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# Liver-directed gene therapy: Quantitative evaluation of promoter elements by using *in vivo* retroviral transduction

($\alpha_1$-antitrypsin)

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**ABSTRACT** **Liver-directed gene therapy will be applicable to many inherited diseases. Although various protocols have been devised for *in vivo* delivery of retrovirus, comparison of hepatocyte transduction frequencies has been difficult due to variations in retroviral titer and a paucity of DNA data. We have previously reported an *in vivo* rat hepatocyte transduction technique which involves 70% hepatectomy followed 24 hr later by portal vein injection of retrovirus during hepatic in-flow occlusion. In this study, we employed this method and concentrated retroviral preparations to achieve transduction of up to 15% of hepatocytes as determined by a quantitative PCR assay. As an initial step toward identifying promoters which lead to high-level long-term expression of retroviral transduced genes, we used our *in vivo* delivery system to compare the Moloney murine leukemia virus long terminal repeat (LTR) promoter with the promoter for the large subunit of murine RNA polymerase II (Pol-II). Human $\alpha_1$-antitrypsin (hAAT) was used as the reporter gene to facilitate long-term analysis of expression. Serum hAAT levels were higher for the Pol-II promoter (143 ng/ml) than for the LTR promoter (50 ng/ml). This difference was consistent with the higher transduction frequency observed for the Pol-II-hAAT vector. Although serum hAAT expression was sustained for up to 1 year in six of eight Pol-II-hAAT-transduced rats and three of five LTR-hAAT-transduced rats and was proportional to hAAT mRNA level and proviral DNA frequency, *in vivo* expression was significantly lower than in transduced tissue culture cells. We conclude that a high frequency of *in vivo* transduction can be achieved by using retroviral vectors and our rapid transduction protocol, but transduced gene expression remains a serious problem. The quantitative assays described herein will facilitate *in vivo* comparisons of gene regulatory elements.**

Liver-directed gene therapy could revolutionize the treatment of many inherited hematologic and metabolic diseases (1–3). Although both *ex vivo* (4, 5) and *in vivo* (6–17) approaches have been used to transfer genes into mammalian hepatocytes, widespread application in humans awaits a simplified delivery system and identification of elements that maximize expression.

The *ex vivo* approach requires hepatocyte harvest, *in vitro* transduction with retrovirus, and reimplantation via the portal circulation. Chowdhury *et al.* (4) used this technique to ameliorate hypercholesterolemia for up to 6 months in Watanabe rabbits by transducing hepatocytes with a low density lipoprotein receptor gene. Using a similar *ex vivo* protocol, Kay *et al.* (5) delivered a human $\alpha_1$-antitrypsin (hAAT) gene to canine hepatocytes. Although serum hAAT at concentrations up to 1000 ng/ml was observed at 7 days after transduction, expression declined shortly thereafter, presumably due to inactivation of the cytomegalovirus (CMV) promoter. The *ex vivo* technique's major liabilities include the potential for contamination during large-scale hepatocyte culture ($\approx 10^8$ hepatocytes per kg of recipient body mass) and the relatively low proportion ($\approx$2%) of the hepatocyte mass that can be safely reintroduced.

*In vivo* delivery of foreign genes to hepatocytes has been accomplished by using: (*i*) intravenous injection of plasmid DNA complexed with liposomes (12) or asialoglycoproteins (13); (*ii*) intraportal (14, 16) or intravenous (15) injection of recombinant adenovirus; (*iii*) projectile delivery of DNA complexed to gold particles (17); and (*iv*) intraportal or intraparenchymal injection of retroviral vectors (5–11). Although expression from plasmid and adenoviral vectors declines rapidly due to loss of the unintegrated DNA (12–17), retroviral vectors efficiently integrate into target cell chromosomal DNA of hepatocytes which have been induced to replicate by a variety of techniques (7–11). However, technical comparisons and evaluations of promoter strength *in vivo* have been difficult because of (*i*) differences in the reporter genes and retroviral vectors employed and (*ii*) the limited data available on proviral DNA transduction frequency and mRNA expression.

