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Retroviral Vector Sequences May Interact with Some Internal Promoters and Influence Expression

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ABSTRACT

Although retroviral vectors show promise for gene therapy, their expression in animals has been low. An improved understanding of how promoters function from a retroviral vector should facilitate the design of improved vectors. In this study, liver-specific promoters were cloned into a retroviral vector and expression from the retroviral long terminal repeat (LTR) and the internal promoter was analyzed. In addition, oligomerized liver-specific transcription factor binding sites were placed upstream of each promoter in an attempt to increase expression further. Additional oligomerized binding sites only increased expression slightly or inhibited expression in hepatoma cells, suggesting that this is not an effective way to increase expression from a retroviral vector. Unexpectedly, the liver-specific albumin promoter was expressed at high levels from a retroviral vector in fibroblasts, suggesting that retroviral elements functioned as an enhancer. Furthermore, the addition of HNF-4 binding sites adjacent to the albumin promoter inhibited both the LTR and albumin promoter in fibroblasts, an effect that was probably mediated by inhibitory proteins present in nonhepatic cells that can bind to HNF-4 sites. These results suggest that both positive and negative influences can be transmitted between the LTR and the albumin promoter. In contrast, the liver-specific human α_1-antitrypsin promoter did not appear to interact with the LTR by either of these criteria. Retroviral vectors have sequences that may inhibit expression of the LTR and some internal promoters *in vivo*. We hypothesize that internal promoters that do not interact with the LTR in tissue culture will be resistant to inhibitory effects of retroviral sequences *in vivo*.

OVERVIEW SUMMARY

Although retroviral vectors can result in long-term expression in hepatocytes of animals, they are poorly expressed *in vivo*. An understanding of how an internal promoter functions from a retroviral vector should lead to the development of improved vectors. The results presented here provide evidence that some promoters such as the albumin promoter can interact with retroviral elements. This interaction results in the activation of the liver-specific albumin promoter in fibroblast-derived cells and the transmission of a negative influence located just upstream of the albumin promoter to the LTR promoter. In contrast, other promoters such as the α_1-antitrypsin promoter do not appear to interact with retroviral sequences. There are several elements in and adjacent to the LTR that inhibit expression in some cells. We hypothesize that an inhibitory effect will only be transmitted to internal promoters which can interact with the LTR.

INTRODUCTION

RETROVIRAL VECTORS CAN BE TRANSFERRED into hepatocytes of animals by using *ex vivo* or *in vivo* gene transfer methods (Hatzoglou *et al.*, 1990; Kaleko *et al.*, 1991; Kay *et al.*, 1992a,b, 1993; Rettinger *et al.*, 1994; Hafenrichter *et al.*, 1994). Although expression is stable for over 2 years, the level of expression per integrated copy has been disappointingly low. It was initially presumed that strong viral promoters would result in high-level expression in hepatocytes *in vivo*, because they are extremely potent in primary hepatocytes (Ponder *et al.*, 1991). It was found, however, that the cytomegalovirus (CMV) and the long terminal repeat (LTR) promoters were expressed poorly from a retroviral vector in hepatocytes of animals (Kaleko *et al.*, 1991; Kay *et al.*, 1992a,b, 1993; Rettinger *et al.*, 1994). Similarly, the SV40 and the CMV promoters are relatively inactive in fibroblasts *in vivo* (Palmer *et al.*, 1991; Scharfmann *et al.*, 1991). Although expression from housekeeping promoters is more stable *in vivo* (Scharfmann *et al.*,

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1991), the level of expression has been low, presumably because they are relatively weak promoters. Strong liver-specific promoters might result in higher-level expression from a retroviral vector in hepatocytes of animals. Little is known, however, regarding whether or not such promoters can be packaged into a retroviral vector and how well they are expressed *in vivo*.

Our laboratory is interested in using retroviral vectors for hepatic gene therapy. This study was performed to improve our understanding of how promoters function from a retroviral vector, which is currently the best way to achieve stable transfer of genes into the livers of animals. Two liver-specific promoters, mouse albumin (mAlb) and human α_1-antitrypsin (hAAT), and one ubiquitously expressed promoter, the promoter for the large subunit of RNA polymerase II (Pol-II), were inserted into an internal position of a retroviral vector upstream of a reporter gene. Oligomers containing binding sites for liver-specific transcription factors were placed immediately upstream of the internal promoter, and expression from both the LTR and the internal promoter was analyzed. These results provide insights into how the addition of liver-specific transcription factor binding sites affects the expression of different promoters from a retroviral vector, and into how these promoters interact with the LTR.

MATERIALS AND METHODS

Cloning of vectors containing basal promoters

LTR-ΔLTR is derived from the Moloney murine leukemia virus retroviral vector LNL6 (Miller and Rosman, 1989) and has been described previously (Hafenrichter *et al.*, 1994). A DNA fragment containing nucleotides −347 to +56 of the hAAT promoter and a DNA fragment containing nucleotides −810 to +8 of the mouse albumin (mAlb) promoter were cloned upstream of the hAAT cDNA to create hAATΔLTR and mAlbΔLTR, respectively, as described (Hafenrichter *et al.*, 1994). Pol-II-ΔLTR contains the 700-bp promoter for the large subunit of RNA polymerase II (Ahearn *et al.*, 1987) upstream of the hAAT cDNA in a 3′ LTR enhancer-deleted retroviral vector. It was created by ligating the 4.5-kb *Pvu* I–*Xho* I fragment of Pol-II-hAAT [contains the 5′ portion of the retroviral vector and the Pol-II promoter in an LTR-intact backbone (Rettinger *et al.*, 1994)], with the 2.4-kb *Xho* I–*Pvu* I fragment of LTRΔLTR [contains the 3′ portion of this 3′ LTR-deleted retroviral vector (Hafenrichter *et al.*, 1994)].

Addition of transcription factor binding sites upstream of hAAT, mAlb, and Pol-II promoters

Complementary oligonucleotides containing an HNF-1 binding site were synthesized as shown in Fig. 1. These were treated with kinase, annealed, and ligated in a 30-fold molar excess into the *Eco* RI site of pSP72 (Promega, Madison WI). HNF-1_2-SP72 contains two copies of the HNF-1 binding site as direct repeats. A 103-bp DNA fragment containing the HNF-1 binding sites was removed from HNF-1_2 -SP72 by *Bgl* II/*Bam* HI digestion. Complementary oligonucleotides with an HNF-4 binding site were synthesized as shown in Fig. 1. These were treated with kinase, annealed, and ligated in a 10-fold molar excess into the *Sal* I site of the pSP72-derived vector MC3-BP.

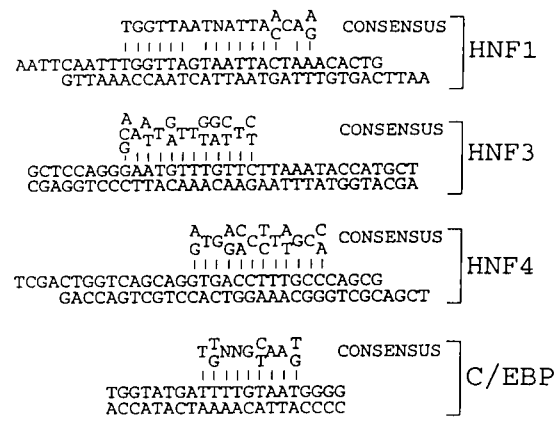

FIG. 1. DNA sequences of liver-specific transcription factor binding sites. A monomer of each of the liver-specific transcription factor binding sites that were analyzed in this study are shown in the orientation in which they occur in the retroviral vector. The HNF-1 binding site contains nucleotides −358 to −342 of the human albumin promoter (Frain *et al.*, 1990). The HNF-4 binding site contains nucleotides −66 to −87 of the Apo C3 promoter (Costa *et al.*, 1990). The HNF-3 binding site is derived from the mouse albumin enhancer (DiPersio *et al.*, 1991). The C/EBP binding site is derived from the D-site at nucleotides −116 to −95 of the mouse albumin promoter (Mueller *et al.*, 1990). Consensus sequences for HNF-1 (Frain *et al.*, 1989), HNF-3 (Overdier *et al.*, 1994), HNF-4 (Sladek *et al.*, 1990), and C/EBP (McKnight, 1992) binding sites are shown.

Although HNF-4_2-MC3-BP contains three copies of the HNF-4 binding site as direct repeats, there is a 1-bp deletion in the 5′ copy (5′-CAGG_GACCTTT-3′), as determined by DNA sequencing, resulting in a construct with only two functional HNF-4 sites. HNF-4_2-MC3-BP was digested with *Hinf* I, which cuts just 3′ of the insert, blunt-ended with Klenow, ligated with *Pst* I linkers, and digested with *Pst* I, which also cuts just upstream of the insert, to generate a 107-bp fragment containing two functional copies of the HNF-4 binding site. This was cloned into the *Pst* I site of HNF-4_2-MC3-BP to generate HNF-4_4-MC3-BP. The 255-bp fragment containing four functional copies of the HNF-4 binding site was removed from HNF-4_4-MC3-BP by *Bam* HI/*Bgl* II digestion. $p_{(D)9}$-CAT contains nine copies of the D-site of the mouse albumin promoter (Mueller *et al.*, 1990), which were removed as a 232-bp fragment by *Bam* HI/*Bgl* II digestion. Three and one-half copies of the HNF-3 binding site from the mouse albumin enhancer were amplified from pAT2-eG3x (DiPersio *et al.*, 1991) by using the polymerase chain reaction as described previously (Hafenrichter *et al.*, 1994) and cloned into pSP72 to create HNF-3_3-SP72. A 237-bp band containing three HNF-3 binding sites was removed from HNF-3_3-SP72 by *Bam* HI/*Bgl* II digestion. All transcription factor binding sites were sequenced to demonstrate that they contained the correct sequence as direct repeats. mAlbΔLTR, hAATΔLTR, and Pol-IIΔLTR were each linearized with *Bgl* II, which cuts immediately upstream of the internal promoter. The fragments containing the oligomerized liver-specific transcription factor binding sites de-

scribed above were inserted to create the retroviral vectors containing additional transcription factor binding sites.

