Quantitative Evaluation of Liver-Specific Promoters From Retroviral Vectors After In Vivo Transduction of Hepatocytes

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Hepatic gene therapy could be used to treat a number of inherited blood diseases such as hemophilia or thrombophilia. Although liver-directed retroviral transduction can result in long-term gene expression in vivo, the low level of protein production has limited its clinical application. We reasoned that the insertion of liver-specific promoters into retroviral vectors would increase gene expression in vivo. The 347-bp human α_1-antitrypsin (hAAT), the 810-bp murine albumin (mAlb), the 490-bp rat phosphoenolpyruvate carboxykinase (rPEPCK), and the 596-bp rat liver fatty acid binding protein promoters were inserted into a Moloney murine leukemia retroviral backbone containing the hAAT reporter gene. Vectors that produced appropriately sized RNA and hAAT protein in vitro were tested in vivo by transducing regenerating rat livers. Long-term serum expression of the hAAT reporter gene was normalized to retroviral transduction efficiency as determined by using a polymerase chain reaction-based assay of genomic DNA from transduced rat livers. The hAAT, mAlb, and rPEPCK promoters were, respectively, 35-, 8-, and 0.02-fold as strong as the previously studied constitutive Pol-II promoter. We conclude that the hAAT promoter resulted in the highest expression from a retroviral vector and may result in therapeutically significant expression of other clinically significant blood proteins.

HEPATIC GENE THERAPY could dramatically alter the treatment of many inherited hematologic diseases. The liver synthesizes a myriad of proteins that play pivotal roles in hemostasis, metabolism, and protection against infection. Although some genetic diseases such as hemophilia can be treated by repeated infusion of the deficient protein, this is expensive, carries a risk of viral infection, and only temporarily ameliorates the disease manifestations. Liver transplantation can correct serum protein deficiencies,[1] but is limited by donor organ availability and the need for immunosuppression. The transfer of a functional gene into the cells of a genetically deficient individual should correct the clinical manifestations by providing long-term production of the gene product.

The liver is an attractive organ for gene therapy of serum protein deficiencies because it performs the proper posttranslational modifications necessary for full activity for many serum proteins, such as the γ-carboxylation of coagulation factors, and the rich blood supply of the liver bathing the transduced hepatocytes facilitates the secretion of protein products into the bloodstream. Both ex vivo and in vivo methods have been used to transfer genes into mammalian hepatocytes. The ex vivo approach[2-5] requires hepatocyte harvest, in vitro transduction with retrovirus, and reintroduction of the transduced hepatocytes into the portal circulation. The in vivo delivery of foreign genes to hepatocytes in situ has been accomplished by injecting DNA,[6-9] adenoviral vectors,[10-14] or retroviral vectors[15-24] into the liver parenchyma, peripheral veins, or the portal vein. Of these, only retroviral vectors result in long-term expression because of their ability to integrate into chromosomal DNA.

A major limitation to achieving successful gene therapy with retroviral vectors in the liver has been the low level of expression observed. For example, hepatic gene therapy experiments using retroviral vectors containing the human factor IX[20] or human α_1-antitrypsin[18,22,24] (hAAT) genes have resulted in expression of the therapeutic gene at only 0.1% of the normal level. One explanation for the low level of expression observed is that transcription from a retroviral vector is inadequate. To identify promoters that may function well in the liver in vivo, plasmids containing a variety of promoters have been introduced into primary hepatocytes by using optimized lipofection.[25] These experiments showed that the human cytomegalovirus (CMV) immediate early promoter was much stronger than other viral, cellular, or liver-specific promoters tested. These results led Armentano et al[26] to place the CMV promoter into an LNL-6–based retroviral vector, which indeed led to high-level expression of a human factor IX gene in cultured primary hepatocytes. Similarly, Wilson et al[27] determined that the viral Moloney murine leukemia virus (Mo-MLV) long terminal repeat (LTR) promoter was better expressed from a retroviral vector than three other promoters, including the chicken β-actin promoter, in rabbit hepatocytes.

Although it was logical to assume that promoter function in primary hepatocytes in vitro would correlate well with promoter function in liver cells in vivo, this, unfortunately, is not the case. Although the CMV promoter was expressed well from either a plasmid or a retroviral vector in vitro,[25,26] it was shut off rapidly in vivo using either an ex vivo or an in vivo approach to transfer the vector into the hepatocytes of dogs or mice.[4,18] Similarly, Wilson et al[2] found that the retrovirus in which the LTR promoter drives the expression of the low-density lipoprotein receptor (LDL-R) gene led to only short-term improvement in the hypercholesterolemia of Watanabe rabbits, although rejection of the allogeneic

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Submitted April 13, 1994; accepted July 22, 1994.

Supported by grants from the National Institutes of Health (R29-DK44593), the American Lung Association, the Markey Foundation, and the American Liver Foundation awarded to K.P.P. and the Surgical Infection Society awarded to D.G.H.

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0006-4971/94/8410-0027$3.00/0

hepatocytes was cited as the most likely reason for the loss of expression. Similarly, we recently quantitated expression from the Mo-MLV LTR promoter and determined that it was only 1% as active in hepatocytes in vivo as in cultured hepatoma cells in vitro.[24] In contrast, although the chicken β-actin gene was poorly expressed in primary hepatocytes,[27] this promoter has led to long-term expression of the LDL-R gene in Watanabe rabbits[3] and is currently being used in human gene therapy trials.[5] Thus, we conclude that expression of a promoter in cultured hepatocytes correlates poorly with expression in hepatocytes in vivo. This result is not totally surprising, because the constellation of transcription factors present in replicating hepatocytes in vitro is almost certainly quite different from those present in quiescent hepatocytes in vivo. Similar results have been observed in primary fibroblasts by Palmer et al[28] and Scharfman et al,[29] who determined that the CMV, SV40, and Mo-MLV LTR promoters are expressed well in vitro, but are shut off upon returning the cells to animals.

Although it is clear that the relative strengths of promoters need to be compared in vivo, few studies have undertaken quantitative comparisons of promoter strength. Unfortunately, studies which use a single promoter with a particular reporter gene cannot be easily compared with those that study another reporter gene, because of potential differences in protein half-life, RNA stability, and vector construction. We have recently developed quantitative assays for normalizing gene expression to in vivo transduction efficiency and have used these techniques to compare the relative in vivo strengths of the Mo-MLV LTR promoter with the promoter for the large subunit for murine RNA polymerase II (Pol-II).[24] In this study, we use our quantitative techniques to directly compare liver-specific promoters for their ability to direct transcription from a retroviral vector in vivo. We determine that the hAAT promoter is stronger than the Pol-II, murine albumin (mAlb), or phosphoenolpyruvate carboxykinase (PEPCK) promoters in vivo.

