

## Liver-Directed Gene Therapy: Evaluation of Liver Specific Promoter Elements

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Liver-directed gene therapy could dramatically alter the therapy of many inherited hematologic and metabolic diseases. We have developed a rapid, reliable, low-mortality method for the *in vivo* delivery in rats of retroviral vectors 24 hr after 70% hepatectomy by intraportal injection during hepatic in-flow occlusion (HIFO). Using the human  $\alpha_1$ -antitrypsin (hAAT) reporter gene, we found *in vivo* that up to 10% of hepatocytes integrated the provirus, and serum hAAT protein levels were sustained for up to 1 year. Despite high *in vivo* transduction efficiencies, gene expression at the mRNA level is disappointingly low compared to *in vitro* transduced NIH 3T3 or Hepa A1 tissue culture cells. In this report, LNL-6-derived retroviral vectors (RV) were combined with one of two strong, liver-specific promoters, murine albumin or human  $\alpha_1$ -antitrypsin, an upstream insertion of a trimer of hepatocyte nuclear factor-3 (HNF-3) binding sites, and the hAAT reporter gene. HNF-3 has been demonstrated to increase *in vitro* transcriptional activity [23]. Twenty-four hours after 70% hepatectomy, 10-fold concentrated (by methotrexate-resistant titer) RV-producing cell supernatant was given intraportally during a 3-min HIFO. Serum hAAT levels as quantitated with a human specific ELISA were sustained for over 40 weeks with all of the liver-specific promoter constructions. However, the hAAT protein production with the murine albumin promoter retroviral constructs decreased with time, but was sustained at levels approximately 80% of the initial serum peak levels with the constructs containing the hAAT promoter. We conclude that the retroviral vectors containing liver-specific promoters can lead to sustained, transduced gene expression and the quantitative evaluation of *in vivo* promoter strengths is essential for the development of high expressing retroviral vectors necessary for hepatic gene therapy in humans. © 1994 Academic Press, Inc.

### INTRODUCTION

The liver synthesizes many serum proteins which play pivotal roles in hemostasis, metabolism, and protection

against infection. Although these deficiencies can be treated by repeated infusions of the particular protein, this is expensive, carries an associated risk of viral infection, and only temporarily ameliorates the disease manifestations. Liver transplantation has been used to correct serum protein deficiencies [1] but is limited by donor organ availability and requires immunosuppression. The transfer of a functional gene into the cells of a genetically deficient individual should correct the clinical manifestations of the disease by providing long-term production of the deficient gene product. Accordingly, the liver has been a primary target organ for somatic gene therapy. Both *ex vivo* [2, 3, 16, 41] and *in vivo* [4-12] methods have been used to transfer genes into mammalian hepatocytes.

The *ex vivo* approach requires hepatocyte harvest, *in vitro* transduction with retrovirus, and reintroduction of the transduced hepatocytes into the portal circulation. While Chowdhury *et al.* [2] and Kay *et al.* [3] have achieved reasonable expression of low-density lipoprotein receptor (LDL-R) and human  $\alpha_1$ -antitrypsin (hAAT) genes, respectively, limitations to *ex vivo* gene therapy include the potential for contamination during large-scale hepatocyte culture ( $10^8$  hepatocytes/kg recipient body wt), limitation of the number of hepatocytes which can be reintroduced (1-2%) due to the potential for portal venous obstruction, and the requirement of a partial hepatectomy to harvest hepatocytes.

*In vivo* delivery of foreign genes to hepatocytes *in situ* has been accomplished by several approaches. The intravenous injection of liposome-coated [4] or asialoglycoprotein-coated [5] plasmid DNA or adenoviral vectors [45] has resulted in gene expression *in vivo*, which usually declines over time due to the instability of the extrachromosomal DNA. Retroviral vectors have been delivered to the liver *in vivo* by intraparenchymal [6] or intraportal injection [7, 8], asanguineous liver perfusion [9, 10], and hepatic inflow occlusion/portal vein injection [11, 12]. Since effective retroviral integration into the hepatocyte genome requires cell division, partial hepatectomy [11, 12], hepatic ischemia [10], and carbon tet-

rachloride injury [8] have been used to induce hepatocyte replication.

