

Sustained correction of bleeding disorder in hemophilia B mice by gene therapy

LILI WANG*, KAZUAKI TAKABE†, SCOTT M. BIDLINGMAIER‡, CHARLES R. ILL‡, AND INDER M. VERMA*§

*Laboratory of Genetics and †Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, San Diego, CA 92186-5800; and ‡Division of Gene Therapy, Immune Response Corporation, Carlsbad, CA 92008

Contributed by Inder Verma, January 19, 1999

ABSTRACT Mice generated by disrupting the clotting factor IX gene exhibit severe bleeding disorder and closely resemble the phenotype seen in hemophilia B patients. Here we demonstrate that a single intraportal injection of a recombinant adeno-associated virus (AAV) vector encoding canine factor IX cDNA under the control of a liver-specific enhancer/promoter leads to a long-term and complete correction of the bleeding disorder. High level expression of up to 15–20 $\mu\text{g/ml}$ of canine factor IX was detected in the plasma of mice injected with 5.6×10^{11} particles of an AAV vector for >5 months. The activated partial thromboplastin time of the treated mice was fully corrected to higher than normal levels. Liver-specific expression of canine factor IX was confirmed by immunofluorescence staining, and secreted factor IX protein was identified in the mouse plasma by Western blotting. All treated mice survived the tail clip test without difficulty. Thus, a single intraportal injection of a recombinant adeno-associated virus vector expressing factor IX successfully cured the bleeding disorder of hemophilia B mice, proving the feasibility of using AAV-based vectors for liver-targeted gene therapy of genetic diseases.

Hemophilia B is an X-linked bleeding disorder resulting from a defect or deficiency in blood coagulation factor IX. Recently, three hemophilia B knockout mouse models that closely resemble the null phenotype of the human disease have been generated by gene targeting (1–3). All of these hemophilia B mice have no detectable factor IX mRNA or protein and exhibit severe hemophilic phenotypes with spontaneous bleeding in the foot pads, spontaneous lethal internal bleeding, and nonstop bleeding after tail clipping. The mouse model systems accurately reflect the pathophysiology of human hemophilia B. Recombinant adenoviral vectors expressing murine or human factor IX upon injection in hemophilia B mice (129 and CD-1 strains) achieved only temporary correction of the bleeding diathesis because of the rapid formation of neutralizing antibodies to murine or human factor IX (2, 4). Although correction in the C57BL/6 strain lasted >12 weeks without detection of antibodies to human factor IX (hFIX), the level of expression of factor IX declined during the time course of 12 weeks (4), possibly because of cytotoxic T lymphocyte response to adenoviral-transduced cells.

Adeno-associated virus (AAV) is promising for *in vivo* gene therapy because the vector is based on a nonpathogenic virus and can infect both dividing and nondividing cells. Additionally, the vector is devoid of any viral coding sequences that reduce the risk of undesirable host immune responses. Several recent reports have demonstrated the persistent expression of transgenes in immunocompetent animals after delivery of AAV vectors into various tissues (5–11). And more recently, 1% of normal canine factor IX levels have been achieved in

hemophilia B dogs by recombinant AAV (rAAV) vectors targeting liver or muscle (12, 13). In this study, we explored the feasibility of using AAV vector for liver-directed gene therapy in hemophilia B mice. We generated an AAV vector encoding canine factor IX (cFIX) driven by a strong liver-specific enhancer/promoter (LSP) and demonstrated the complete cure of the bleeding disorder in hemophilia B mice.