Tissue culture

The amphotropic retroviral packaging cell line GP + AM12 (Markowitz *et al.*, 1988) and NIH-3T3 fibroblast cells (Jainchill *et al.*, 1969) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% supplemented calf serum (Hyclone, Logan, UT), penicillin (100 U/ml)/streptomycin (100 μg/ml) (P/S) at 37°C. Hepa1A cells (Darlington, 1987) were maintained in 75% minimal essential medium (MEM), 25% Waymouth's medium (Hazelton, St. Lenexa, KS), 10% supplemented serum, and P/S. For selection, cells were given the appropriate basal medium with 10% dialyzed calf serum (Sigma Chemical, St. Louis, Mo.) and 250 n*M* methotrexate (MTX; Sigma Chemical).

Transfection of GP+AM12 cells and infection of NIH-3T3 and Hepa1A cells

Ten micrograms of retroviral vector DNA was transfected into 50% confluent GP+AM12 packaging cells by using the calcium phosphate method (Miller and Rosman, 1989). In some cases, packaging cell lines were selected with methotrexate, and virus from resistant clones was used to transduce cells. In other cases, the retrovirus was collected from a transient transfection of packaging cells. Conditioned medium was collected from the packaging cells after a 12-hr incubation, passed through a 0.45-μm filter, and 100–500 μl of retrovirus added to 20,000 NIH-3T3 or Hepa1A cells on a 24-well plate at 50% confluency. Polybrene (Sigma Chemical) was present at a final concentration of 8 μg/ml. Twelve hours after addition of the virus, the cells received fresh medium, and 26 hr after the addition of virus, the cells were trypsinized and the entire contents transferred to a 10-cm plate and selected with Mtx. Controls consisted of noninfected cells. Plates containing 5–100 colonies were analyzed further. For each plate, up to six colonies were picked and assayed for hAAT protein in the supernatant by using an hAAT ELISA (Hafenrichter *et al.*, 1994). When the majority of the colonies produced hAAT, all colonies on an individual plate were pooled and grown on 10-cm plates.

Isolation of RNA

Cells were washed with PBS and suspended in 4 ml of 4 *M* guanidinium-thiocyanate (Fluka Biochemika, Buchs, Switzerland) and total RNA isolated as described (Chomczynski and Sacchi, 1987). In some cases, mRNA was hybrid selected with an oligo(dT)-Sepharose column (Collaborative Research Inc., Bedford, MA) as recommended by the supplier.

Northern Blotting of RNA

RNA was electrophoresed through a 1% formaldehyde denaturing agarose gel, transferred to a nitrocellulose membrane (BA-S-NC, Schleicher & Schuell, Keene, NH), hybridized with probe, and washed as recommended by the manufacturer. All DNA fragments used as a template to prepare radiolabeled probes were gel-purified and labeled to a specific activity of 10^8 cpm/μg with [α-^{32}P]dCTP using a random primer labeling kit (New England Nuclear, Boston, MA). A 592-bp *Xho* I–*Eco* RI fragment of the IRES DNA (Ghattas *et al.*, 1991) was used to detect retroviral transcripts. A 980-bp *Eco* RI–*Sal* I fragment containing 596 nucleotides from the 3′ end of the human 28S rRNA was removed from pES-28S (Parker and Bond, 1989) and used to detect 28S. IRES and 28S rRNA probes were hybridized to membranes at 42°C in 50% formimide and the final wash performed at 65°C with 0.1 × SSC (15 m*M* NaCl, 15 m*M* sodium citrate). For quantitation of mRNAs for liver-specific transcription factors in hepatoma and control cells, polyadenylated RNA was electrophoresed, transferred, and hybridized with a 2-kb *Xho* I fragment of mouse HNF-1α obtained from mHNF-1-BS (Kuo *et al.*, 1990), a 1.6-kb *Bam* HI fragment of rat HNF-3α obtained from pH3αBamHI (Lai *et al.*, 1990), a 2.3-kb fragment of rat HNF-4 obtained from pLEN4S (Sladek *et al.*, 1990), or a 900-bp *Pst* I fragment of rat C/EBPα obtained from pMSV-C/EBP-wt (Friedman *et al.*, 1989). Membranes were hybridized at 42°C in 50% formimide, and the final wash was performed at 60°C with 0.1× SSC.

RNase protection assay

hAAT-ΔLTR was digested with *Bgl* II (cuts at the 5′ end of the hAAT promoter) and with *Pvu* II (cuts at +230 of the hAAT cDNA) and cloned into the RNA expression vector pSP72 to create PB-hAAT-SP72. Pol-IIΔLTR was digested with *Hinc* II (cuts at −132 of the Pol-II promoter) and with *Pvu* II (cuts at +230 of the hAAT cDNA) and cloned into pSP72 to generate PBH-Pol-II-SP72. PB-hAAT-SP72 was linearized with *Bst* EII (cuts at −80 of the hAAT promoter) and PBH-Pol-II-SP72 was linearized with *Bsp* 1286 I (cuts at +633 of the pSP72 vector). Each was transcribed with SP6 RNA polymerase and [α-^{32}P]GTP (Melton *et al.*, 1984) to generate antisense probes with a specific activity of 6×10^8 cpm/μg that span the internal promoter transcription initiation site. To generate the "positive sense" RNAs, PB-hAAT-SP72 and PBH-Pol-II-SP72 were linearized with *Xho* I (cuts downstream of the hAAT cDNA), transcribed with T7 RNA polymerase in the presence of tracer amounts of [α-^{32}P]GTP, and the 645- and 938-nucleotide bands, respectively, were gel-purified on a 4% polyacrylamide/8 *M* urea gel and eluted. For RNase protection assays, 1×10^5 cpm of the antisense probe was mixed with 10 μg of total RNA per sample under standard conditions (Melton *et al.*, 1984), heated to 80°C, and slow-cooled to 49°C over 2 hr. When less than 10 μg of sample RNA was added, the total amount of RNA was brought to 10 μg by using yeast tRNA. After 3–16 hr of incubation, samples were digested with 1 unit/ml of RNase T_1 (Calbiochem, La Jolla, CA) and 1 μg/ml of RNase A (United States Biochemical, Cleveland, OH) for 60 min at 37°C, extracted with phenol, and precipitated with ethanol. Protected fragments were electrophoresed on a 6% polyacrylamide/8 *M* urea/0.5× Tris-borate EDTA gel, and bands were quantitated on a Betascope 630 two-dimensional Beta counter (Betagen, Waltham, MA).

RESULTS

Generation of retroviral vectors

Retroviral vectors containing three different basal promoters with or without additional oligomerized liver-specific tran-

scription factor binding sites were transfected into an amphotropic retroviral packaging cell line and used to transduce fibroblast and hepatoma cells in tissue culture. Expression was quantitated by an RNase protection assay on RNA from pools of singly transduced cells that were obtained by methotrexate selection. The Mtx^R gene is translated from the downstream position of a dicistronic mRNA by virtue of the internal ribosome entry site sequence (IRES) and should be expressed equally well from LTR- or internal promoter-initiated transcripts. Quantitation of expression requires that cells are transduced with only one copy of the retrovirus and that a pool of cells be observed, because individual integration sites will affect the level of expression. Because the number of Mtx^R colonies per plate was 10-fold less than the number of cells infected, it is unlikely that an individual cell was transduced with more than one copy of the retrovirus. Conversely, it is unlikely that the pools were contaminated with uninfected cells: Although a small number of Mtx^R colonies were occasionally observed in nontransduced cells, expression was only quantitated from plates where the transduced cells had >10-fold more colonies than noninfected cells. Furthermore, plates were only used if >83% of the colonies expressed hAAT. Finally, Southern blot analysis of pools of transduced cells demonstrated one copy of the retrovirus per cell (data not shown).

hAAT Promoter

The hAAT promoter is expressed primarily in the liver (Shen *et al.*, 1987). It contains HNF-1 and HNF-4 binding sites, both of which are critical for expression *in vitro* (Li *et al.*, 1988; Monaci *et al.*, 1988). Retroviral vectors containing the basal hAAT promoter with or without additional transcription factor binding sites are shown in Fig. 2. Pools of singly transduced cells were selected with Mtx, and RNA from these cells was analyzed by an RNase protection assay, as diagrammed in Fig. 3A. Expression in Hepa1A cells is shown in Fig. 3B, while expression in NIH-3T3 cells is shown in Fig. 3C. Quantitation of the results for both cell lines was determined by 2-dimensional phosphorimaging and is shown in Fig. 3D.

The hAAT promoter represents only 15% of all transcripts in hAATΔLTR-transduced Hepa1A cells (Fig. 3B, lanes 6–8), suggesting that the hAAT promoter is weaker than the ΔLTR in these cells. Addition of HNF-3 sites led to a seven-fold increase in the absolute number of transcripts initiated from the hAAT promoter. In contrast, addition of HNF-1 sites decreased expression from the hAAT promoter to a level that was only 15% that observed for hAAT-ΔLTR. Addition of HNF-4 sites had no significant effect upon expression. Addition of C/EBP sites led to ~two-fold increase in expression for the sample shown, although RNA from three other pools tested contained aberrantly sized bands on Northern blot and RNase protection assays, suggesting that rearrangements occurred (data not shown). Addition of HNF-1, HNF-3, or HNF-4 sites had no statistically significant effect upon the number of LTR-initiated transcripts in Hepa1A cells as compared with Hepa1A cells transduced with hAATΔLTR. Thus, the positive and negative effect observed after addition of HNF-3 and HNF-1 sites, respectively, upon the hAAT promoter was not transmitted to the LTR promoter.

As expected, the liver-specific hAAT promoter was inactive from hAAT-ΔLTR in NIH-3T3 cells, and the addition of liver-specific transcription factor binding sites did not activate it, as shown in Fig. 3C. Addition of transcription factor binding sites also had no significant effect upon the number of LTR-initiated transcripts in NIH-3T3 cells. Northern blot analysis demonstrated that samples from both cell lines had similar amounts of RNA, and confirmed the relative proportions of LTR- and hAAT promoter-initiated transcripts (data not shown).