In attempts to increase gene expression, retroviral vectors containing strong viral promoters have been used. While these promoter constructs are extremely active in tissue culture cells, they are poorly expressed in hepatocytes and *in vivo* attenuate with time [2, 3, 7, 12–14, 45]. Therefore, the use of cellular promoters has been proposed as a means of providing sustained expression [2, 7, 11]. While insertion of the promoter for the large subunit of murine RNA polymerase II (Pol-II) into retroviral vectors containing the hAAT reporter gene sustained expression for over 1 year, decreases in serum hAAT levels also occurred. This was felt to likely be due to the loss of function from the LTR and not from the internally placed Pol-II promoter [11].

Therefore, in this study, the expression of retroviral vectors containing the strong, liver-specific 810-bp murine albumin (mAlb) and the 347-bp hAAT promoters plus the trimers of the DNA binding sequence for hepatocyte nuclear factor-3 (HNF-3) were measured *in vivo* by serum hAAT production. *In vitro*, HNF-3 increased the hAAT transcription from the mAlb [23], while *in vivo*, its presence appeared to exert a stabilizing influence on long-term serum hAAT protein expression.

## MATERIALS AND METHODS

**Construction of retroviral vectors.** The Moloney murine leukemia virus (Mo-MLV)-based LNL-6 retroviral vector (LTR-hAAT) contains the LTR promoter, the hAAT cDNA reporter gene, the encephalomyocarditis virus internal ribosome entry site (IRES), and the mutant dihydrofolate reductase gene and was cloned as previously described [11]. In order to quantitatively evaluate the mAlb and hAAT liver-specific promoters, a retroviral vector was constructed which contained a 178-bp deletion in the enhancer region of the 3' LTR (Fig. 1A). Transfection of packaging cells leads to the integrated vector in the packaging cells (Fig. 1B), which transcribes mRNA from the LTR and the internal promoters (arrows). Injection of preparations of the retroviral vector produced by the packaging cells into rats leads to the integrated provirus within the target cell genome (Fig. 1B), which predominantly transcribes mRNA from the internal promoter (arrow). Thus, as a result of the 3' enhancer deletion, the majority of the transcripts will originate from the internal promoter, thus facilitating quantitative comparisons of liver specific promoter elements. Therefore, the LTR-hAAT- $\Delta$ LTR retroviral vector (RS 32392-hAAT) which contains a 178-bp deletion in the 3' LTR enhancer region was created by removal of the sequences from the 5' *Pvu*II site to the *Xba*I of LTR-hAAT sites as described [25]. This deletion will subsequently be referred to as the  $\Delta$ LTR deletion.

**Construction of mAlb-hAAT- $\Delta$ LTR (Fig. 2).** To create mAlb-SP72, the 810-bp fragment of the mouse

albumin promoter was obtained from pAT2 (from Dr. Kenneth Zaret, Brown University, Providence, RI) by *Hpa*II digestion (which cuts just downstream from the transcription start site) and blunt end formation with T4 polymerase. The *Bam*HI linkers were ligated, digested with *Bam*HI and *Eco*RI, and ligated into the *Eco*RI/*Bam*HI site of SP72. A *Bgl*II/*Bam*HI fragment of mAlb-SP72 containing the 810-bp albumin promoter was ligated into the unique *Bgl*II site of hAAT- $\Delta$ LTR to create mAlb-hAAT- $\Delta$ LTR.