MATERIALS AND METHODS

rAAV Vector Construction. The AAV vector AAV-LSP-cFIX is depicted in Fig. 1a. The vector plasmid pAAV-LSP-cFIX was derived from plasmid pAAV-MFG-cFIX, which was constructed by cloning a 3.52-kilobase blunt-ended *NheI* to *BamH* fragment containing the Moloney murine leukemia virus 5' long terminal repeat and the canine factor IX cDNA sequence and the poly(A) site of bovine growth hormone into *XbaI*-digested and blunt-ended pSub 201 (containing only AAV terminal repeats) (14). pAAV-LSP-cFIX was constructed by replacing the murine leukemia virus 5' long terminal repeat of pAAV-MFG-cFIX with a liver-specific enhancer/promoter. pAAV-MFG-cFIX was digested with *NheI* (blunt-ended) and *NcoI*, and the backbone was ligated with an 845-bp *PstI* (blunt-ended) to *NcoI* fragment containing a liver-specific enhancer/promoter from pCY-601 (15). In brief, the 845-bp liver specific promoter (LSP) contains the thyroid hormone-binding globulin promoter sequences (–382 to +3), two copies of $\alpha 1$ -microglobulin/bikunin enhancer sequences (–2,804 through –2,704), and a 71-bp leader sequence as described (15). A 580-bp *HindIII* to *EcoRI* fragment (blunt-ended) containing the intervening sequences (IVS) 2 of human β -globin gene from pMD.G (16) was inserted in the *BglII* site (blunt-ended), which is just in front of the ATG start codon of canine FIX. Plasmid pAAV-LSP-GFP was constructed based on pAAV-MFG-GFP by replacing the cFIX and poly(A) of pAAV-MFG-cFIX with a 1-kilobase *NcoI* to *AflIII* (blunt-ended) fragment containing the GFP coding sequence and SV40 Poly(A) from pEGFP-N1 (CLONTECH). pAAV-LSP-GFP then was constructed by replacing the MFG promoter of pAAV-MFG-GFP [treated with endonucleases *NheI* (blunt-ended) and *NcoI*] with an 855-bp *EcoRV* and *NcoI* fragment containing the liver-specific enhancer/promoter from pCY-601.

AAV vectors were made by three plasmids cotransfection methods as described by Xiao *et al.* (17). Vectors were recovered from two sequential CsCl gradients and were dialyzed against sterile PBS. The physical vector titer was determined by a quantitative dot-blot assay (18). Titers were routinely in the range of $1\text{--}6 \times 10^{12}$ genomes/ml.

Abbreviations: AAV-adeno-associated virus; rAAV, recombinant AAV; cFIX, canine factor IX; LSP, liver-specific promoter; GFP, green fluorescent protein; aPTT, activated partial thromboplastin time; hFIX, human factor IX.

§To whom reprint requests should be addressed at: Laboratory of Genetics, The Salk Institute, P.O. Box 85800, San Diego, CA 92186-5800. e-mail: verma@salk.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

PNAS is available online at www.pnas.org.