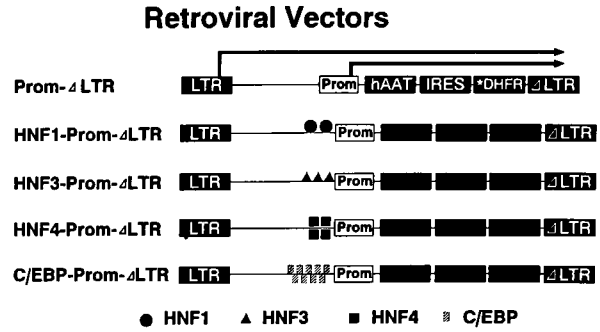

FIG. 2. Retroviral vectors containing internal promoters with or without additional liver-specific transcription factor binding sites. The basal promoters analyzed in this study were the α_1-antitrypsin (hAAT) promoter, the mouse albumin promoter (mAlb), and the promoter for the large subunit of RNA polymerase II. These were placed upstream of the human α_1-antitrypsin (hAAT) reporter gene, in the position marked "Prom" in the diagram. The internal ribosome entry site (IRES) (Ghattas *et al.*, 1991) allows translation of the downstream mutant dihydrofolate reductase (*DHFR) gene, which confers resistance to methotrexate. The oligomerized HNF-1, HNF-3, HNF-4, or C/EBP binding sites shown in Fig. 1 were placed immediately upstream of the hAAT promoter to create HNF-1-hAATΔLTR, HNF-3-hAATΔLTR, HNF-4-hAATΔLTR, and C/EBP-hAATΔLTR, respectively. Similarly, the oligomerized HNF-1, HNF-3, HNF-4, or C/EBP binding sites were placed upstream of the Pol-II or the mAlb promoter to generate the corresponding retroviral vectors. All retroviral vectors were present on a pBR322-derived plasmid that allowed growth in bacterial cells, which is not shown in this diagram.

Pol-II promoter

The Pol-II promoter is the promoter for the large subunit of RNA polymerase II, and hence is expressed in all cells (Ahearn *et al.*, 1987). Although the promoter has not been studied extensively, sequence analysis demonstrates putative SP1 binding sites. Retroviral vectors containing the Pol-II promoter with or without additional liver-specific transcription factor binding sites are shown in Fig. 2. RNA from pools of singly transduced cells was analyzed for expression by using the RNase protection assay diagrammed in Fig. 4A. Because the Pol-II promoter is non-liver-specific, expression from both the LTR and the internal Pol-II promoter is observed in both Hepa1A (Fig. 4B) and NIH-3T3 cells (Fig. 4C). In Hepa1A cells, addition of HNF-3 or C/EBP binding sites had no significant effect upon transcription from the Pol-II promoter. As was noted for the C/EBP-hAATΔLTR construct, some of the pools of C/EBP-Pol-IIΔLTR-transduced cells exhibited abnormal sized bands on Northern blot or RNase protection assay, suggesting that a

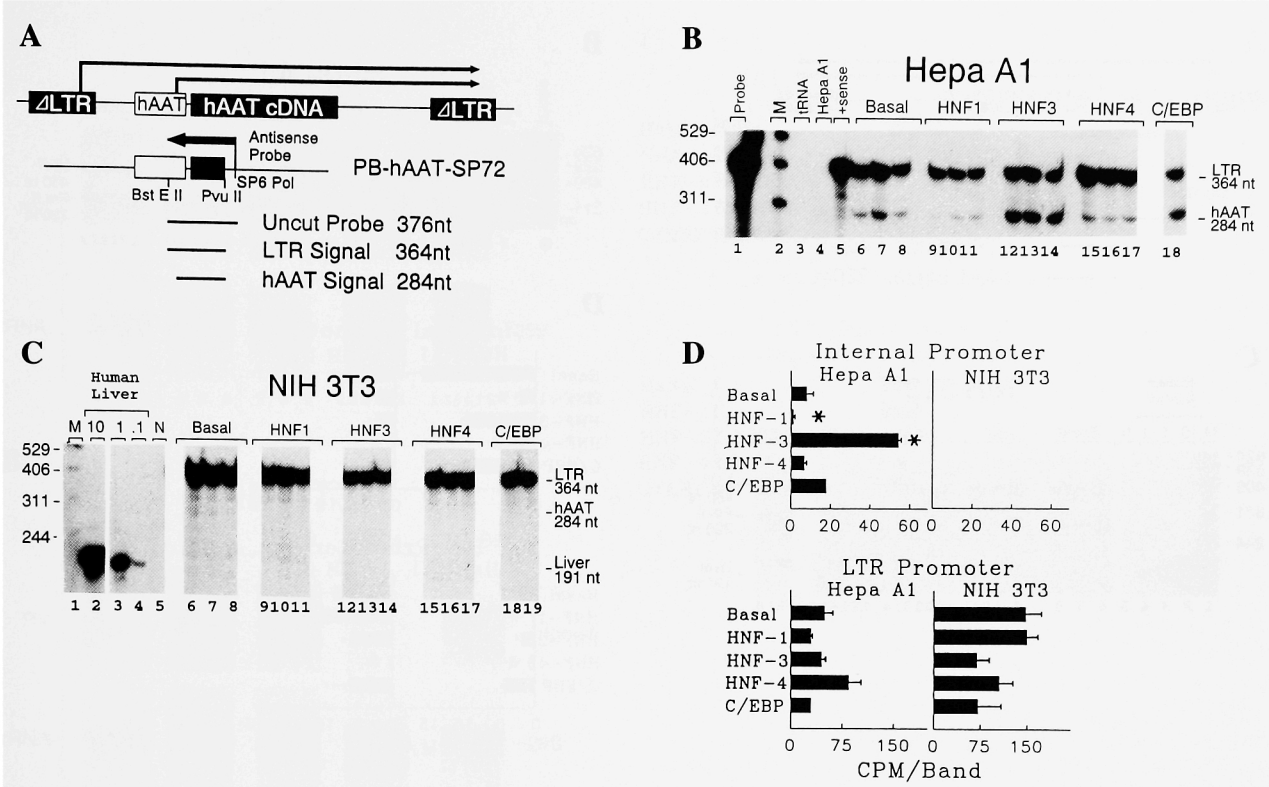

FIG. 3. Effect of addition of liver-specific transcription factor binding sites upon expression from a retroviral vector containing the hAAT promoter. A. RNA probe used for an RNase protection assay to differentiate LTR-initiated from hAAT promoter-initiated transcripts. A fragment containing the hAAT promoter and hAAT cDNA was removed from the retroviral vector and cloned into an RNA expression vector. This was used to generate a complementary RNA probe that can differentiate between LTR-initiated and hAAT promoter-initiated transcripts as shown. SP6 represents the initiation site for SP6 RNA polymerase. B. RNase protection assay of RNA from Hepa1A cells. RNA was hybridized with the probe shown in A, digested with single strand-specific RNases, and electrophoresed on a 6% denaturing polyacrylamide gel. Lane 1, Undigested probe; lane 2, end-labeled *Msp* I-digested pBR322 DNA markers (M), with the sizes indicated on the left in nucleotides. Negative controls include yeast tRNA (lane 3) and RNA from uninfected Hepa1A cells (lane 4). Lane 5, 100 pg of positive sense RNA, which marks the position of LTR-initiated transcripts. The samples shown in lanes 6–8 (hAATΔLTR), lanes 9–11 (HNF-1-hAATΔLTR), lanes 12–14 (HNF-3-hAATΔLTR), lanes 15–17 (HNF-4-hAATΔLTR), and lane 18 (C/EBP-hAATΔLTR) represent RNA samples from pools of singly transduced Hepa1A cells. The gel was exposed for 20 hr at −70°C. C. RNase protection assay of RNA from NIH-3T3 cells. Lane 1, DNA markers; lanes 2–4, a 191-nucleotide band that results after incubation with 10 μg, 1 μg, or 0.1 μg of normal human liver RNA; lane 5 (N), RNA from uninfected NIH-3T3 cells. The samples shown in lanes 6–8 (hAATΔLTR), lanes 9–11 (HNF-1-hAATΔLTR), lanes 12–14 (HNF-3-hAATΔLTR), lanes 15–17 (HNF-4-hAATΔLTR), and lanes 18–19 (C/EBP-hAATΔLTR) represent RNA samples from pools of singly transduced NIH-3T3 cells. The position of LTR-initiated, hAAT promoter-initiated, and RNA from human liver RNA are shown on the right. The gel was exposed for 20 hr at −70°C. D. Quantitation of the amounts of LTR-initiated and hAAT promoter-initiated transcripts in transduced Hepa1A and NIH-3T3 cells. The RNase protection assays shown in B and C were performed simultaneously. A 2-dimensional phosphorimager was used to quantitate the signal for LTR-initiated and hAAT promoter-initiated transcripts. The average cpm for each signal ± the standard error of the mean (SEM) is shown. The signal for vectors containing additional transcription factor binding sites was compared with the signal observed for the same promoter in the same cell type for the basal retroviral vector which contains the hAAT promoter without additional transcription factor binding sites. (*) Statistically significant difference ($p < 0.05$), as determined by using Student's *t*-test.

rearrangement had occurred (these samples are not shown here). Vectors containing additional HNF-1 or HNF-4 sites initiated only 14% and 20% as many transcripts from the Pol-II promoter, respectively, as compared with the basal Pol-II promoter in Hepa1A cells. The effect of addition of transcription factor binding sites upon expression from the LTR promoter in Hepa1A cells was similar to the effect upon the Pol-II promoter: addition of HNF-3 and C/EBP sites had no significant effect upon expression, whereas addition of HNF-1 and HNF-4 sites resulted in expression which was only 10% and 20%, respectively, that observed for the LTR promoter in Pol-IIΔLTR-transduced Hepa1A cells.

