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CMV-β-Actin Promoter Directs Higher Expression from an Adeno-Associated Viral Vector in the Liver than the Cytomegalovirus or Elongation Factor 1α Promoter and Results in Therapeutic Levels of Human Factor X in Mice

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ABSTRACT

Although AAV vectors show promise for hepatic gene therapy, the optimal transcriptional regulatory elements have not yet been identified. In this study, we show that an AAV vector with the CMV enhancer/chicken β-actin promoter results in 9.5-fold higher expression after portal vein injection than an AAV vector with the EF1α promoter, and 137-fold higher expression than an AAV vector with the CMV promoter/enhancer. Although induction of the acute-phase response with the administration of lipopolysaccharide (LPS) activated the CMV promoter/enhancer from the context of an adenoviral vector in a previous study, LPS resulted in only a modest induction of this promoter from an AAV vector *in vivo*. An AAV vector with the CMV-β-actin promoter upstream of the coagulation protein human factor X (hFX) was injected intravenously into neonatal mice. This resulted in expression of hFX at 548 ng/ml (6.8% of normal) for up to 1.2 years, and 0.6 copies of AAV vector per diploid genome in the liver at the time of sacrifice. Neonatal intramuscular injection resulted in expression of hFX at 248 ng/ml (3.1% of normal), which derived from both liver and muscle. We conclude that neonatal gene therapy with an AAV vector with the CMV-β-actin promoter might correct hemophilia due to hFX deficiency.

OVERVIEW SUMMARY

Optimization of gene expression from an AAV vector might allow higher levels of expression to be achieved, which will be necessary for effective gene therapy for some genetic deficiencies. It might also allow a lower dose of vector to be administered, which would reduce the risk of insertional mutagenesis or germ line transmission. Neonatal gene transfer might reduce the chance of inducing an immune response, and would lead to a more immediate correction of a genetic disease than would transfer into adults. We demonstrate here that the CMV-β-actin promoter is expressed well from an AAV vector in the liver. Neonatal intravenous administration of an AAV vector that expresses the coagulation protein factor X from this promoter results in therapeutic levels of factor X for more than 1 year in mice. Neonatal gene therapy with an AAV vector may allow effective gene therapy to be achieved for hemophilia.

INTRODUCTION

GENE THERAPY could be used to correct a variety of genetic deficiencies in the liver. Adeno-associated virus (AAV) vectors can transduce nondividing cells and have no apparent toxicity (Büeler, 1999). In contrast, although Moloney murine leukemia virus retroviral vectors can transfer genes into ~5% of hepatocytes and achieve stable and therapeutic levels of expression of blood proteins (Le *et al.*, 1997; Cai *et al.*, 1998), they transduce only dividing cells.

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Lentiviral vectors also require replicating cells for efficient transfer into the liver (Park *et al*., 2000), although they can transduce some nondividing cells. Adenoviral vectors can transduce almost 100% of nonreplicating hepatocytes, but are limited by their toxicity and short-term duration of expression (Ilan *et al*., 1999).

The advantages of AAV vectors have led to their use for hepatic gene therapy (Patijn and Kay, 1999). AAV vectors contain a ≤4.8-kb single-stranded DNA genome (Büeler, 1999). They bind to heparan sulfate proteoglycan on the surface of cells (Summerford *et al*., 1998), with either an integrin (Summerford *et al*., 1999) or the fibroblast growth factor receptor (Qing *et al*., 1999) serving as a coreceptor. After intravenous (Ponnazhagan *et al*., 1997) or intraportal (Snyder *et al*., 1997, 1999) injection, a higher copy number is observed in the liver than in other organs, suggesting that the liver has better flow and/or contact with the blood, has more receptors, and/or is more conducive to some later step of infection than are cells from other organs. Injection of high titers ($\sim 1 \times 10^{11}$ particles) of AAV vector into the portal vein can result in the accumulation of an average of three to five copies per liver cell, and expression in ~5% of hepatocytes (Miao *et al*., 1998, 2000). The transduced cells contain ~100 copies of the AAV vector as high molecular weight concatemers that can be either integrated or episomal (Miao *et al*., 1998; Nakai *et al*., 1999). Expression increases slowly over approximately 2 months, which is likely due to the rate at which the single-stranded genomic DNA is converted into the double-stranded DNA that is recognized by transcription factors. Expression from an AAV vector in the liver has been stable for more than 1 year in mice, and for more than 2 years in dogs.

Although expression from an AAV vector is stable, the expression per copy has been relatively low. Increasing expression should allow lower doses of AAV to be effective, which would reduce the risk of insertional mutagenesis or integration into the germ line. Previous studies have observed moderate expression from the elongation factor 1α (EF1α) promoter (Nakai *et al*., 1998), high (Xiao *et al*., 1998) or low (Daly *et al*., 1999b) activity from the cytomegalovirus (CMV)-β-actin promoter, and moderate (Xiao *et al*., 1998) or low (Snyder *et al*., 1997; Nakai *et al*., 1998) activity from the CMV promoter in livers of adults. In addition, the CMV promoter might be activated by induction of the acute-phase response with lipopolysaccharide (LPS), which occurs from the context of an adenoviral vector. We therefore tested these promoters for expression from the liver *in vivo*, and determined whether LPS could activate them.

Human factor X (hFX) deficiency results in a hemophilia that is comparable in severity to hemophilia A or B (Roberts and Hoffman, 2000). The absence of purified hFX makes this hemophilia more difficult to treat than the more common hemophilias (Lechler, 1999). Normal hFX levels are 8 μg/ml, and achieving 10% of normal (0.8 μg/ml) would correct most of the bleeding manifestations of this disorder. Once the CMV-β-actin promoter was identified as being able to promote high-level and stable expression from an AAV vector *in vivo*, we tested its ability to direct expression of hFX in normal mice. This resulted in the expression of stable and therapeutic levels of hFX in mice for more than 1 year.