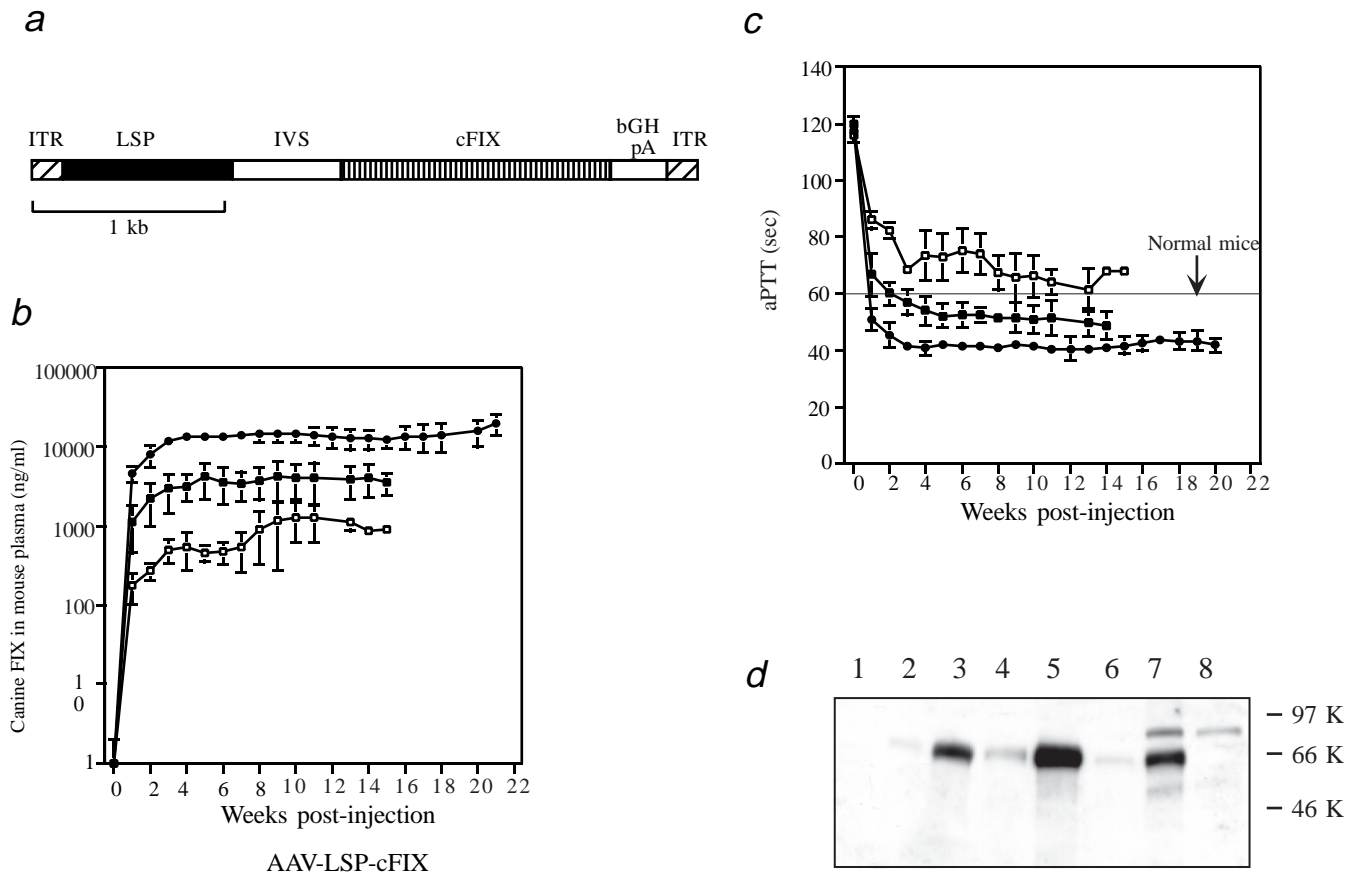


FIG. 1. Long-term expression of functional canine factor IX in hemophilia B mice. (a) Schematic drawing of rAAV-LSP-cFIX vector. (b) ELISA assays. Recombinant AAV-LSP-cFIX was delivered as a single intraportal injection into the liver of adult hemophilic C57BL/6 mice in a dose of 2×10^{11} ($n = 4$, ■) or 5.6×10^{11} ($n = 5$, ●) vector genomes or into the liver of adult hemophilic 129 mice at a dose of 2×10^{11} particles ($n = 3$, □). Mice were bled weekly after vector administration. The canine factor IX concentration in the mouse plasma was determined by an ELISA assay. (c) Functional factor IX activity in the mouse plasma was determined by an *in vitro* aPTT assay (same symbols as in b). The line in the middle shows the aPTT for normal mice. (d) Western blot analysis. Plasma from C57BL/6 hemophilic mice 4 months postinjection with 2.8×10^{11} particles or 5.6×10^{11} particles of AAV-LSP-cFIX was analyzed by Western blotting. Lanes: 1, naive mouse, (0.1 μ l of plasma); 2 and 3, plasma from mouse injected with 2.8×10^{11} particles (0.01 and 0.1 μ l, respectively); 4 and 5, plasma from mouse injected with 5.6×10^{11} particles (0.01 and 0.1 μ l, respectively); 6 and 7, normal canine plasma (0.01 and 0.1 μ l, respectively) (Sigma); 8, 0.1 μ l of plasma from a hemophilic dog (a gift from T. Nichols of the University of North Carolina, Chapel Hill).

In Vitro Transduction of HepG2 Cells. HepG2 cells were seeded in a 12-well plate at a density of 2.5×10^5 cells/well. Twenty-four hours later, 5×10^9 vector genomes of AAV-LSP-cFIX were added to the wells with 1 ml of fresh medium (Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1 μ g/ml of vitamin K₁). Twenty-four-hour conditioned media were collected on day 2 postinfection and were assayed for cFIX by ELISA.

Animal Procedures. All animal procedures were performed in accordance with protocols approved by the Institute Animal Care and Use Committee. Hemophilia B carrier females were bred with normal male mice of either C57BL/6 or 129 background for several generations. Hemophilic offspring were identified by activated partial thromboplastin time (aPTT) assays (2). Hemophilia B mice used in this study were in F4 or F5 generation and 8–10 weeks old (16–25 grams). Food was withdrawn from mice 4 hours before surgery. Fifteen to twenty micrograms of purified human FIX (Enzyme Research Laboratories, South Bend, IN) diluted in PBS were injected intravenously into mice 1–2 hours before the surgery. By 48-hour postinfusion, <50 ng/ml of human factor IX could be detected in the plasma. Under isofluorane anesthesia, a midline incision was made followed by displacing the intestine. Intraportal injection was performed through ileocecal vein (2–3 mm proximal from the branch of marginal vein) by using a Hamilton syringe together with a 30G needle. Injection was

