HUMAN GENE THERAPY 7:637–645 (March 20, 1996)
Mary Ann Liebert, Inc.

Liver-Directed Gene Therapy: A Retroviral Vector with a Complete LTR and the ApoE Enhancer-α_1-Antitrypsin Promoter Dramatically Increases Expression of Human α_1-Antitrypsin *In Vivo*

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ABSTRACT

Hepatic gene therapy could improve the treatment of many inherited disorders. Although retroviral vectors result in long-term expression in hepatocytes *in vivo*, their low level of expression currently precludes most clinical applications. Four copies of the liver-specific apolipoprotein E (ApoE) enhancer were placed upstream of the human α_1-antitrypsin (hAAT) promoter in either orientation into a retroviral vector with a complete long terminal repeat (LTR) and the hAAT cDNA to generate ApoE(+)hAAT-LTR and ApoE(−)hAAT-LTR. In addition, the ApoAI promoter was placed upstream of the hAAT cDNA in a similar retroviral vector backbone. Amphotropic retroviral vectors were transferred into regenerating rat liver cells *in vivo* by intraportal injection. ApoE(−)hAAT-LTR and ApoE(+)hAAT-LTR led to average hAAT levels of 5 μg/ml (0.5% of normal levels of a very abundant protein), and 2.5 μg/ml, respectively, which was stable for at least 10 months after transduction. This level of serum hAAT was >25-fold higher than what was observed from the ApoAI promoter used in this study. Serum levels of hAAT were >15-fold higher than what was observed from retroviral vectors containing the hAAT cDNA that were analyzed previously by this lab. In some cases, improved expression was due to the promoter chosen. In other cases, the increase in expression was primarily due to the higher titers obtained by using a retroviral backbone with an intact LTR as opposed to a vector with a deletion in the LTR. The increased expression levels observed from this enhancer/promoter combination in an intact retroviral backbone may enable one to achieve therapeutic levels of clinically important genes from a retroviral vector in liver cells of animals.

OVERVIEW SUMMARY

Low levels of expression from retroviral vectors *in vivo* are a limiting factor in the successful application of gene therapy to the treatment of genetic disorders. Three retroviral vectors containing liver-specific transcriptional elements were tested for their ability to direct expression of the serum protein human α_1-antitrypsin (hAAT) gene from rat liver cells. The apolipoprotein E enhancer-hAAT promoter combination resulted in high-level expression of the hAAT reporter gene *in vivo* when placed into a retroviral vector with an intact LTR. The level of serum hAAT observed was >15-fold higher than what was observed with all previously tested retroviral vectors. This retroviral vector may significantly improve the overall levels of expression of other clinically important genes from retroviral vectors that have been transferred into liver cells by using *ex vivo* or *in vivo* techniques.

INTRODUCTION

LIVER-DIRECTED GENE THERAPY could revolutionize treatments for inherited hematologic and metabolic disorders such as phenylketonuria, human α_1-antitrypsin (hAAT) deficiency, familiar hypercholesterolemia (FH), and hemophilia. Although both adenoviral vectors and plasmid DNAs have been expressed from hepatocytes in animals (Ledley, 1993), only

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retroviral vectors result in stable expression. Both *in vivo* and *ex vivo* approaches have been used to transfer retroviral vectors into mammalian hepatocytes. *In vivo* delivery of retroviral vectors has been accomplished by induction of liver cell replication by performing a partial hepatectomy and injection of retrovirus into the liver *via* a closed perfusion system (Ferry *et al.*, 1991; Rozga *et al.*, 1992; Cardoso *et al.*, 1993), simple injection into the portal vein (Hatzoglou *et al.*, 1990; Kay *et al.*, 1992b, 1993; Rettinger *et al.*, 1993, 1994; Kolodka *et al.*, 1993, 1995; Hafenrichter *et al.*, 1994), or direct injection into the liver parenchyma (Kaleko *et al.*, 1991). Although long-term expression of hAAT (Kay *et al.*, 1992b; Kolodka *et al.*, 1993; Rettinger *et al.*, 1994; Hafenrichter *et al.*, 1994) and Factor IX (FIX; Kay *et al.*, 1993) has been achieved in rats, mice, and dogs by using *in vivo* hepatic gene therapy, the level of expression is subtherapeutic at <0.1% of normal. Because the transduction efficiency was as high as 10% in some of these studies, these results suggested that the expression per provirus was low.

The *ex vivo* approach requires hepatocyte harvest, *in vitro* transduction with a retrovirus, and reimplantation *via* the portal vein (Chowdhury *et al.*, 1991; Kay *et al.*, 1992a; Grossman *et al.*, 1994). This approach has been successfully used to transfer a low-density lipoprotein (LDL) receptor gene into the Watanabe rabbit, an animal model for FH, resulting in a partial decline in the serum cholesterol (Chowdhury *et al.*, 1991). *Ex vivo* retrovirus-mediated hepatic gene therapy is currently being used to treat human patients with FH. Although a 19% decline in the serum cholesterol was noted in one patient (Grossman *et al.*, 1994), it is unlikely that this decrease will prevent the development of atherosclerosis (Brown *et al.*, 1994). A possible reason for this inadequate response is that expression from the β-actin promoter used in this study was low *in vivo*. The marked discrepancy between the number of transduced cells returned to the patient and the frequency of positive cells identified by *in situ* hybridization analysis suggests that the expression was below detectable levels in most transduced cells (Grossman *et al.*, 1994).