MATERIALS AND METHODS

AAV vectors with the human α1-antitrypsin (hAAT) cDNA

CMV-hAAT-AAV is the same vector that was designated as AAV-C-AT in a previous study (Song *et al*., 1998). It contains nucleotides (nt) −522 to +72 of the CMV promoter, where +1 corresponds to the transcriptional initiation site (this sequence is identical to nt 620 to 1213 of the CMV sequence with accession number GI 59800; Hennighausen and Fleckenstein, 1986). EF1α-hAAT-AAV is the same vector designated as AAV-E-AT in a previous study (Song *et al*., 1998). It contains 450 bp of the upstream sequence from the human EF1α promoter, 34 bp of exon 1, 943 bp of intron 1, and 11 bp of exon 2 (nt 127 to 1561 for the EF1α sequence with accession number GI 181962; Uetsuki *et al*., 1989). CMV-β-actin-hAAT-AAV was constructed by placing the CMV-β-actin cassette (Daly *et al*., 1999a) upstream of the hAAT cDNA. It contains nt −706 to −188 of the CMV enhancer (nt 436 to 954 of GI 59800) and the 1345-nt chicken β-actin promoter (nt 1 to 1345 of the sequence with accession number GI 2171233; Miyazaki *et al*., 1989; Niwa *et al*., 1991). The latter contains 278 nt of the chicken β-actin promoter, 90 nt of exon 1, 917 nt of a hybrid chicken β-actin/rabbit β-globin intron, and 55 nt of exon 3 from rabbit β-globin. Vectors were packaged (Hauswirth *et al*., 2000) after cotransfection with the plasmid pDG (Grimm *et al*., 1998), which contains the AAV *rep* and *cap* genes and the adenoviral genes necessary to support AAV vector production, into 293 cells. Cells were disrupted by freeze–thaw lysis, and virions were purified by iodixanol gradient ultracentrifugation followed by heparin–Sepharose column chromatography (Hauswirth *et al*., 2000). The ratio of particle number (assessed by competitive PCR) to infectious units (assessed by infectious center assay) (Hauswirth *et al*., 2000) was 100:1.

AAV vector with the CMV-β-actin promoter and the hFX cDNA

The AAV vector plasmid pCAG (Daly *et al*., 1999a) was partially restricted with *Eco*RI and a 1.5-kb *Eco*RI fragment of the hFX cDNA was inserted. This hFX cDNA contains a Thr-to-Arg mutation at amino acid −2 in order to improve processing in nonhepatic cells, and a mutation that results in loss of the internal *Eco*RI site without any amino acid changes (Rudolph *et al*., 1996). Virions were prepared from 293 cells after cotransfection of vector and the helper plasmid pIM45 (Pereira *et al*., 1997), and superinfection with adenovirus serotype 5 (Ad5) at a multiplicity of infection (MOI) of 2. They were purified over two cesium chloride gradients and the final product was incubated at 56°C for 45 min to inactivate residual adenovirus as described (Daly *et al*., 1999a). The level of infectious adenovirus was <100 plaque-forming units/ml, as determined by the absence of a cytopathic effect on 293 cells. The particle number was determined by dot blot (Hauswirth *et al*., 2000), and the particle-to-infectious unit ratio was 100:1.

Analysis for transcription factor-binding sites

The programs TFSEARCH: Searching Transcription Factor Binding Sites (http://pdap1.trc.rwcp.or.jp/research/db/

TFSEARCH.html) and the Transcription Element Search System (TESS) (http://www.cbil.upenn.edu/tess/index.html) were used to analyze promoters for transcription factor-binding sites. Only sites for transcription factors that are present in the liver under normal or induced conditions are mentioned here.

Animal procedures

For all procedures in adults, mice were anesthesized with metophane. For experiments in adult mice, the portal veins of 6-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were injected with 100 μl of phosphate-buffered saline (PBS) containing approximately 1×10^{11} particles of AAV. Hemostasis was achieved with a small piece of Gelfoam (Pharmacia & Upjohn, Mountain View, CA). Some mice were injected intraperitoneally with LPS from *Escherichia coli* serotype O111:B4 (Sigma, St. Louis, MO) dissolved in pyrogen-free water with 0.9% NaCl. Experiments with neonatal C57BL/6 mice involved one intravenous injection of ~100 μl containing 1.7×10^9 particles into the superficial temporal vein on day 3 after birth (Sands and Barker, 1999), or four intramuscular injections of 3 μl each containing a total of 2×10^8 particles into the anterior and posterior portions of each upper leg. Retroorbital blood was allowed to clot for serum, and was drawn through a heparinized capillary tube and anticoagulated with a 1:10 volume of 3.8% trisodium citrate for plasma.

ELISA

For analysis of hAAT levels by enzyme-linked immunosorbent assay (ELISA), a polyclonal goat anti-hAAT antibody (Atlantic Antibodies, Scarborough, NJ) was used for capture, and the same antibody coupled to horseradish peroxidase (HRP) was used for detection (Rettinger *et al.*, 1994). Standards were created by serial dilutions of Calibrator 4 (Atlantic Antibodies), and the assay was sensitive to 0.5 ng/ml. For analysis of hFX levels, mouse monoclonal antibodies 5 and 1066 were obtained from J. Miletich (Rudolph *et al.*, 1996) and ELISA was performed as described previously (Le *et al.*, 1997). Standards were created by diluting purified hFX (Hematologic Technologies, Essex Junction, VT) and the assay was sensitive to 6 ng/ml. For detection of anti-hFX antibodies, ELISA plates were coated with 100 μl of sodium bicarbonate buffer containing hFX at 1 μg/ml. ELISA was performed as described (Le *et al.*, 1997) except that an HRP-coupled anti-mouse IgG antibody (Sigma) was used at a 1:120 dilution to detect murine antibodies.

Isolation of DNA and RNA

Approximately one-third of the liver and all muscle from the upper leg were homogenized in guanidinium (1 ml per 100 mg of sample); half was used for preparation of RNA, and the other half for preparation of genomic DNA (Rettinger *et al.*, 1994). RNA was treated with DNase I (Boehringer Mannheim, Indianapolis, IN) at 0.1 unit/μg of RNA, according to the instructions of the manufacturer. The concentrations of RNA and DNA were determined by measuring the optical density (OD) at 260 nm.

Southern blot for AAV vector sequences

Southern blot involved digestion of 15 μg of genomic DNA, electrophoresis on a 0.7% agarose gel, and transfer to an Optitran reinforced nitrocellulose membrane (Schleicher & Schuell, Keene NH). For detection of the hAAT-containing AAV vector sequences, DNA was digested with *Eco*RV (cuts at +75 nt of the hAAT cDNA) and *Bam*HI (cuts at +882 nt), and probed with the same fragment. For detection of hFX-containing AAV vector sequences, DNA was digested with *Apa*I, which cuts at +535 and +1293 nt of the hFX cDNA, and probed with the same fragment. Standards included dilutions of hAAT- or hFX-containing plasmid DNA digested with the same enzyme, or DNA from NIH 3T3 cells that were transduced with a single copy of a retroviral vector expressing the appropriate cDNA. PCR was used to detect vector DNA sequences for some samples, using the primers and conditions noted below.

