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Long-term expression of human coagulation factor VIII and correction of hemophilia A after *in vivo* retroviral gene transfer in factor VIII-deficient mice

(gene therapy/retroviral vector/pseudotyping)

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ABSTRACT **Hemophilia A is caused by a deficiency in coagulation factor VIII (FVIII) and predisposes to spontaneous bleeding that can be life-threatening or lead to chronic disabilities. It is well suited for gene therapy because a moderate increase in plasma FVIII concentration has therapeutic effects. Improved retroviral vectors expressing high levels of human FVIII were pseudotyped with the vesicular stomatitis virus G glycoprotein, were concentrated to high-titers (10^9–10^{10} colony-forming units/ml), and were injected intravenously into newborn, FVIII-deficient mice. High-levels (≥200 milliunits/ml) of functional human FVIII production could be detected in 6 of the 13 animals, 4 of which expressed physiologic or higher levels (500–12,500 milliunits/ml). Five of the six expressers produced FVIII and survived an otherwise lethal tail-clipping, demonstrating phenotypic correction of the bleeding disorder. FVIII expression was sustained for >14 months. Gene transfer occurred into liver, spleen, and lungs with predominant *FVIII* mRNA expression in the liver. Six of the seven animals with transient or no detectable human FVIII developed FVIII inhibitors (7–350 Bethesda units/ml). These findings indicate that a genetic disease can be corrected by *in vivo* gene therapy using retroviral vectors.**

Hemophilia "A" is well suited for gene therapy because coagulation factor VIII (FVIII) levels in the blood plasma are therapeutic over a wide range, although unusually high FVIII expression levels may increase thrombotic risk (1–3). Gene therapy has the potential to provide life-long correction of the bleeding disorder. Effective gene therapy for hemophilia A requires the use of vectors that do not express immunogenic or toxic viral antigens and that can lead to long-term production of therapeutic FVIII levels (1, 2). Retroviral vectors derived from Moloney murine leukemia virus meet that requirement by virtue of their stable chromosomal integration and lack of viral genes. Initial efforts to develop hemophilia A gene therapy by using retroviral vectors containing a B domain-deleted *FVIII* cDNA were hampered by low titer [10^2-10^3 colony-forming units (cfu)/ml] and low levels of FVIII (4–8).This inhibition was relieved by including the native Moloney murine leukemia virus *env* intron upstream of the *FVIII* cDNA, which resulted in a 1,000-fold increased titer (10^5 cfu/ml) and 100- to 1,000-fold increased FVIII production (4, 9, 10). Despite these improvements, the maximal vector dose remained too low for efficient *in vivo* gene transfer.

In the present study, an intron-based retroviral vector (MFG-FVIIIΔB) (9, 10) that drives a B domain-deleted *FVIII* cDNA from the Moloney murine leukemia virus long terminal repeat was pseudotyped with the vesicular stomatitis virus G-protein (VSV-G), allowing concentration of viral vector particles to very high titer. To achieve this, a high-titer packaging cell line was used (293GPG) (11) that expressed VSV-G in a conditional, tetracycline-regulated manner. This high-titer vector was injected into young FVIII-deficient mice with targeted inactivation of the *FVIII* gene, which mimic the clinical hemophilia A phenotype, leading to efficient gene transfer in liver, spleen, and lungs. Hepatic expression of FVIII caused long-term supraphysiologic and physiologic functional FVIII production and correction of the bleeding disorder. Thus, stable correction of hemophilia A was achieved by retroviral gene therapy, which may be applicable to the treatment of other diseases.