Increased expression from a retroviral vector will be critical for the successful application of hepatic gene therapy techniques in humans regardless of the delivery system chosen. Although viral promoters such as the retroviral long terminal repeat (LTR) and the cytomegalovirus (CMV) promoter are quite active in primary hepatocytes *in vitro* (Ponder *et al.*, 1991), they are expressed at low levels from hepatocytes in animals (Kaleko *et al.*, 1991; Kay *et al.*, 1992a,b; Rettinger *et al.*, 1994). A similar inactivity of viral promoters has been observed in fibroblasts *in vivo* (Palmer *et al.*, 1991; Scharfmann *et al.*, 1991). This inactivity might reflect an absence of positive-acting transcription factors in mitogenically quiescent cells of animals, or the presence of inhibitors of transcription. Although ubiquitously expressed cellular promoters led to long-term expression from a retroviral vector in hepatocytes (Rettinger *et al.*, 1994) and fibroblasts (Scharfmann *et al.*, 1991) *in vivo*, expression remained low.

A logical approach to increase expression from a retroviral vector in hepatocytes *in vivo* is to utilize liver-specific promoters and enhancers. Kay *et al.* (1992b) determined that an albumin promoter/enhancer was stronger *in vivo* than the CMV promoter, although it underwent an as yet unmapped 2-kb deletion while being amplified in the packaging cells (Mark Kay, personal communication), and the overall expression was still relatively low. We recently determined that the human α_1 antitrypsin promoter was expressed at higher levels *in vivo* than the albumin, phosphoenolpyruvate carboxykinase, LTR, and Pol II promoters, after normalization to the number of copies delivered (Hafenrichter *et al.*, 1994). A caveat to this result was the fact that the hAAT promoter was in a retroviral vector with a deletion in the 3′ enhancer, resulting in a packaging cell line of low titer. The purpose of this study was to confirm that the hAAT promoter would result in high-level expression from an enhancer-intact retroviral vector of high titer, and to attempt to further increase expression by adding the strong liver-specific apolipoprotein E (ApoE) enhancer (Schachter *et al.*, 1993; Simonet *et al.*, 1993) upstream of the hAAT promoter. Finally, the apolipoprotein AI (ApoAI) promoter (Sastry *et al.*, 1988; Walsh *et al.*, 1989) was also tested for its ability to be expressed from a retroviral vector *in vivo*.

MATERIALS AND METHODS

Construction of retroviral vectors

ApoE(+)hAAT-LTR and ApoE(−)hAAT-LTR: LTR-hAAT (Hafenrichter *et al.*, 1994) is derived from LNL-6 (Miller and Rosman, 1989), a Moloney murine leukemia virus (MoMLV)-based retroviral vector. hAAT-LTR was generated by cloning the 403-bp hAAT promoter upstream of the hAAT cDNA of LTR-hAAT, as described previously (Hafenrichter *et al.*, 1994). pJS-406 (Jonathan Smith, Rockefeller University) contains four copies of the 156 bp ApoE enhancer (Schachter *et al.*, 1993). The 650-bp insert was removed from pJS-406 by *Kpn* I digestion, and cloned into the *Kpn* I site of pSP72 (Promega, Madison WI) to create ApoE-SP72. The orientation of the insert was determined by DNA sequencing with T7 and SP6 primers. After *Bgl* II and *Bam* HI digestion of ApoE-SP72 (cuts on either end of the insert in the polylinker), the 700-bp fragment was cloned into the *Bgl* II site upstream of the hAAT promoter of hAAT-LTR in both orientations, to create ApoE(+)hAAT-LTR and ApoE(−)hAAT-LTR.

ApoAI-LTR: p256-AI-CAT contains the 256-bp human ApoAI promoter (Sastry *et al.*, 1988). p256-AI-CAT was digested with *Hga* I (cuts at +5 relative to the transcription initiation site) and blunt-ended by the Klenow fragment of DNA polymerase. After digestion with *Kpn* I (cleaves at the 5′ end of the promoter), the 261-bp DNA fragment was cloned into the *Kpn* I and *Sma* I sites of pSP72 to generate ApoAI-SP72. ApoAI-SP72 was digested with *Bam HI*, which cuts on both sides of the promoter, and the 270-bp ApoAI promoter was cloned into the *Bgl* II site of LTR-hAAT to generate ApoAI-LTR.

Creation of Retroviral Producer Cell Lines: The amphotropic GP + envAM12 murine fibroblast (Markowitz *et al.*, 1988) and NIH-3T3 fibroblast (Jainchill *et al.*, 1969) lines were maintained in Dulbecco's modified Eagle's medium (DMEM). The mouse hepatoma line Hepa1A (Darlington, 1987) was maintained in 75% minimal essential medium with 25% Waymouths MAB medium (MEM). Complete media contained 10% supplemented heat-inactivated calf serum (Hyclone Laboratories, Logan, UT), penicillin (100 U/ml), and strepto-

mycin (100 μg/ml) (DMEM). After calcium phosphate transfection of GP + AM12 cells with retroviral vector DNAs (Miller and Rosman, 1989), cells were selected for 14 days with complete DMEM containing 250 n*M* methotrexate (Mtx) and dialyzed calf serum (Sigma Chemical, St. Louis, MO). Approximately 50 Mtx-resistant (Mtx®) clones were screened for their relative titers by infection of NIH-3T3 cells and quantitation of hAAT protein in the supernatant of the transduced cells, as described previously (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994).