$\approx 200 \mu$ l/2–3 min. A small piece of Gelfoam (Amersham Pharmacia) was attached and pressed to the injection site for 3 min immediately after the injection. The abdomen was closed layer-to-layer by using 4–0 silk suture (Sherwood-Davis & Geck, St. Louis) after no bleeding was confirmed. After the operations, animals were allowed to drink 20% glucose solution instead of water for 4 days. Blood samples were collected at 7-day intervals from the retro-orbital plexus into 1/10 volume of 3.2% citrate. Tail clip assay was performed at various time points after the injection as described with slight modifications (19). Under isofluorane anesthesia, mouse tail was pulled through a hole with 1.5-mm diameter until snug and was cut with a No. 11 surgical blade to ensure the wound of identical cross section between each mouse tail (4).

Assays for Antigen Levels and Biological Activity of Canine FIX. Canine FIX antigen in mouse plasma was determined by ELISA as described (20). Mouse samples were diluted 1:100 to 1:4,000 depending on the cFIX expression level in mice. Pooled normal canine plasma (Sigma) was used as a factor IX standard (5 μ g/ml). Clotting activity of cFIX in the treated mice was determined by aPTT assays as described (2).

Western Blot Analysis. The presence of canine FIX in the plasmas of treated mice was demonstrated by Western blot analysis as described (2). Mouse or canine plasmas were diluted 1:10 and 1:100 in sample loading buffer. The primary antibody used here was an affinity-purified rabbit anti-cFIX antibody (Enzyme Research Laboratories; diluted 1:1,000).

Immunofluorescence Staining. Liver lobes were excised from the dead animal and were fixed with 4% cold paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C and then were saturated in 30% sucrose in 0.1 M phosphate buffer. The fixed tissues were frozen in O.C.T. embedding compound before sectioning. Sections first were rinsed in PBS (pH 7.4) for 10 min and then were blocked in PBS with 5% donkey serum, 2% BSA, and 0.2% Triton X-100 for 20 min. Sections subsequently were incubated for 2 hours with an affinity-purified rabbit anti-cFIX antibody (Enzyme Research Laboratories; diluted 1:500). After three washes (10 min each) in PBS, the secondary antibody donkey anti-rabbit FITC (The Jackson Laboratory; diluted 1:100) was applied for 90 min. All incubation steps were at room temperature. For GFP expression, sections were rinsed in PBS for 10 min and were air-dried and mounted. Fluorescence microscopy was performed with a Zeiss Axiovert 25.

RESULTS

In Vitro Transduction of HepG2 Cells. AAV-LSP-cFIX, an AAV vector containing a liver-specific promoter (LSP) transcribing canine factor IX (Fig. 1*a*), was constructed to target liver, the natural site for factor IX production. Human hepatoma (HepG2), a liver-derived cell line transduced by AAV-LSP-cFIX at a multiplicity of infection of 10^4 vector genomes/cell, secreted canine factor IX in the media at 48 ng/ 10^5 cells/24 hours. No canine factor IX was detected in the media of mock infected HepG2 cells or in HeLa and human embryonic kidney (293) cells infected with same amount of virus. This suggested that the liver-specific promoter in AAV-LSP-cFIX is only active in liver-derived cell line but not in HeLa or 293 cells.

Sustained and High Level Expression of Biologically Active cFIX in Hemophilia B Mice by a Single Intraportal Injection of AAV-LSP-cFIX. To determine whether AAV-LSP-cFIX can produce substantial amount of factor IX *in vivo*, adult immunocompetent hemophilia B mice in C57BL/6 or 129 genetic backgrounds were injected with 2×10^{11} or 5.6×10^{11} particles of AAV-LSP-cFIX via the portal vein. Factor IX was detected at 0.13–6.25 $\mu\text{g/ml}$ in the plasma of treated mice within the first week and increased continuously during the next 3 weeks, reaching a plateau of 1–20 $\mu\text{g/ml}$ (Fig. 1*b*). The hemophilic C57BL/6 mice ($n = 5$) that received 5.6×10^{11} rAAV particles had the highest level of canine FIX expression ($15.8 \pm 5.7 \mu\text{g/ml}$, 20 weeks after injection), approximately three times higher than the ones that received 2×10^{11} particles ($n = 4$; Fig. 1*b*). The canine FIX expression was relatively less efficient in hemophilic 129 mice ($n = 3$; 1 $\mu\text{g/ml}$), which is at least 3–4 \times less than the C57BL/6 mice that received the same amount of virus. Furthermore, it took them longer to reach the plateau of factor IX expression.