Reverse transcriptase polymerase chain reaction for detection of RNA

For detection of hAAT sequences, Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) were used to amplify 2 μg of DNase I-treated total RNA in a volume of 50 μl, according to the instructions of the manufacturer. The top primer was from nt 177 to 199 of the hAAT cDNA (5′-TTCAACAAGATCACCCCCAACCT-3′) and the bottom primer was from nt 569 to 551 (5′-GGCCTCTTCGGTGTCC-CCG-3′), and 35 cycles of PCR (94°C for 1 min, 62°C for 1 min, and 72°C for 1 min) were performed after reverse transcription according to the instructions of the manufacturer. For detection of hFX mRNA sequences, the top primer was from nt 61 to 82 (5′-GGGGAAAGTCTGTTCATCCGCA-3′) of the hFX cDNA, and the bottom primer was from nt 580 to 561 (5′-CCTCCCCGCTGCTGCTGGTG-3′). Reverse transcription was performed with 2 μg of RNA, the above-described primers, and 1 unit of avian myeloblastosis virus (AMV) RT at 42°C for 15 min. Samples were then amplified with 35 cycles of PCR (92°C for 1.5 min, 61°C for 1 min, and 72°C for 1 min) in a volume of 50 μl, using a buffer containing 16 m*M* ammonium sulfate, 20 m*M* Tris (pH 8.55), 3.3 m*M* $MgCl_2$, bovine serum albumin (0.15 mg/ml), a 100 μM concentration of each dNTP, and 0.5 units of *Taq* polymerase (Perkin-Elmer, Norwalk, CT). For both reactions, standards were generated by mixing human liver RNA with mouse liver RNA. RT-PCR products were electrophoresed on a 1.5% agarose gel and transferred to a membrane, and Southern blot was performed with full-length cDNA probes and a high-stringency final wash. To demonstrate that the RNA was of good quality, RT-PCR was performed with mouse β-actin primers as described (Watson *et al.*, 1992) followed by ethidium bromide staining.

IL-6 bioassay

Interleukin 6 (IL-6)-dependent T1165 cells (Larisa *et al.*, 1996; kindly provided by R Schreiber) were maintained in RPMI 1640 containing 10% fetal bovine serum, 1% sodium pyruvate, 0.1% 2-mercaptoethanol, 1% L-glutamine, 1% penicillin/streptomycin, and IL-6 at 30 ng/ml. Cells (30,000) were added to a 96-well plate in medium without IL-6. Mouse serum samples or a known concentration of IL-6 was added, and cell survival was determined 40 hr later after staining with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Carmichael *et al.*, 1987). The sensitivity of the assay was 0.1 ng/ml of IL-6.

Electrophoretic mobility shift assay

Liver nuclear extracts were prepared as described in detail (Gao *et al.*, 1999) and the protein concentration was determined by the Bradford assay (Bio-Rad, Hercules, CA). Double-stranded oligonucleotide probes were end labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP (>4000 Ci/mmol). The top strand for NF-κB was TCGA-GGGCTGGGGATTCCCCATCTC from the class I major histocompatibility complex enhancer element H2-κB (Ballard *et al.*, 1989). Activity was normalized to the level in samples from normal mice that were injected with PBS after subtraction of background, which was the signal present in a sample that did not receive any protein.

Statistical analysis

Statistical analysis between two groups of animals was performed with the program Instat from GraphPad software (San Diego, CA), using the Student *t* test.

RESULTS

Comparison of various promoters from an AAV vector for expression in the liver

AAV vectors that contained hAAT cDNA were used to compare the efficacy of different promoters *in vivo*, and are diagrammed in Fig. 1A. Since hAAT is a secreted protein, expression can be monitored over time by measuring hAAT levels in blood. Although both the CMV-hAAT-AAV and EF1α-hAAT-AAV vectors contain the TK-neo transcription unit in order to facilitate titering, the larger size of the CMV-β-actin promoter precluded the incorporation of this second transcription unit. It is possible that this difference in the backbone might affect expression, although the weakness of the TK promoter makes promoter interference less likely. Similar titers of these vectors were injected into the portal vein of normal adult C57BL/6 mice, and blood was tested for hAAT levels by immunoassay, as shown in Fig. 1B. The first data point was obtained 2 months after transduction, as previous studies have shown that expression from an AAV vector reaches peak levels at about that time. The CMV-β-actin promoter directed the highest level of expression, with an average of 1725 $\pm$ 87 ng/ml, and expression was stable for 300 days. This expression was higher than from the EF1α promoter (195 $\pm$ 36 ng/ml), or the CMV promoter (8 $\pm$ 1.5 ng/ml).

To document that higher expression was due to a more active promoter, the DNA copy number in the liver was determined by Southern blot of genomic DNA. As shown in Fig. 2A, CMV-hAAT-AAV-, CMV-β-actin-hAAT-AAV-, and EF1α-hAAT-AAV-transduced mice had 4.1 $\pm$ 0.7, 6.45 $\pm$ 0.6, and 6.9 $\pm$ 0.2 copies of AAV vector per cell, respectively. After correction for differences in the copy number of AAV vector in the liver, expression from the CMV-β-actin promoter was 9.5-fold higher than from the EF1α promoter, and 137-fold higher than from the CMV promoter/enhancer. Analysis of mRNA by RT-PCR demonstrated that RNA levels correlated well with serum protein levels, as shown in Fig. 2B. This confirms the hypothesis that the higher levels of protein that are