In vitro *transduction of NIH-3T3 and Hepa1A cells with retroviral vectors containing liver-specific promoters*

Conditioned medium from confluent retroviral packaging cells was filtered through a 0.45-μm filter and polybrene was added to a final concentration of 8 μg/ml. Various dilutions of the conditioned medium were added to 20,000 NIH-3T3 or Hepa 1A cells present on a 24-well plate at 50% confluency. Forty-eight hours later, the transduced cells were trypsinized and the entire contents transferred to a 10-cm plate in complete medium containing 250 n*M* Mtx. After 14 days of selection, colonies were counted to determine the Mtx® retroviral titer, pooled together, and cultured to confluency. Pools of transduced cells were analyzed for hAAT production by culturing the cells for 6 hr and assaying the medium for hAAT by ELISA. After collection of the supernatants, the transduced cells were trypsinized, counted on a hemocytometer, and used to obtain RNA for further analysis, as described previously (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994).

In vivo *Hepatocyte Transduction Protocol:* Adult male Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing 200–275 grams, received standard NIH and institutional approved care with Purina rodent chow and tap water *ad libitum*. Rats received a 70% partial hepatectomy as described (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994). Twenty-four hours later, conditioned medium was collected from packaging cells and concentrated by ultracentrifugation. Polybrene was added to a final concentration of 8 μg/ml, and *in vivo* hepatocyte transduction accomplished by portal vein injection of the retrovirus without occlusion of the distal portal vein or hepatic artery.

Northern Blot Analysis: Ten micrograms of RNA was electrophoresed on a 1% agarose gel in the presence of formaldehyde (Sambrook *et al.*, 1989), and transferred to an Optitran nitrocellulose membrane (Schleicher and Schuell, Keene, NH). One lane was cut from the gel and stained with ethidium bromide to identify the position of 18S (2 kb) and 28S (5 kb) rRNA. Membranes were hybridized with the 1.3-kb hAAT cDNA probe labeled to a specific activity of 3×10^9 cpm/μg DNA by random primer extension (Sambrook *et al.*, 1989).

PCR Amplification of Proviral DNA Sequences: A 30-cycle multiplex PCR technique was employed, using one primer set that amplified proviral (IRES) DNA and one primer set that amplified the rat genomic liver fatty acid binding protein (LFABP) DNA, as described previously (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994). Amplified DNA was electrophoresed through a 2% agarose/1× Tris-ammonium acetate gel, transferred to an Optitran nitrocellulose membrane, and hybridized with a 217-bp *Hind* III/*Kpn* I fragment of the IRES labeled by random primer extension. After quantitation, the membrane was stripped and reprobed with a radiolabeled 620-bp LFABP probe. Radioactivity was quantitated on a Betascope 630 two-dimensional Beta counter (Betagen, Waltham, MA).

RESULTS

Development of retroviral vectors containing liver-specific promoters

In an attempt to identify transcriptional elements that function well from a retroviral vector *in vivo*, the ApoAI promoter (Sastry *et al.*, 1988; Walsh *et al.*, 1989) was placed directly upstream of the hAAT cDNA in a retroviral vector designated ApoAI-LTR, and the ApoE enhancer (Shachter *et al.*, 1993; Simonet *et al.*, 1993) was placed in either orientation upstream of the hAAT promoter to create ApoE(+)hAAT-LTR and ApoE(−)hAAT-LTR, as shown in Fig. 1. The plus designates an orientation identical to that observed for the endogenous gene, where the minus refers to the opposite orientation. The LTR indicates that the complete LTR from LNL-6 (a hybrid between the Mo-MLV and the Moloney murine sarcoma virus) was present in the 3′ position, in contrast to some previous studies in which an enhancer-deleted LTR was used (Hafenrichter *et al.*, 1994). All vectors contain the hAAT cDNA as a reporter gene and a mutant dihydrofolate reductase (*DHFR) gene, which confers Mtx resistance. Translation of *DHFR from the downstream position of a dicistronic mRNA is initiated by the internal ribosome entry site (IRES) sequence (Ghattas *et al.*, 1991).

All three retroviral vectors were transfected into GP + AM12 amphotropic retroviral packaging cells and high titer clones isolated. *In vitro* Mtx® titers for the clones utilized in this study varied from 1 to 10×10^5 cfu/ml, as summarized in Table 1. The constructs containing the ApoE enhancer upstream of the hAAT promoter resulted in the production of 50–75 ng

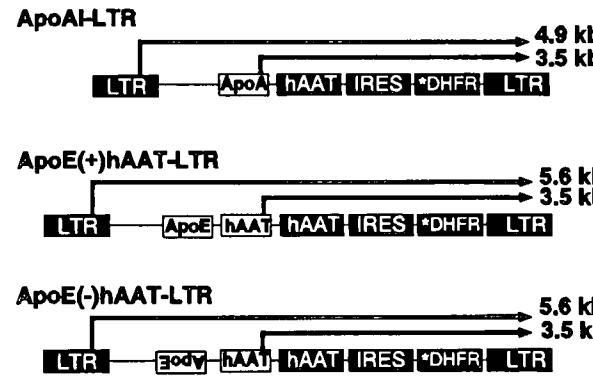

FIG. 1. Retroviral vectors containing liver-specific transcriptional elements. Transcriptional elements were placed immediately upstream of the hAAT cDNA in a retroviral vector that also contained an IRES upstream of the *DHFR gene. Arrows indicate that transcription can initiate from the LTR or the internal promoter, with the size of the transcript indicated on the right. Either transcript results in an mRNA that can be translated into both proteins.