MATERIALS AND METHODS

Construction of Retroviral Vectors

LTR-hAAT. LTR-hAAT is a retroviral vector constructed from the Mo-MLV derivative LNL-6.[30] It contains a 1.3-kb hAAT cDNA, the encephalomyocarditis virus internal ribosome entry site (IRES), and a mutant, methotrexate resistant (Mtx^R) dihydrofolate reductase (*DHFR) gene.[24] The LTR-ΔLTR retroviral vector contains a 178-bp deletion in the 3′ LTR enhancer region from the *Pvu* II site at position 3312 to the *Xba* I site at position 3488 (numbers refer to the locations on LNL-6).

rPEPCK-LTR. The rat cytosolic PEPCK promoter[31] was cut at the *Xba* I site at −490 relative to the transcription start site, blunt-ended with T4 DNA polymerase, and ligated with *Bam*HI linkers. After *Bam*HI and *Bgl* II digestion (cuts at +73), the 560-bp DNA fragment was cloned into the *Bgl* II site of LTR-hAAT to create rPEPCK-LTR.

rLFABP-LTR. The rat LFABP promoter[32] was cut at −596 with *Sal* I, blunt-ended, and ligated with *Bgl* II linkers. After *Bam*HI (cuts at +21) and *Bgl* II digestion, the 620-bp fragment was cloned into the *Bgl* II site of LTR-hAAT to create rLFABP-LTR.

mAlb-ΔLTR. To create the cloning intermediate, mAlb-SP72, the mouse albumin promoter present in pAT2[33] was digested with *Hpa* II (which cuts 8 nt downstream from the transcription start site), blunt-ended, and ligated with *Bam*HI linkers. After digestion with *Bam*HI and *Eco*RI (cuts at −810), the 820-bp fragment was ligated into the *Eco*RI/*Bam*HI site of pSP72 (Promega, Madison, WI) to create mAlb-SP72. mAlb-SP72 was cut with *Bgl* II and *Bam*HI and the 840-bp fragment was ligated into the unique *Bgl* II site of LTR-ΔLTR to create mALB-ΔLTR.

hAAT-ΔLTR. The DNA fragment containing the hAAT promoter[34] was digested with *Sma* I, which cuts at +56, and ligated with *Bcl* I linkers. After *Bcl* I and *Bgl* II (cuts at −347) digestion, the 403-bp fragment was cloned into the *Bgl* II site of LTR-ΔLTR to create hAAT-ΔLTR.

HNF-3-mAlb-ΔLTR and HNF-3-hAAT-ΔLTR. pAT-2eG3x contains 3.5 copies of the DNA sequence that binds to the HNF-3 transcription factor (5′-GCTCCAGGGAATGTTTGTTCTTAAATA CCATGCT-3′) upstream of the 810-bp mAlb promoter in pUC18.[33] The polymerase chain reaction (PCR) was used to amplify a 198-bp DNA containing the HNF-3 binding sites as follows: an oligonucleotide containing an *Eco*RI site and identity with the top strand of the pUC18 sequence 5′ to the HNF-3 sites (5′-GTCGAATTCGCA-TGCGTCGACGGAAACAGCTATGACCATGA-3′) and an oligonucleotide with an *Eco*RI site and identity with the bottom strand at the 5′ end of the mAlb promoter (5′-CAGGAATTCAGTCAA-GACTACATCCAAAT-3′) were synthesized. The oligonucleotides were used in a 25-cycle PCR reaction with pAT-2eG3x, as described previously,[24] with denaturation at 94°C for 2 minutes, annealing at 45°C for 2 minutes, and extension at 72°C for 2 minutes. The PCR products were digested with *Eco*RI and cloned into the *Eco*RI site of pSP72 to create $(HNF\text{-}3)_3$pSP72. A 237-bp $(HNF\text{-}3)_3$ fragment was removed from $(HNF\text{-}3)_3$pSP72 by *Bgl* II and *Bam*HI digestion and was cloned into the unique 5′ *Bgl* II sites of mAlb-ΔLTR and hAAT-ΔLTR to create HNF-3-mAlb-ΔLTR and HNF-3-hAAT-ΔLTR, respectively.

Creation of Retroviral Producer Cell Lines

The amphotropic GP+*env*AM12[35] and ecotropic GP+E-86[36] murine fibroblast lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated calf serum (Hyclone Laboratories, Logan, UT), penicillin (10^5 U/L), and streptomycin (10^5 μg/L). After calcium phosphate transfection with retroviral vector DNAs,[30] cells were cultured for 14 days in 250 nmol/L methotrexate- (Sigma Chemical, St Louis, MO) supplemented DMEM with dialyzed calf serum. Supernatant from 48 Mtx^R clones were screened for the production of retrovirus by infecting NIH 3T3 cells and measuring hAAT production 72 hours later. High-titer clones were proven to be free of replication competent helper virus by amplification and marker rescue assay.[30] For the rPEPCK-LTR retroviral vector, both GP+E-86 and GP+AM-12 packaging cell lines were transfected and the best clones were subjected to "ping-pong" cocultivation[37] for 4 weeks. Amphotropic cells were then selected by treating the cocultures with 200 μg/mL hygromycin-B (Sigma Chemical), which eradicated the ecotropic cells.

In Vitro Transduction of NIH 3T3 and Hepa A1 Cells With Retroviral Vectors Containing the Liver-Specific Promoters

Conditioned medium from confluent retroviral packaging cells was filtered (0.45 μm), spiked with polybrene to a final concentration of 8 μg/mL, and added at various dilutions to 50% confluent murine NIH 3T3 fibroblasts[38] or murine Hepa A1 hepatoma cells.[39] Forty-eight hours later, the transduced cells were trypsinized, diluted 1:20, and administered fresh DMEM supplemented with 250 nmol/L Mtx. After 14 days of selection, Mtx^R colonies were counted to determine the Mtx^R retroviral titer,[30] pooled together, and cultured to confluence

in Mtx-supplemented DMEM. The transduced cells were analyzed for hAAT production by culturing the cells for 6 hours in hormonally defined medium[25] (HDM). Supernatants were assayed for hAAT activity by enzyme-linked immunosorbent assay (ELISA).[24] After the collection of the supernatants, the transduced cell monolayers were trypsinized, counted on a hemocytometer, and used to obtain either RNA or DNA for further analysis, as described previously.[24] Where indicated, cells transduced with rPEPCK-LTR were stimulated for 6 hours with 0.5 mmol/L dibutyryladenosine 3′:5′-cyclic monophosphate (Bt_2cAMP), 1 mmol/L theophylline, and 1 μmol/L dexamethasone[40] in 75% tyrosine-free minimum essential medium and 25% tyrosine-free Waymouth's medium, and nonstimulated cells were incubated in the basal medium without any hormones. Southern blot was performed on DNA from cells transduced with each of the vectors by using restriction enzymes that cut within the LTRs; all vectors resulted in DNA of the expected size.

In Vivo Hepatocyte Transduction Protocol

Adult male Sprague-Dawley rats (SASCO, Omaha, NE), weighing 200 to 275 g, received standard National Institutes of Health-approved institutional care with rodent chow and tap water ad libitum. Rats received a 70% partial hepatectomy, as described.[24] Twenty-four hours later, packaging cell conditioned medium was concentrated by ultracentrifugation and spiked to a final concentration of 8 μg/mL polybrene, and in vivo hepatocyte transduction was accomplished by portal vein injection of retrovirus during hepatic-inflow occlusion.[24]

Northern Blotting of Cellular RNA

Ten micrograms of total cellular RNA was electrophoresed on a 1% formaldehyde denaturing agarose gel[41] and transferred to a nitrocellulose membrane (BA-S-NC; Schleicher and Schuell, Keene, NH). Ethidium bromide staining and UV visualization showed that the RNA isolated was of good quality and loaded in equal amounts. The 592-bp *Xho* I/*Eco*RI fragment of the IRES DNA[42] was labeled with α-^{32}P-dCTP by random primer extension[41] and hybridized with the nitrocellulose membrane at 42°C with 50% formamide and washed according to the manufacturer's instructions.