METHODS

Retroviral Vector Production and Titration. The MFG-FVIIIΔB vector (9) was produced and titrated as described (10). To generate the corresponding VSV-G pseudotyped vectors (VSV_{293}-FVIII), subconfluent 293GPG packaging cells (11) were transduced successively with conditioned medium containing the MFG-FVIIIΔB vector, polybrene (8 μg/ml), and tetracycline (1 μg/ml). Individual clones were obtained by limiting dilution. Clones that expressed the highest levels of FVIII as measured with a functional COAtest (10) were induced in the absence of tetracycline and were screened for viral production by RNA dot blot analysis (10). A random-primed vector-specific probe was derived by PCR as described (10) using the MFG-FVIIIΔB plasmid as target and primers spanning a 418-bp region within the packaging sequence (5′-GGGCCAGACTGTTACCACTCCC-3′ and 5′-GGCGC-CTAGAGAAGGAGTGAGGG-3′). Serially diluted viral vector supernatants with known functional titer ($G418^R$ cfu/ml) based on VSV-G pseudotyped vectors containing a neo^R gene (LXSN) (12) were used as standards. Functional titer was determined by transduction of NIH 3T3 cells (4) and was expressed in G418-resistant colony-forming units per milliliter. To exclude the presence of rearranged proviral sequences, clones were subjected to Southern blot analysis (4). After expansion of the highest producer clone in the presence of tetracycline, semiconfluent cells were seeded in a 10-tray

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Abbreviations: FVIII, coagulation factor VIII; VSV-G, vesicular stomatitis virus G glycoprotein; RT, reverse transcriptase; cfu, colony-forming unit; kb, kilobase.
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cell-factory (Nunc, Roskilde, Denmark). When the plates were 80–90% confluent, VSV-G protein expression was induced by growing the cells in medium without tetracycline. The supernatant was harvested at 24- or 48-hr intervals over 2 weeks, was frozen immediately on dry ice before storing at −80°C, and was filtered by using a 0.45-μm filter before use. Viral concentration was carried out by centrifugation (13), and titer and yield of concentrated vector preparations were determined by RNA dot blot analysis (10).

Animal Studies. FVIII-deficient mice containing a disruption of the murine *FVIII* gene in exon 17 (14) were backcrossed with C57BL/6 mice over five generations. To obtain FVIII-KO/SCID mice (generated by B. Vanzieleghem, University of Leuven), FVIII-deficient mice were crossed with SCID mice (15), which are characterized by lack of functional T and B-cells and are unable to mount a specific immune response to foreign antigens. Genotyping and phenotypic characterization of the offspring was performed as described (14) and confirmed that all FVIII-deficient mice used contained the disrupted murine *FVIII* gene. Two- to three-day-old homozygous female and hemizygous male FVIII-deficient littermates were injected intravenously over two consecutive days with concentrated VSV_{293}-FVIII vector at a total dose equivalent to $0.9–1.4 \times 10^8$ cfu and were supplemented with polybrene (40 μg/ml). The animals were injected with 100 μl of concentrated vector preparations per day in the retro-orbital plexus by using a 30-gauge needle and a 1-ml syringe. Similarly, FVIII-deficient littermates were injected with PBS and polybrene as control. After injection, topical thrombin was applied to arrest bleeding. Plasma samples were obtained from each mouse by retro-orbital bleeding for functional human FVIII determination. To assess phenotypic correction, a 1-cm section of the tail was clipped, and survival was monitored. Experiments were approved by the Animal Ethical Commission of the University of Leuven.

Analysis of FVIII Expression. Biologically active human FVIII was quantified in citrated plasma samples in triplicate from mice by using FVIII COAtests (Chromogenix, Molndal, Sweden) as described (4). Plasma from FVIII-deficient mice spiked with human plasma derived FVIII (Octapharma, Langenfeld, Germany) of known activity was used as standard. One unit corresponded to 200 ng FVIII/ml (100%) and the sensitivity of the assay was ≈20–30 milliunits/ml. Physiologic FVIII concentrations were defined as 100–200 ng/ml.

Detection of Anti-FVIII Antibodies. Human FVIII-specific antibodies were detected by ELISA as described (16) with some modifications. Plates were coated with 3 units/ml human plasma-derived FVIII. Serially diluted monoclonal mouse anti-human FVIII antibody (mAb 18) was used as a control (17) with a sensitivity of 0.5 ng/ml. The human FVIII inhibitory antibody titer in the serum samples that scored positive in ELISA was determined with a Bethesda assay as described (18). Inhibition of FVIII activity by serially diluted mouse plasma was measured by using a functional FVIII COAtest. The detection limit of the assay was 0.2–0.4 Bethesda units/ml.