We recently described a rapid method for the *in vivo* delivery of retroviral vectors by using 70% hepatectomy in rats followed 24 hr later by portal vein injection of retrovirus during hepatic in-flow occlusion (11). In this study, we employed retroviral vectors carrying the hAAT gene to optimize our delivery system and to evaluate long-term expression from the Moloney murine leukemia virus (Mo-MLV) long terminal repeat (LTR) promoter and the promoter for the large subunit of murine RNA polymerase II (Pol-II). Although up to 15% of hepatocytes could be transduced *in vivo* and both promoters led to hAAT expression for up to 1 year in most animals, expression was significantly attenuated in hepatocytes *in vivo* as compared with fibroblast and hepatoma cell lines in culture. We conclude that a major focus of gene therapy research should be to identify promoters which function well *in vivo* and that our simple *in vivo* delivery system will facilitate quantitative *in vivo* comparison of various promoters and may ultimately be used for hepatic gene therapy.

## MATERIALS AND METHODS

**Creation of Retroviral Producer Cell Lines.** Retroviral vectors were transfected (18) into GP+E-86 ecotropic retroviral

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Abbreviations: hAAT, human $\alpha_1$-antitrypsin; CMV, cytomegalovirus; Mo-MLV, Moloney murine leukemia virus; LTR, long terminal repeat; Pol-II, RNA polymerase II; LFABP, liver fatty acid-binding protein; IRES, internal ribosome entry site; Mtx, methotrexate; $^R$, resistance or resistant; cfu, colony-forming unit.

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packaging cells (19) and colonies were selected with 250 nM methotrexate (Mtx; Sigma) (20). Transduced GP+E-86 cells were cocultured for 8 days with GP+*env*AM12 fibroblasts (21) with Polybrene (Sigma) at 8 μg/ml. Amphotropic colonies were selected with hygromycin B at 100 μg/ml (22) and 250 nM Mtx and screened by infecting NIH 3T3 cells and measuring hAAT production 72 hr later. Mtx-resistance ($Mtx^R$) titers were determined on NIH 3T3 cells (18), and high-titer clones were proven free of replication-competent helper virus (18).

***In Vivo* Hepatocyte Transduction.** Ten to 15 ml of medium was collected from confluent 15-cm plates of amphotropic packaging cells, centrifuged at 500 × *g*, passed through a 0.45-μm-pore cellulose acetate filter, spiked with Polybrene (40 μg/ml, final concentration), and stored on ice until use. To obtain concentrated retrovirus, the conditioned medium was ultracentrifuged at 15,000 rpm in a Beckman SW28 rotor for 90 min, after which the pellets were resuspended in fresh medium, refiltered, and treated as above. Adult male Sprague–Dawley rats (Sasco, Omaha, NE), weighing 200–275 g, received standard institutional animal care. Twenty-four hours after 70% hepatectomy, *in vivo* hepatocyte transduction was performed as described (11).

**Total Liver RNA and DNA Isolations.** One gram of liver was homogenized in 10 ml of 4 M guanidinium thiocyanate (Fluka)/25 mM sodium citrate/0.5% Sarkosyl/0.1 M 2-mercaptoethanol. RNA was isolated from half of the sample by the acid pH phenol/chloroform extraction method (23). DNA was isolated from the other portion in a room devoid of retrovirus and plasmid DNA by neutral pH phenol/chloroform/isoamyl alcohol (PCA) extraction, proteinase K digestion, and RNase A digestion (ref. 24, pp. E3–E4, 7.71–7.78, and 9.16–9.21, respectively). DNA was chromatographed on a Sephadex G-25 column (Pharmacia), and the early fractions containing high molecular weight DNA were quantitated by fluorometry (25).

**RNase Protection Assay.** Antisense RNA probes with specific activity of $6 \times 10^8$ cpm/μg of RNA and 100 μg of total RNA per sample were used in an RNase protection assay as described (ref. 24, pp. 10.13–10.37), with the following exceptions: (*i*) for the Pol-II-hAAT-BP probe only, samples were denaturated at 85°C, then slowly cooled to the hybridization temperature over 2 hr; (*ii*) samples were digested for 1 hr at 37°C with RNase A (United States Biochemical) at 20 μg/ml and RNase T1 (Calbiochem) at 20 units/ml; and (*iii*) no proteinase K digestion was performed. Protected fragments were electrophoresed on a 6% polyacrylamide/8 M urea/0.5× Tris–borate–EDTA gel (ref. 24, pp. 6.36–6.39).