PCR Amplification of Proviral DNA Sequences

A 30-cycle multiplex PCR technique was employed using primer sets that amplified both proviral (IRES) and rat genomic (liver fatty acid binding protein [LFABP]) DNA sequences, as described previously.[24] Amplified DNA sequences were electrophoresed through a 2% agarose/1× Tris-ammonium acetate gel,[41] transferred to BA-S-NC nitrocellulose membrane, and hybridized with a 217-bp *Hin*dIII/*Kpn* I fragment of the IRES labeled to a specific activity of 3×10^9 cpm/μg DNA 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 Packaging Cell Lines With Retroviral Vectors Containing Liver-Specific Promoters

To date, expression from retroviral vectors has been disappointingly low in hepatocytes in vivo. We reasoned that the insertion of strong, liver-specific promoters might increase expression and selected four promoters for evaluation based on their ability to direct expression in the livers of transgenic mice or in hepatoma cells. We also evaluated the effect of inserting three hepatocyte nuclear factor-3 (HNF-3) binding sites upstream of the mAlb and hAAT promoters, because this increased expression from the mAlb promoter in hepatoma cells.[33] We decided to clone the promoters into a retroviral vector with a deletion in the enhancer region of the 3′ LTR. Such a deletion is transferred to the 5′ LTR after one round of retroviral infection, resulting in a ~90% decrease in LTR-initiated transcription.[43,44] This facilitates the in vitro analysis of internal promoter function, because high levels of transcription from the LTR can interfere with the expression from the internal promoter.

Retroviral vectors (Fig 1) were transfected into GP+*env*AM12 amphotropic retroviral packaging cells. After Mtx selection, 48 clones for each vector were screened for their ability to program NIH 3T3 cells to express hAAT. Although adequate expression was achieved with the vectors containing the hAAT and mAlb promoters in transduced NIH 3T3 cells (Table 1), both the rLFABP-ΔLTR and rPEPCK-ΔLTR vectors resulted in negligible hAAT protein production (data not shown). We therefore placed the rLFABP and rPEPCK promoters in a retroviral vector with an intact 3′ LTR to facilitate isolation of high-titer clones. The rLFABP-LTR and rPEPCK-LTR vectors were transfected into GP+*env*AM12 cells and the best clones selected as described above. In addition, the rPEPCK-LTR vector was transfected into the ecotropic GP+E-86 packaging cells and the technique of ecotropic/amphotropic "ping-pong" cocultivation[37] was performed to generate high-titer packaging cell lines. The GP+*env*AM12 clones remaining after hygromycin B and Mtx selection were screened for hAAT protein production after infection of NIH 3T3 cells.

Mtx^R Retroviral Titers and Protein Expression From Liver-Specific Retroviral Vectors in Singly Transduced Tissue Culture Cells

The best amphotropic packaging cell line for each of the retroviral vectors was used to infect NIH 3T3 fibroblasts and Hepa A1 hepatoma cells at various dilutions. After selection with Mtx, colonies were counted to determine the Mtx^R retroviral titers, as shown in Table 1. The rLFABP-LTR packaging cell lines produced insignificant numbers of Mtx^R colonies. The rPEPCK-LTR construct had a high titer, as expected for a vector that underwent "ping-pong" amplification, whereas the hAAT and mAlb promoter-containing constructs had lower titers, as is generally observed after direct transfection into amphotropic packaging cell lines without "ping-pong" amplification.

Resistant cells should each contain a single copy of integrated retrovirus when they are infected at a low multiplicity of infection. Such singly transduced cells were used to quantitate hAAT protein production by performing a timed supernatant collection and normalizing to the number of cells on the plate. It should be noted that both LTR- and internal promoter-initiated transcripts can result in hAAT protein production. hAAT-ΔLTR produced a similar amount of hAAT protein in transduced NIH 3T3 and Hepa A1 cells, whereas HNF-3-hAAT-ΔLTR resulted in a threefold and fourfold increase in hAAT protein production in the NIH 3T3 and Hepa A1 cells, respectively. Although mAlb-ΔLTR resulted in high levels of hAAT protein production in NIH

3T3 cells, much lower expression was observed in the Hepa A1 cells. The addition of HNF-3 binding sites to the mAlb promoter had little effect on expression in NIH 3T3 cells, but increased hAAT protein expression by 35-fold in the hepatoma cells. The rLFABP-LTR vector had no detectable

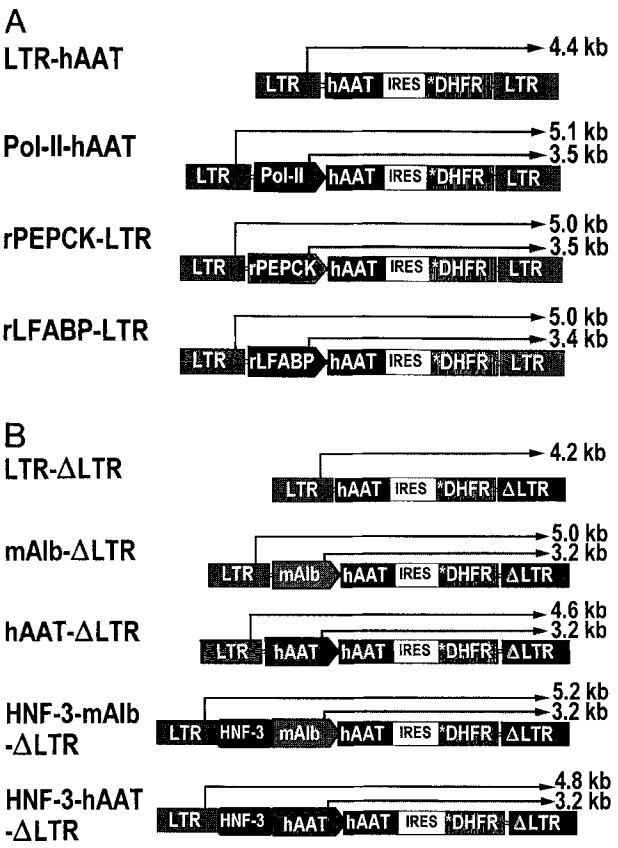

Fig 1. Retroviral vectors containing liver-specific promoters. (A) Retroviral vectors containing an intact 3′ LTR. LTR-hAAT contains the 1.3-kb hAAT cDNA and the encephalomyocarditis virus IRES sequence upstream of the mutant, Mtx^R *DHFR.[24] Transcription initiating from the retroviral LTR promoter results in a 4.4-kb transcript, as indicated by the arrow. The 700-bp murine Pol-II promoter,[24] the 490-bp rat PEPCK promoter,[31] and the 596-bp rat LFABP promoter[32] were placed immediately upstream from the hAAT cDNA in LTR-hAAT to create Pol-II-hAAT (as described previously[24]), rPEPCK-LTR, and rLFABP-LTR, respectively; transcription can initiate from either the LTR promoter (resulting in transcripts of 5.1, 5.0, and 5.0 kb, respectively) or from the internal promoter (resulting in transcripts of 3.5, 3.5, and 3.4 kb, respectively), as depicted by the arrows. (B) Retroviral vectors containing a deletion in the 3′ LTR (ΔLTR). The LTR-ΔLTR retroviral vector contains a 178-bp deletion in the enhancer of the 3′ LTR. Transcription initiating at the LTR promoter results in a 4.2-kb RNA (arrow). The 810-bp mAlb promoter[33] and the 347-bp hAAT promoter[34] were each inserted upstream from the hAAT cDNA in LTR-ΔLTR to create mAlb-ΔLTR and hAAT-ΔLTR, respectively; transcription initiating from the LTR results in 5.0- and 4.6-kb RNAs, respectively, whereas transcription initiating from the internal promoters results in a 3.2-kb RNA (arrows). The HNF-3-mAlb-ΔLTR and HNF-3-hAAT-ΔLTR vectors were created by inserting three HNF-3 binding site sequences[33] upstream of the internal promoter of mAlb-ΔLTR and hAAT-ΔLTR, respectively; transcription initiating from the retroviral LTR promoter results in 5.2- and 4.8-kb RNAs, respectively, whereas transcription initiating from the mAlb or hAAT internal promoters results in a 3.2-kb RNA.