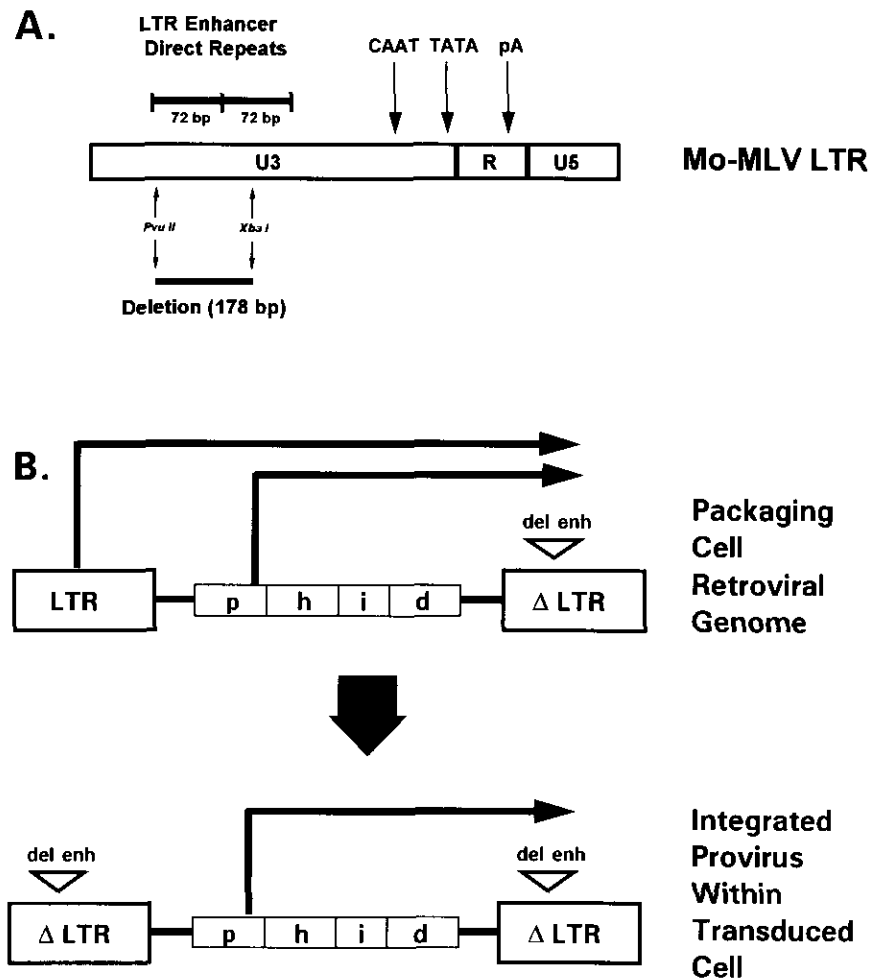
**Construction of hAAT-hAAT- $\Delta$ LTR (Fig. 2).** The 347-bp fragment of the hAAT promoter from the *Bgl*II site at -347 to the *Sma*I site at +10 of the hAAT gene 3E was *Bcl*I linker ligated. After *Bcl*I and *Bgl*II digest (which cuts at -347), the DNA was cloned into the *Bgl*II site of hAAT- $\Delta$ LTR, regenerating the unique 5' *Bgl*II site, and creating hAAT-hAAT- $\Delta$ LTR.

**Insertion of HNF-3 binding sites (Fig. 2).** A 200-bp fragment containing HNF-3 binding site trimers were obtained from Dr. Kenneth Zaret and was digested with *Eco*RI and cloned into the *Eco*RI site of pSP72. The HNF-3 fragment was removed by digesting with *Bam*HI and *Bgl*II and placed into the mAlb and hAAT promoter containing retroviral vectors via the unique 5' *Bgl*II site.

**Creation of retroviral producer cell lines.** The amphotropic GP+*env*AM12 [26] murine fibroblast line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated calf serum (HyClone Laboratories, Logan, UT), penicillin ( $10^5$  U/liter), streptomycin ( $10^5$  mg/liter) (DMEM). After calcium phosphate transfection with retroviral vector DNA, the GP+*env*AM12 cells were cultured for 14 days in 250 nM methotrexate (Sigma Chemical, St. Louis, MO) supplemented DMEM. Supernatant from 50 methotrexate-resistant (Mtx<sup>R</sup>) GP+*env*AM12 clones were screened for the production of retrovirus by infecting NIH 3T3 cells and measuring hAAT production 72 hr later. Mtx<sup>R</sup> titers were determined on NIH 3T3 cells [27], and high-titer clones were proven free of replication competent helper virus by amplification and marker rescue assay [27].