**PCR Amplification of Proviral DNA Sequences.** Internal ribosome entry site primers IRES-1 (5′-GTCTTCTTGACGAGCATTCC-3′, nucleotides 160–179) and IRES-2 (5′-ACCTTCTGGGCATCCTTCA-3′, nucleotides 471–451) amplified a 311-bp fragment from retroviral DNA. Liver fatty acid-binding protein primers LFABP-1 (5′-GCATTGCTAGAGATGTGATTCACATGTC-3′, nucleotides 270–298) and LFABP-2 (5′-CCACAGCTGACCACAACAGCTCTG-3′, nucleotides 623–599) amplified a 353-bp fragment from rat genomic DNA (26). Each 50 μl multiplex PCR reaction mixture (27) included 50 ng of DNA, 20 mM Tris·HCl at pH 8.55, 16 mM $(NH_4)_2SO_4$, 3.3 mM $MgCl_2$, bovine serum albumin at 150 μg/ml, 2 mM deoxynucleoside triphosphates, 25 pmol of each oligonucleotide primer, and 1.25 units of *Thermus aquaticus* DNA polymerase (Perkin–Elmer). Thirty cycles were performed with denaturation at 94°C for 2 min and annealing/extension at 65°C for 2 min. Amplified DNA sequences were electrophoresed through a 2% agarose/1× Tris ammonium acetate gel (ref. 24, pp. 6.3–6.8), blotted to BA-S-NC nitrocellulose membranes (Schleicher & Schuell), and hybridized with a radiolabeled IRES DNA probe (ref. 24, p. 10.14). After quantitation, the membrane was stripped and rehybridized with an LFABP probe.

## RESULTS

**Production of Retroviral Vectors.** Previous studies demonstrated our ability to deliver retrovirus to regenerating hepatocytes by the hepatic in-flow occlusion/portal vein injection method (11). To facilitate quantitation of long-term expression, we constructed two retroviral vectors carrying the cDNA for a serum protein, hAAT (Fig. 1). In LTR-hAAT, expression of hAAT was driven by the Mo-MLV LTR. In Pol-II-hAAT, transcription could proceed from either the LTR or the internally located Pol-II promoter. The IRES (30) allowed translation of the mutant murine dihydrofolate reductase gene to occur in a cap-independent fashion and ensured that it could be translated equally well from either a full-length or an internally initiated transcript. Amphotropic producer clones had $Mtx^R$ titers of $1 \times 10^6$ colony-forming units (cfu)/ml (for Pol-II-hAAT) and $2 \times 10^5$ cfu/ml (for LTR-hAAT) as determined on NIH 3T3 cells. After transduction with Pol-II-hAAT or LTR-hAAT at low multiplicity of infection, pools of $Mtx^R$ Hepa A1 cells expressed hAAT at 126 and 491 ng/24 hr per $10^6$ cells, respectively. Southern blots of DNA from transduced NIH 3T3 fibroblasts demonstrated appropriate-sized bands for vector DNA (data not shown).

**Effect of Retrovirus Concentration on *in Vivo* Hepatocyte Transduction.** We reasoned that *in vivo* transduction frequency might be improved by increasing the number of retroviral particles injected. Our ultracentrifugation protocol resulted in a 24-fold concentration by volume, and a 10-fold increase in $Mtx^R$ titers (cfu/ml). When delivered *in vivo*, concentrated retrovirus yielded a 7-fold (Pol-II-hAAT) or 31-fold (LTR-hAAT) increase in serum hAAT as compared with an unconcentrated batch of the same viral preparation (Fig. 2), demonstrating that *in vivo* transduction frequencies are proportional to the number of retroviral particles delivered. Although the cellular Pol-II promoter appeared to yield higher serum hAAT expression (143 ± 47 ng/ml; mean ± SEM) than the viral LTR promoter (50 ± 25 ng/ml), the difference was not statistically significant [$P > 0.10$; Student's *t* test for unpaired data (two-tailed) (31)]. These levels represent 1/7000 (Pol-II) and 1/20,000 (LTR) of the serum hAAT level in normal humans (≈1 mg/ml) (32). Portal vein injection of concentrated Pol-II retrovirus into rats at the time of partial hepatectomy, or into rats which did not receive a hepatectomy, resulted in no detectable hAAT at 1 week after injection (data not shown), demonstrating that serum hAAT is dependent upon replication of hepatocytes at the time of injection. Control rats which received a β-galactosidase-