TABLE 1. *IN VITRO* EVALUATION OF RETROVIRAL VECTORS

Retroviral vector	*Mtx^R titer*	*Expression in NIH-3T3 cells (ng/24 h per million cells)*	*Expression in Hepa1A cells (ng/24 h per million cells)*
Apo AI-LTR	1×10^5 cfu/ml	155 ± 29.8	93 ± 1
ApoE(−)hAAT-LTR	10×10^5 cfu/ml	75.3 ± 7.9	71 ± 6.6
ApoE(+)hAAT-LTR	4×10^5 cfu/ml	74.5 ± 5.2	59 ± 8.3

Mtx^R titers for each retroviral vector were tested on NIH-3T3 cells. Pools of singly transduced NIH-3T3 and Hepa1A cells were obtained and grown to confluence. A timed collection was analyzed for hAAT production and normalized to cell number. Data is presented as the average ± SEM.

of hAAT/24 hr per million cells in pools of singly transduced NIH-3T3 and Hepa1A cells. The ApoAI-LTR construct resulted in expression that was slightly higher in both cell lines. These levels of expression are similar to that observed for other retroviral vectors containing an identical backbone but different internal promoters (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994).

Long-term expression of retroviral vectors after in vivo *transduction of rats*

The expression from these retroviral vectors in the liver *in vivo* was determined. Conditioned medium from each packaging cell line was used to transduce regenerating rat liver cells *in vivo*. Serum was obtained from transduced rats and analyzed in duplicate for hAAT levels by ELISA (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994), as shown in Fig. 2A. hAAT was undetectable in all rats prior to transduction, demonstrating the specificity of the antibody for the human protein. The ApoE(−)hAAT retroviral vector led to the highest level of expression, with an average serum hAAT of 5 μg/ml. The construct in which the apo E enhancer was inserted in the opposite orientation [ApoE(+)hAAT-LTR] led to slightly lower average expression at 2.5 μg/ml. Expression in ApoAI-LTR-transduced rats was considerably lower, with an average of 0.1 μg/ml. Expression in all rats was stable for 14 weeks. To assess the longevity of expression better, the 3 rats with the highest level of expression from each of the ApoE enhancer-containing vectors were followed longer. As shown in Fig. 2B, all 6 maintained stable levels of serum hAAT for up to 10 months after transduction.

Analysis of RNA from cells transduced in vitro *and* in vivo *with retroviral vectors*

The retroviral vectors used in this study contain an internal promoter and the LTR promoter. Identification of which promoter functions *in vivo* may further our understanding of how promoters function from retroviral vectors in animals. The Northern blot analysis shown in Fig. 3 demonstrates that for all three retroviral constructs, the major RNA in transduced Hepa1A cells is full-length (lanes 2–4). This suggests that the LTR promoter is the major promoter used and that large deletions have not occurred. RNA from both the packaging cells and transduced NIH-3T3 cells were also primarily full length (data not shown). Although the internal promoter was responsible for <5% of all retroviral transcripts in hepatoma cells *in vitro*, it represents ~50% of all transcripts in liver RNA from