We next determined the functional FIX activity in the mouse plasma by an aPTT assay. The baseline clotting time of the hemophilia B mice in this assay is 116.7 ± 4.7 sec, and that of normal mice is 59.6 ± 3.2 sec. After a single intraportal injection of AAV-LSP-cFIX, the aPTT clotting time of all treated mice gradually decreased during the first 4 weeks to different levels, depending on the mouse strain and the amount of virus injected, and then maintained at that level for the duration of the experiment (Fig. 1*c*). The effect was most dramatic in the hemophilic C57BL/6 mice that received 5.6×10^{11} rAAV particles: the clotting time decreased beyond the normal level (≈ 40 –42 sec). As with the amount of secreted factor IX, the dosage effect was observed, and the clotting time of the hemophilic C57BL/6 mice that received 2×10^{11} particles shortened to the normal range. The clotting time of the hemophilic 129 mice injected with 2×10^{11} particles was shortened, although not to the normal level but close to the level that is observed in the heterozygote carrier mice ($69.7 \pm$

3.9 sec), thus emphasizing the role of the mouse strain in gene expression.

To demonstrate the presence of canine FIX in the plasmas of the treated mice, Western blot analyses were performed. A prominent 64-kDa protein band was seen in plasma from two treated mice at the level consistent with ELISA results (Fig. 1*d*). One mouse (Fig. 1*d*, lane 3) expressed canine factor IX at a level equivalent to that seen in normal canine plasma (Fig. 1*d*, lane 7) 4 months after infusion with 2.8×10^{11} particles of AAV-LSP-cFIX. Another mouse (Fig. 1*d*, lane 5) expressed a 3- to 4-fold higher concentration of canine factor IX than that in normal canine 4 months postinfusion with 5.6×10^{11} particles.

Detection of cFIX in Mouse Liver by Immunofluorescence Staining. The presence of canine factor in mouse liver was confirmed by immunofluorescence studies on liver sections from a hemophilic C57BL/6 mouse 4 months postinjection with 2.8×10^{11} rAAV particles. Fig. 2*a* and *b* shows that canine factor IX was largely detected in the linings of sinusoids, central and hepatic veins of the liver, rather than in hepatocytes, as previously reported for human FIX expressed in liver (21). This discrepancy may be attributable to different antibodies used to detect factor IX. The positive staining in the linings of sinusoids was seen evenly throughout the entire liver section, presumably because the sinusoids are networked. Canine FIX was not detected in liver sections from untreated mice (Fig. 2*c*). Because factor IX is secreted, it may be difficult to detect it in hepatocytes. To test whether intraportal injection leads to expression in hepatocytes, we generated an rAAV vector containing the green fluorescent protein (GFP) reporter gene under the control of the liver-specific promoter/enhancer (AAV-LSP-GFP). Fig. 2*d* and *e* shows expression of GFP in the hepatocytes 18 weeks after injection with 3×10^{11} AAV-LSP-GFP particles intraportally into adult C57BL/6 mice. GFP-expressing cells were evenly distributed throughout the liver; however, the level of expression was not even among the expressing cells. We estimate that $\approx 10\%$ of the hepatocytes were expressing GFP. We therefore conclude that *in vivo* transduction with rAAV containing the liver specific promoter can direct long term transgene expression in the liver.

Long-term phenotype correction of murine hemophilia B. Mice expressing 1–20 $\mu\text{g/ml}$ of canine factor IX in plasma appeared phenotypically normal because they did not bleed spontaneously or bleed profoundly after scratches or ear tagging. To show directly that expression of biologically active canine factor IX in the treated mice resulted in correction of the bleeding disorder, a tail clip survival study was performed on untreated and treated hemophilia B mice, as well as normal C57BL/6 mice (Table 1). All of the normal C57BL/6 mice readily survived tail clipping with no evidence of distress. Three of eleven untreated mice survived tail clipping, but all three mice were lethargic at 24 hours. In contrast, all mice that received AAV-LSP-cFIX survived tail clipping with no evidence of distress at 15–22 weeks postinjection. These data show long-term phenotypic correction of murine hemophilia B with a single intraportal injection of AAV viral vectors.