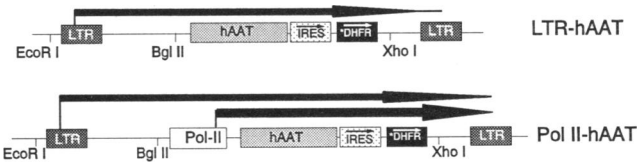


FIG. 1. Construction of retroviral vectors. RS8391 is an LNL6-derived retroviral vector which contains the IRES upstream of a mutant dihydrofolate reductase gene, *DHFR. After partial digestion with *Eco*RI, a 1.3-kb *Eco*RI fragment containing the hAAT cDNA (28) was inserted 5′ to the IRES to produce LTR-hAAT. pHB-II (29) was digested with *Hin*dIII (cuts at the 5′-end of the Pol-II promoter), blunted, ligated with *Bgl* II linkers, and cut with *Bgl* II and *Bam*HI (cuts 90 bp 3′ to the transcriptional start site). This 700-bp fragment was inserted into the *Bgl* II site of LTR-hAAT to create Pol-II-hAAT. Arrows indicate that transcription can initiate from the LTR or from the internal Pol-II promoter.

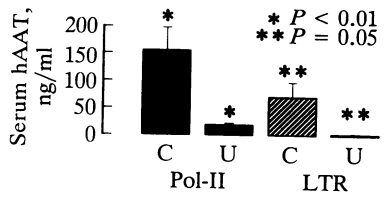


FIG. 2. Effect of retrovirus concentration on *in vivo* hepatocyte transduction. Retrovirus was concentrated 10-fold by ultracentrifugation and spiked with Polybrene at a final concentration of 40 μg/ml. *In vivo* hepatocyte transductions were performed with 5 ml of concentrated or unconcentrated virus. Serum hAAT was measured at 1 week after transduction and plotted (mean ± SEM) for rats receiving concentrated (C) or unconcentrated (U) Pol-II-hAAT or LTR-hAAT retrovirus. *P* values are for each C vs. U. Long-term survival was 85% for animals receiving concentrated or unconcentrated retrovirus.

containing retrovirus had no detectable serum hAAT (data not shown).

**Long-Term hAAT Expression After *in Vivo* Transduction.** Rats which received concentrated retrovirus in the above experiment were followed long term for serum hAAT expression (Fig. 3). Of the six surviving Pol-II-hAAT rats, two had hAAT decline to <3 ng/ml, while the other four maintained detectable levels; the average serum hAAT at 1 week was 122.6 ± 46.6 ng/ml, and at 1 year it was 37.7 ± 13.2 ng/ml, representing a statistically insignificant ($P > 0.10$) decrease to 31% of the initial level. Serum hAAT fell to <3 ng/ml in two of five rats that received LTR-hAAT retrovirus. For these five animals, the average serum hAAT at 1 week was 50 ± 25 ng/ml and at 1 year was 13.98 ± 9.714 ng/ml. This represents a statistically insignificant ($P > 0.11$) decline to 27.8% of the initial level of expression.

**Liver hAAT mRNA Levels in Transduced Rodents.** To quantitate the relationship between hAAT mRNA and serum protein levels, we performed an RNase protection assay on liver RNA isolated from transduced rats (Fig. 4). Standards were created by using various amounts of human liver RNA (*B*, lanes 4–13). Pools of LTR-hAAT-transduced $Mtx^R$ Hepa A1 cells had 20% as much hAAT mRNA per μg of total RNA as human liver (*B*, lanes 14 and 15), while Pol-II-hAAT-transduced Hepa A1 cells had 100% as much (*C*, lanes 3 and 4). This demonstrated that cultured cells carrying a single copy of retrovirus per diploid genome expressed nearly as much hAAT mRNA per μg of total RNA as human liver cells *in vivo*. RNA isolated from nontransduced rat liver had no detectable hAAT mRNA (*C*, lane 5).

The hAAT signal in 100 μg of liver RNA from LTR-hAAT rats (Fig. 4*B*, lanes 16–19) or Pol-II-hAAT rats (*C*, lanes 4–14) was as high as that found in 0.01 or 0.05 μg, respectively, of human liver RNA, leading to an estimated hAAT RNA level of 1/10,000 or 1/2000, respectively, that of normal human liver. This leads us to conclude that low levels of hAAT mRNA are responsible for the low hAAT protein production.