Table 1. The Production of hAAT Protein by the Liver-Specific Retroviral Vectors

Promoter	Mtx^R Titer on NIH 3T3 Fibroblasts (cfu/mL)	hAAT Protein Production in Transduced NIH 3T3 Cells* (ng hAAT/10^6 cells/24 h)	hAAT Protein Production in Transduced Hepa A1 Cells* (ng hAAT/10^6 cells/24 h)
mAlb	60,000	351.6 ± 55.2	7.1 ± 0.47
HNF-3-mAlb	10,000	356.0 ± 159	247.5 ± 37.0
hAAT	10,000	63.4 ± 11.7	50.3 ± 10.0
HNF-3-hAAT	10,000	192.0 ± 41	221.0 ± 52.0
rPEPCK	2,000,000	4.6 ± 2†	108.6 ± 6.0†
		6.3 ± 1‡	137.0 ± 19.8‡

* hAAT protein production by the retroviral transduced NIH 3T3 or Hepa A1 cells was measured in a 6-hour collection from confluent cell cultures in HDM and was normalized to the number of cells present.

† hAAT protein production by the transduced NIH 3T3 and Hepa A1 cells was measured in a 6-hour collection from confluent cell cultures in basal medium, without any additives, and was normalized to the number of cells present.

‡ hAAT protein production by the retroviral transduced NIH 3T3 and Hepa A1 cells was measured in a 6-hour collection from confluent cell cultures in basal medium supplemented with 0.5 mmol/L Bt_2-cAMP, 1 μmol/L dexamethasone, and 1 mmol/L theophylline and was normalized to the number of cells present.

activity in either cell type (data not shown). Although the fibroblasts transduced with rPEPCK-LTR produced much less hAAT than the other promoters in NIH 3T3 cells, expression was similar to the other promoters in the hepatoma cells. Because the rPEPCK promoter is induced by the addition of the Bt_2-cAMP and theophylline,[31,40,45] transduced fibroblasts and hepatoma cells were tested for their hormonal responsiveness. As shown in Table 1, stimulated fibroblasts and hepatoma cells increased hAAT protein production by 1.37- and 1.26-fold, respectively.

Analysis of RNA From Packaging and Transduced Cells

When constructing a retroviral vector with an internal promoter, it is important to document that full-length genomic transcripts are being produced and that no rearrangements have occurred during transduction and to assess the level of expression from the internal promoter. RNA from packaging cell lines for each liver-specific promoter was analyzed by Northern blot. LTR-initiated transcripts are predicted to range from 5.2 to 4.2 kb in size, whereas the internal promoter-initiated transcripts are predicted to range from 3.5 to 3.2 kb, depending on the particular vector, as shown in Fig 1. The major bands for the packaging cells containing hAAT-ΔLTR and HNF-3-hAAT-ΔLTR were derived from the LTR promoter, as expected in the fibroblast-derived packaging cells (Fig 2B, lanes 6 and 7, and Fig 2A, lane 11). The minor short band of 3.5 kb may be derived from a splice product that uses the 5′ retroviral splice site[30] and a putative 3′ splice site (UCUUCUUCUCCCCAG) at position +260 within the hAAT cDNA, which closely matches the consensus 3′ splice site [$(Py)_nNPyAG$].[46] The presence of such a spliced product is suspected because of the fact that an identical sized band is observed for all pro-

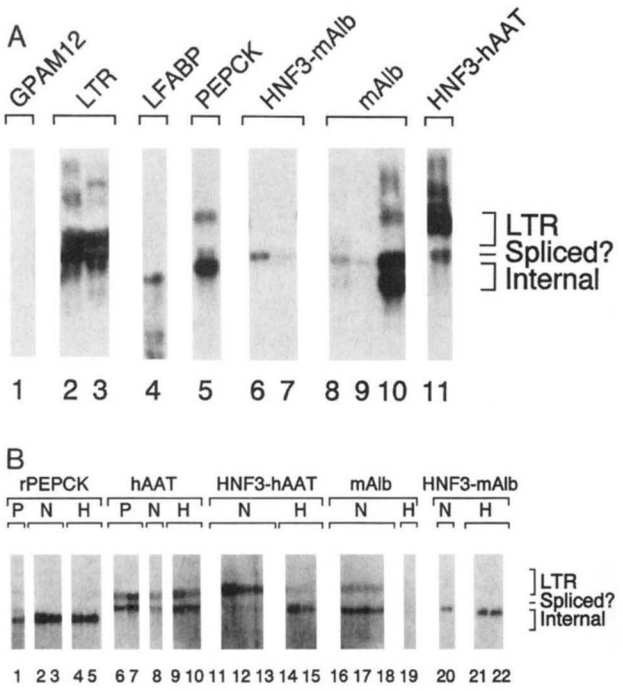

Fig 2. Northern blot analysis of RNA from packaging and transduced cells. (A) Northern blot analysis of RNA from packaging cell lines. RNA was isolated from the packaging cell lines and Northern blot analysis was performed using the IRES probe, which detects RNAs derived from either the retroviral LTR or the internal promoter. The diagram of the retroviral vectors and the predicted lengths of the LTR and internal promoter derived transcripts are shown in Fig 1A and B. Control (GP+AM12) packaging cells have no signal (lane 1). RNA from different retroviral packaging cell clones containing the LTR-hAAT (lanes 2 and 3), rLFABP-LTR (lane 4), rPEPCK-LTR (lane 5), HNF-3-mAlb-ΔLTR (lanes 6 and 7), mAlb-ΔLTR (lanes 8 through 10), and HNF-3-hAAT-ΔLTR (lane 11) retroviral vectors are shown. (B) Northern blot analysis of RNA from packaging cells and transduced cells. NIH 3T3 fibroblasts or Hepa A1 hepatoma cells were transduced at a low multiplicity of infection and singly infected pools were selected. Northern blot analysis of total RNA was performed by using the IRES probe, which detects RNAs initiated from either the LTR or the internal promoter. Packaging cell lines (P) are shown in lanes 1, 6, and 7; transduced NIH 3T3 cells (N) are shown in lanes 2, 3, 8, 11 through 13, 16 through 18, and 20; and transduced Hepa A1 cells (H) are shown in lanes 4, 5, 9, 10, 14, 15, 19, 21, and 22. RNAs from cells containing the rPEPCK-LTR (lanes 1 through 5), hAAT-ΔLTR (lanes 6 through 10), HNF-3-hAAT-ΔLTR (lanes 11 through 15), mAlb-ΔLTR (lanes 16 through 19), and HNF-3-mAlb-ΔLTR (lanes 20 through 22) retroviral vectors are shown.

moter constructs that produce full-length RNA, as well as for the LTR-hAAT construct, which lacks an internal promoter (Fig 2A, lanes 2 and 3). The mAlb-ΔLTR (Fig 2A, lanes 8 through 10) and HNF-3-mAlb-ΔLTR (Fig 2A, lanes 6 and 7) packaging cells produced only a small amount of full-length RNA and a large amount of a 3.2-kb RNA, which is consistent with initiation from the internal mAlb promoter. Similarly, the rPEPCK-LTR packaging cells produced moderate amounts of full-length RNA (Fig 2A, lane 5) and larger amounts of a 3.5-kb band, which is consistent with initiation of transcription from the internal rPEPCK promoter. The rLFABP-LTR packaging cells (Fig 2A, lane 4) produced no observable full-length RNA and a small amount of a 3.4-kb RNA, which is consistent with initiation from the internal promoter, and a large amount of RNA less than 1.5 kb in size, which is consistent with an RNA processing event.