In NIH-3T3 cells, the addition of HNF-1, HNF-3, or C/EBP sites had no significant effect upon initiation from either promoter. Again, addition of C/EBP sites resulted in some rearrangements as shown in lane 18 of Fig. 4C (this sample was not used for quantitation of expression). Surprisingly, addition of HNF-4 sites dramatically decreased expression from both the

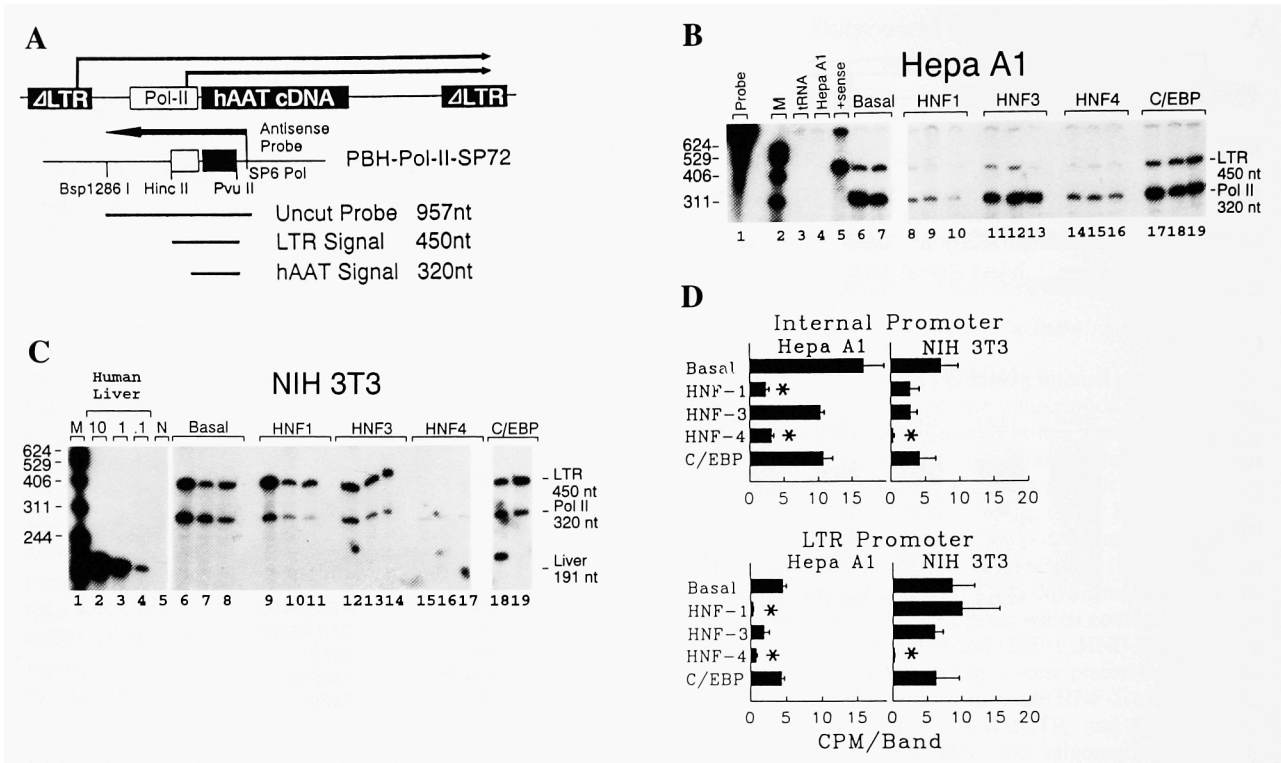

FIG. 4. Effect of addition of liver-specific transcription factor binding sites upon expression from the Pol-II promoter. A. RNA probe used for an RNase protection assay to differentiate LTR-initiated from internal Pol-II promoter-initiated transcripts. The antisense RNA probe spans the initiation site for the Pol-II promoter, and can be used to differentiate LTR-initiated from Pol-II-initiated transcripts, as shown. B. RNase protection assay of RNA from transduced Hepa1A cells. RNA was analyzed by RNase protection assay as diagrammed in A. Lane 1, Undigested probe; lane 2, DNA markers. Negative controls are shown in lane 3 (yeast tRNA) and lane 4 (RNA from uninfected Hepa1A cells). Lane 5, 100 pg of positive sense RNA, which serves as a marker for the position of LTR-initiated transcripts. The samples shown in lanes 6–7 (Pol-II-ΔLTR), lanes 8–10 (HNF-1-Pol-II-ΔLTR), lanes 11–13 (HNF-3-Pol-II-ΔLTR), lanes 14–16 (HNF-4-Pol-II-ΔLTR), and lanes 17–19 (C/EBP-Pol-II-ΔLTR) represent RNA samples from pools of singly transduced Hepa1A cells. The gel was exposed 20 hr at −70°C. C. RNase protection assay of RNA from transduced NIH-3T3 cells. Lane 1, DNA markers; lanes 2–4, 10 μg, 1 μg, and 0.1 μg of normal human liver RNA; lane 5, RNA from uninfected NIH-3T3 cells. The samples shown in lanes 6–8 (Pol-II-ΔLTR), lanes 9–11 (HNF-1-Pol-II-ΔLTR), lanes 12–14 (HNF-3-Pol-II-ΔLTR), lanes 15–17 (HNF-4-Pol-II-ΔLTR), and lanes 18–19 (C/EBP-Pol-II-ΔLTR) represent RNA from singly transduced NIH-3T3 cells. The positions of LTR-initiated, Pol-II promoter-initiated, and human liver transcripts are shown on the right. The gel was exposed 20 hr at −70°C. D. Quantitation of LTR-initiated and Pol-II promoter-initiated transcripts in transduced Hepa1A and NIH-3T3 cells. The RNase protection assays using RNA isolated from Hepa1A and NIH-3T3 cells shown in B and C were performed simultaneously. Expression levels were quantitated as described in Fig. 3D.

LTR promoter (1% of the amount of LTR-initiated transcripts as was seen in Pol-IIΔLTR-transduced cells) and the Pol-II promoter (6% of the amount of Pol-II-initiated transcripts as was seen in Pol-IIΔLTR transduced cells). Northern blot analysis demonstrated that similar amounts of RNA were present in each sample, and confirmed the results regarding the amounts of LTR- and Pol-II promoter-initiated transcripts.

mAlb promoter

The mouse albumin promoter is expressed specifically in the liver. It contains binding sites for the liver-specific transcription factors HNF-1 and C/EBP, and for the ubiquitous transcription factors NF-Y (binds to CCAAT sites) and NF-1 (Maire *et al.*, 1989). Retroviral vectors containing the mAlb promoter with or without additional liver-specific transcription factor binding sites are shown in Fig. 2. RNA from pools of singly transduced cells was analyzed for expression by Northern blot, as shown in Figs. 5, A and B. Northern blot analysis is shown here, because the levels of RNA in individual samples were not homogeneous, making it necessary to correct for RNA loading by normalization to rRNA. A 3.2-kb band whose size is consistent with initiation from the internal mAlb promoter represented >90% of all transcripts in Hepa1A cells (Fig. 5A) and >75% of all transcripts in NIH-3T3 cells (Fig. 5B). This was quite surprising, because the mAlb promoter was not expressed in fibroblast-derived lines in other studies (Friedman *et al.*, 1989; DiPersio *et al.*, 1991). However, both RNase protection and S1 nuclease assays with probes that spanned the mAlb initiation site demonstrated that indeed this transcript represents initiation from the mAlb promoter (data not shown).

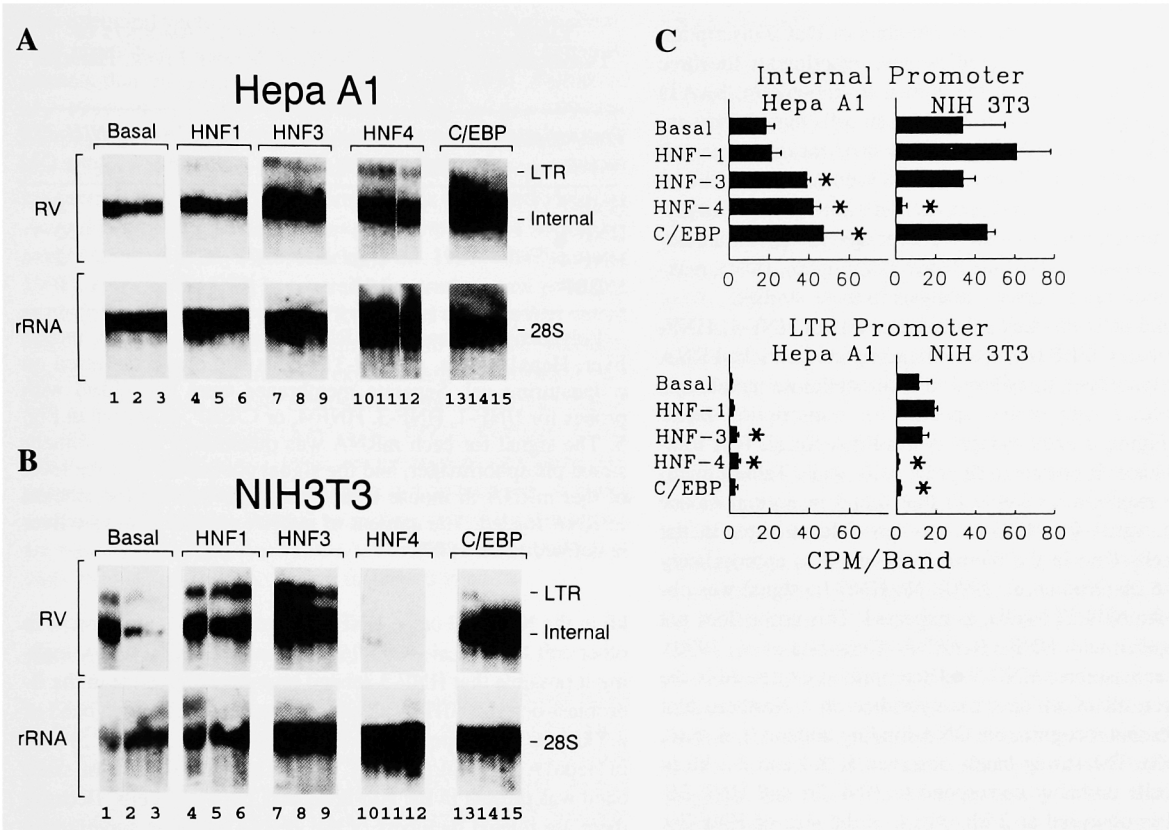

FIG. 5. Effect of addition of liver-specific transcription factor binding sites upon expression from the mAlb promoter. A. Northern blot analysis of RNA from transduced Hepa1A cells. Ten micrograms of total RNA from each sample was electrophoresed and transferred to a nitrocellulose membrane. The top panel shows the results obtained when the blots were hybridized with a probe specific for the IRES (RV). The 5- to 5.2-kb LTR-initiated (LTR) and 3.2-kb mAlb promoter-initiated (internal) transcripts are identified on the right. The membrane was stripped and rehybridized with a probe specific for the 28S rRNA for normalization purposes, as shown in the bottom panel (rRNA). The samples shown in lanes 1–3 (mAlbΔLTR), lanes 4–6 (HNF-1-mAlbΔLTR), lanes 7–9 (HNF-3-mAlbΔLTR), lanes 10–12 (HNF-4-mAlbΔLTR), and lanes 13–15 (C/EBP-mAlbΔLTR) represent RNA from pools of singly transduced Hepa1A cells. B. Northern blot analysis of RNA from transduced NIH-3T3 cells. The samples shown in lanes 1–3 (mAlbΔLTR), lanes 4–6 (HNF-1-mAlbΔLTR), lanes 7–9 (HNF-3-mAlbΔLTR), lanes 10–12 (HNF-4-ΔLTR), and lanes 13–15 (C/EBP-ΔLTR) represent RNA from pools of singly transduced NIH-3T3 cells. C. Quantitation of LTR-initiated and mAlb promoter-initiated transcripts of Hepa1A and NIH-3T3 cells transduced with the mAlb promoter-containing retroviral vectors. The signal for each transcript on the Northern blots was quantitated by using a 2-dimensional phosphorimager, and normalized to the intensity of the 28S rRNA signal. The signal for vectors containing additional transcription factor binding sites was compared with the signal observed for the same promoter in the same cell type for the basal retroviral vector containing the mAlb promoter without additional transcription factor binding sites. (*) Statistically significant difference ($p < 0.05$).