***In vivo* hepatocyte transduction protocol.** Age-matched adult male Sprague-Dawley rats (SASCO, Omaha, NE) weighing 200–275 g, received rodent chow and tap water *ad libitum*. Standard NIH care with a 12-h light-dark cycle was provided. Twenty-four hours after 70% hepatectomy, *in vivo* hepatocyte transduction was accomplished by temporarily occluding the hepatic artery and portal vein with microvascular clips and injecting 5 ml of cold concentrated retrovirus-conditioned medium into the proximal portal vein via a 30-gauge needle, as described previously [11]. Hepatic inflow occlusion time was 3 min.

**hAAT ELISA.** hAAT was measured by sandwich ELISA [28] using the IgG fraction of goat anti-hAAT (Atlantic Antibodies, Scarborough, NJ; Lot No. 60206) as the first and the second antibody. The second anti-



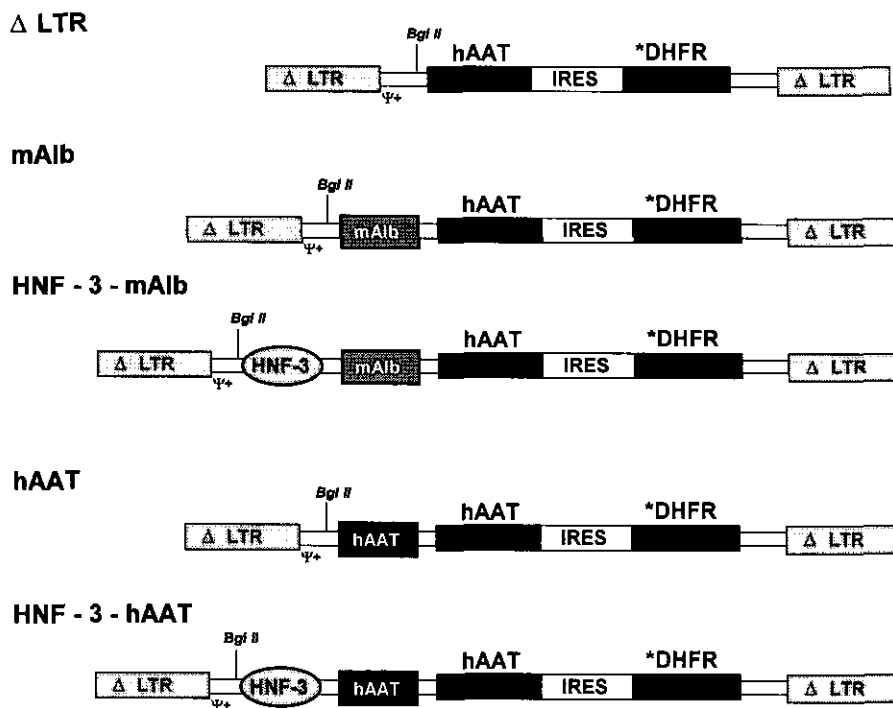
**FIG. 1.** (A) The structure of the intact Moloney murine leukemia virus (Mo-MLV) LTR is shown with the relative positions of the elements depicted: the 72-bp direct repeats of the enhancer, the polyadenylation signal sequence (pA), and the CAAT and the TATA transcriptional elements. A deletion which encompasses portions of the two tandem repeat regions of the LTR enhancer was created by restriction enzyme digestion, as described under Materials and Methods. (B) The retroviral genome which is present in the packaging cell line, containing the 3' LTR deletion (shown by del enh and  $\Delta$ LTR), which is transferred to the 5' LTR after transduction of the target cell. The promoter (p), hAAT reporter gene (h), IRES sequences (i), and mutant dihydrofolate reductase gene (d) are abbreviated for clarity. In the packaging cell line genome, transcription can initiate from the LTR or the internal promoter. As a result of the LTR enhancer deletion, transcription from the integrated provirus genome predominantly initiates from the internal promoter [25, 35].

body was labeled with horseradish peroxidase (Conju-Quick; Midwest Scientific, St. Louis, MO), and developed with 3,3',5,5'-tetramethylbenzidine dichloride substrate (Sigma). The lower sensitivity limit of the assay was <3 ng/ml hAAT.