Northern blot analysis was also performed on RNA from singly infected pools of murine fibroblast-derived NIH 3T3 and murine hepatoma-derived Hepa A1 cells. As shown in Fig 2B, hAAT-ΔLTR (lane 8) and HNF-3-hAAT-ΔLTR (lanes 11 through 13) transduced NIH 3T3 fibroblasts and hAAT-ΔLTR transduced hepatoma cells (lanes 9 and 10) had two major bands. The more abundant 4.6- and 4.8-kb bands are the full-length, LTR-derived RNA, whereas the shorter ~3.2-kb band could represent either the internally initiated RNA or the putative splice product discussed above. In contrast, the major band for HNF-3-hAAT-ΔLTR transduced Hepa A1 cells is ~3.2 kb, which suggests that the internal promoter is preferentially used. Indeed, an RNase protection assay confirmed that the internal hAAT promoter was not used from either construct in the fibroblast-derived cells. In contrast, Hepa A1 cells initiated 10% and 50% of transcripts from the internal promoter, respectively, after transduction with hAAT-ΔLTR and HNF-3-hAAT-ΔLTR (data not shown). Thus, retroviral vectors containing the hAAT promoter led to only LTR-initiated transcription in both packaging cells and transduced fibroblasts, although the internal promoter was appropriately expressed in hepatoma cells.

There is a paucity of all transcripts in the Hepa A1 cells transduced with mAlb-ΔLTR (Fig 2B, lane 19), which is consistent with the low hAAT protein production. We have no explanation for the low level of expression of the basal mAlb promoter in the hepatoma cells, because we expected the albumin promoter to be quite active in the hepatoma cells. The addition of the HNF-3 binding sites in HNF-3-mAlb-ΔLTR did lead to the expected increase in the amount of the shorter transcript in Hepa A1 cells (lanes 21 and 22), which we hypothesize is derived from the internal promoter. Because the mouse albumin promoter is not expressed in nonhepatoma cells,[47-51] we were surprised to observe that in both mAlb-ΔLTR (lanes 16 through 19) and HNF-3-mAlb-ΔLTR (lanes 20 through 22) transduced NIH 3T3 cells, the major band is 3.2 to 3.5 kb, which is consistent with initiation from the internal promoter or with a putative spliced product, whereas only a small amount of the 5.0- to 5.2-kb full-length LTR-initiated transcript is present. Because shorter transcripts were unexpected in the fibroblast cells, we considered that they might have resulted from an RNA processing event or vector rearrangement and performed further analyses. Both RNase protection and S1 nuclease assays confirmed that indeed the internal promoter was preferentially used in both fibroblast-derived and hepatoma cells (data not shown). Because previous studies have shown that the mAlb promoter can be dramatically enhanced in nonhepatoma cells by the SV40[33,47] or adenovirus[51] enhancers, we hypothesize that transcriptional elements that remain in the LTR after the deletion of the 75-bp repeats may act upon the mAlb promoter to increase transcription. It is unclear why the mAlb promoter should be preferentially expressed over the LTR.

For the rPEPCK-LTR transduced NIH 3T3 and Hepa A1 cells (Fig 2B, lanes 2 through 5), the major 3.2-kb band observed is consistent with initiation from the internal promoter and there is little difference in the levels of RNA in hormonally stimulated cells as compared with the nonstimulated cells. It is unlikely that the shorter transcripts in transduced cells are caused by a rearrangement that occurred during transduction, because DNA from cells transduced with all of the vectors was analyzed by Southern blot and shown to be full length (data not shown). This finding suggests that the shorter RNA species are indeed derived from the internal promoter of an intact provirus. This is similar to the results of Hatzoglou et al,[40,45] who placed the same rPEPCK promoter upstream of the neomycin phosphotransferase gene in an Mo-MLV retroviral vector and observed a marked predominance of rPEPCK-initiated transcripts as compared with LTR-initiated transcripts in G418 selected cells. Thus, the rPEPCK promoter is preferentially expressed over the LTR in the rPEPCK-LTR retroviral vector. The fact that the expression was quite low in the rPEPCK-ΔLTR retroviral vector leads us to hypothesize that the retroviral LTR may exert an enhancer effect on the internal rPEPCK promoter.

Long-Term Expression of Liver-Specific Promoters After In Vivo Transduction

One of the principal goals of gene therapy is stable expression of the transduced gene in vivo. We used conditioned medium from each of the retroviral vector packaging cell lines discussed above to transduce regenerating rat liver cells in vivo. The transduced rats were followed for serum hAAT protein expression by ELISA. hAAT was undetectable in all rats before transduction, demonstrating the specificity of the antibody for the human protein. The hAAT-ΔLTR construct (Fig 3A) resulted in an average expression of 43.0 ng/mL for three animals and expression was quite stable, as serum hAAT protein levels at 40 weeks posttransduction were 80% of their posttransduction week-1 levels. Rats transduced with the HNF-3-hAAT-ΔLTR construct (Fig 3B) had an average serum level of 23.2 ng/mL for two animals and maintained 88% of their posttransduction week-1 values at 40 weeks. Rats transduced with the mAlb-ΔLTR construct (Fig 3C) initially had mean hAAT levels of 148.5 ng/mL for two animals, but by 40 weeks posttransduction, expression had decreased to 48% of posttransduction week-1 values. Although the HNF-3-mAlb-ΔLTR construct (Fig 3D) resulted in lower early levels of hAAT protein in the serum (mean 16.7 ng/mL for 2 animals) as compared with mAlb-ΔLTR, expression similarly decreased to 46% at 40 weeks. Despite the high titer of the rPEPCK-LTR vector (Table 1), the mean serum hAAT protein level at posttransduction week 1 was 19.3 ng/mL for two animals and had decreased to 13% of this level by 20 weeks posttransduction (Fig 3E).

PCR Quantitation of Proviral DNA in the Livers of Transduced Rats

To compare in vivo promoter strengths, expression must be normalized to transduction efficiency. We isolated genomic DNA from rat liver biopsy specimens from at least two animals for each promoter and used a multiplex PCR technique to amplify proviral (IRES) and rat genomic (LFABP) sequences. We minimized the potential inaccuracies of the semiquantitative PCR technique for the estimation of transduction efficiency by (1) isolating nontransduced control rat DNA simultaneously with the transduced samples to control for contamination; (2) using primers that amplify a rat genomic sequence (LFABP) simultaneously with the retroviral (IRES) sequence to control for PCR efficiency and DNA loading; (3) analyzing the samples from all of the transduced rats, controls, and standard curve in the same PCR reaction and blots; and (4) performing the PCR reactions and hybridizations multiple times on the rat liver DNA samples and reporting the mean transduction efficiency of at least four independent analyses.