The effect of addition of liver-specific transcription factor binding sites upon expression in Hepa1A cells is shown in Fig. 5A. Addition of HNF-1 sites did not alter the number of mAlb promoter-initiated transcripts. Addition of HNF-3 sites increased the number of mAlb-initiated transcripts in Hepa1A cells by 50% after normalization for the loading efficiency to 28S rRNA. Addition of HNF-4 and C/EBP sites led to a statistically significant ~two-fold increase in the number of mAlb promoter-initiated transcripts. Addition of HNF-3 and HNF-4 sites significantly increased the number of LTR-initiated transcripts in Hepa1A cells, although these transcripts still represent only 10% of all transcripts.

Addition of HNF-1 or HNF-3 binding sites had no significant effect upon the number of transcripts that initiated from either the mAlb or the LTR promoter in NIH-3T3 cells. Addition of C/EBP sites had no effect upon initiation from the mAlb promoter, although it decreased the number of LTR-initiated transcripts by 80%. In contrast, addition of HNF-4 sites dramatically decreased the number of both LTR- and mAlb promoter-initiated transcripts, to a level that was ~10% that observed in mAlbΔLTR-transduced cells for both promoters. This is similar to the strong inhibition observed for the Pol-II promoter-containing construct after the addition of HNF-4 sites in fibroblast-derived cells.

Quantitation of mRNA for liver-specific transcription factors

A trivial explanation for the inability to activate transcription with a particular binding site is that the hepatoma cell line

used does not contain sufficient amounts of that transcription factor. Hepa1A cells were used in these experiments for three reasons. First, the choice of the human α_1-antitrypsin (hAAT) reporter gene precluded the use of human cells that already express hAAT. Second, other commonly used rat hepatoma cell lines such as H4II cells (Darlington, 1987) could not be infected with amphotropic retroviral vectors (data not shown). Finally, Hepa1A cells express a variety of liver-specific genes including mouse albumin and α_1-antitrypsin (Darlington, 1987), making them good candidates for analysis in these studies.

We tested cells for their relative amounts of HNF-1, HNF-3, HNF-4, and C/EBP mRNAs. Polyadenylated-enriched RNA was electrophoresed, transferred to a nitrocellulose membrane, and hybridized with probes specific for transcription factor mRNAs. Figure 6 demonstrates that mRNA for all four transcription factors is present in Hepa1A cells, while Table 1 quantitates the expression relative to that found in normal mouse liver. The signal for HNF-1α was two-fold stronger in the Hepa1A cells than in the mouse liver, and was appropriately sized at 3.6 kb (Kuo *et al.*, 1990). No HNF-1α signal was observed in the NIH-3T3 cells, as expected. This probe does not cross-hybridize with HNF-1β mRNA (Cereghini *et al.*, 1990). There are at least three HNF-3-related proteins expressed in the human liver, all of which cross-hybridize on a Northern blot with probes that recognize the DNA-binding domain (Lai *et al.*, 1990, 1993). The strong bands observed at 3.4 and 2.2 kb in Hepa1A cells probably correspond to HNF-3α and HNF-3β. No signal is observed at 2 kb, which is the size of HNF-3γ. Surprisingly, a signal was observed with the HNF-3 probe at 4 kb in the NIH-3T3 cells. HNF-3 related proteins are present in other cell types (Lai *et al.*, 1993; Kaestner *et al.*, 1994), making it possible that HNF-3-related proteins are present in the fibroblast-derived NIH-3T3 cells. An appropriately sized band of 4.5 kb was recognized by the HNF-4 probe (Sladek *et al.*, 1990) in Hepa1A cells. Surprisingly, a small amount of a similar-sized band was present in the non-hepatoma NIH-3T3 cells. Because there are related members of the steroid hormone superfamily that can bind to HNF-4 sites, it is possible that the mRNA for such a protein could cross-hybridize with our probe. The C/EBPα probe recognized an appropriately sized band at 2.7 kb (Landshultz *et al.*, 1988) in Hepa1A cells that co-migrated with the major band from mouse liver. No signal was observed with the C/EBPα probe in the NIH-3T3 cells, as expected. The mRNAs for C/EBPβ, C/EBPγ, and C/EBPδ are of a different size and do not cross-hybridize with the C/EBPα probe (Thomassin *et al.*, 1992).

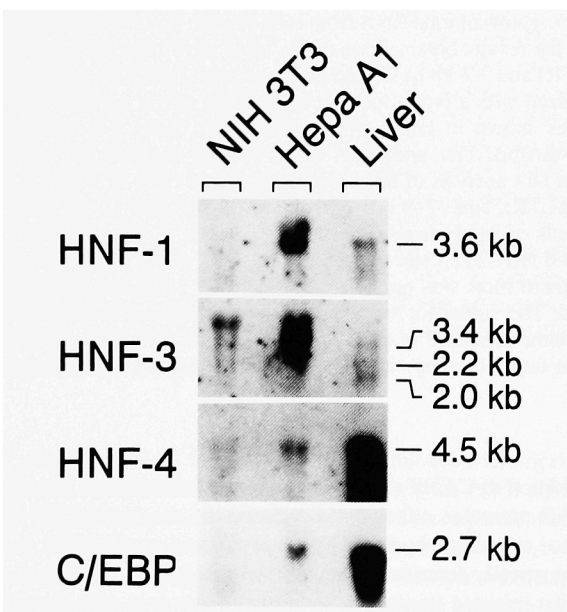

FIG. 6. Analysis of liver-specific transcription factor mRNA levels in NIH-3T3 cells, Hepa1A cells, and mouse liver. Twenty micrograms of poly(A)-enriched RNA isolated from NIH-3T3 and Hepa1A cells, and 10 μg of poly(A)-enriched RNA from mouse liver was electrophoresed through a denaturing agarose gel and transferred to a nitrocellulose membrane. Separate blots were hybridized with a mouse HNF-1α, a rat HNF-3α, a rat HNF-4, and a rat C/EBPα probe. Expression was quantitated by analysis with a 2-dimensional phosphorimager and corrected for the amount of RNA loaded.

TABLE 1. SUMMARY OF THE RELATIVE AMOUNTS OF TRANSCRIPTION FACTOR MRNAS IN MOUSE LIVER, HEPA1A CELLS, AND NIH-3T3 CELLS

Transcription factor	*Mouse hepatocytes (%)*	*Hepa1A cells (%)*	*NIH-3T3 cells (%)*
HNF-1	100	182	0
HNF-3	100	620	55
HNF-4	100	7	2
C/EBP	100	7	0

Polyadenylated-enriched RNA was isolated from mouse liver, Hepa1A cells, or NIH-3T3 cells and electrophoresed on a denaturing gel. Separate membranes were hybridized with probes for HNF-1, HNF-3, HNF-4, or C/EBP, as shown in Fig. 5. The signal for each mRNA was quantitated by a 2-dimensional phosphorimager, and the signal compared with the level of that mRNA in mouse liver after correction for the amount of RNA loaded. The amount of mRNA present in mouse liver is defined to be 100%.

We conclude that mRNAs for the liver-specific transcription factors analyzed in this study are present in the Hepa1A cells, albeit at varying levels as compared with the mouse liver. In other hepatoma-derived cell lines, C/EBPα mRNA levels are usually <10% that observed in hepatocytes of animals, HNF-1 mRNA levels are usually similar, and HNF-3 and HNF-4 mRNA levels are variable (Friedman *et al.*, 1989; Cereghini *et al.*, 1990; Herbst *et al.*, 1991; Kuo *et al.*, 1992). Although the presence of liver-specific transcription factor mRNAs in Hepa1A cells suggests that functional transcription factors will be produced, it is possible that accessory transcription factors and/or post-translational modifications are necessary for a particular transcription factor to function.

DISCUSSION

Although retroviral vectors show promise for use in hepatic gene therapy, they have only achieved relatively low levels of expression *in vivo*. This could reflect attenuation of the LTR, attenuation of an internal promoter, or attenuation of both, because either transcript can usually result in an RNA that is translated into a protein product. There are several potential reasons

why retroviral vectors might be poorly expressed *in vivo*. First, viral promoters including the LTR are generally less active in animals than in cultured cells (Kaleko *et al.*, 1991; Palmer *et al.*, 1991; Scharfmann *et al.*, 1991; Kay *et al.*, 1992a,b; Rettinger *et al.*, 1994). This might reflect the absence of positive-acting transcription factors or the presence of negative-acting factors. In addition, LTR promoters are often methylated *in vivo*, although it is unclear if this is a primary or a secondary event (Jaenisch *et al.*, 1985; Kaleko *et al.*, 1991; Challita *et al.*, 1994). Because the LTR is generally the predominant promoter in tissue culture, loss of such LTR-initiated transcription would result in an overall decline in expression in animals. Although internal promoters can increase expression from retroviral vectors in hepatocytes *in vivo*, expression remains less than that of the endogenous promoter (Kay *et al.*, 1992b; Rettinger *et al.*, 1994; Hafenrichter *et al.*, 1994), and the reasons why some promoters function better than others is not understood. A better understanding of how internal promoters and transcription factor binding sites function from a retroviral vector should provide insights into how to design improved vectors.

Rationale for the experimental approach

In these experiments, expression was analyzed from pools of Mtx^R cells. The alternative approach of normalizing expression to DNA copy number in nonselected cells was precluded by the low titers of the enhancer-deleted retroviral vectors (Hafenrichter *et al.*, 1994) and the low sensitivity of Southern blot analysis. The method used here may not accurately reflect the average level of expression from individual integration events, because Mtx selection requires that mutant dihydrofolate reductase (*DHFR) by expressed at levels sufficient to result in Mtx^R. The presence of the LTR, which is expressed from all basal constructs, ensures that colonies of Mtx^R cells should be isolated after transduction with each construct, regardless of the strength of the internal promoter. We did note that the addition of HNF-4 binding sites inhibited both promoters in some cases. Colonies derived from transduction with these HNF-4-containing vectors had lower levels of RNA and hAAT protein production than all individual colonies containing the basal promoter, but were still resistant to Mtx. This implies that *DHFR production was not limiting for the basal constructs. It is possible, however, that these results underestimate the degree of repression observed after the addition of HNF-4 binding sites because of the fact that only Mtx^R colonies were analyzed.