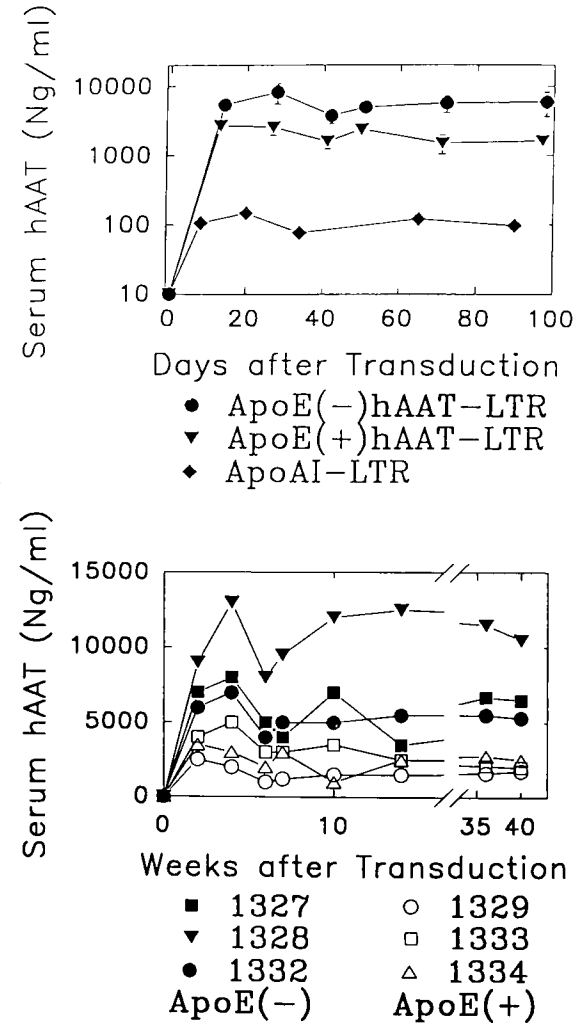

FIG. 2. A. Average serum hAAT levels in rats transduced *in vivo* with ApoE(+)hAAT-LTR, ApoE(−)hAAT-LTR, and ApoAI-LTR retroviral vectors. Rats were transduced with conditioned medium containing approximately 2×10^7 cfu of each retroviral vector. The average serum hAAT ± the SEM are shown for ApoE(+)hAAT-LTR ($n = 5$), ApoE(−)hAAT-LTR ($n = 5$), and ApoAI-LTR ($n = 6$) transduced rats. B. Long-term evaluation of serum hAAT levels in some ApoE(+)hAAT-LTR and ApoE(−)hAAT-LTR rats. The 3 rats from A with the highest level of serum hAAT from the ApoE(+)hAAT-LTR and the ApoE(−)hAAT-LTR groups were followed for an additional 6 months. The serum hAAT levels observed for individual rats at each time point are shown. Expression has remained at stable levels for a total of 10 months to date.

rats transduced *in vivo* with ApoE(−)hAAT-LTR and ApoE(+)hAAT-LTR, as shown in lanes 6–11. This demonstrates that transcription has shifted significantly from the LTR to the internal promoter. The combination of LTR- and ApoE-hAAT-initiated retroviral transcripts in rats transduced with the ApoE enhancer/hAAT promoter constructs is somewhat less than 1% of the signal observed in normal human liver, a result that is consistent with the relative levels of hAAT protein in serum. The initiation site for the ApoAI-LTR-transduced rats could not be determined by Northern blot analysis, as the level of RNA was below detectable levels (lanes 12–14).

PCR quantitation of proviral DNA in the livers of transduced rats

To compare retroviral vectors of different titers for their *in vivo* promoter strength, expression must be normalized to transduction efficiency. Genomic DNA was isolated from the livers of the 3 rats with the highest level of expression for each retroviral vector. A multiplex PCR technique was performed to determine the proviral copy number, as shown in Fig. 4. The potential inaccuracies of the semi-quantitative PCR technique for estimating transduction efficiency were minimized by: (i) utilizing one set of primers that amplifies a rat genomic sequence (LFABP) simultaneously with a set of primers that amplifies the retroviral (IRES) sequence, to control for PCR efficiency and DNA loading; (ii) analyzing the samples from transduced rats, controls, and the standard curve on the same day; and (iii) performing the reaction three times.

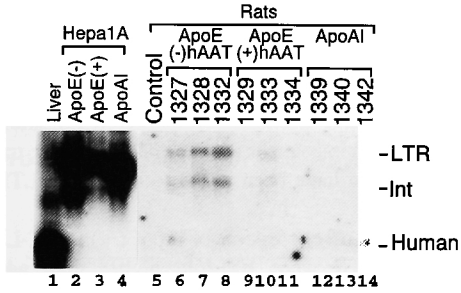

FIG. 3. Northern blot analysis of RNA from cells transduced with the retroviral vectors *in vitro* and *in vivo*. RNA was isolated from normal human liver, from Hepa1A cells transduced with retroviral vectors *in vitro*, or from rat livers 2 months after transduction with retroviral vectors *in vivo*. Northern blot analysis was performed using 10 μg of total cellular RNA and a radiolabeled hAAT cDNA probe, and a 6-day autoradiogram was obtained. Lane 1 shows RNA obtained from a normal human liver. The position of the 1.3-kb hAAT mRNA is marked on the right by the label Human. Lanes 2, 3, and 4 show pools of RNA from Hepa1A cells transduced with 1 copy per cell of ApoE(−)hAAT-LTR, ApoE(+)hAAT-LTR, and ApoAI-LTR, respectively. The position of the 4.9- to 5.6-kb LTR-initiated transcripts (LTR) and the 3.5-kb internal promoter-initiated transcripts (Int) are shown on the right. Lane 5 (control) shows RNA obtained from a nontransduced rat liver. Lanes 6–8, 9–11, and 12–14 show RNA obtained from rats transduced with ApoE(−)hAAT-LTR, ApoE(+)hAAT-LTR, and ApoAI-LTR, respectively. The animal number is listed above each lane. Ethidium bromide staining demonstated that the samples in lane 9 and 11 were underloaded.

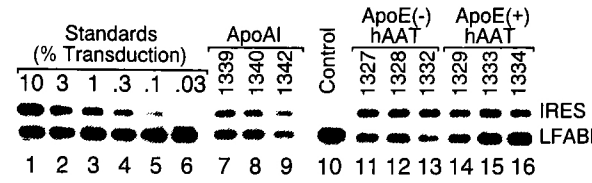

FIG. 4. PCR quantitation of the *in vivo* retroviral transduction efficiency in livers of transduced rats. Liver DNA was isolated 2 months after transduction and multiplex PCR was performed. Amplified sequences were electrophoresed, transferred to a nylon membrane, and hybridized with the IRES probe for retroviral sequences (18-hr autoradiogram), stripped, and reprobed with the LFABP probe for rat genomic sequences (1-hr autoradiogram). Standards (lanes 1–6) contain DNA from singly transduced NIH-3T3 cells diluted with DNA from a nontransduced rat liver. The number above each lane represents the percentage of the DNA which was derived from singly transduced NIH-3T3 cells. Lanes 7–9 represent DNA from rats transduced with ApoAI-LTR. The animal number is listed above each lane. Lane 10 shows DNA from a nontransduced rat that was isolated simultaneously with the transduced rat samples. Lanes 11–13 and 14–16 show DNA derived from rats transduced with ApoE(−)hAAT-LTR and ApoE(+)hAAT-LTR, respectively.