For semiquantitative assessment of retroviral gene frequency, PCR standards were created by diluting DNA isolated from a transduced NIH 3T3 cell clone that was shown to contain a single copy of retroviral DNA with varying amounts of nontransduced rat liver DNA (Fig 4A, lanes 1 through 6), as described previously.[24] The signal for each sample was quantitated by using a Betagen two-dimensional imager and a standard curve was constructed by correcting the IRES signal for the LFABP signal. Nontransduced control rat liver samples, isolated simultaneously with the transduced rat liver samples, showed a signal for LFABP but not for IRES, as expected (Fig 4B, lane 12).

The proviral integration frequencies of DNA from transduced rats were determined by comparing the ratios of the IRES/LFABP signals with those of the standard curve. Based on the mean of at least four independent PCR-based comparisons, the in vivo transduction efficiency of the mAlb-ΔLTR (lanes 13 and 14) vector ranged from 0.32% to 0.5% for individual rats, whereas the transduction efficiency for the HNF-3-mAlb-ΔLTR construct (lanes 15 and 16) was one-sixth as high at 0.055% to 0.082%. Transduction with the hAAT-ΔLTR construct (lanes 17 through 19) resulted in 0.034% to 0.065% of hepatocytes containing the provirus, and those transduced with the HNF-3-hAAT-ΔLTR construct (lanes 20 and 21) showed a 0.038% to 0.050% transduction frequency. The rPEPCK-LTR construct (lanes 10 and 11) resulted in transduction of 5.3% to 6.9% of hepatocytes. It is noteworthy that the observed variations in retroviral transduction efficiency (Table 2) are quite consistent with variations in the Mtx^R titer of these retroviral vectors (Table 1).

Comparison of the Relative Promoter Strengths of the Liver-Specific Retroviral Constructs

The strengths of the liver-specific internal promoters were compared by normalizing the serum hAAT protein levels measured at the time of liver biopsy for the proviral integration frequency (Table 2). As shown in Fig 5, the mean normalized hAAT protein expression for the mAlb-ΔLTR and HNF-3-mAlb-ΔLTR vectors was similar at 141.5 and 158.2 ng/mL hAAT/1% proviral integration, respectively. The hAAT-ΔLTR and HNF-3-hAAT-ΔLTR vectors resulted in similar levels of normalized expression at 657 and 590 ng/

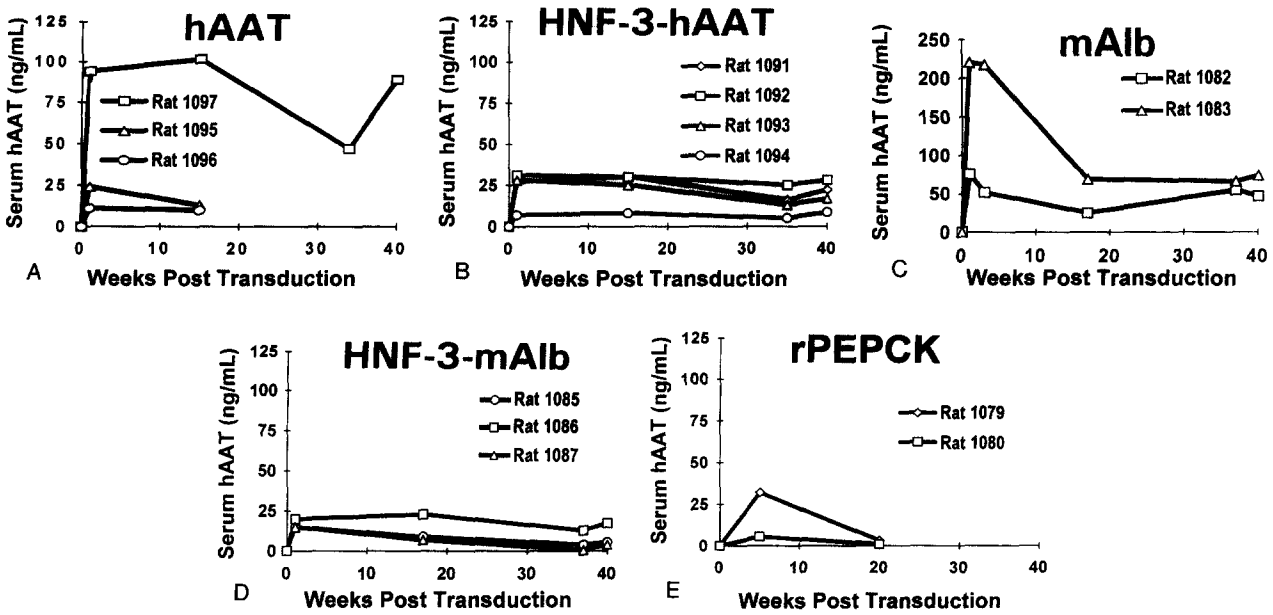

Fig 3. Long-term in vivo expression in rats transduced with retroviral vectors containing the liver-specific promoters. Rats were transduced with conditioned medium from each of the liver-specific retroviral vector packaging cell lines. Expression of the hAAT reporter gene was quantitated in rat sera by a human-specific ELISA and is depicted for individual rats during the course of the experiment. All rats had no hAAT expression before transduction. The number of retroviral particles delivered to the estimated 3 × 10^8 hepatocytes in the regenerating rat livers are based on the Mtx^R retroviral titers for the injectate on NIH 3T3 cells and are as follows: hAAT-ΔLTR (5 × 10^5 cfu), HNF-3-hAAT-ΔLTR (5 × 10^5 cfu), mAlb-ΔLTR (3 × 10^6 cfu), HNF-3-mAlb-ΔLTR (5 × 10^5 cfu), and rPEPCK-LTR (1 × 10^8 cfu).

mL hAAT/1% proviral integration, respectively. Thus, in contrast to the results observed in vitro, the addition of the three HNF-3 binding sites to the basal mAlb or hAAT promoters did not enhance in vivo promoter strength. The rPEPCK-LTR vector resulted in a much lower normalized expression (0.33 ng/mL hAAT/1% proviral integration). Thus, the mAlb, hAAT, and rPEPCK promoter containing constructs were 8-, 35-, and 0.02-fold as strong, respectively, as the Pol-II promoter, which had a normalized expression of 18.3 ng/mL hAAT/1% proviral integration when analyzed simultaneously with these samples.

DISCUSSION

The liver is particularly well suited as a target organ for gene therapy of blood protein deficiencies. The large endothelial fenestrations of the hepatic sinusoids permit direct contact of retroviral vectors with hepatocytes. Partial hepatectomy results in a coordinated burst of replication of healthy hepatocytes, allowing the efficient chromosomal integration of retroviruses, whereas the long lifespan of quiescent hepatocytes enhances the long-term expression of a transduced gene. Furthermore, the liver can perform necessary posttranslational modifications and secreted products have ready access to the vasculature. We and others have shown that intraportal injection of retroviral vectors results in selective transduction of up to 10% of hepatocytes in vivo and no expression is observed in other organs[18,20,24] or in the liver in the absence of hepatocyte replication.[17,24] The major limiting problem to hepatic gene therapy is at the level of expression from a retroviral vector in vivo. Herein, we report our in vitro and in vivo results using the hAAT, mAlb, rPEPCK, and the rLFABP liver-specific promoters to express genes from retroviral vectors in hepatocytes.