In addition to the Mtx^R gene, the retroviral vectors contained a deletion in the enhancer region of the 3′ LTR. Because the LTR promoter is strong in tissue culture cells, and because the phenomenon of "promoter interference" exists (Ghattas *et al.*, 1991), the transcriptional elements in the 3′ LTR were weakened by deleting a 178-bp fragment containing the 72-bp repeats in the enhancer. Because of the life cycle of a retrovirus, the sequences in the 3′ LTR appear in both the 5′ and the 3′ LTR of the integrated provirus after infection of a cell. An identical enhancer deletion resulted in ~90% decrease in LTR-initiated transcripts (Soriano *et al.*, 1991). Although crippling of the LTR was necessary to increase the relative levels of expression from the internal promoter, a caveat to the conclusions presented here is that LTR/internal promoter interactions might differ for an enhancer-intact retroviral vector.

Effect of addition of HNF-1 sites

HNF-1 sites are located within 150 nucleotides of the transcriptional start site of several liver-specific promoters (Frain *et al.*, 1989; Kuo *et al.*, 1990; Crabtree *et al.*, 1992) and can direct expression of a reporter gene when placed upstream of a TATA box in HNF-1-expressing cells (Kuo *et al.*, 1990; Denecke *et al.*, 1993). Overexpression of HNF-1 decreased expression of some (Denecke *et al.*, 1993) but not all (Babajko *et al.*, 1993) HNF-1-dependent promoters, suggesting that high levels of HNF-1 might "squelch" expression by binding a titratable factor. In addition, HNF-1 repressed the activity of several HNF-4-dependent promoters that did not contain an HNF-1 site (Tian and Schibler, 1991; Kuo *et al.*, 1992; Kritis *et al.*, 1993).

In our experiments, two upstream HNF-1 sites dramatically decreased expression from the internal promoter of hAAT- and Pol-II-promoter containing constructs in hepatoma cells, although there was no effect upon the mAlb promoter. This negative effect was dependent upon HNF-1, because no effect was observed in NIH-3T3 cells, which do not contain HNF-1. For the Pol-II promoter-containing construct only, this negative effect was transmitted to the LTR, implying an interaction between the LTR and the internal Pol-II promoter, as will be discussed below. We hypothesize that incorrectly positioned HNF-1 can inhibit transcription of some promoters. The strong predilection of HNF-1 sites for the proximal promoter region suggests that they function optimally from that position. Furthermore, inhibition of the HNF-4-dependent hAAT promoter (see Fig. 7) by aberrantly placed HNF-1 sites is consistent with the fact that overexpression of HNF-1 can inhibit HNF-4-dependent promoters. The absence of a negative effect of HNF-1 sites upon the albumin promoter was not surprising because the endogenous albumin enhancer has HNF-1 sites in a far upstream position, and overexpression of HNF-1 stimulated the mouse albumin promoter in tissue culture cells, in contrast to its negative effect upon several HNF-4-dependent promoters at the same level of HNF-1 expression (Kritis *et al.*, 1993). Aberrantly positioned HNF-1 may exert a negative effect upon SP1, which probably binds to the Pol-II promoter.

Effect of addition of HNF-3 sites

HNF-3 sites are present in the promoter or the enhancer region of several liver-specific genes (Lai *et al.*, 1990, 1993). Multimerized HNF-3 binding sites increase expression when placed upstream of a basal TATA box (Pani *et al.*, 1992) or the 800-bp mouse albumin promoter (DiPersio *et al.*, 1991) in HNF-3-expressing cells. In our experiments, addition of HNF-3 sites activated the hAAT and the mAlb promoters in hepatoma cells, both of which are liver-specific promoters that do not contain an HNF-3 site, as diagrammed in Fig. 7. Although the magnitude of activation of the mAlb promoter was less than that observed by DiPersio *et al.* (1991), we hypothesize that this difference may simply be due to the fact that the basal mAlb promoter is already extremely active from the retroviral vector in our experiments, making any additional effect less apparent. Addition of HNF-3 sites had no effect upon expression of the Pol-II promoter, suggesting that HNF-3 requires an interaction with some other liver-specific transcription factor such as HNF-1 for a positive effect.

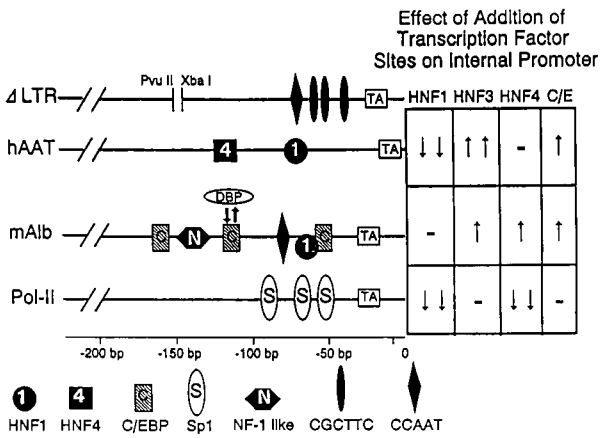

FIG. 7. Diagram of the promoters analyzed in this study and the effect of addition of transcription factor binding sites upon their expression in Hepa1A cells. Sequences that are known to play a role in the activation of the promoters used in this study are shown on the left. The MoMLV LTR (Majors, 1990) used here has a 178-bp deletion in the 72-bp direct repeats, which removes both copies of the core sequence that binds strongly to C/EBP (Hafenrichter *et al.*, 1994). The CCAAT sequence that binds weakly to C/EBP (Landschultz *et al.*, 1988) and the TATA box are shown. The sequence CGCTTC is functionally important for transcription (Landschultz *et al.*, 1988; Majors, 1990), but the protein(s) that bind to it have not yet been identified. The position of HNF-1 and HNF-4 binding sites on the proximal hAAT promoter are shown (Li *et al.*, 1988; Monaci *et al.*, 1988). Sequences between −347 and −150 increase transcription three-fold in hepatoma cells (Shen *et al.*, 1987), although proteins that bind to this region have not been identified. Several proteins that bind to the proximal 170 bp of the mouse albumin promoter are shown (Maire *et al.*, 1989). Sequences further upstream contribute little to expression in hepatoma cells. The mouse Pol-II promoter has three sites that closely resemble SP1 binding sites near the TATA box, although protein binding has not been demonstrated (Ahearn *et al.*, 1987). On the right, the effect of the addition of liver-specific transcription factor binding sites upon expression from the internal promoter in hepatoma cells is shown based upon the results of Figs. 3–5. HNF-1 (HNF1), HNF-3 (HNF3), HNF-4 (HNF4), or C/EBP sites (C/E) were placed upstream of the basal promoter, and the number of transcripts that initiate from the internal promoter in Hepa1A cells was determined. (↑) Increase in internal promoter-initiated transcripts as compared with the basal promoter in Hepa1A cells; (↓) decrease; (−) no significant alteration.

Effect of addition of C/EBP sites

C/EBPα is the original leucine zipper protein, which was first isolated by virtue of its ability to bind to both the CCAAT box and the core enhancer of a retroviral LTR (Landschultz *et al.*, 1988). Additional family members (Crabtree *et al.*, 1992; McKnight, 1992) can form hetero- or homodimers with themselves or other family members, and can bind to related sequences. C/EBP sites can activate a TATA box in C/EBP-expressing cells (Mueller *et al.*, 1990), and are found in both the promoter and enhancer regions of liver-specific genes.

In our experiments, the addition of nine C/EBP sites led to a two-fold increase in transcription from the mAlb promoter. Although addition of C/EBP sites stimulated expression of the hAAT promoter in some experiments, frequent rearrangements were observed for this construct, casting doubt upon the validity of the results. Frequent rearrangements were also observed for the C/EBP-Pol-IIΔLTR construct. We conclude that C/EBP sites had a slightly positive effect upon expression of liver-specific promoters, but that it led to frequent rearrangements of some retroviral vectors. The presence of an AUG sequence in the C/EBP binding site may lead to premature translational initiation of LTR-initiated transcripts, thus inhibiting translation of the downstream hAAT mRNA into protein. Although the AUG in the C/EBP binding site would not affect expression of hAAT protein for the mAlb-containing vectors where the internal promoter is predominant, selective pressures for rearrangements might occur for vectors in which the LTR is the major promoter used.

Effect of addition of HNF-4 sites

HNF-4 is an orphan member of the steroid hormone receptor superfamily, and no ligand has been identified to date (Sladek *et al.*, 1990). HNF-4 sites play a critical role in the expression of a number of liver-specific promoters (Li *et al.*, 1988). HNF-4 sites placed upstream of a TATA box activated expression in HNF-4-expressing cells (Sladek *et al.*, 1990; Mietus-Snyder *et al.*, 1992). Other members of the steroid hormone family can bind to HNF-4 sites. The retinoic acid receptor α (RXRα) activates transcription in the presence of retinoic acid (Nakshatri and Chambon, 1994). In contrast, the binding of apoA1 regulatory protein 1 (ARP-1) or Ear3/Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) results in inhibition of transcription (Mietus-Snyder *et al.*, 1992; Nakshatri and Chambon, 1994). ARP-1 is present in limited amounts in hepatoma and intestinal cells, but in much larger amounts in a variety of other cell types, where it may play a critical role in inhibiting expression of liver- and intestine-specific genes. The mechanism of inhibition by ARP-1 is two-fold. ARP-1 competes with HNF-4 and/or RXRα in binding to HNF-4 sites present on liver-specific promoters or enhancers, although the ARP-1 DNA-binding domain alone cannot exert the full inhibitory effect (Ge *et al.*, 1994). In addition, ARP-1 strongly inhibited an HNF-3-dependent promoter that did not contain an HNF-4 site, although it had less effect upon an SP1-dependent promoter (Paulweber *et al.*, 1993).