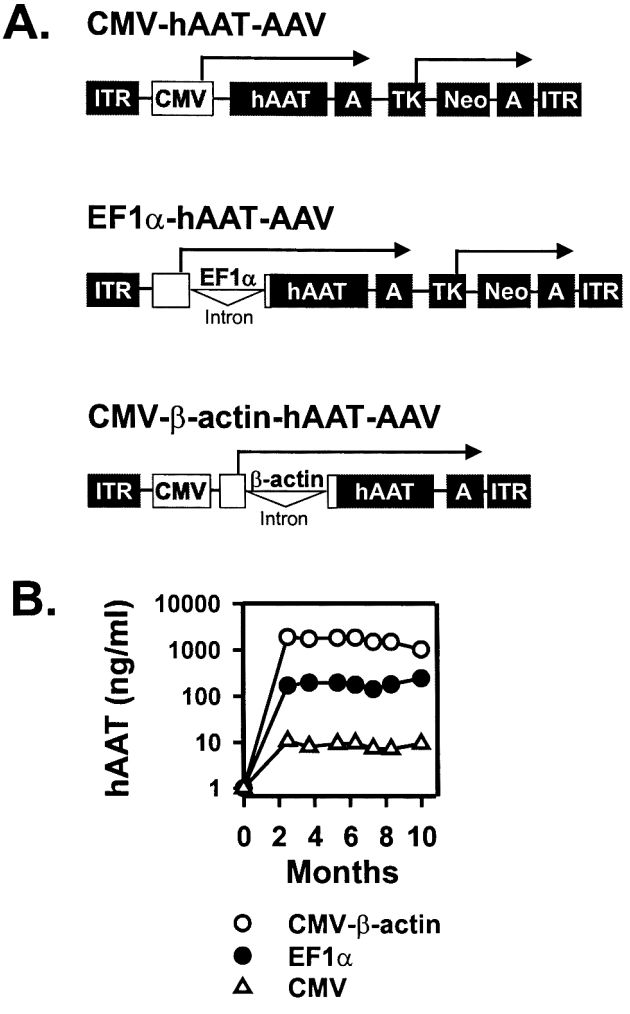

FIG. 1. (**A**) AAV vectors containing hAAT cDNA. AAV vectors containing hAAT cDNA that were used in this study are shown. All vectors contained the inverted terminal repeats (ITRs) at both the 5′ and the 3′ end. CMV-hAAT-AAV is 3.7 kb and contains the 594-bp cytomegalovirus promoter and enhancer (CMV), the 1.3-kb human α_1-antitrypsin cDNA (hAAT), and a polyadenylation site (A). It also contains a second transcription unit with the thymidine kinase (TK) promoter, the neomycin resistance gene (Neo), and a polyadenylation site (A). EF1α-hAAT-AAV is 4.4 kb and contains the 1434-bp EF1α promoter with its splice site. It is otherwise identical to CMV-hAAT-AAV. CMV-β-actin-hAAT-AAV is 3.86 kb and contains the 518-bp CMV enhancer, and the 1345-bp chicken β-actin promoter with a hybrid splice site, the hAAT cDNA, and a polyadenylation site (A). (**B**) Expression of hAAT in serum after injection of AAV vectors expressing hAAT into the portal vein of mice. Six-week-old C57BL/6 mice were injected in the portal vein with 1.43×10^{11} particles of CMV-hAAT-AAV ($n = 4$), 1.27×10^{11} particles of EF1α-hAAT-AAV ($n = 3$), or 1.17×10^{11} particles of CMV-β-actin-hAAT-AAV ($n = 4$). Serum was obtained at various times after transduction, and tested for hAAT levels by immunoassay. The average hAAT levels $\pm$ SEM are shown. All samples were assayed simultaneously at the completion of the experiment to avoid interassay variation.

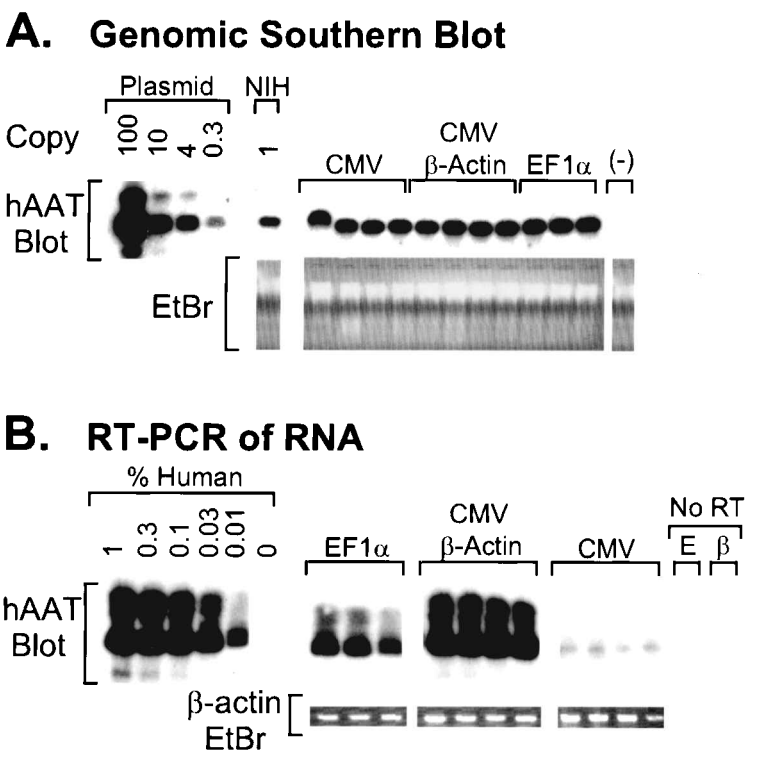

FIG. 2. DNA copy number and RNA levels in the liver after transduction with hAAT-expressing AAV vectors. The animals whose expression data are shown in Fig. 1 were killed 300 days after transduction, and DNA and RNA were isolated from the liver. (**A**) Genomic Southern blot. All samples were digested with *Eco*RV and *Bam*HI, which results in a 805-bp band from the hAAT cDNA that is identical for all vectors and plasmids. For mice that were transduced with CMV-hAAT-AAV (CMV), CMV-β-actin-hAAT-AAV (CMV β-Actin), EF1α-hAAT-AAV (EF1α), or a control (–) that received the CMV-β-actin-hFX vector that encodes a different cDNA, 15 μg of DNA obtained from the liver was digested. Standards represent 15 μg of NIH 3T3 cells that were transduced with a single copy of an hAAT-expressing retroviral vector (NIH-1 copy), or the EF1α-hAAT-AAV plasmid (Plasmid) that was diluted to give the indicated copy number per diploid genome. The top region shows the result of the Southern blot with the 805-nt hAAT probe (hAAT-blot), while the bottom region shows the result of ethidium bromide staining of the digested DNA prior to transfer (EtBr). The copy number was determined by phosphorimaging and comparison with the standard curve. (**B**) Analysis of RNA by RT-PCR. RNA obtained from the liver was amplified by RT-PCR with primers specific for the hAAT cDNA, and a Southern blot was performed (hAAT blot). Standards were generated by mixing human liver RNA with non-transduced mouse liver RNA at the indicated ratio. Samples from one EF1α-hAAT-AAV-transduced (E) and one CMV-β-actin-hAAT-AAV-transduced (β) mouse were also amplified by PCR without prior reverse transcription (No RT), to demonstrate that the signal derived from RNA and not from contaminating DNA. All samples were also amplified with mouse β-actin cDNA-specific primers and stained with ethidium bromide after electrophoresis (β-actin EtBr) to demonstrate that they contained amplifiable RNA.

observed in the CMV-β-actin-hAAT-AAV-transduced mice was due to the presence of more mRNA. We conclude that the vector with the CMV-β-actin promoter results in much higher levels of expression than do the vectors with the other promoters, and that this is likely due to increased levels of transcription.