The above RNase protection assay does not differentiate between LTR- and Pol-II-initiated transcripts. To define the transcription initiation site, a second RNase protection assay was used in which the probe spanned the Pol-II initiation site (Fig. 4*D*). More than 90% of transcripts initiate from the LTR promoter in the packaging cell line (Fig. 4*E*, lane 3), or in transduced cells (data not shown). In contrast, RNA derived from a Pol-II-hAAT-transduced rat liver showed similar levels of initiation from the Pol-II promoter and from the LTR. Thus Pol-II-initiated transcripts represent a much higher fraction of total transcripts *in vivo* as compared with *in vitro*.

**PCR Quantitation of Proviral DNA in Transduced Rodents.** To quantitate transduction efficiency, we isolated genomic DNA from rat liver biopsy samples and used multiplex PCR to amplify proviral (IRES) and rat genomic (LFABP) DNA sequences, and amplification was followed by Southern blotting (Fig. 5). As expected, nontransduced rat liver DNA had a signal after probing with LFABP but not IRES (lane 2). To create standards with known proportions of retroviral DNA, DNA was isolated from an LTR-hAAT-transduced NIH 3T3 clone which contained (by Southern blot) a single copy of proviral DNA per cell, and this was diluted with normal rat genomic DNA (lanes 2–9). For these standards, the ratio of IRES to LFABP signal increased linearly as the relative copy number of LTR-hAAT DNA increased. The rat genomic DNA signal (LFABP) was not saturated when 50 ng of DNA was added to the PCR mixture, as both the IRES and LFABP signals increased proportionately when the mass of NIH 3T3 cell DNA (containing 0.3% LTR-hAAT DNA) was increased from 25 to 100 ng (lanes 8 and 9).

PCRs were also performed on liver DNA from transduced rats (Fig. 5, lanes 10–14). The ratio of IRES to LFABP signal was compared with the standard curve to determine the integration frequency of proviral DNA. In contrast to the relatively low amounts of hAAT protein and mRNA, proviral DNA frequency varied from 5% to 15% for the Pol-II-hAAT rats. Transduction frequencies for the LTR-hAAT rats were somewhat lower at 0.2–1.0%. Table 1 summarizes the amounts of proviral DNA, hAAT mRNA, and serum hAAT observed in several transduced rodents. These results indicated that (*i*) high transduction frequencies could be attained with our method and (*ii*) the lower expression in the LTR-hAAT rats as compared with the Pol-II-hAAT rats was due to less frequent proviral integration.

## DISCUSSION

Hepatic gene therapy is increasingly attractive, given the recent success of both *ex vivo* (4, 5) and *in vivo* (6–17) transduction protocols. Its widespread application would be facilitated by a delivery system which avoids large-scale hepatocyte culture or a complicated asanguineous liver perfusion system. We have previously reported an *in vivo* hepatocyte transduction protocol which entails 70% hepatectomy followed 24 hr later by portal vein injection of a retroviral vector during hepatic in-flow occlusion (11). In this study, we used vectors bearing the hAAT gene in the

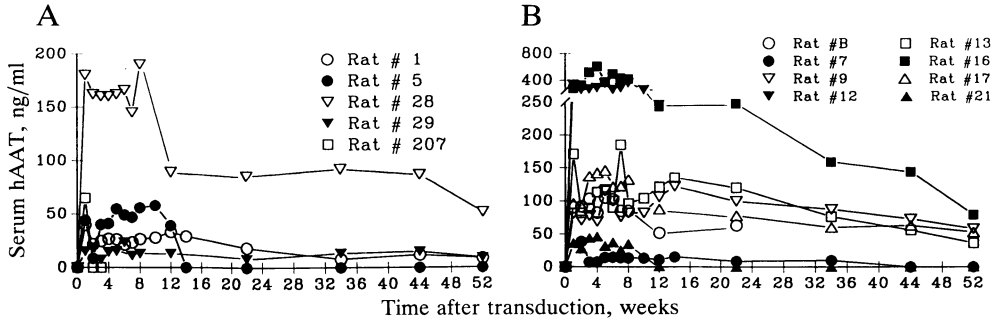


FIG. 3. Long-term hAAT expression after *in vivo* hepatocyte transduction. Rats which received concentrated retrovirus (Fig. 2) were followed long term for serum hAAT expression. (*A*) Rats received $1 \times 10^7$ cfu of LTR-hAAT vector ($n = 5$). (*B*) Rats received $5 \times 10^7$ cfu of Pol-II-hAAT vector ($n = 8$).