PCR standards were created by diluting DNA isolated from NIH-3T3 cells that contained a single copy of retroviral DNA with varying amounts of nontransduced rat liver DNA, as described previously (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994). A standard curve was constructed by correcting the IRES signal for the LFABP signal. The proviral integration frequencies of DNA from transduced rats were determined by comparing the ratio of the IRES/LFABP signal to those of the standard curve. The ApoE(−)hAAT-transduced rats had a transduction efficiency of 6%, the ApoE(+)hAAT transduced rats had a transduction efficiency of 3%, and the ApoAI-LTR transduced rats had a transduction efficiency of 3.7%. This transduction efficiency is similar to that determined in previous studies after delivery of a similar number of retroviral particles (Hafenrichter *et al.*, 1994; Rettinger *et al.*, 1994). In addition, DNA obtained from rats 13 and 16, which were transduced previously with the Pol-II-LTR retroviral vector (Rettinger *et al.*, 1994) were included in these assays (data not shown). The calculated transduction efficiency on these assays was similar to that obtained on previous PCR-based assays.

Comparison of expression with previously studied retroviral vectors

The work presented here is a continuation of our efforts to identify a retroviral vector that results in high-level expression *in vivo*. Because we have previously analyzed several retroviral vectors with an identical hAAT cDNA-IRES-*DHFR casette, it is germane to compare expression levels obtained here with those observed in previous studies. Figure 5A demonstrates that the absolute level of expression (ng of hAAT per ml of serum) observed for ApoE(−)hAAT-LTR and ApoE(+)hAAT-LTR is greater than 15-fold greater than what we observed with all previously analyzed retroviral vectors.

The overall level of expression of hAAT in serum will de-

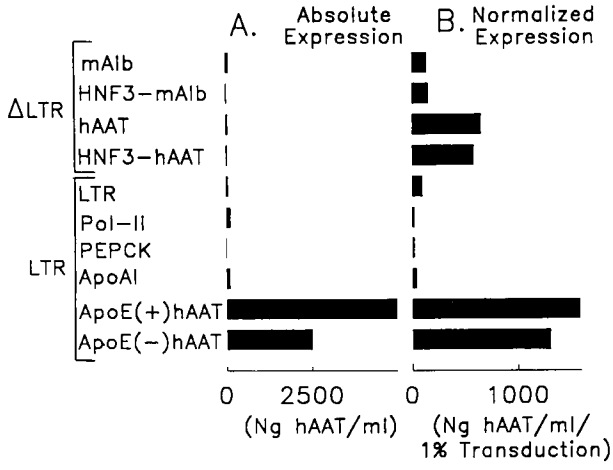

FIG. 5. Comparison of absolute and relative levels of expression from different retroviral vectors. A. Absolute expression. The serum hAAT level in ng/ml for several different retroviral vectors that have been analyzed by this lab is shown. Constructs designated ΔLTR have a 178-bp deletion in the enhancer region of the 3′ LTR, which is transferred to both the 5′ and 3′ LTRs after retroviral transduction. Constructs designated LTR have the complete hybrid Mo-MLV/Moloney murine sarcoma virus LTR from LNL-6. hAAT-ΔLTR contains the 402-bp hAAT promoter (Hafenrichter *et al.*, 1994), and mAlbΔLTR contains the 700-bp mouse albumin promoter (Hafenrichter *et al.*, 1994). HNF-3 indicates that three copies of the hepatocyte nuclear factor 3 binding site were placed upstream of the basal promoter. LTR-LTR contains the LTR without an internal promoter (Rettinger *et al.*, 1994), Pol-II-LTR contains the promoter for the large subunit of RNA polymerase II (Rettinger *et al.*, 1994), while PEPCK-LTR contains the 560-bp phosphoenolpyruvate carboxykinase promoter (Hafenrichter *et al.*, 1994). Except for the above-mentioned changes in the promoter and the LTR, all vectors were present in the same retroviral vector backbone with the hAAT cDNA-IRES-*DHFR casette. All constructs were analyzed using the same ELISA assay and standards as was used in this study. B. Normalized expression. To control for the fact the retroviral titers varied by as much as 100-fold, expression was normalized for the percentage of hepatocytes that were transduced. This was determined by using the same PCR assay and standards as was used in this study. The data are expressed in ng/ml per 1% transduction efficiency and were obtained by dividing the serum hAAT (Fig. 2 and Table 2 in this study) by the percentage of hepatocytes that were transduced (Fig. 4 and Table 2 in this study).

pend upon both the relative efficacy of the promoter chosen and the percentage of hepatocytes that are modified. To facilitate direct comparison of different retroviral vectors of varying titer, the expression was normalized to a 1% transduction efficiency for all constructs, as shown in Table 2. Both constructs containing the ApoE enhancer led to high levels of expression after normalization to the copy number. For ApoE(−)hAAT-LTR the average expression was 1588 ng/ml per 1% transduction efficiency and for ApoE(+)hAAT-LTR the average expression was 1309 ng/ml per 1% transduction efficiency. Average expression from ApoAI-LTR was considerably lower at 35.7 ng/ml per 1% transduction efficiency. Although the normalized expression of the ApoE enhancer-containing retroviral vectors is still higher than all other vectors that were studied previously, a significant part of the improvement in expression was due to the fact a complete LTR led to much higher titers than was observed for the retroviral vectors with a deletion in the 3′ LTR.