DISCUSSION

For a variety of metabolic diseases, a major concern for gene therapy is sustained production of the therapeutic gene product. Recombinant AAV vectors have been shown quite promising for the therapeutic gene delivery in liver and skeletal muscle (8–12, 22). To generate enough gene products, a strong promoter is desirable so that less vector would be required to be administered. Several promoters have been compared for their strength *in vivo* after rAAV delivery into the mouse liver, and highest expression of reporter gene was noted with the albumin promoter and the 5' long terminal repeat of Moloney murine leukemia virus (MFG) (11). Snyder *et al.* (8, 12) have

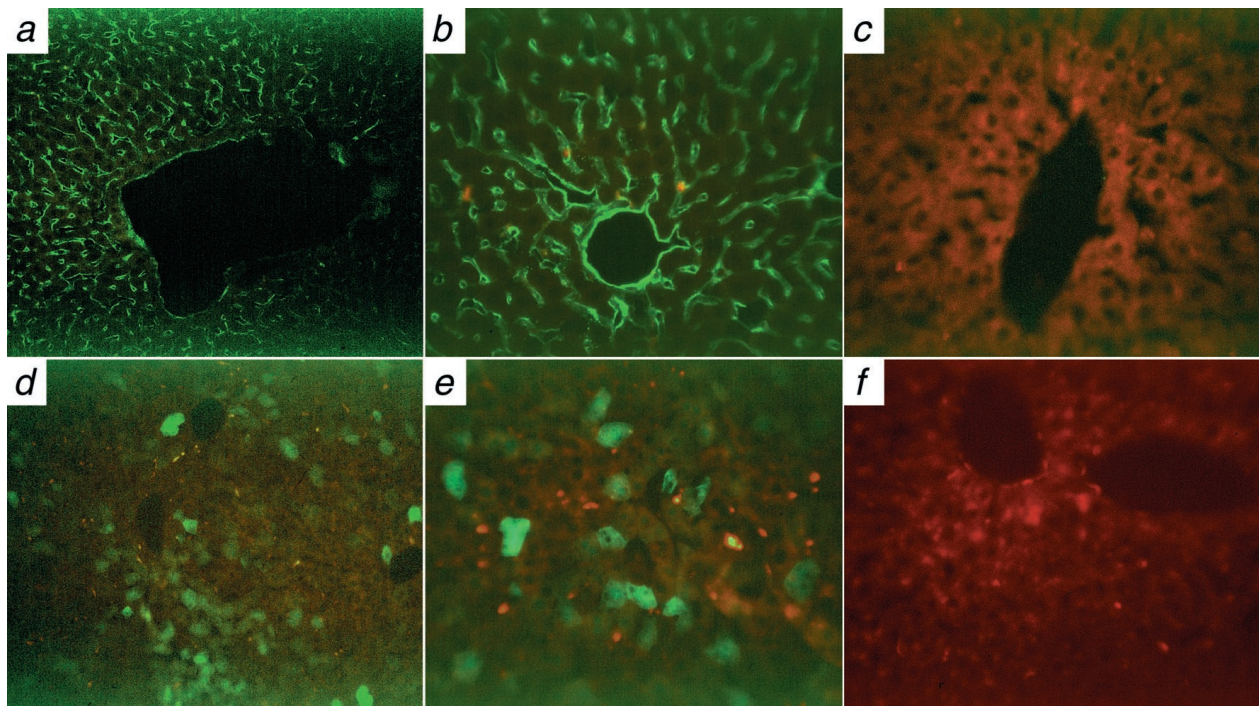


FIG. 2. Detection of canine factor IX in the mouse liver by immunofluorescence staining. AAV-LSP-cFIX (2.8×10^{11} particles in $200 \mu\text{l}$) was injected intraportally into hemophilic C57BL/6 mice. Four months after the injection, one mouse was killed, and the liver was analyzed for canine factor IX by immunofluorescence staining. Plasma level of canine factor IX was $6.5 \mu\text{g/ml}$ at the time the mouse was killed. Shown is AAV-transduced liver demonstrating the secreted canine factor IX in the linings of sinusoids and central vein (*a*, $\times 20$; *b*, $\times 40$). (*c*) Control naive mouse ($\times 40$). (*d-f*) Transduction of mouse liver by AAV-LSP-GFP. Particles (3×10^{11}) in $150 \mu\text{l}$ were injected intraportally into C57BL/6 mice. Eighteen weeks after the injection, one mouse was killed, and the liver was analyzed for GFP expression by immunofluorescence microscopy. Hepatocytes positive for GFP expression were identified in the AAV-transduced liver. [*d*, $\times 20$; *e*, $\times 40$; *f* (control naive mouse), $\times 40$].