There are two potential problems in generating retroviral vectors containing an internal promoter. First, because only the full-length genomic RNA intermediate can transfer genetic information, the presence of an RNA processing signal will preclude its faithful transmission. Indeed, attempts to make a packaging cell line with the LFABP promoter[53] were unsuccessful, presumably due to a perfect consensus polyadenylation signal [AATAAA(N_{18})GGTTTTGAGTT] present at position −472 to −438,[32] which closely matches the consensus polyadenylation signal [AAUAAA($N_{15\text{-}25}$) $GU_{[Rich]}$].[46] A second problem in retroviral construction is that of promoter interference.[42] Indeed, we found that retroviral constructs with the PEPCK or the mAlb promoter resulted in transcription that primarily initiated from the internal promoter in both fibroblast or hepatoma cells in vitro. Even in the packaging cell lines, there was very little full-length genomic RNA, which should make it difficult to obtain the high-titer packaging cell lines that are necessary to optimize in vivo gene expression. We hypothesize that enhancer elements in the LTR are acting primarily on the internal promoter, because the PEPCK promoter was virtually inactive in an LTR-deleted vector and the mAlb promoter is inactive in nonhepatoma cells from a plasmid that lacks retroviral elements.[33,47-51] If indeed the LTR was critical in increasing expression, the loss of LTR enhancer activity[16,24] may be responsible for the marked attenuation in vivo with the mAlb- and PEPCK-containing constructs.

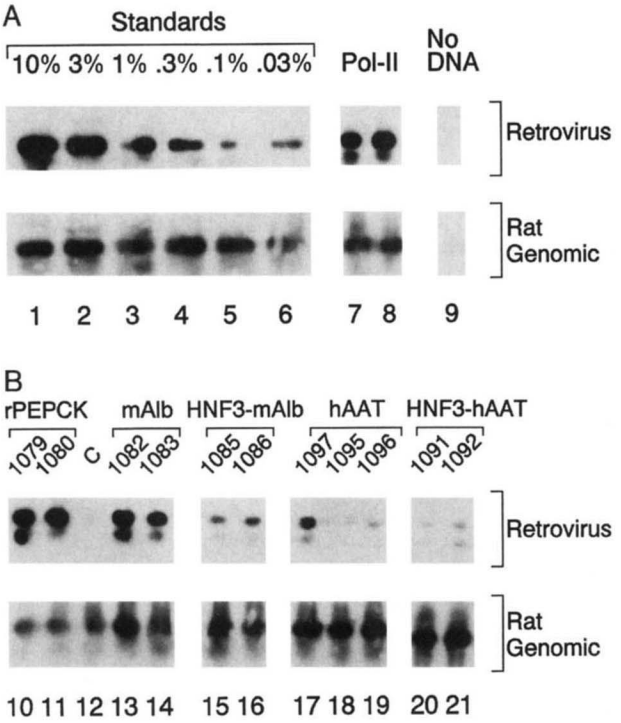

Fig 4. PCR quantitation of in vivo retroviral integration frequency in livers of the transduced rats. Liver DNA was isolated from biopsy specimens from the transduced rats at either 16 to 18 or 40 weeks posttransduction. Multiplex PCR using retroviral (IRES) and rat genomic (LFABP) primers was performed simultaneously on all samples. Amplified sequences were electrophoresed, transferred to a nylon membrane, and hybridized first to an IRES probe (4-hour autoradiogram), stripped, and reprobed with a LFABP probe (6-hour autoradiogram). Standards (lanes 1 through 6) were prepared by mixing various concentrations of DNA derived from NIH 3T3 cells containing a single copy of retroviral DNA with nontransduced rat liver DNA. Lane 9 represents a PCR mixture devoid of DNA, whereas lane 12 represents DNA from a nontransduced rat liver that was simultaneously isolated with the transduced rat liver samples. Lanes 7 and 8 represent Pol-II-hAAT transduced rat liver samples that had been studied previously.[24] The DNA from the livers of rats transduced with the rPEPCK-LTR (lanes 10 and 11), mAlb-ΔLTR (lanes 13 and 14), HNF-3-mAlb-ΔLTR (lanes 15 and 16), hAAT-ΔLTR (lanes 17 through 19), and HNF-3-hAAT-ΔLTR (lanes 20 and 21) retroviral vectors are shown. The specific rat used in each assay is indicated above the sample.

Of the promoters that resulted in retroviral vectors which could be packaged and used to transduce new cells, the hierarchy of promoter strength is hAAT>mAlb>Pol II>>> PEPCK. The low activity of the PEPCK promoter was surprising, because it directs expression in transgenic mice[54] and in rat hepatocytes when delivered as plasmid DNA complexed with galactosylated poly (L-lysine).[9] However, in the transgenic mouse study, the use of a heterologous reporter gene precluded quantitative comparison with the endogenous gene. In addition, Hatzoglou et al[15] reported relatively low levels of expression from a retroviral vector containing a PEPCK promoter in rats, and Cheng et al[8] reported low expression from a plasmid construct containing the rPEPCK promoter delivered to rat livers by using a DNA ballistic

Table 2. Comparison of In Vivo Promoter Strengths of the Retroviral Constructs

Rat No.	Promoter	hAAT Protein in Serum at Time of Liver Biopsy* (ng/mL)	Proviral Integration Frequency† (% of hepatocytes containing provirus)	Normalized Serum Expression‡ (ng/mL hAAT/1% proviral integration)
1082	mAlb	74.0	0.540 ± .120	137.0 ± 30.4
1083	mAlb	46.8	0.320 ± .100	146.0 ± 44.9
1085	HNF-3-mAlb	5.6	0.055 ± .016	101.8 ± 28.8
1086	HNF-3-mAlb	17.6	0.082 ± .023	214.8 ± 60.5
1095	hAAT§	10.0	0.034 ± .007	294.1 ± 64.7
1096	hAAT§	12.6	0.042 ± .015	300.0 ± 109
1097	hAAT	89.5	0.065 ± .014	1,377.0 ± 293
1091	HNF-3-hAAT	22.2	0.050 ± .030	444.0 ± 269
1092	HNF-3-hAAT	28.0	0.038 ± .017	736.8 ± 338
1079	rPEPCK§	3.2	6.90 ± 1.04	0.46 ± 0.07
1080	rPEPCK§	1.0	5.30 ± 1.80	0.19 ± 0.06
13	Pol-II§	100.0	5.36 ± 1.98	18.3 ± 6.9

* hAAT protein level in the serum of rats transduced with the liver-specific retroviral vectors at the time of liver biopsy (see Fig 3).

† Percentage of hepatocytes containing provirus as determined by at least four separate experiments performed in duplicate ± the standard error of the mean[52] (see Fig 4 for a representative example).

‡ Ratio of the serum hAAT protein level in the transduced rats to the proviral integration frequency.

§ Liver biopsy and serum samples were obtained at approximately 16 to 18 weeks posttransduction. All other samples were obtained approximately 40 weeks posttransduction.

device. It is possible that details of vector construction and/or the particular cDNA used might influence expression in vivo.