Attempt to activate the CMV promoter with LPS

Previous studies have demonstrated that the CMV promoter is active in the liver from a retroviral vector during liver regeneration (Rettinger *et al.*, 1993) or from an adenoviral vector during liver regeneration or inflammation (Löser *et al.*, 1998). We therefore tested the ability of LPS to activate the CMV promoter from an AAV vector in the liver *in vivo*. LPS results in the production of IL-6, which causes phosphorylation and activation of the transcriptional activity of STAT3 (signal transducer and activator of transcription 3) in the liver. LPS also results in activation of the DNA-binding activity of NF-κB by inducing degradation of its inhibitor, I-κB (Hatada *et al.*, 2000). A 0.63-mg/kg dose of LPS induces the systemic inflammatory response syndrome in C57BL/6 mice, as it resulted in high levels of IL-6 in blood at 3 hr (Fig. 3A), and NF-κB DNA-binding activity in liver at 1.5 hr (Fig. 3B and 3C). The binding to the NF-κB probe was specific, as it was readily competed with an excess of unlabeled probe (data not shown). Although somewhat higher levels of IL-6 were observed with the higher doses of LPS, these were not significantly different from the values observed for animals that received 0.63 mg/kg, and the higher doses resulted in some deaths. We therefore chose a dose of 0.63 mg/kg for subsequent studies.

The mice whose expression data are shown in Fig. 1 were treated intraperitoneally with LPS at 0.63 mg/kg 8.5 months af-

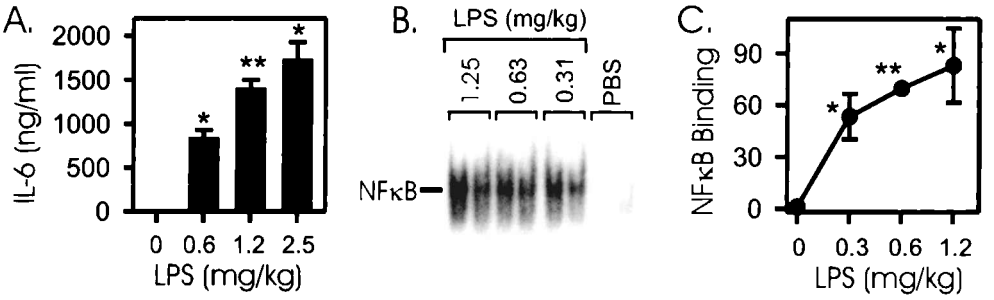

FIG. 3. Effect of LPS on serum IL-6 levels and liver NF-κB DNA-binding activity in C57BL/6 mice. Three-month-old C57BL/6 mice were injected intraperitoneally with varying doses of LPS. (**A**) Serum IL-6 levels. Serum obtained 3 hr after injection of LPS was tested for IL-6 levels, using a bioassay. The average levels for two animals at each dose of LPS are shown. Normal mice had low IL-6 levels in their serum (2.4 $\pm$ 0.5 ng/ml). IL-6 levels in LPS-treated mice were compared for statistically significant differences from those in normal mice, using the Student *t* test. $^{*}p$ Value of 0.05 to 0.005; $^{**}p$ value of 0.005 to 0.0005. (**B**) Liver NF-κB EMSA. Nuclear extracts obtained 1.5 hr after administration of PBS or the indicated dose of LPS were incubated with a radiolabeled NF-κB probe in an EMSA. Only the more slowly migrating bands that represent binding of protein to the probe are shown here. (**C**) Quantitation of the NF-κB DNA-binding activity. The amount of NF-κB DNA-binding activity was quantitated with a phosphorimager for two animals from each group, normalized to the level found in the normal mice that received an injection of PBS, and plotted as noted in (**A**).

ter transduction, and hAAT levels in serum were determined, as shown in Fig. 4. LPS resulted in a 2-fold increase in expression from the CMV promoter. Although this was significantly higher than the prestimulation values, and remained elevated for 7 days after LPS treatment, the absolute level of expression remained low. LPS also resulted in a 2.5-fold increase in expression from the EF1α promoter, and a 2.7-fold increase in expression from the CMV-β-actin promoter. We conclude that a modest stimulation of gene expression occurred from the CMV, as well as the other promoters, in response to LPS.

Expression of therapeutic levels of hFX from an AAV vector with the CMV-β-actin promoter in mice

The above-described data led us to conclude that the CMV-β-actin promoter directs the highest level of expression of the promoters that were tested. We therefore tested this promoter for its ability to direct expression of hFX in mice. An AAV vector with the CMV-β-actin promoter upstream of the hFX cDNA was constructed, as diagrammed in Fig. 5A. This AAV vector was injected intravenously or intramuscularly into normal neonatal mice for two reasons. First, their smaller size allowed less vector to be used. Second, we hypothesized that neonates might be less likely to develop antibodies than young adults. Intravenous injection was used, as it was previously demonstrated that intravenous injection into neonatal animals primarily transduced the liver (Daly *et al.*, 1999b). Intramuscular injection was also tested, as this approach has been used to express factor IX (FIX) in mice (Hagstrom *et al.*, 2000), dogs (Herzog *et al.*, 1999), and humans (Kay *et al.*, 2000).