reported expression of up to $2 \mu\text{g/ml}$ of hFIX in mice after intraportal delivery of 8.4×10^{10} particles of AAV-MFG-hFIX whereas Nakai *et al.* (21) reported expression of $0.7\text{--}3.2 \mu\text{g/ml}$ of hFIX in mice after intraportal delivery of 2.7×10^{11} particles of AAV-EF1 α -hFIX. We have shown that the use of a liver-specific enhancer/promoter to transcribe the cFIX gene allows both high and sustained expression. Sustained expression of $15\text{--}20 \mu\text{g/ml}$ of cFIX has been achieved in hemophilia mice after a single intraportal delivery of 5.6×10^{11} particles of AAV-LSP-cFIX. In our hand, LSP is at least two-fold stronger than the MFG (L.W. and I.V., unpublished data). Furthermore, the amount of the factor IX protein appears to be proportional to the amount of the rAAV vector delivered, thus allowing regulation of the concentration of the therapeutic gene product. In contrast to what has been observed by others—that rAAV genomes were converted to high-molecular-weight DNA as integrated or multiple concatamers

with time (5, 11, 22, 23)—our analysis suggested that the majority of rAAV genomes persist in liver in episomal form 4–5 months postinjection (data not shown).

We also evaluated the effect of different mouse strains on cFIX expression after administration of same amount of vectors. So far, C57BL/6 is the only immunocompetent mouse strain that has been reported for sustained expression of factor IX. Our data showed that the levels of expression of factor IX protein differed (up to 4-fold) in different genetic backgrounds of hemophilia B mice. Of interest, hemophilia B mice in 129 genetic background on intraportal injection with AAV-LSP-cFIX did not generate antibodies to factor IX, but they generated very high levels antibodies to factor IX after intravenous or intramuscular injection of recombinant adenoviral vectors carrying factor IX (L.W. and I.V., unpublished data). Thus, the genetic background, the nature of the vector, and the route of injection will dictate the extent of the immune response generated against a transgenic protein. Preliminary experiments in the dog model system deficient in factor IX protein show shortened whole blood clotting time 85 days postinjection with rAAV (AAV-LSP-cFIX) vector (L.W. and T. Nichols, unpublished data). The future challenge will involve not only sustained expression, but also immunological tolerance of the transgenic protein.

Table 1. Phenotype correction of murine hemophilia B

Mouse strain	Dose of AAV-LSP-cFIX, particles/animal	Weeks postinjection	Survivors
C57BL/6	None	-	6/6
Hemophilia B-C57BL/6	None	-	*3/11
Hemophilia B-129	None	-	0/3
Hemophilia B-C57BL/6	5.6×10^{11}	21	5/5
Hemophilia B-C57BL/6	2×10^{11}	15	4/4
Hemophilia B-129	2×10^{11}	15	3/3

Hemophilic mice in C57BL/6 and 129 strains were treated with a single intraportal injection of AAV-LSP-cFIX with the indicated amount. Factor IX plasma level and activity were measured by ELISA and aPTT assays. Fourteen or twenty weeks after the treatment, mouse tail was clipped, and the mouse survival rate was recorded 24 hours later. Normal C57BL/6 and untreated hemophilia mice served as controls. *All three survivors were lethargic at 24 hours.

We thank members of the Verma lab and Dr. Wylie Vale for their interest in this work, Drs. N. Somia and M. Weitzman for critical review of the manuscript, and G. Pao for the help on the immunofluorescence staining. We are grateful to Drs. X. Xiao and R. J. Samulski for Ad-free AAV production system, Dr. T. Nichols for the hemophilic dog plasma, K. Suter for expert assistance on all animal procedures, and B. Coyne for help in preparing the manuscript. This work was supported by grants to I.M.V. from the March of Dimes, Berger Foundation, Valley Foundation, and the National Institutes of Health. K. Takabe is supported by the Yoshida Scholarship Foundation. I.M.V. is an American Cancer Society Professor of Molecular Biology.