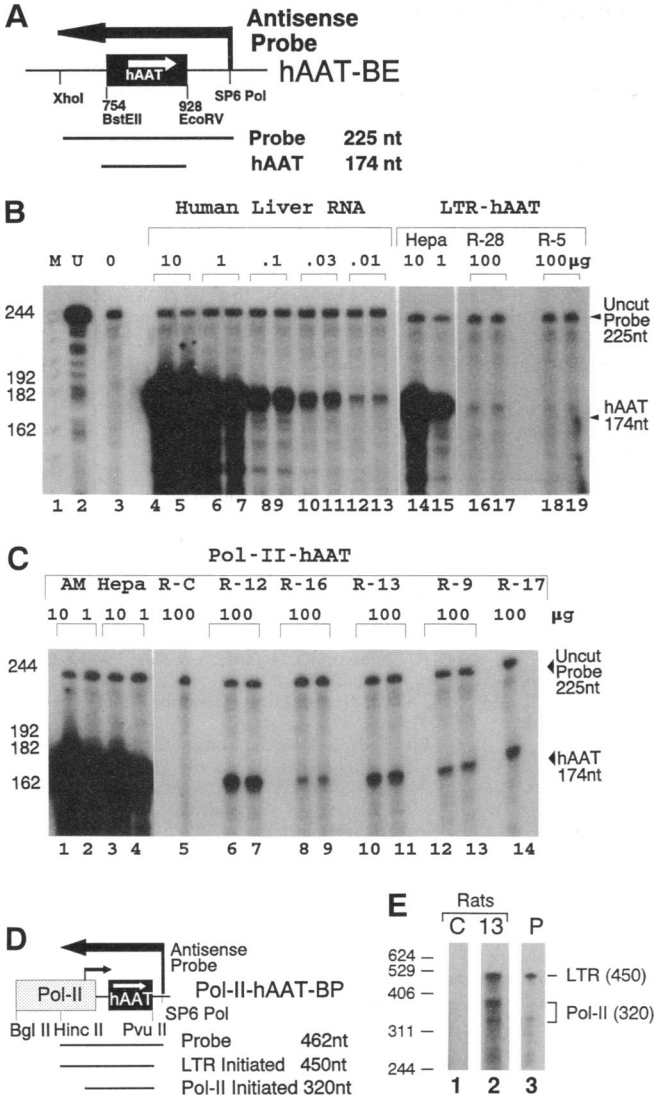


FIG. 4. RNase protection assay of RNA from rat liver and cultured cells. (*A*) Schematic diagram of the hAAT-BE probe. An antisense RNA probe specific for hAAT results in a 174-nt protected fragment after hybridization with hAAT mRNA and digestion with single-strand-specific RNases. (*B*) Protection assay using the hAAT-BE probe. Total rat liver RNA was isolated from biopsy samples obtained at 10–14 weeks after transduction and hybridized with the hAAT-BE probe. A single RNase protection assay was performed for all samples. The amount of specific RNA used is designated above each lane; the total RNA used in the protection assay was brought up to 100 µg by adding tRNA if necessary. Lane 1 shows molecular weight DNA markers (length in nt shown on the left), lane 2 depicts the undigested probe, and lane 3 shows probe which was hybridized with 100 µg of yeast tRNA and digested with RNase. Lanes 4–13 represent various amounts of human liver RNA standards. RNA obtained from a pool of Hepa A1 cells containing one copy of LTR-hAAT retrovirus per cell was used in lanes 14 and 15. Samples of liver RNA from two rats transduced *in vivo* with the LTR-hAAT vector are shown in lanes 16–19. (*C*) Protection assay using the hAAT-BE probe. Lanes 1 and 2 represent GP+*env*AM12-derived RNA, and lanes 3 and 4 show a pool of single-copy Pol-II-hAAT-transduced Hepa A1 cell RNA. Control rat liver RNA is shown in lane 5, and lanes 6–14 represent liver RNA from five rats transduced *in vivo* with the Pol-II hAAT vector. (*D*) Schematic diagram of the Pol-II-hAAT-BP probe. Transcripts which initiate from the LTR promoter result in a 450-nt protected band after RNase protection, while those which initiate from the internal Pol-II promoter generate a 320-nt protected band. (*E*) RNase protection assay using Pol-II-hAAT-BP probe. The probe shown in *D* was used in an RNase protection assay. The Pol-II-hAAT retroviral packaging cell line contained primarily full-length (LTR-initiated) transcript (lane 3). RNA from a control rat (C; lane 1) had no protected band, while RNA from a Pol-II retroviral transduced rat (rat 13; lane 2) had approximately equal levels of initiation from the LTR and the internal promoter.