DISCUSSION

The liver is an excellent target organ for gene therapy for many genetic disorders. It can perform necessary post-translational modifications of coagulation proteins, and secreted products have ready access to the vasculature. Although we and others have demonstrated that *ex vivo* and *in vivo* hepatic gene therapy results in long-term expression of genes from retroviral vectors in animals for up to 2 years, a major limitation to the successful application of these techniques is the relatively low level of expression observed.

This study demonstrates that the ApoE(−)hAAT-LTR retroviral vector resulted in an average serum hAAT of 5 μg/ml (~0.5% of the level in normal human serum) when a transduction efficiency of ~6% of hepatocytes was achieved, suggesting that the expression per transduced cell was ~10% that of the endogenous hAAT gene. Expression was stable for 10 months in this study. Because previous studies have demonstrated stable expression from other promoters for up to 2 years (Rettinger *et al.*, 1994; Ponder, 1996), it is likely that expression will be maintained. This level of serum hAAT protein production was >15-fold higher than what was achieved in this and previous studies from retroviral vectors of similar titer with an identical backbone containing the LTR alone, or a retroviral vector with an internally placed Pol-II, PEPCK, or ApoAI promoter in addition to the LTR. Because there were no differences other than the promoter, these data suggest that the ApoE enhancer:hAAT promoter combination functions much better from a retroviral vector *in vivo* than the other transcriptional elements that have been tested to date in an LTR-intact vector.

Absolute levels of expression of ApoE(−)hAAT-LTR and ApoE(+)hAAT-LTR were 100-fold higher than what was achieved from all previously studied vectors with a deletion in the 3′ LTR, although the normalized level of expression was only ~three-fold higher than what was observed for hAAT-ΔLTR and HNF-3-hAAT-ΔLTR (Hafenrichter *et al.*, 1994). This illustrates the point that the higher titers achieved with a complete LTR are critical for achieving a high transduction efficiency in animals when using an *in vivo* delivery system, as was used in this study. Although the exact contribution of the ApoE enhancer cannot be determined because of the differences in the retroviral backbone, the data suggest that it improved expression ~three-fold, assuming that inclusion of an intact LTR had no effect upon expression.

The retroviral vectors used in this study contain a complete LTR and an internal promoter. Identification of which promoter(s) functions is necessary to understand why the ApoE(−)hAAT-LTR and ApoE(+)hAAT-LTR vectors result in higher expression *in vivo* than previously tested vectors. Internal promoter-initiated transcripts represented <5% of all retroviral mRNAs in Hepa1A cells *in vitro*. The low percentage of transcripts that initiate from the internal promoter for

ApoE(+)hAAT-LTR and ApoE(−)hAAT-LTR constructs in hepatoma cells is similar to what was observed for a retroviral vector which contains the hAAT promoter alone (Hafenrichter *et al.*, 1994). In contrast, internal promoter-initiated transcripts represented ~50% of all retroviral mRNAs for the ApoE-hAAT-containing retroviral vectors in hepatocytes *in vivo*. This shift in the promoter used is at least partially due to attenuation of the LTR. We previously demonstrated that the LTR was 1% as active in hepatocytes *in vivo* as in fibroblasts or hepatoma cells in culture (Rettinger *et al.*, 1994), while Fig. 3 demonstrates that LTR-initiated transcripts are less abundant in the liver than what was observed in singly transduced cultured cells, even after correction for the lower transduction efficiency. The shift in the ratio of LTR-initiated- to internal promoter-initiated-transcripts is probably also due to activation of the internal hAAT promoter *in vivo*. Activation of the internal promoter might reflect a loss of promoter interference, a phenomenon in which promoters which are immediately downstream of the LTR are inhibited (Emerman and Temin, 1986; Ghattas *et al.*, 1991). Alternatively, activation of the internal hAAT promoter might be due to the fact that hepatocytes in animals have higher levels of several transcription factors as compared with cultured hepatoma cells (Wu *et al.*, 1996).