1. Lin, H. F., Maeda, N., Smithies, O., Straight, D. L. & Stafford, D. W. (1997) *Blood* **90**, 3962–3966.
2. Wang, L., Zoppe, M., Hackeng, T. M., Griffin, J. H., Lee, K.-F. & Verma, I. M. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 11563–11566.
3. Kundu, R. K., Sangiorgi, F., Wu, L. Y., Kurachi, K., Anderson, W. F., Maxson, R. & Gordon, E. M. (1998) *Blood* **92**, 168–174.
4. Kung, S. H., Hagstrom, J. N., Cass, D., Tai, S. J., Lin, H. F., Stafford, D. W. & High, K. A. (1998) *Blood* **91**, 784–790.
5. Xiao, X., Li, J. & Samulski, R. J. (1996) *J. Virol.* **70**, 8098–8108.
6. Kessler, P. D., Podsakoff, G. M., Chen, X., McQuiston, S. A., Colosi, P. C., Matelis, L. A., Kurtzman, G. J. & Byrne, B. J. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 14082–14087.
7. Fisher, K. J., Jooss, K., Alston, J., Yang, Y., Haecker, S. E., High, K., Pathak, R., Raper, S. E. & Wilson, J. M. (1997) *Nat. Med.* **3**, 306–312.
8. Snyder, R. O., Miao, C. H., Patijn, G. A., Spratt, S. K., Danos, O., Nagy, D., Gown, A. M., Winther, B., Meuse, L., Cohen, L. K., *et al.* (1997) *Nat. Genet.* **16**, 270–276.
9. Murphy, J. E., Zhou, S., Giese, K., Williams, L. T., Escobedo, J. A. & Dwarki, V. J. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 13921–13926.
10. Song, S., Morgan, M., Ellis, T., Poirier, A., Chesnut, K., Wang, J., Brantly, M., Muzyczka, N., Byrne, B. J., Atkinson, M., *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**, 14384–14388.
11. Xiao, W., Berta, S. C., Lu, M. M., Moscioni, A. D., Tazelaar, J. & Wilson, J. M. (1998) *J. Virol.* **72**, 10222–10226.
12. Snyder, R. O., Miao, C., Meuse, L., Tubb, J., Donahue, B. A., Lin, H. F., Stafford, D. W., Patel, S., Thompson, A. R., Nichols, T., *et al.* (1999) *Nat. Med.* **5**, 64–70.
13. Herzog, R. W., Yang, E. Y., Couto, L. B., Hagstrom, J. N., Elwell, D., Fields, P. A., Burton, M., Bellinger, D. A., Read, M. S., Brinkhous, K. M., *et al.* (1999) *Nat. Med.* **5**, 56–63.
14. Samulski, R. J., Chang, L. S. & Shenk, T. (1987) *J. Virol.* **61**, 3096–3101.
15. Ill, C. R., Yang, C. Q., Bidlingmaier, S. M., Gonzales, J. N., Burns, D. S., Bartholomew, R. M. & Scuderi, P. (1997) *Blood Coagul. Fibrinolysis* **8**, Suppl. 2, S23–S30.
16. Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M. & Trono, D. (1996) *Science* **272**, 263–267.
17. Xiao, X., Li, J. & Samulski, R. J. (1998) *J. Virol.* **72**, 2224–2232.
18. Snyder, R. O., Xiao, X. & Samulski, R. J. (1996) in *Current Protocols in Human Genetics*, eds Dracopoli, N., Haines, J., Krof, B., Moir, D., Morton, C., Seidman, C., Seidman, J. & Smith, D. (Wiley, New York), Vol. 1, pp. 1–24.
19. Connelly, S., Andrews, J. L., Gallo, A. M., Kayda, D. B., Qian, J., Hoyer, L., Kadan, M. J., Gorziglia, M. I., Trapnell, B. C., McClelland, A., *et al.* (1998) *Blood* **91**, 3273–3281.
20. Axelrod, J. H., Read, M. S., Brinkhous, K. M. & Verma, I. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5173–5177.
21. Nakai, H., Herzog, R. W., Hagstrom, J. N., Walter, J., Kung, S. H., Yang, E. Y., Tai, S. J., Iwaki, Y., Kurtzman, G. J., Fisher, K. J., *et al.* (1998) *Blood* **91**, 4600–4607.
22. Herzog, R. W., Hagstrom, J. N., Kung, S. H., Tai, S. J., Wilson, J. M., Fisher, K. J. & High, K. A. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 5804–5809.
23. Miao, C. H., Snyder, R. O., Schowalter, D. B., Patijn, G. A., Donahue, B., Winther, B. & Kay, M. A. (1998) *Nat. Genet.* **19**, 13–15.