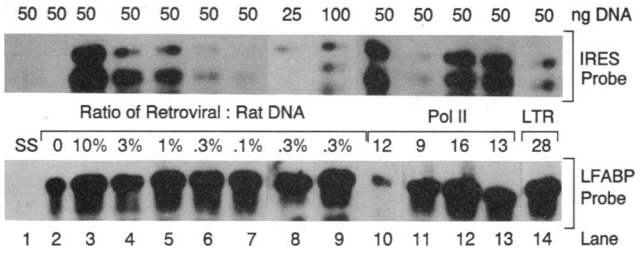


FIG. 5. PCR assessment of provirus integration frequency. Liver DNA was isolated from rats 4–5 months after *in vivo* transduction. Multiplex PCR was performed with IRES (detects retroviral sequences) and LFABP (detects rat chromosomal DNA sequences) oligonucleotide primers. Amplified sequences were electrophoresed and transferred to a nylon membrane, where they were hybridized first with an IRES probe (48-hr autoradiogram) and then with an LFABP probe (6-hr autoradiogram). Lane 1 represents control (salmon sperm) DNA; lanes 2–9 represent the PCR standards prepared by mixing various proportions of single-copy LTR-hAAT DNA with nontransduced rat liver DNA. Lanes 10–13 show DNA obtained from rats transduced with the Pol-II-hAAT retrovirus. Lane 14 shows DNA from a rat which was transduced with the LTR-hAAT retrovirus.

optimization of our delivery system and the quantitation of transduction frequency and long-term gene expression.

Initial experiments were aimed at optimizing *in vivo* transduction frequencies. A 10-fold increase in *in vitro* retroviral titers after concentration led to a similar enhancement of *in vivo* hAAT expression for both the LTR-hAAT and Pol-II-hAAT vectors (Fig. 2). No hAAT expression was observed if retrovirus was delivered when hepatocytes were not dividing, demonstrating that expression is due to transduced liver cells. These results indicate that delivery of concentrated retrovirus is safe and that the amount of retrovirus delivered to the regenerating liver is a limiting factor.

For the Pol-II-hAAT retrovirus, we estimate that $5 \times 10^7$ retroviral particles were delivered to approximately $3 \times 10^8$ regenerating hepatocytes, giving a calculated ratio of retroviral particles to hepatocytes of 1:6. DNA analysis demonstrated that Pol-II-hAAT vectors achieved an average provirus integration frequency of 10% (Fig. 5; Table 1). Although these transduction frequencies were determined by using PCR, we believe they are valid because (*i*) internal standards were used to normalize for PCR efficiency; (*ii*) Southern blots of genomic DNA from rats 12 and 13 (without PCR) confirmed a provirus DNA frequency of ≈10% (data not shown); and (*iii*) these frequencies are consistent with our results with a retrovirus of lower titer containing the β-galactosidase gene (11). Our transduction efficiency is higher than that reported for intraparenchymal injection (0.6%) (7), portal vein injection without occlusion of incoming blood flow (1%) (10), and the *ex vivo* approach (1%) (4, 5), and it is similar to the efficiencies attained with a complicated asanguineous liver perfusion technique (1–5%) (8, 9).

The second major goal of these studies was to develop methods of quantitating expression from different promoter elements *in vivo*. Although the Pol-II-hAAT retroviral vector yielded 3- to 5-fold higher serum hAAT and liver hAAT mRNA levels than LTR-hAAT, these differences reflect the higher transduction frequency observed for Pol-II-hAAT, which is consistent with its 5-fold higher *in vitro* titers. Although the level of expression was maintained in most animals for at least 1 year, we estimated that expression per integrated copy of retrovirus was only 1% as high *in vivo* as in transduced tissue culture cells *in vitro*.