## RESULTS

*In vitro* evaluation of liver-specific promoter constructions. Retroviral constructs were transfected into amphotropic GP+envAM-12 packaging cells and selected for Mtx resistance. With retroviral supernatants from highest titer GP+envAM-12 clone producing packaging cell lines for each of the four promoter constructs, NIH 3T3 fibroblasts and Hepa A1 hepatoma cells were trans-

duced at a low multiplicity of infection. After selection with methotrexate, resistant cells should have a single copy of the retrovirus integrated, which facilitates *in vitro* comparison of promoters. Pools of transduced Mtx<sup>R</sup> Hepa A1 and NIH 3T3 cells were analyzed for their production of hAAT protein (Table 1). For the mAlb promoter constructs, the insertion of the HNF-3 binding sites produced threefold lower amounts of hAAT protein on the NIH 3T3 fibroblasts, which are devoid of liver-specific transcription factors, but no change in hAAT protein production was measured in the hepatoma cells. The hAAT-hAAT- $\Delta$ LTR and HNF-3-hAAT-hAAT- $\Delta$ LTR promoter constructs demonstrated similar production of hAAT protein by the NIH 3T3 cells *in vitro* (Table 1). We could not quantitate the hAAT production by the mAlb-hAAT- $\Delta$ LTR-transduced Hepa A1



**FIG. 2.** Description of retroviral vectors containing the liver-specific promoter constructions. The LNL-6-derived retroviral vector backbone (RS3891) [11], with a 3' LTR enhancer deletion, is abbreviated as  $\Delta$ LTR. The murine albumin promoter was inserted into the *Bgl*II restriction site to create Alb-hAAT- $\Delta$ LTR (abbreviated here as mAlb), and a trimer of hepatocyte nuclear factor-3 binding sites was inserted into the regenerated *Bgl*II restriction site upstream from the mAlb promoter, creating HNF-3-mAlb-hAAT- $\Delta$ LTR (abbreviated as HNF-3-mAlb). The hAAT promoter containing vectors hAAT-hAAT- $\Delta$ LTR and HNF-3-hAAT-hAAT- $\Delta$ LTR (abbreviated hAAT and HNF-3-hAAT, respectively) were created in a similar fashion, and are described under Materials and Methods.

cells due to insufficient titer and the low infectability of the Hepa A1 cells.

Amphotropic packaging cell clones had Mtx<sup>R</sup> retroviral titers determined on NIH 3T3 and Hepa A1 cells as estimated by limiting dilution analysis of unconcentrated

supernatant [11] for the mAlb-hAAT- $\Delta$ LTR- and the hAAT-hAAT- $\Delta$ LTR-containing constructs ranging from 10,000 to 60,000 colony forming units (cfu)/ml.

*Long-term expression of liver-specific promoters after in vivo transduction.* One of the principal goals of gene therapy is long-term expression of the transduced gene. Rats that were transduced via the intraportal injection of 24-fold concentrated (by volume and 10-fold by Mtx<sup>R</sup> titer) packaging cell supernatant containing the retroviral construct mAlb-hAAT- $\Delta$ LTR during hepatic inflow occlusion, initially expressed high levels of serum hAAT in two of three rats, but by 40 weeks post-transduction, expression had decreased to 48% of post-transduction Week 1 values (Fig. 3). The insertion of the HNF-3 binding sites into the mAlb promoter construct resulted in lower early levels of hAAT protein in the serum, but expression and degree of attenuation (46%) comparable to those of the mAlb-hAAT- $\Delta$ LTR vector transductions over the 40-week study period.

