–S30.
- ²⁷Vielhaber, E., Jacobson, D. P., Ketterling, R. P., Liu, J. Z., and Sommer, S. S. (1993). A mutation in the 3' untranslated region of the factor IX gene in four families with hemophilia B. *Hum. Mol. Genet.* 2: 1309–1310.
- ²⁸Chen, S. H., Schoof, J. M., Weinmann, A. F., and Thompson, A. R. (1995). Heteroduplex screening for molecular defects in factor IX genes from haemophilia B families. *Br. J. Haematol.* 89: 409–412.
- ²⁹de la Salle, C., Charmanier, J. L., Baas, M. J., Schwartz, A., Wiesel, M. L., Grunebaum, L., and Cazenave, J. P. (1993). A deletion located in the 3' nontranslated part of the factor IX gene responsible for mild haemophilia B [letter]. *Thromb. Haemost.* 70: 370–371.
- ³⁰Kaleko, M., Kayda, D., Sakhujia, K., Mehaffey, M., and McClelland, A. (1995). Genomic sequences increase adenoviral vector-mediated factor IX expression 1,900 fold and enable sustained expression *in vivo*. *J. Cell. Biochem.* 21: A366.
- ³¹Kurachi, S., Deyashiki, Y., Takeshita, J., and Kurachi, K. (1999). Genetic mechanisms of age regulation of human blood coagulation factor IX. *Science* 285: 739–743.
- ³²Kay, M. A., Baley, P., Rothenberg, S., Leland, F., Fleming, L., Ponder, K. P., Liu, T., Finegold, M., Darlington, G., Pokorny, W., et al. (1992). Expression of human alpha 1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. *Proc. Natl. Acad. Sci. USA* 89: 89–93.
- ³³Schmidt, E. V., Christoph, G., Zeller, R., and Leder, P. (1990). The cytomegalovirus enhancer: A pan-active control element in transgenic mice. *Mol. Cell. Biol.* 10: 4406–4411.
- ³⁴Ponder, K. P., Dunbar, R. P., Wilson, D. R., Darlington, G. J., and Woo, S. L. (1991). Evaluation of relative promoter strength in primary hepatocytes using optimized lipofection. *Hum. Gene Ther.* 2: 41–52.
- ³⁵Isom, I., Georgoff, I., Salditt-Georgieff, M., and Darnell, J. E., Jr. (1987). Persistence of liver-specific messenger RNA in cultured hepatocytes: Different regulatory events for different genes. *J. Cell Biol.* 105: 2877–2885.
- ³⁶Budker, V., Zhang, G., Knechtle, S., and Wolff, J. A. (1996). Naked DNA delivered intraportally expresses efficiently in hepatocytes. *Gene Ther.* 3: 593–598.
- ³⁷Liu, F., Song, Y., and Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* 6: 1258–1266.
- ³⁸Zhang, G., Budkar, V., and Wolff, J. A. (1999). High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* 10: 1735–1737.
- ³⁹Long, G. L., Chandra, T., Woo, S. L., Davie, E. W., and Kurachi, K. (1984). Complete sequence of the cDNA for human alpha 1-antitrypsin and the gene for the S variant. *Biochemistry* 23: 4828–4837.
- ⁴⁰Armentano, D., Thompson, A. R., Darlington, G., and Woo, S. L. (1990). Expression of human factor IX in rabbit hepatocytes by retrovirus-mediated gene transfer: Potential for gene therapy of hemophilia B. *Proc. Natl. Acad. Sci. USA* 87: 6141–6145.
- ⁴¹Le, M., Okuyama, T., Cai, S. R., Kennedy, S. C., Bowling, W. M., Flye, M. N., and Ponder, K. P. (1997). Therapeutic levels of functional human factor IX in rats after retroviral mediated hepatic gene therapy. *Blood* 89: 1254–1259.
- ⁴²Dang, Q., Walker, D., Taylor, S., Allan, C., Chin, P., Fan, J., and Taylor, J. (1995). Structure of the hepatic control region of the human apolipoprotein E/C-I gene locus. *J. Biol. Chem.* 270: 22577–22585.
- ⁴³Miller, A. D. (1990). Retrovirus packaging cells. *Hum. Gene Ther.* 1: 5–14.
- ⁴⁴Bray, G. L., Weinmann, A. F., and Thompson, A. R. (1986). Calcium-specific immunoassays for factor IX reduced levels of antigens in patients with vitamin K disorders. *J. Lab. Clin. Med.* 107: 269–278.
- ⁴⁵Yant, S. R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z., and Kay, M. A. (2000). Somatic integration and long-term therapeutic transgene expression in normal and hemophilic mice using plasmid DNA encoding a DNA transposon system. *Nat. Genet.*, in press.
- ⁴⁶Shachter, N. S., Zhu, Y., Walsh, A., Breslow, J. L., and Smith, J. D. (1993). Localization of a liver-specific enhancer in the apolipoprotein E/C-I/C-II gene locus. *J. Lipid Res.* 34: 1699–1707.
- ⁴⁷Wu, X., Holschen, J., Kennedy, S. C., and Ponder, K. P. (1996). Retroviral vector sequences may interact with some internal promoters and influence expression. *Hum. Gene Ther.* 7: 159–171.
- ⁴⁸Okuyama, T., Huber, R. M., Bowling, W., Pearline, R., Kennedy, S. C., Flye, M. W., and Ponder, K. P. (1996). Liver-directed gene therapy: A retroviral vector with a complete LTR and the ApoE enhancer-alpha 1-antitrypsin promoter dramatically increases expression of human alpha 1-antitrypsin *in vivo*. *Hum. Gene Ther.* 7: 637–645.
- ⁴⁹Simonet, W. S., Bucay, N., Lauer, S. J., and Taylor, J. M. (1993). A far-downstream hepatocyte-specific control region directs expression of the linked human apolipoprotein E and C-I genes in transgenic mice. *J. Biol. Chem.* 268: 8221–8229.
- ⁵⁰Emerman, M., and Temin, H. M. (1986). Quantitative analysis of gene suppression in integrated retrovirus vectors. *Mol. Cell. Biol.* 6: 792–800.
- ⁵¹Ghantas, I. R., Sanes, J. R., and Majors, J. E. (1991). The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. *Mol. Cell. Biol.* 11: 5848–5859.
- ⁵²Wu, J. C., Merlino, G., and Fausto, N. (1994). Establishment and characterization of differentiated, nontransformed hepatocyte cell lines derived from mice transgenic for transforming growth factor alpha. *Proc. Natl. Acad. Sci. USA* 91: 674–678.
- ⁵³Poort, S. R., Rosendaal, F. R., Reitsma, P. H., and Bertina, R. M. (1996). A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 88: 3698–3703.
- ⁵⁴Howard, T. E., Marusa, M., Channell, C., and Duncan, A. (1997). A patient homozygous for a mutation in the prothrombin gene 3'-untranslated region associated with massive thrombosis [see comments]. *Blood Coagul. Fibrinolysis* 8: 316–319.