In our experiments, the addition of HNF-4 sites upstream of the Pol-II and the mAlb promoter decreased transcription from both the internal promoter and the retroviral LTR in the fibroblast-derived NIH-3T3 cells. This inhibition is probably due to ARP-1, which can bind to the HNF-4 site used in this study (Costa *et al.*, 1990). The inhibition of the mAlb and Pol-II promoters suggests that ARP-1 can inhibit promoters that do not contain HNF-3 sites. The negative effect upon the LTR promoter leads us to draw two conclusions. First, ARP-1 can inhibit the LTR promoter. Second, the HNF-4 sites present ~1 kb downstream of the initiation site of the LTR promoter must interact with the LTR promoter in some fashion. Intriguingly, HNF-4 sites had no effect upon transcription from the LTR promoter in HNF-4-hAATΔLTR-transduced fibroblasts, although the distance between the HNF-4 sites and the LTR was identical. Thus, the hAAT promoter did not interact with the LTR by these criteria.

Addition of HNF-4 sites had a variable effect upon expression in hepatoma cells. HNF-4 sites led to a two-fold increase in expression from the mAlb promoter, but had no effect upon the hAAT promoter. Because the hAAT promoter already contains an HNF-4 site, while the mAlb promoter does not (Fig. 7), it is possible that additional sites do not exert any additional benefit. Surprisingly, addition of HNF-4 sites inhibited expression from the Pol-II and LTR promoter in HNF-4-Pol-IIΔ-transduced hepatoma cells, a result that is similar to that observed in NIH-3T3 cells. This inhibition could be mediated by ARP-1; previous studies have demonstrated that ARP-1 is expressed in hepatoma cell lines, although Hepa1A cells have not been specifically studied. It is possible that a liver-specific promoter preferentially binds HNF-4 over ARP-1 because of interactions between HNF-4 and other liver-specific transcription factors. We therefore hypothesize that ARP-1 or a related protein binds to the HNF-4 sites upstream of the Pol-II promoter and inhibits expression of both the Pol-II and the LTR promoter in hepatoma cells.

The addition of oligomerized liver-specific transcription factor binding sites is not an effective way to increase expression from a retroviral vector

The original purpose of this study was to increase expression from an internal promoter of a retroviral vector by adding more transcription factor binding sites. Although these results have provided insights into the how specific transcription factors interact with other elements of a promoter, we have not achieved our original goal. HNF-3 sites had the most potent activation in Hepa1A cells, increasing expression from the hAAT promoter by seven-fold. Although the addition of HNF-3 sites would appear promising for use in retroviral vectors for hepatic gene therapy, evaluation of these vectors in rat livers *in vivo* demonstrated that HNF-3 sites only increased expression from an hAAT or mAlb promoter-containing construct slightly (Hafenrichter *et al.*, 1994). It is unclear why this discrepancy exists between the *in vivo* and the *in vitro* results, but it emphasizes the point that it is essential to test retroviral vectors *in vivo* for relative levels of expression. We conclude that the addition of oligomerized transcription factor binding sites is not a promising approach to increase the level of expression from retroviral vectors. This conclusion is not totally surprising, given the complexities of promoter structure and the probable need for more precise spacing than what was achieved here.

Implications for the design of retroviral vectors for gene therapy

A second goal of this study is to understand better how promoters and transcription factor binding sites function from a retroviral vector. It has been difficult to achieve high-level expression from a retroviral vector *in vivo*. This partly reflects the fact that the LTR promoter itself is poorly expressed in many cell types. For example, although the LTR can lead to long-term expression *in vivo*, the relative levels of expression of hAAT and factor IX were quite low as compared with normal animals (Kay *et al.*, 1993; Rettinger *et al.*, 1994). In other studies, the LTR was completely inactive when introduced into preimplantation embryos (Jaenisch *et al.*, 1985; Stewart *et al.*, 1987) or into fibroblasts after birth (Palmer *et al.*, 1991). Although near-therapeutic levels of less abundant proteins can be achieved from the LTR of a retroviral vector, the absolute level of expression is probably still relatively low (Lynch *et al.*, 1992; Correll *et al.*, 1994). This poor expression could be due to the absence of positive-acting transcription factors, or to the presence of inhibitory factors. Regions of the Moloney murine leukemia virus (Mo-MLV) that inhibit expression in embryonal carcinoma cells include: (i) the upstream conserved region (UCR) at the 5′ end of the LTR (Challita *et al.*, 1995); (ii) the 72-bp repeats in the LTR enhancer (Grez *et al.*, 1991); and (iii) the tRNA binding site (Challita *et al.*, 1995). It is unclear, however, if these regions can inhibit expression of promoters in animals.

Although the LTR is less active in quiescent cells *in vivo* than in rapidly dividing cells in tissue culture, data regarding the level of expression from an internal promoter of a retroviral vector are murky. Some *in vivo* experiments suggest that an internal promoter is inhibited from a retroviral vector (Richards and Huber, 1993; McCune and Townes, 1994), while others have noted expression (Stewart *et al.*, 1987; Correll *et al.*, 1994). Our experiments suggest that the LTR and the internal promoter interact for the mAlb and Pol-II promoter-containing constructs, but not for the hAAT promoter-containing construct. We believe that the absence of an interaction between the LTR and internal promoter might be critical for achieving high-level long-term expression, if, in fact, negative elements within the LTR can inhibit an internal promoter. Indeed, the hAAT promoter, which does not interact with the LTR, was considerably stronger *in vivo* than the mAlb, Pol-II, or PEPCK promoters, all of which do interact with the LTR (Hatzoglou *et al.*, 1990; Hafenrichter *et al.*, 1994; T. Okuyama and K.P. Ponder, submitted). The addition of HNF-4 sites might help to determine if an internal promoter interacts with the LTR. In addition, it might ensure that expression of retroviral vectors containing liver-specific promoters only occurs in the liver, a theoretical advantage when using *in vivo* delivery methods for hepatic gene therapy.

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REFERENCES

AHEARN, J.M., BARTOLOMEI, M.S., WEST, M.L., CISEK, L.J., and CORDEN, J.L. (1987). Cloning and sequence analysis of the mouse genomic locus encoding the largest subunit of RNA polymerase II. J. Biol. Chem. **262,** 10695–10705.

BABAJKO S., TRONCHE, F., and GROYER, A. (1993). Liver-specific expression of human insulin-like growth factor binding protein 1: Functional role of transcription factor HNF1 in vivo. Proc. Natl. Acad. Sci. **90,** 272–276.

CEREGHINI, S., YANIV, M., and CORTESE, R. (1990). Hepatocyte dedifferentiation and extinction is accompanied by a block in the synthesis of mRNA coding for the transcription factor HNF1/LFB1. EMBO J. **9,** 2257–2263.

CHALLITA, P.M., and KOHN, D.B. (1994). Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated methylation in vivo. Proc. Natl. Acad. Sci. USA **91,** 2567–2571.

CHALLITA, P-M, SKELTON, D., EL-KHOUEIRY, A., YU, X.-J., WEINBERG, K., and KOHN, D.B. (1995). Multiple modifications in cis elements of the long terminal repeat of retroviral vectors lead to increased expression and decreased DNA methylation in embryonic carcinoma cells. J. Virol. **69,** 748–755.

CHOMCZYNSHI, P., and SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162,** 156–159.

CORRELL, P.H., COLILLA, S., and KARLSSON, S. (1994). Retroviral vector design for long-term expression in murine hematopoietic cells in vivo. Blood **84,** 1812–1822.

COSTA, R.H., VAN DYKE, T.A., YAN, C., KUO, F., and DARNELL, J.E. (1990). Similarities in transthyretin gene expression and differences in transcription factors: Liver and yolk sac compared to choroid plexus. Proc Natl. Acad. Sci. USA **87,** 6589–6593.

CRABTREE, G.R., SCHIBLER, U., and SCOTT, M.P. (1992). Transcriptional regulatory mechanisms in liver and midgut morphogenesis of vertebrates and invertebrates. In *Transcriptional Regulation.* K.R. Yamamoto and S.L. McKnight, eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

DARLINGTON, G. (1987). Liver cell lines. Methods Enzymol. **151,** 19–38.

DENECKE, B., BARTKOWSKI, S., SENKEL, S., KLEIN-HITPASS, L., and RYFFEL, G.U. (1993). Chimeric liver transcription factors LFB1 (HNF1) containing the acidic activation domain of VP16 act as positive dominant interfering mutants. J. Biol. Chem. **268,** 18076–18082.

DiPERSIO, C.M., JACKSON, D.A., and ZARET, K.S. (1991). The extracellular matrix coordinately modulated liver transcription factors and hepatocyte morphology. Mol. Cell. Biol. **11,** 4405–4414.

FRAIN, M., SWART, G., MONACI, P., NICOSIA, A., STAMPFI, S., FRANK, R., AND CORTESE, R. (1989). The liver-specific transcription factor LF-B1 contains a highly diverged homeobox DNA binding domain. Cell **59,** 145–157.

FRAIN, M., HARDON, E., CILIBERTO, G., and SALA-TREPAT, J.M. (1990). Binding of a liver-specific factor to the albumin gene promoter and enhancer, Mol. Cell. Biol. **10,** 991–999.

FRIEDMAN, A.D., LANDSCHULTZ, W.H., and McKNIGHT, S.L. (1989). CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. Genes & Dev. **3,** 1314–1322.

GE, R., RHEE, M., MALIK, S., and KARATHANASIS, S.K. (1994). Transcriptional repression of apolipoprotein AI gene expression by orphan receptor ARP-1, J. Biol. Chem. **18,** 13185–13192.

GHATTAS, I.R., SANES, J.R., and MAJORS, J.E. (1991). The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. Mol. Cell. Biol. **11,** 5848–5859.

GREZ, M., ZORING, M., NOWOCK, J., and ZIEGLER, M. (1991). A single point mutation activated the Moloney murine leukemia virus long terminal repeat in embryonic stem cells. J. Virol. **65,** 4691–4698.

HAFENRICHTER, D.G., WU, X., RETTINGER, S.D., KENNEDY, S.C., FLYE, M.W., and PONDER, K.P. (1994). Quantitative evaluation of liver-specifc promoters from retroviral vectors after in vivo transduction of hepatocytes. Blood **84,** 3394–3404.

HATZOGLOU, M., LAMERS, W., BOSCH, F., WYNSHAW-BORIS, A., and HANSON, R. (1990). Hepatic gene transfer in animals using retroviruses containing the promoter for phosphoenolpyruvate carboxykinase. J. Biol. Chem. **265,** 17285–17293.

HERBST, R.S., NIELSCH, U., SLADEK, F., LAI, E., BABISS, L.E., and DARNELL, J.E., Jr. (1991). Differential regulation of hepatocyte-enriched transcription factors explains changes in albumin and transthyretin gene expression among hepatoma cells. New Biol. **3,** 289–296.