Plasma obtained from CMV-β-actin-hFX-AAV-transduced mice was tested for hFX levels, as shown in Fig. 5B. Intravenous injection into neonates resulted in average hFX levels of 548 $\pm$ 153 ng/ml for up to 1.2 years after transduction. This represents 6.8% of normal hFX levels, which should have a major therapeutic effect in patients with hemophilia due to hFX deficiency. The apparent fluctuation in the plasma levels was

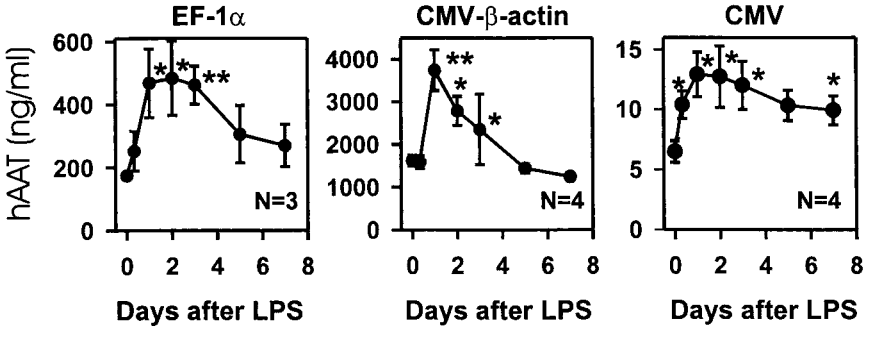

FIG. 4. Effect of LPS on hAAT levels in hAAT-AAV-transduced mice. The same mice whose expression data are shown in Fig. 1 were injected with LPS (0.63 mg/kg) 8.5 months after transduction, when they were 11 months old. Serum was tested for hAAT levels by immunoassay, and plotted as the average $\pm$ SEM. Data at each time point after LPS administration were compared with the prestimulation value by the Student *t* test. Values that were significantly different from the prestimulation values are indicated as noted in Fig. 3A.

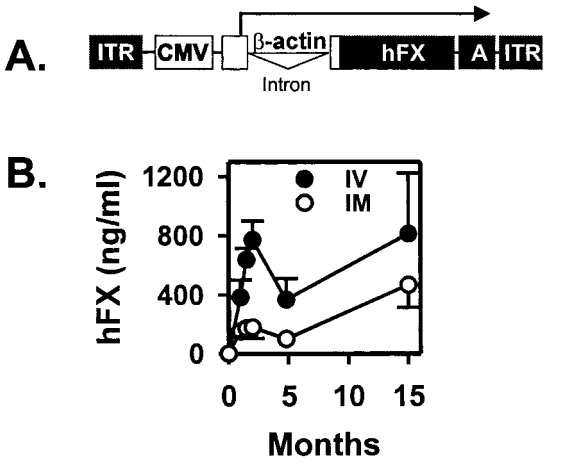

FIG. 5. (**A**) Diagram of the CMV-β-actin-hFX-AAV vector. The 1.5-kb human factor X (hFX) cDNA was inserted into a vector that is otherwise identical to the CMV-β-actin-hAAT-AAV vector shown in Fig. 1A. (**B**) Expression of hFX in mice after intramuscular or intravenous injection into neonatal C57BL/6 mice. Three neonatal (3-day-old) C57BL/6 mice were injected intravenously with 1.7×10^9 particles of CMV-β-actin-hFX-AAV via the temporal vein, or intramuscularly with 2×10^8 particles. Plasma was obtained at various times after transduction, and assayed for hFX levels by an immunoassay. Note that samples were not assayed simultaneously at the end of the experiment because of insufficient amounts of plasma. The average hFX level ± SEM is shown.

probably due to the fact that all samples were not assayed simultaneously because of the limited amounts of plasma and the low sensitivity of the hFX assay. Intramuscular injection of an 8-fold lower dose resulted in somewhat lower levels of expression, with an average of 247 ± 74 ng/ml. This represents 3.1% of normal, which should result in a significant therapeutic effect in patients with hemophilia.

DNA was analyzed from transduced animals in order to determine the AAV vector copy number. As shown in Fig. 6A, mice that received AAV by intravenous injection had 0.68 ± 0.16 copies per diploid genome in the liver. Somewhat surprisingly, mice that received intramuscular injection of AAV also had readily detectable levels of AAV vector in the liver on a genomic Southern blot, which was calculated to be 0.34 ± 0.14 copies per cell. Since genomic Southern blot was not performed on samples from the muscle because of the lower yield of DNA, PCR was used to determine whether the muscle contained the AAV vector, as shown in Fig. 6B. The signal for hFX DNA sequences was normalized to that for mouse β-glucuronidase sequences to control for amplification efficiency. Using this method, the amount of AAV vector in the liver was calculated to be 1.5 ± 0.3 copies per diploid genome for mice that received an intravenous injection of CMV-β-actin-FX-AAV, which was ~3-fold higher than in the liver for mice that received the vector intramuscularly (0.46 ± 0.08 copies). For mice that received the vector intramuscularly, the copy number in the muscle was similar (0.57 ± 0.12 copies) to that found in the liver for the same animals. To determine which organs expressed the AAV vector, RT-PCR was performed on RNA from muscle or liver. Although the levels of hFX sequences in the