With the hAAT promoter, seven of seven rats expressed the hAAT gene for over 16 weeks. Attenuation of serum hAAT protein levels was less than that with the mAlb constructs, since the hAAT-hAAT- $\Delta$ LTR-transduced rats produced 80% of their Week 1 post-transduction levels at 16 weeks post-transduction. Rats transduced with the HNF-3-hAAT-hAAT- $\Delta$ LTR construct

**TABLE 1**

***In Vitro* Transduced Gene Expression of the Retroviral Vectors Containing Liver-Specific Promoters**

Promoter construction	hAAT protein production (ng/24 hr/10 <sup>6</sup> cells)	
	NIH 3T3 cells	Hepa A1 cells
mAlb	113	*
HNF-3-mAlb	37.5	*
hAAT	447	203
HNF-3-hAAT	459	217

*Note.* NIH 3T3 (ATCC No. 1658) [46] fibroblasts and Hepa A1 [47] hepatoma cells were transduced at a low multiplicity of infection with GP+*env*AM-12 packaging cell supernatants from each of the four liver-specific promoter constructions. The transduced NIH 3T3 and Hepa A1 cells underwent methotrexate selection, and the hAAT production (ng/24 hr/10<sup>6</sup> cells) was quantitated by ELISA. Asterisks (\*) denote nondetectable hAAT production due to insufficient numbers of transduced Hepa A1 cells after MTX selection (see Results).

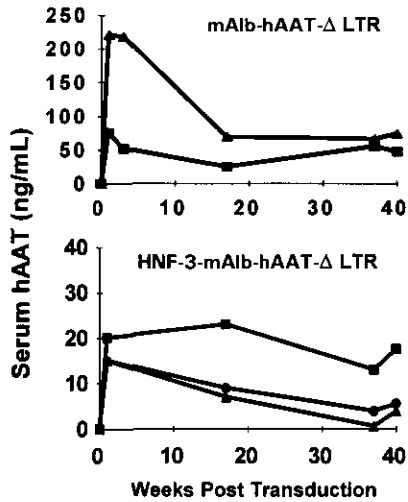


FIG. 3. Long-term *in vivo* gene expression of the rats transduced with the retroviral vectors containing the liver-specific murine albumin promoter constructs.

maintained 88% of their Week 1 post-transduction values at 40 weeks (Fig. 4).

#### DISCUSSION

The liver is an excellent target organ for *in situ* retroviral gene therapy. Portal venous and hepatic arterial blood bathes hepatocytes through large fenestrations in the sinusoidal endothelium, permitting direct contact of hepatocytes with large particles such as retroviruses. Although retroviral infection of the resting liver is inefficient due to the requirement of cell division for the genomic integration of retroviruses, partial hepatectomy results in a coordinated burst of hepatocyte cell division, with up to 44% of hepatocytes proliferating at 25 hr posthepatectomy [36]. The subsequent long lifespan of quiescent hepatocytes should facilitate stable and long-term expression of a transduced gene. In addition, many of the proteins deficient in inherited metabolic and hematologic diseases are synthesized within the liver, where they undergo post-translational modifications necessary for full functional activity.

Although a number of investigators have used retroviral vectors to transduce up to 0.5–10% of hepatocytes of animals, the inability to achieve high levels of expression remains the single major obstacle to successful hepatic gene therapy. In attempts to increase gene expression, retroviral vectors containing strong viral promoters have been used. In general, viral promoters, which are extremely active in tissue culture cells, are expressed poorly by retroviral vectors *in vivo*. For example, the cytomegalovirus promoter *in vitro* demonstrates high levels of gene expression in hepatocytes [3, 7, 12, 13, 14], but *in vivo* gene expression rapidly attenuates [3, 7]. Possible mechanisms responsible for the decrease in

gene expression with viral promoters include DNA methylation [8, 17], negative regulatory proteins [18, 19, 20, 21], and the absence of transcription factors in quiescent hepatocytes [15, 22].

The use of cellular promoters has been proposed as a means of solving the viral promoter attenuation problem. Chowdhury *et al.* [2] utilized a chicken  $\beta$ -actin promoter to direct the gene for LDL-R and observed no attenuation *in vivo* after 3 months, although levels of *in vivo* transduction were not quantified. We have used our retroviral transduction method [11, 12] to compare the ability of retroviral vectors containing the native LTR retroviral promoter or an inserted cellular Pol-II promoter to express the hAAT at the mRNA and serum protein levels [11]. While reasonable transduction frequencies of 10–15% were obtained, RNA levels were much lower than those for transduced tissue culture cells, but sustained serum protein expression was achieved for over 1 year. This suggests that *in vivo* transcription may be inappropriately low from the construct.