PCR. Genomic DNA was extracted from different organs by phenol/chloroform extraction (10). To determine relative transduction efficiencies in the various organs, PCR was performed on 200 ng of DNA by using primers specific for the *FVIII* cDNA spanning the B-domain deletion (5′-GATGAGAACCGAAGCTGG-3′ and 5′-GTCAAACTCATCTTTAGTGGGTGC-3′) and β-actin specific primers as described (10). PCR was performed by using AmpliTaq Gold (Perkin–Elmer) by denaturation for 10 min at 95°C, followed by 30 cycles for *FVIII* (and 28 cycles for β-actin) of 1 min at 95°C, 1 min at 59°C, 2 min at 72°C, and a final extension for 5 min at 72°C, yielding a 1.1-kilobase (kb) *FVIII* (B domain-deleted)-specific PCR product and a 0.2-kb β-actin specific product. Serially diluted DNA obtained from a producer clone containing five integrated *FVIII*-proviral copies (10) was used as a standard to calculate the average vector copy number per diploid genomic equivalent in the various organs. A constant amount of DNA (200 ng) was maintained in the standard by adding spleen DNA from a FVIII-deficient mouse. Amplified products were separated by gel electrophoresis on 1.5% agarose gels. The intensities of the PCR-amplified vector-specific *FVIII*-fragment relative to the standard were quantified with a Stratagene Eagle Eye II and NIH IMAGE 1.61/PPC software (http://rsb.info.nih.gov/nih-image/downloda.html) after background subtraction and β-actin normalization.

To determine which organ expressed FVIII, total RNA was purified by using Trizol followed by reverse transcriptase (RT)–PCR as described (10). Potentially contaminating residual genomic DNA was first eliminated by using DNase I (Life Technologies, Grand Island, NY). The same PCR conditions were used as above except for a different primer pair that specifically primed in *FVIII* exon 23 (C1 region) (5′-TCTTCTTTGGCAATGTGGATTCAT-3′) and the retroviral vector backbone (5′-GTTGAGTCAAAACTAGAGCCTGGACC-3′). Amplified RT-PCR products also were separated by gel electrophoresis on 1.5% agarose gels.

Inhibition of Gene Transfer. Viral vector producer cells were grown in the presence or absence of tetracycline to repress or induce VSV-G expression, respectively. The conditioned medium was concentrated 1,000-fold by centrifugation and was incubated at 4°C for at least 30 min with or without 10% (vol/vol) anti-VSV-G mAb (I1, 1–2 mg/ml ascites) (19) before transducing COS-7 cells (10^5 cells/ml) or injection into FVIII-deficient neonates. *In vitro* transductions were performed by centrifuging vector and target cells at $1,400 \times g$ for 1 hr (32°C) with polybrene (8 μg/ml). Cells were incubated at 32°C and were washed the next day. Conditioned medium was collected to determine FVIII activity using a FVIII COAtest. DNA was extracted from the transduced COS-7 cells and from FVIII-deficient recipient mice injected with the inactivated vector preparations and was subjected to FVIII-specific PCR to determine relative gene transfer efficiencies.

RESULTS

Generation of High-Titer VSV-G Pseudotyped Retroviral Vectors. To generate the VSV-G pseudotyped MFG-FVIIIΔB vector, 293GPG packaging cells were first transduced with the MFG-FVIIIΔB vector and subsequently were cloned by limiting dilution. The titer of the VSV-G pseudotyped MFG-FVIIIΔB vector (designated as VSV_{293}-FVIII) produced by the highest producer clone was equivalent to $1.8 \pm 0.7 \times 10^6$ cfu/ml (mean ± SEM, $n = 7$) and was significantly increased (30-fold, $P < 0.001$) compared with amphotropic MFG-FVIIIΔB produced by Ψ-CRIP cells ($6 \pm 3 \times 10^4$ $G418^R$ cfu/ml, mean ± SEM, $n = 23$) (8, 9). In addition, the VSV_{293}-FVIII vector could be concentrated 1,000-fold further by centrifugation, yielding final average titers equivalent to $1.1 \pm 0.4 \times 10^9$ cfu/ml with yields of 63 ± 9% (mean ± SEM, $n = 7$). The active FVIII concentration in the concentrated viral vector preparations corresponded to 350 ± 100 milliunits/ml (mean ± standard deviation, $n = 3$).