Conflicting results have been obtained by other investigators regarding the ability to achieve long-term expression from a retroviral vector in animals. A muscle creatine kinase enhancer upstream of the CMV (Dai *et al.*, 1992) or the β-actin (Yao *et al.*, 1994) promoter was expressed well in myoblasts *in vivo*. Similarly, an internal thymidine kinase promoter was active in many organs including the liver after transduction of preimplantation embryos, while the LTR was inactive (Stewart *et al.*, 1987). In contrast, albumin (Richards and Huber, 1993) and β-globin (McCune and Townes, 1994) promoter/enhancers were inactive in several lines of transgenic mice when integrated with adjacent retroviral sequences, even if the enhancer region of the LTR was deleted (McCune and Townes, 1994). The reason why some internal promoters function well from a retroviral vector *in vivo* while others do not is unclear. Retroviral sequences from the upstream conserved region (Flanagan *et al.*, 1992), the enhancer (Tsukiyama *et al.*, 1989; Akgun *et al.*, 1991), or the tRNA primer binding site (Akgun *et al.*, 1991; Yamauchi *et al.*, 1995) can inhibit expression of the LTR in embryonic carcinoma cells. Some of these sequences bind to inhibitory proteins, and may be responsible for the poor expression observed from the LTR *in vivo* (Kaleko *et al.*, 1991; Scharfmann *et al.*, 1991; Rettinger *et al.*, 1994), and may inhibit an adjacent promoter. Promoters that are not inhibited by adjacent retroviral sequences might bind transcription factors that can negate an inhibitory factor, or might somehow be insulated from these negative effects. We previously demonstrated that the hAAT promoter was not influenced by retroviral sequences *in vitro* (Wu *et al.*, 1996). In contrast, the albumin and the Pol-II promoters were influenced by retroviral sequences *in vitro*, and were considerably less active *in vivo*. This led us to hypothesize that the hAAT promoter is insulated from the inhibitory effects of a retroviral vector, although further experiments will need to be performed to confirm this hypothesis.

Implications for gene therapy

There are two major limiting factors to the use of retroviral vectors for hepatic gene therapy for treating human genetic deficiencies. The first relates to the method of delivering the retrovirus to hepatocytes of animals. The *ex vivo* approach requires a partial hepatectomy to obtain hepatocytes, extensive *in vitro* culture, and injection of a large number of cells into the portal vein. The *in vivo* approach eliminates the need for culturing cells, although it still requires a major surgical procedure to induce liver cell replication, a necessary prerequisite for retroviral transduction. Although the mortality of a 70% hepatectomy is relatively low and could be justified for life-threatening genetic deficiencies, simplification of the delivery system will be

TABLE 2. SUMMARY OF RESULTS FOR INDIVIDUAL RATS ANALYZED IN THIS STUDY

Construct	*Rat number*	*Average serum hAAT [ng/ml ± SD]*	*Percent of liver cells transduced (% ± SD)*	*Normalized expression (ng/ml per 1% transduction efficiency ± SD)*
ApoE(−)hAAT-LTR	1,327	6,587 ± 2743	2.7 ± 1	2,439 ± 1381
	1,328	10,875 ± 3722	7.3 ± 2.1	1,489 ± 662
	1,332	5,412 ± 808	8.0 ± 2.7	677 ± 249
ApoE(+)hAAT-LTR	1,329	1,631 ± 436	5.3 ± 3.3	308 ± 208
	1,333	3,137 ± 945	2.5 ± 1.7	1,255 ± 933
	1,334	2,531 ± 711	1.2 ± 0.2	2,109 ± 686
ApoAI-LTR	1,339	177 ± 52	5.3 ± 3.2	33 ± 22
	1,340	125 ± 20	3.8 ± 2.2	33 ± 19
	1,342	90 ± 29	2.2 ± 0.2	41 ± 13

Rats were transduced *in vivo* with the retroviral vector. Serum was obtained at various times after transduction, and the average level of hAAT determined. The percentage of hepatocytes that were transduced with the retroviral vector was determined for the three rats of each group shown in Fig. 2 with the highest level of hAAT. This was determined by performing three separate PCR reactions (each in duplicate) on DNA from a rat liver biopsy obtained at 2 months after transduction (Fig. 4) and calculating the average transduction efficiency. The normalized expression was determined by dividing the serum hAAT level by the transduction efficiency.

necessary before these techniques can be routinely applied in humans. A second technical obstacle has been the relatively low level of expression achieved from retroviral vectors in hepatocytes *in vivo*. This study demonstrates that placing the ApoE enhancer-hAAT promoter combination into a retroviral vector with a complete LTR results in expression that is >15-fold higher than other retroviral vectors that were previously studied. Although 5 μg/ml of hAAT in serum would not be sufficient to prevent the clinical manifestations of α_1-antitrypsin deficiency, a 10-fold increase in the overall expression by either delivering more retroviral particles to the liver or by further improvements in the level of expression from the retroviral vector would be. Furthermore, this promoter-enhancer might allow therapeutic levels of expression of a coagulation protein to be achieved. A previous study that utilized the LTR promoter to express a canine FIX gene from a retroviral vector in FIX-deficient dogs achieved 0.1% of normal FIX levels in serum (Kay *et al.*, 1993). If indeed the ApoE-hAAT promoter works as well upstream of other cDNAs as it does upstream of the hAAT cDNA, the 15-fold increase in expression over that observed from the LTR promoter would be predicted to have a significant therapeutic effect upon bleeding in these hemophiliac dogs.

ACKNOWLEDGMENTS

We thank Jonathan Smith of the Rockefeller University for plasmid constructs, Sanae Okuyama for assistance in DNA sequencing, and Mark Sands for helpful comments on the manuscript.

This work was supported by the National Institutes of Health (R29-DK44593) and the March of Dimes awarded to K.P.P.

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Received for publication August 18, 1995; accepted after revision December 5, 1995.

This article has been cited by:

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