The efficacy of liver-specific promoters to drive high-level, sustained reporter gene expression has been demonstrated in cultured hepatoma cell lines, hepatocytes, and in transgenic mouse systems [14, 41, 44]. Kay *et al.* [7] inserted the liver-specific murine albumin promoter/enhancer construction into a retroviral vector with intact LTR sequences which directed the expression of the hAAT reporter gene. Serum hAAT protein levels averaged 500 ng/ml and gene expression was sustained for at least 6 months, suggesting that this promoter was 5- to 10-fold stronger than the Pol-II or Mo-MLV LTR promoters. This promoter construct had a 2.0-kb deletion (M. Kay, personal communication). In contrast, transduction with the liver-specific phosphoenolpyruvate carboxykinase promoter containing retroviral vectors

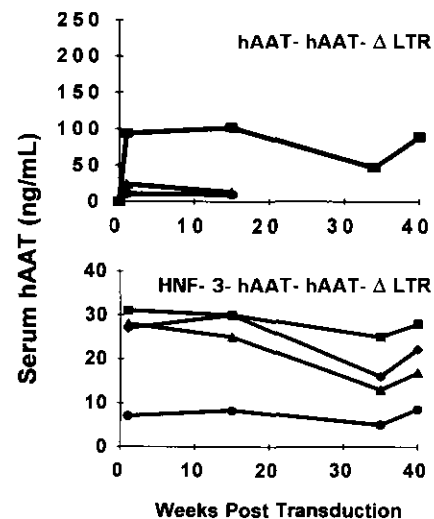


FIG. 4. Long-term *in vivo* gene expression of the rats transduced with the retroviral vectors containing the liver-specific human  $\alpha_1$ -antitrypsin promoter constructs.

led to extremely low levels of human growth hormone reporter gene expression [6]. Therefore, we employed retroviral vectors containing either the strong, liver-specific murine albumin or human  $\alpha_1$ -antitrypsin promoters to increase and stabilize hAAT gene expression in the transduced rats.

Attempts to utilize tissue-specific regulatory sequences in retroviral vectors may be complicated by the presence of powerful regulatory elements in the LTR regions which may interfere with the functions of the tissue specific signals [32]. To limit this problem of variation in gene expression by affected internal promoters, Yu *et al.* [31] used a self-inactivating (SIN) vector in which the enhancer and the "CAAT box" were deleted from the LTRs. Yee *et al.* [32] employed a disabled retroviral vector in which the enhancer, the CAAT box, and the transcriptional start site were deleted from the LTR regions. Increased transcription derived from the internal promoters was demonstrated with each of these constructions.

A Mo-MLV-based retroviral vector with an internal promoter (Pol-II) inserted can initiate transcription from either the 5' LTR or the internal Pol-II promoter [11]. To diminish the possible influence of viral transcription on the quantitation of gene expression from this internally placed promoter, we employed the  $\Delta$ LTR Mo-MLV-based retroviral vector which creates a deletion of the 5' LTR enhancer upon integration into the target cell genome [25]. The 178-bp *PvuII/XbaI* fragment (Fig. 1) which was removed, encompasses most of the two 75-bp tandem repeats comprising the LTR enhancer [32]. The U3 region in the 3' LTR of an infectious retroviral RNA serves as a template for the formation of both U3 regions during replication of retroviruses [34]. Therefore, a deletion in the U3 enhancer region of the 3' LTR will be transferred to both LTRs in the provirus. This reduces the transcriptional activity of the LTR promoter by as much as 85–90% [25, 35], but exerts minimal effects upon an internally placed promoter [33]. In addition, the deletion does not appear to effect the stability of proviral expression, as this vector backbone has been used by Kay *et al.* [7] and Chowdhury *et al.* [2] to drive gene expression controlled by internally placed promoters for at least 3 and 6 months, respectively.