JAENISCH, R., SCHNIEKE, A., and HARBERS, K. (1985). Treatment of mice with 5-azacytidine efficiently activates fetal retroviral genomes in different tissues. Proc. Natl. Acad. Sci. USA **82,** 1451–1455.

JAINCHILL, J.L., AARONSON, S.A., and TODORO, G.J. (1969). Murine sarcoma and leukemia viruses: Assay using clonal lines of contact inhibited mouse cells. J. Virol. **4,** 549–553.

KAESTNER, K.H., HIEMISCH, H., LUCKOW, B., and SCHUTZ, G. (1994). The HNF-3 gene family of transcription factors in mice: gene structure, cDNA sequence, and mRNA distribution. Genomics **20,** 377–385.

KALEKO, M., GARCIA, J.V., and MILLER, A.D. (1991). Persistent gene expression after retroviral gene transfer into liver cells in vivo. Hum. Gene Ther. **2,** 27–32.

KAY, M.A., BALEY, P., ROTHERNBERG, S., LELAND, F., FLEMING, L., PONDER, K.P., LIU, T.J., FINEGOLD, M., DARLINGTON, G., POKORNY, W., and WOO, S.L.C. (1992a). Expression of human α_1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. Proc. Natl. Acad. Sci. USA **89,** 89–93.

KAY, M.A., LI, Q., LIU, T.-J., LELAND, F., TOMAN, C., and WOO, S.L.C. (1992b). Hepatic gene therapy: persistent expression of human α-1-antitrypsin in mice after direct gene delivery in vivo. Hum. Gene Ther. **3,** 641–647.

KAY, M.A., ROTHENBERG, S., LANDEN, C.N., BELLINGER, D.A., LELAND, F., TOMAN, C., FINEGOLD, M., THOMPSON, A.R., READ, M.S., BRINKHOUS, K.M., and WOO, S.L.C. (1993). In vivo gene therapy of hemophilia B: Sustained partial correction in Factor IX deficient dogs. Science **266,** 117–119.

KRITIS, A.A., KITISTAKI, E., BARDA, D., ZANNIS, V.I., and TALIANIDIS, I. (1993). An indirect negative autoregulatory mechanism involved in hepatocyte nuclear factor-1 gene expression. Nucleic Acids Res. **21,** 5882–5889.

KUO, C.J., CONLEY, P.B., HSIEH, C.-L., FRANCKE, U., and CRABTREE, G.R. (1990). Molecular cloning, functional expression, and chromosomal localization of mouse hepatocyte nuclear factor 1. Proc. Natl. Acad. Sci. USA **87,** 9838–9842.

KUO, C.J., CONLEY, P.B., CHEN, L., SLADEK, F.M., DARNELL, J.E., and CRABTREE, G.R. (1992). A transcriptional hierarchy involved in mammalian cell-type specification. Nature **355,** 457–461.

LAI, E., PREZIOSO, V.R., SMITH, E., LITVIN, O., COSTA, R.H., and DARNELL, J.E., Jr. (1990). HNF-3α, A hepatocyte-enriched transcription factor of novel structure is regulated transcriptionally. Genes & Dev. **4,** 1427–1436.

LAI, E., CLARK, K.L., BURLEY, S.K., and DARNELL, J.E. (1993). Hepatocyte nuclear factor 3/forkhead or "winged helix" proteins: a family of transcription factors of diverse biologic function. Proc. Natl. Acad. Sci. USA **90,** 10421–10423.

LANDSCHULTZ, W.H., JOHNSON, P.E., ADASHI, E.Y., GRAVES, B.J., and McKNIGHT, S.L. (1988). Isolation of a recombinant copy of the gene encoding C/EBP. Genes & Dev. **2,** 786–800.

LI, Y., SHEN, R.-F., TSAI, S.Y., and WOO, S.L.C. (1988). Multiple hepatic trans-acting factors are required for in vitro transcription of the human alpha-1 antitrypsin gene. Mol. Cell. Biol. **8,** 4362–4369.

LYNCH, C.M., CLOWES, M.M., OSBORNE, W.R.A., CLOWES, A.W., and MILLER, A.D. (1992). Long-term expression of human adenosine deaminase in vascular smooth muscle cells of rats: a model for gene therapy. Proc. Natl. Acad. Sci. USA **89,** 1138–1142.

MAIRE, P., WUARIN, J., and SCHIBLER, U. (1989). The role of *cis*-acting promoter elements in tissue-specific albumin gene expression. Science **244,** 343–346.

MAJORS J. (1990). The structure and function of retroviral long terminal repeats. Curr. Topics Microbiol. Immunol. **157,** 49–92.

MARKOWITZ, D., GOFF, S., and BANK, A. (1988). Construction and use of a safe and efficient amphotropic packaging cell line. Virology **167,** 400–406.

McKNIGHT, S.L. (1992). CCAAT/enhancer binding protein. In *Transcriptional Regulation.* K.R. Yamamoto and S.L. McKnight eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

McCUNE, S.L., and TOWNES, T.M. (1994). Retroviral vector sequences inhibit human beta-globin gene expression in transgenic mice. Nucleic Acids Res. **22,** 4477–4483.

MELTON, D.A., KRIEG, P.A., REBAGLIATI, M.R., MANIATAS, T., ZINN, K., and GREEN, M.R. (1984). Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12,** 7035–7042.

MIETUS-SNYDER, M., SLADEK, F.M., GINSBURG, G.S., KUO, F., LADIAS, J.A.A., DARNELL, J.E., and KARATHANASIS, S.K. (1992). Antagonism between apolipoprotein A1 regulatory protein 1, Ear3/COUP-TF, and hepatocyte nuclear factor 4 modulates apolipoprotein CIII gene expression in liver and intestinal cells. Mol. Cell. Biol. **12,** 1708–1718.

MILLER, A.D., and ROSMAN, G.J. (1989). Improved retroviral vectors for gene transfer and expression. Biotechniques **7,** 980–990.

MONACI, P., NICOSIA, A., and CORTESE, R. (1988). Two different liver specific factors stimulate in vitro transcription from the human α1-antitrypsin promoter, EMBO J. **7,** 2075–2087.

MUELLER, C.R., MAIRE, P., and SCHIBLER, U. (1990). DBP, a liver enriched transcriptional activator, is expressed late in ontogeny and its tissue specificity is determined posttranscriptionally. Cell **61,** 279–291.

NAKSHATRI, H., and CHAMBON, P. (1994). The directly repeated RG(G/T)TCA motifs of the rat and mouse cellular retinol-binding protein II genes are promiscuous binding sites for RAR, RXR, HNF-4, and ARP-1 homo- and heterodimers. J. Biol. Chem. **269,** 890–902.

OVERDIER, D.G., PORCELLA, A., and COSTA, R.H. (1994). The DNA binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino acid residues adjacent to the recognition helix. Mol. Cell. Biol. **14,** 2755–2766.

PALMER, T.D., ROSMAN, G., OSBORNE, W., and MILLER, A.D. (1991). Genetically modified skin fibroblasts persist long after transplantation but gradually inactivate introduced genes. Proc. Natl. Acad. Sci. USA **88,** 1330–1334.

PANI, L., OVERDIER, D.G., PORCELLA, A., QIAN, X., LAI, E., and COSTA, R.H. (1992). Hepatocyte nuclear factor 3 beta contains two transcriptional activation domains, one of which is novel and conserved with the Drosophila fork head protein. Mol. Cell. Biol. **12,** 3723–3732.

PARKER, K.A., and BOND, U. (1989). Analysis of pre-rRNAs in heat shocked HeLa cells allow identification of the upstream termination site of human polymerase I transcription. Mol. Cell. Biol. **9,** 2500–2512.

PAULWEBER, B., SANDHOFER, F., and LEVY-WILSON, B. (1993). The mechanism by which the human apolipoprotein B gene reducer operates involves blocking of transcriptional activation by hepatocyte nuclear factor 3. Mol. Cell. Biol. **13,** 1534–1546.

PONDER, K.P., DUNBAR, R., WILSON, D., DARLINGTON, G., and WOO, S.L.C. (1991). Evaluation of relative promoter strength in primary hepatocytes using optimized lipofection. Hum. Gene Ther. **2,** 41–52.

RETTINGER, S.D., KENNEDY, S.C., WU, X., SAYLORS, R.L., HAFENRICHTER, D.G., FLYE, M.W., and PONDER, K.P. (1994). Liver-directed gene therapy: Quantitative evaluation of promoter elements by using in vivo retroviral transduction. Proc. Natl. Acad. Sci. USA **91,** 1460–1464.

RICHARDS, C.A., and HUBER, B.E. (1993). Generation of a transgenic model for retrovirus-mediated gene therapy for hepatocellular carcinoma is thwarted by the lack of transgene expression. Hum. Gene Ther. **4,** 143–150.

SCHARFMANN, R., AXELROD, J.H., and VERMA, I.M. (1991). Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants. Proc. Natl. Acad. Sci. USA **88,** 4626–4630.

SHEN, R.F., LI, Y., SIFERS, R., WANG, H., HARDICK, C., TSAI, S., and WOO, S.L.C. (1987). Tissue-specific expression of the human α_1-antitrypsin gene is controlled by multiple cis-regulatory elements. Nucleic Acids Res.. **15,** 8399–8415.

SLADEK, F.M., ZHONG, W. LAI, E., and DARNELL, J.E. Jr. (1990). Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes & Dev. **4,** 2353–2365.

SORIANO P., FRIEDRICH, G., and LAWINGER, P. (1991). Promoter interactions in retrovirus vectors introduced into fibroblasts and embryonic stem cells. J. Virol. **65,** 2314–2319.

STEWART, C.L., SCHUETZE, S., VANEK, M., and WAGNER, E.F. (1987). Expression of retroviral vectors in transgenic mice obtained by embryonic infection. EMBO J. **2,** 383–388.

THOMASSIN, H., HAMEL, D., BERNIER, D., GUERTIN, M., and BELANGER, L. (1992). Molecular cloning of two C/EBP related proteins that bind to the promoter and enhancer of the alpha 1-fetoprotein gene. Nucleic Acids Res. **20,** 3091–3098.

TIAN, J.-M., and SCHIBLER, U. (1991). Tissue-specific expression of the gene encoding hepatocyte nuclear factor 1 may involve hepatocyte nuclear factor 4. Genes & Dev. **5,** 2225–2234.

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