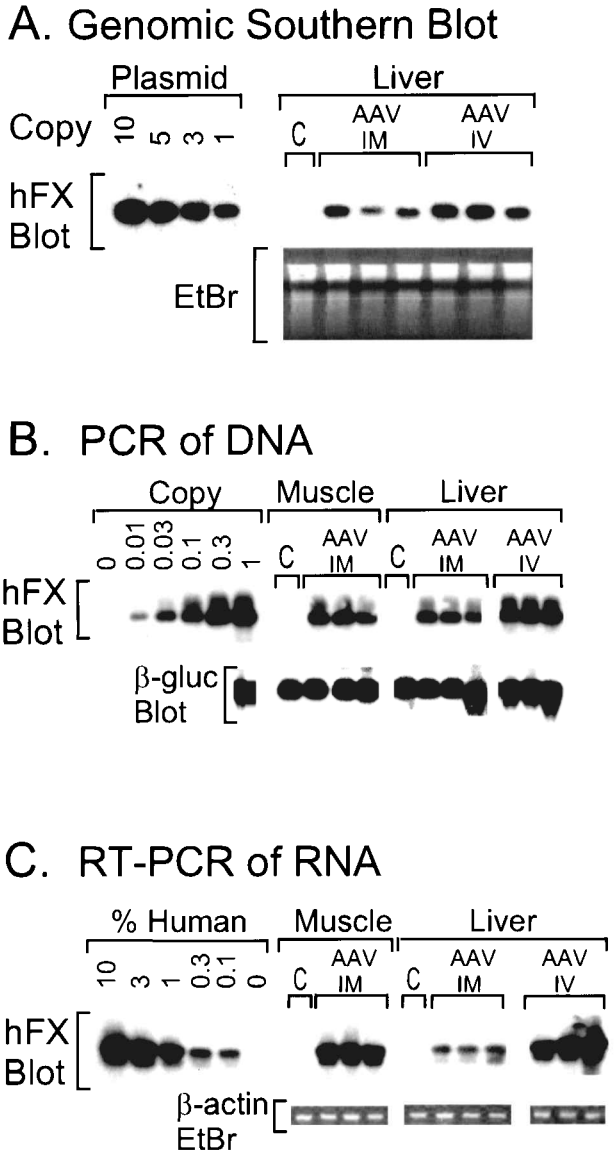

FIG. 6. Evaluation of the DNA copy number and RNA levels after transduction with CMV-β-actin-hFX-AAV. (**A**) Southern blot analysis. DNA was obtained from the liver 1.2 years after transduction for the animals whose expression data are shown in Fig. 5. Fifteen micrograms of DNA was digested with *Apa*I, which results in a 758-nt band derived from the hFX cDNA, and the Southern blot was performed with the same hFX probe. Standards represent dilutions of a plasmid (Plasmid) containing the hFX cDNA at the indicated copy number. The top region shows the results of the Southern blot (hFX Blot), while the bottom region shows the results of ethidium bromide staining of the digested samples prior to transfer (EtBr). (**B**) PCR of DNA to assess the DNA copy number. One microgram of DNA from muscle or liver was amplified with hFX or mouse β-glucuronidase-specific primers, and the Southern blot was performed. The controls (C) are from a nontransduced mouse. (**C**) RT-PCR of RNA from liver or muscle. Two micrograms of RNA was amplified by RT-PCR with either hFX-specific primers followed by Southern blot (hFX-Blot), or with mouse β-actin-specific primers followed by ethidium bromide staining (β-actin EtBr). The standard curve was generated by mixing human liver RNA with mouse RNA at the indicated ratio.

liver appeared to be much higher for animals that received the vector intravenously than for those that received it intramuscularly, the poor quality of the standard curve and the absence of a quantitative competitor precludes precise quantitation. For mice that received AAV vector by intramuscular injection, the levels of RNA appeared to be higher in the muscle than in the liver, although precise quantitation was not possible.

DISCUSSION

The CMV-β-actin promoter directs the highest level of expression from an AAV vector in the liver in vivo

In this study, we determined that an AAV with the CMV-β-actin promoter is expressed at levels that are 9.5-fold higher than an AAV vector with the EF1α promoter, and 137-fold higher than an AAV vector with the CMV promoter. This resulted in expression of hAAT at 1725 ± 87 ng/ml, which was maintained at stable levels for 300 days. The stable expression observed here in adults differs from the results reported by Daly *et al*. (1999b), who observed that human β-glucuronidase activity in the liver and serum were ~50-fold lower at 12 to 16 weeks of age than they were at 2 to 4 weeks of age after transfer into neonatal mice. This apparent decline in their study could simply reflect the fact that the promoter is expressed better in growing animals than in adults. Indeed, an AAV vector with nt −580 to −220 of the CMV enhancer and a substantially shorter region (nt −275 to +1) of the chicken β-actin promoter (Xiao *et al*., 1998), and an adenoviral vector with an identical CMV-β-actin promoter to that used here (Kiwaki *et al*., 1996), were expressed well in the liver long term *in vivo*. We therefore conclude that this promoter can direct stable expression in the liver in adult animals.

The AAV vector with EF1α promoter exhibited intermediate levels of expression, with an average of 195 ± 36 ng/ml, and expression that was 14-fold higher than from an AAV vector with the CMV promoter. The moderate levels of expression that we observed with this promoter are consistent with another study that found that a slightly longer EF1α promoter directed similar levels of expression of human factor IX at a similar dose of AAV vector (Nakai *et al*., 1998), and exhibited expression that was >100-fold higher than from the CMV promoter. Although we found that the vector with the CMV-β-actin promoter resulted in expression that was 9.5-fold higher than from the vector with the EF1α promoter, it remains possible that differences in the backbone of the two vectors could account for part or all of this difference. Expression of this promoter is probably due primarily to several Sp1 sites (Nielson *et al*., 1998).

The CMV promoter is expressed at low levels from an AAV vector in vivo *and is not efficiently activated by LPS in liver*

The CMV promoter exhibited low expression from an AAV vector in this study, with an average hAAT level of 8 ± 1.5 ng/ml observed for the CMV-hAAT-AAV-transduced mice. Poor expression from an AAV vector with the CMV promoter in this study is consistent with its low expression from an AAV vector (Snyder *et al*., 1997; Nakai *et al*., 1998), a retroviral vector (Kay *et al*., 1992), and an adenoviral vector in the absence of inflammation or liver regeneration (Löser *et al*., 1998), but differs from one report of moderate levels of expression from an AAV vector in the liver (Xiao *et al*., 1998).

Although the CMV promoter is inactive in a quiescent liver from a variety of viral vectors in most studies, it is expressed at high levels from a retroviral vector during liver regeneration (Rettinger *et al*., 1993), or from an adenoviral vector in livers of animals with acute inflammation or regeneration (Löser *et al*., 1998). This led us to test whether the CMV promoter might function as a "stealth promoter" in animals, in which it is quiescent in the normal liver, but strongly activated in response to an inexpensive and easy method for inducing inflammation. Such a stealth promoter might enable one to transfer the vector into an animal, allow the slow conversion to double-stranded DNA to occur, then stimulate expression in order to determine the cell type and frequency of transduction. This could be of particular importance in large animal models in which immunodeficient strains are not available, and robust immune responses to commonly used histochemically visible proteins are often observed (Izembart *et al*., 1999). However, we found that LPS resulted in only a 2-fold increase in expression from the CMV promoter *in vivo* when stimulation was applied 8.5 months after transduction, which would likely be insufficient to be of practical significance.