An advantage of using retroviral vectors is sustained gene expression resulting from the highly efficient retroviral integration into the target cell chromosome. When we followed the serum hAAT expression in rats transduced with the Pol-II vector long term, the serum hAAT protein levels decreased to steady-state levels after 12 weeks, which were an average of 31% of initial Week 1 values [11]. Mapping of the transcription initiation site in these rats suggested that much of the observed attenuation of hAAT gene expression was due to a decrease in the LTR-initiated transcript [11]. The insertion of liver-specific promoters into retroviral vectors with deleted LTR enhancer regions facilitates the assessment of the

long-term gene expression since much of the LTR transcription is inactivated [25]. The *in vivo* gene expression obtained with our mAlb-hAAT- $\Delta$ LTR retroviral vector is consistent with that of Kay *et al.* [7] using the same LTR enhancer deleted retroviral vector backbone, the murine albumin promoter/enhancer, and a lower number of retroviral particles injected into hepatectomized mice. We observed that for the mAlb-hAAT- $\Delta$ LTR vector, the serum hAAT protein expression attenuated to 48% of Week 1 values over the 10-month study period, which is consistent with the decrease in hAAT gene expression over time observed by Kay *et al.* [7] with the liver-specific albumin promoter/enhancer. However, the hAAT-hAAT- $\Delta$ LTR vector-transduced rats in our study demonstrated a minimal (12–20%) decrease over the 40-week study period.

In the liver, multiple *cis*-acting transcription factors contribute to the high level of gene expression in the hepatocyte compared to other cells [43]. Among the transcription factors is the HNF-3 binding activity that consists of three distinct proteins (HNF-3 $\alpha$ , HNF-3 $\beta$ , and HNF-3 $\gamma$ ) each of which have been cloned and shown to be preferentially expressed in the liver and to a lesser extent in a subset of other tissues [42]. Oligomers of HNF-3 binding sites placed upstream of a murine albumin promoter containing vector transfected into hepatoma cells resulted in a 30-fold increase in transcriptional activity compared to the murine albumin promoter alone [23]. *In vivo*, the addition of the HNF-3 binding sites to the mAlb-hAAT- $\Delta$ LTR had no apparent effect on the stability of long-term hAAT protein production. However, for the hAAT promoter constructs, the HNF-3-hAAT-hAAT- $\Delta$ LTR vector resulted in a lower (12%) decrease of *in vivo* gene expression over the basal hAAT-hAAT- $\Delta$ LTR promoter (20%) over the 40-week study period. These results indicate that the hAAT-hAAT- $\Delta$ LTR construct is more stable *in vivo* over time than the mAlb promoter construct, an effect which may be supplemented by the insertion of the HNF-3 binding sites. This may be a result of interactions between the transcription factor binding site within each liver-specific promoter construction [23, 42, 43] or the upstream  $\Delta$ LTR sequence [18, 19, 20, 22, 25].

Several groups have reported low titers using retroviral vectors with deletions in the LTR transcriptional unit [7, 31, 32]. Our titers were substantially lower than those of Soriano *et al.* [25] and Kay *et al.* [7] using the  $\Delta$ LTR deletion in a different retroviral backbone and an internally placed murine albumin promoter/enhancer. Since only full-length retroviral RNAs which contain an intact psi packaging signal, 5' and 3' LTR regions can be effectively packaged into a retroviral particle and subsequently integrate into a target cell chromosome, the initiation of transcripts from the internal promoter may result in lower retroviral titers. Although the retroviral titers of the liver-specific promoter constructions containing the  $\Delta$ LTR deletion was 40- to 100-fold lower

than those of our Pol-II-containing vectors (11), the serum hAAT protein production was only 2- to 7-fold lower in the mAlb and hAAT promoter constructions. This suggests that the liver-specific promoter constructions are between 10- and 14-fold stronger than the constitutive Pol-II promoter.

We conclude that the effect of internally inserted liver-specific promoters and promoter elements *in vivo* is highly dependant upon the individual promoter and retroviral vector utilized. These results demonstrate the importance of *in vivo* comparisons of promoter- and gene-regulatory elements. We are currently analyzing the liver-specific promoter constructs at the proviral DNA level to quantitatively compare the promoter element strengths. Our retroviral transduction method, which allows the rapid assessment of gene regulatory elements and their interactions *in vivo*, should facilitate the development of potent retroviral vectors for the treatment of human disease.

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