Although expression from the mAlb promoter was considerably higher than from the PEPCK promoter, this would result in an hAAT level that is still only 1.4% of normal if

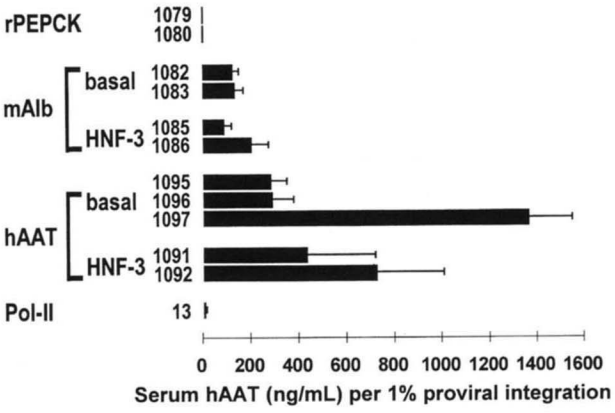

Fig 5. Normalized in vivo promoter strengths of the retroviral constructs. The relative strengths of the liver-specific promoter constructs were compared by normalizing the serum hAAT level measured at the time of liver biopsy (Fig 3) for the percentage of hepatocytes that integrated the provirus as determined by a minimum of four independent PCR-based assays of liver genomic DNA for an individual animal (Fig 4 and Table 2). Results are expressed as the mean of the serum hAAT protein level (ng/mL)/1% proviral integration ± SEM of these determinations for an individual animal.

100% of the cells could be transduced. This is considerably less active than the endogenous mAlb gene, although a quantitative comparison of relative promoter strength is difficult because of the use of a heterologous reporter gene. Relatively low expression is not surprising, as a 2-kb albumin enhancer increases expression 30-fold over that observed with a basal promoter in transgenic mice.[55] Unfortunately, inclusion of this 2-kb enhancer led to inappropriately short RNA in our hands, which precluded the generation of a retroviral vector. Indeed, Kay et al[18] tested an identical promoter/enhancer for expression from a retroviral vector. A packaging cell line obtained after prolonged "ping-pong" amplication was later found to have deletion in the promoter/enhancer region that has not yet been mapped (M. Kay, personal communication, February 1994). Because the level of expression they observed was similar to that seen in our study,[18] we suspect that important enhancer elements were probably deleted. It is possible that a 830-bp[33] or 330-bp[50] enhancer fragment that has activity in tissue culture cells would not undergo rearrangements, but still increase expression in vivo. Alternatively, the high level of initiation from the internal mAlb promoter in a retroviral vector might make it difficult to obtain the high-titer packaging cell lines needed for in vivo transduction. We have therefore chosen not to pursue this promoter further.

The hAAT promoter exhibited the highest level of expression in vivo, with an average of 657 ng/mL hAAT/1% transduction efficiency. Although this is stronger than the others tested, expression is still only ~6.5% that of the endogenous gene. It is possible that more promoter and/or enhancer elements are necessary to maximize expression. Although 2 kb of 5′ flanking sequence directs high-level expression of the genomic hAAT gene,[56,57] shorter promoter constructs have only been tested with a heterologous reporter gene in transgenic mice, making it very difficult to quantitate the absolute level of expression.[34,56] Thus, although the 347-bp fragment of the hAAT promoter tested here has most of the activity of longer promoters in hepatoma cells,[58] it is possible that elements important for expression in vivo were excluded because of the need to use a shorter promoter fragment to create a retroviral vector. Peng et al[59] have also shown that a 732-bp hAAT promoter functions well from a retroviral vector in hepatocytes in vitro, although this construct was never tested in vivo. We are currently adding enhancer elements to attempt to further increase the level of expression. Finally, because promoters function by recruiting transcription factors to the vicinity of the start site, we reasoned that simply adding more binding sites might lead to increased levels of expression in vivo. Despite the fact that we and others[33] have observed that addition of three HNF-3 binding sites increases expression from either the mAlb or the hAAT promoter in hepatoma cells in vitro, this unfortunately had little effect on expression in vivo in this study.

Importance of the LTR Enhancer in the Retroviral Backbone

A potential criticism of our study is the fact that slightly different retroviral vectors were used to test different promoters. Those designated ΔLTR had a 178-bp deletion that included the two 75-bp tandem repeats of the U3 enhancer region in the 3′ LTR; all other constructs had an intact 3′ LTR. Upon reverse transcription of the retroviral RNA genome, the U3 sequences from the 3′ LTR serve as a template for the synthesis of the 5′ LTR, which transfers the deletion to the 5′ LTR in the provirus and weakens the LTR promoter by ~85% to 90%.[43,44] Such a 3′ LTR deletion facilitates the analysis of promoter function in tissue culture cells.[43,60] In addition, it is possible that sequences in the LTR might exert a negative influence on expression from a retroviral vector in vivo. Richards and Huber[61] reported that an albumin promoter/enhancer was inactive in transgenic mice when integrated with retroviral sequences, although their construct was not tested in the absence of the retroviral elements. Furthermore, sequences within the LTR enhancer are responsible for the loss of expression from the LTR promoter in embryonic carcinoma cells[62] and may be involved in the attenuation of expression from the LTR after transduction of preimplantation embryos[63] or adult hepatocytes.[24] In contrast, others believe that the LTR enhancer does not exert a negative influence on an internal promoter. Soriano et al[43] tested retroviral vectors with an intact LTR or a 3′-LTR deletion identical to that used in our study and observed no significant difference in the level of expression from an internal promoter in embryonic stem cells, which do not express the LTR promoter. Stewart et al[63] transduced preimplantation embryos with retroviral vectors and observed that, although the LTR was inactive in vivo in all animals, the internal thymidine kinase promoter was active in the liver. Similarly, Kay et al[18] tested expression from the albumin promoter/enhancer in hepatocytes in vivo from both 3′-deleted and 3′-intact LTR retroviral backbones and observed no significant difference in expression after correction for the number of retroviral particles delivered. Thus, although we believe it is unlikely that the 3′ LTR deletion will significantly effect in vivo expression, we can only formally conclude that the rPEPCK promoter is significantly weaker than the Pol-II promoter in vivo, whereas the hAAT promoter is approximately fourfold stronger than the mAlb promoter.

Implications for Gene Therapy

Although the stability of expression from retroviral vectors is quite promising for use in hepatic gene therapy, low levels of protein production per integrated provirus currently limits the level of in vivo expression. Although in vitro studies are an excellent means of developing and screening retroviral vectors, the discrepancy between in vivo and in vitro comparisons of promoter strengths underscore the importance of quantitative in vivo comparisons. We conclude from our studies that the hAAT promoter is more efficiently expressed in vivo than the other liver-specific, cellular, or viral promoters tested. Furthermore, the absence of initiation from the liver-specific hAAT promoter in the fibroblast-derived packaging cell lines has allowed us to obtain high-titer retroviral vectors by using "ping-pong" amplification that are currently being tested in vivo. We predict that a 10% transduction efficiency will result in a serum hAAT level of 6 μg/mL. Although this level is still insufficient for the gene therapy of hAAT deficiency (at least 150 μg/mL is needed),

it would be in the therapeutic range for other serum protein deficiencies such as factor IX, factor X, or protein C.

ACKNOWLEDGMENT

We thank Richard Hanson and Jin Sung Liu of Case Western University (Cleveland OH), Kenneth Zaret of Brown University (Providence, RI), Jeffrey Gordon of Washington University (St Louis, MO), and Rong-Fong Shen and Savio L.C. Woo of Baylor University (Houston, TX) for supplying plasmid constructs.

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