Table 1. Serum hAAT protein, hAAT mRNA, and proviral DNA in transduced rats

| Rat no. | hAAT protein in serum ng/ml | hAAT protein in serum %§ | RNA, %* | DNA, %† | DNA/RNA‡ |
|---|---|---|---|---|---|
| | | Pol-II-hAAT rats | | | |
| 12 | 270 | 0.027 | 0.03 | >15 | 667 |
| 13 | 100 | 0.01 | 0.02 | 15 | 750 |
| 16 | 250 | 0.025 | 0.01 | 4.6 | 460 |
| 17 | 90 | 0.009 | 0.02 | 6 | 300 |
| | | LTR-hAAT rats | | | |
| 5 | 40 | 0.004¶ | 0.0025 | 0.2 | 80 |
| 28 | 100 | 0.01 | 0.005 | 1 | 200 |

*Percentage of hAAT mRNA in transduced rat liver (or Hepa A1 cells) as compared with normal human liver standards (Fig. 4).
†Percentage of hepatocytes transduced with retrovirus as determined by four separate experiments performed in duplicate (Fig. 5).
‡Ratio of DNA to RNA (column 5 ÷ column 6).
§Percentage relative to human serum (Fig. 3).
¶Fell to zero.

Several investigators have demonstrated that strong viral promoters including the CMV (5, 10, 33) and Mo-MLV LTR (34) can be inactivated *in vivo* in fibroblasts (33, 34) or hepatocytes (5, 10). In contrast, the LTR promoter has directed expression of β-galactosidase (8) and neomycin resistance (7) for up to 100 days in animal livers, although the significant increase in expression upon the addition of 5-azacytidine suggested that the LTR promoter was attenuated (but not inactivated) by DNA methylation *in vivo* (7). Our results suggest that attenuation and/or inactivation of the LTR does indeed occur *in vivo* in rat hepatocytes. For all animals, expression of the LTR in hepatocytes *in vivo* is only ≈1% of that observed in cultured tissue cells. In addition, we noted that two of five LTR-hAAT rats (Fig. 3*A*) lost expression within 3 months after transduction, although PCR analysis of liver DNA revealed proviral DNA frequencies of 0.1–0.3% (data not shown). We conclude that the LTR promoter is a poor choice for achieving high level *in vivo* expression in liver cells.

The use of constitutive cellular promoters might circumvent the viral promoter attenuation problem. For example, Scharfmann *et al.* (33) showed that the dihydrofolate reductase promoter was expressed long term in fibroblasts *in vivo*, while the CMV promoter was not. Similarly, Chowdhury *et al.* (4) observed persistent *in vivo* expression from the chicken β-actin promoter. The Pol-II-hAAT retrovirus used in our studies can initiate transcription from either the 5′ LTR or the internal Pol-II promoter. Fig. 4*E* suggests that much of the overall decrease in expression in Pol-II-hAAT rats is due to attenuation from the LTR promoter and that expression from the internal Pol-II promoter (albeit weak) is relatively constant. This is consistent with the results of Soriano *et al.* (35), who found that an internal promoter was not influenced by inactivation of the upstream LTR promoter in embryonic stem cells.

Liver-specific promoters may further increase *in vivo* expression from a retroviral vector. Kay *et al.* (10) tested *in vivo* hAAT expression from a retroviral vector carrying a 2.8-kb liver-specific murine albumin promoter-enhancer. They demonstrated serum hAAT levels of ≈500 ng/ml, which suggests that the mouse albumin promoter/enhancer construct is 5- to 10-fold stronger than the Pol-II and Mo-MLV LTR promoters, if indeed the provirus integration frequency is 1%. In contrast, the phospho*enol*pyruvate carboxykinase promoter led to low levels of expression from a retroviral (6) or a plasmid (17) vector.

These results lead us to conclude that an hAAT gene can be efficiently delivered to hepatocytes *in vivo* and expressed long term. Although up to 15% of hepatocytes can be transduced, the highest level of expression obtained (≈300 ng/ml) is only a fraction of that required for gene therapy of $\alpha_1$-antitrypsin deficiency. This suggests that increasing expression from a retroviral vector by identifying strong promoters should be a major focus of gene therapy research. The use of our portal vein injection/hepatic in-flow occlusion method will allow rapid assessment of multiple promoters *in vivo* and might be applicable for gene therapy in humans once safety concerns are adequately addressed.

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