The failure to activate the CMV promoter may have been due to modifications of the promoter, such as methylation or histone deacetylation (Hassig and Schreiber, 1997; Lorincz *et al*., 2000), that could reduce its ability to respond to induction by the appropriate transcription factors. Similarly, LPS only partially activated the CMV promoter from an adenoviral vector 1 month after transfer into the liver (Löser *et al*., 1998). It may therefore be difficult to activate promoters in animals that have been silenced for a prolonged period of time. Activation of the CMV-β-actin promoter by 2.7-fold in response to LPS may have been due to the CMV enhancer from nt −522 to −188, which is present in both constructs, and is known to contain two NF-κB sites, three CREB sites, one AP-1 site (Hennighausen and Fleckenstein, 1986; Ghazal *et al*., 1988; Chan *et al*., 1996), and two putative STAT3 sites (at nt −510 and at −390 relative to the transcription initiation site). These transcription factors are induced during inflammation and/or liver regeneration. Alternatively, stimulation of this promoter may have been due to a putative NF-κB and EGR site within the β-actin sequences. Activation of the EF1α by 2.5-fold in response to LPS may have been due to the presence of potential NF-κB sites at nt −310, +710, and +840, an EGR site at +600, or an AP-1 site at +680 based on computer analysis of the EF1α sequences (not shown).

Comparison of transcription factor-binding sites for the CMV enhancer-containing vectors

Since promoters activate genes by binding to proteins that recruit RNA polymerase, the specific binding sites present in a promoter are critical for understanding why a particular promoter functions in a specific organ. The high-level expression from the CMV enhancer-β-actin promoter is in strong contrast to the low-level expression from the CMV promoter/enhancer. Although other differences in the vector such as the presence of a splice site or a second transcription unit could affect ex-

pression, most others have also observed that the CMV enhancer-β actin promoter is expressed much better *in vivo* than the CMV promoter alone. This could be due to the presence of highly active transcriptional elements in the β-actin promoter, or to the presence of inhibitory elements in the portion of the CMV promoter that are not present in the other construct. In addition to the transcription factor-binding sites mentioned above, the CMV enhancer contains three NF-1 sites and one SP1 site, as well as one potential Oct-1 site (nt -280) and one potential C/EBP site (nt -515), based on sequence analysis. These transcription factors are either ubiquitous or are present in the normal liver, and could help to activate transcription. The region from nt -188 to $+73$ is unique to the CMV promoter construct. Although it contains a putative C/EBP site at nt -100, a known Sp1 site at nt -80, and a known TATA box at -25, the two CREB/ATF sites, the one AP1 site, and the two NF-κB sites would be largely unoccupied in the normal liver. The paucity of transcription factors near the start site may lead to the lack of transcription in the normal liver.

It is also possible that the CMV-β-actin promoter is expressed at higher levels due to binding of positive-acting transcription factors to the β-actin portion of the promoter. On the basis of sequence analysis (data not shown), the β-actin promoter from nt -278 to $+1$ contains four putative Sp1 sites, one putative NF-Y site, one putative Oct-1 site, and one putative HNF-3 site, all of which might bind to transcription factors that are present in the normal liver. In addition, the β-actin exon/intron sequences, which have been reported to contain enhancer activity (Niwa *et al*., 1991), contain seven potential Sp1 sites and three potential Oct-1 sites, which could also contribute to transcriptional activity in the normal liver. We hypothesize that this promoter is more active in the liver because the sequences near the start site bind to transcription factors that are present in the normal liver. Further studies will be needed to determine which of these sequences account for the difference in expression between the two promoters.

The CMV-β-actin promoter directs high-level expression of hFX from an AAV vector after injection into neonatal mice

The efficacy of the CMV-β-actin promoter from an AAV vector in the liver *in vivo* was further demonstrated by its ability to direct stable levels of expression of hFX in mice after neonatal injection. In this study, neonatal mice that were injected intravenously with CMV-β-actin-hFX-AAV expressed hFX at 548 ng/ml for up to 1.2 years after transduction. This was associated with 0.68 copies of the AAV vector DNA per diploid genome in the liver, as determined by Southern blot. A similar copy number was achieved in a previous study after intravenous injection into neonates (Nakai *et al*., 1998), while DNA was present in the liver at an undefined level in another study at late times after intravenous injection into neonates (Daly *et al*., 1999b). These data demonstrate that AAV transduction can be achieved in animals with moderate levels of hepatocyte replication, as occurs during normal postnatal growth. The clinical efficiency of this level of expression could not be determined, as these were normal mice that already expressed mouse FX (mFX). Normal mice were used here, as mFX-deficient mice die around birth due to their severe bleeding diathesis (Dewerchin *et al*., 2000). However, we predict that this level (6.8% of normal) should correct most of the bleeding manifestations of hFX deficiency.

In this study, no antibodies were produced to the hFX protein in C57BL/6 mice that received the AAV vector as neonates via either intramuscular or intravenous injection (data not shown), using a mouse IgG-specific ELISA similar to that described previously in rats (Le *et al*., 1997). This could be due to the phenomenon of neonatal tolerance, or to the inability of C57BL/6 mice to generate antibodies to hFX. We favor the latter possibility, as injection of an hFX-expressing retroviral vector into young adult C57BL/6 mice failed to induce anti-hFX antibodies, although injection of the same retroviral vector into similar-aged BALB/c mice induced antibodies with a titer that was as high as 1:3200 (data not shown). We are currently testing additional neonatal and adult adult mice for their immunological response to hFX to better address this issue.

Neonatal intramuscular injection of an 8-fold lower amount of AAV than was used for intravenous injection resulted in expression of hFX at 248 ng/ml, and expression was maintained for up to 1.2 years. This was associated with 0.34 copies of AAV vector per cell in the liver as determined by Southern blot, and a similar copy number in the muscle as determined by PCR. Similarly, both organs contained hFX mRNA, although the level appeared to be higher in the muscle. This demonstrates that intramuscular injection of an AAV vector that expresses hFX can also have a therapeutic effect, and that both liver and muscle may contribute to expression. Intravenous dissemination after intramuscular injection into neonatal mice is likely due to their small size and the inability to avoid blood vessels. This may not occur in adults or in neonatal animals from larger species in which more carefully controlled injections could be performed.

Implications for gene therapy

Our study shows that neonatal intravenous injection of an AAV vector with the CMV-β-actin promoter results in a high copy number in the liver, and stable and therapeutic levels of expression of hFX in blood of adult mice. This demonstrates that the moderate levels of hepatocyte replication that occur in a rapidly growing animal do not preclude transduction with an AAV vector. Neonatal gene transfer would lead to a more rapid correction of the genetic disorder, and might be less likely to stimulate an immune response. It remains to be determined whether neonatal injection can be equally effective in larger animals, and whether germ line transmission occurs. If these issues can be resolved, this approach might be clinically acceptable for treatment of hemophilia in neonates.

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