***In Vivo* FVIII Expression and Phenotypic Correction.** A total of 14 neonatal FVIII-deficient mice were injected with concentrated high-titer VSV_{293}-FVIII vectors. One neonate died after injection because of bleeding at the injection site that could not be arrested by topical thrombin application, but no acute toxicity of the high-titer vector was observed. Nine neonates were injected with PBS as controls. High-level (≥200 milliunits FVIII/ml) of functional human FVIII was detected in 6 of the 13 (46%) animals, 4 of which (31%) had physiologic or supraphysiologic levels (500–12,500 milliunits/ml) (Fig. 1). The functional FVIII activity in plasma was completely and specifically inhibited with polyclonal anti-FVIII antibodies from a hemophilia patient and with a monoclonal anti-human

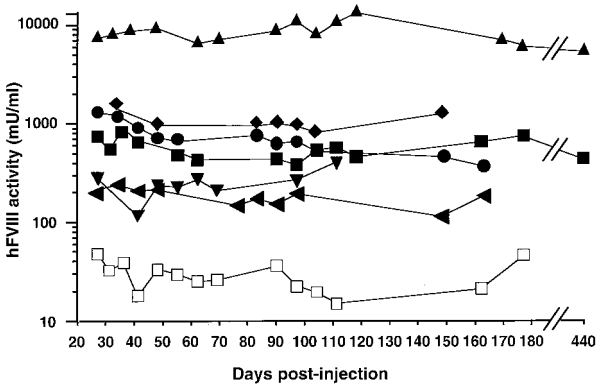

FIG. 1. Functional FVIII expression in hemophilia A mice. Neonatal FVIII-deficient littermates were injected with concentrated VSV_{293}-FVIII vector. Shown are mean plasma FVIII activities ($n = 3$) of recipient mice with prolonged FVIII expression (mice 1–6 in Table 1 are depicted by ▲, ♦, ●, ■, ▼, and ◀, respectively) and negative control mouse injected with PBS (□).The experiment was repeated twice in two independent batches of animals with different vector lots. Mice 1, 4, and 5 (▲, ■ , and ▼) were injected with 1.4×10^8 cfu/ml, and mice 2, 3, and 6, (♦, ●, and ◀) with 0.9×10^8 cfu/ml VSV_{293}-FVIII. Tail-clipping was performed 2 months postinjection except for mice 5 and 6 (3 months). Five of thirteen mice injected with VSV_{293}-FVIII vector (9–13, in Table 1) and all control mice injected with PBS (9 of 9) did not yield detectable FVIII.

FVIII antibody (mAb18) (17). Five of the six (83%) high expressers survived an otherwise lethal tail-clipping, demonstrating phenotypic correction (Table 1) and expressed FVIII for at least 5 months (Fig. 1). Two mice (nos. 1 and 4) were followed-up for >14 months and still expressed stable, high levels of FVIII. Human FVIII-specific inhibitory antibodies were not detected by ELISA in these long-term expressers (Table 1). Despite levels of FVIII corresponding to 280 milliunits/ml in one of the mice (no. 5; Table 1), it did not survive the stringent tail-clipping assay, which is somehow reminiscent of the occurrence of uncontrolled bleeding in mild hemophilia patients, which depends on the nature of the injury.

Expression of FVIII was transient in 2 of the 13 (15%) (Fig. 2 and Table 1) and nondetectable in 5 of the 13 (38%) animals receiving the high-titer vector (Table 1). Six of the seven animals with transient or no detectable human FVIII developed FVIII inhibitors as measured by ELISA and Bethesda

Table 1. Phenotypic correction of murine hemophilia A

Recipient	FVIII activity	Anti-FVIII Ab	Survival
1	6700 ± 23	−	+
2	940 ± 65	−	+
3	650 ± 15	−	+
4	420 ± 8	−	+
5	280 ± 8	−	−
6	190 ± 3	−	+
7	72 ± 4	13 ± 0.3	−
8	54 ± 12	40 ± 0.5	−
9	−	7.0 ± 0.1	−
10	−	56 ± 12	−
11	−	64 ± 17	−
12	−	350 ± 39	−
13	−	−	−
PBS	−	−	−(0/6)

Mice were injected with concentrated VSV_{293}-FVIII (1–13) or PBS. FVIII activity (in mU/ml) at the time of tail-clipping is shown. Anti-FVIII antibodies (Ab) were determined by ELISA and Bethesda assays (in Bethesda units/ml). Undetectable FVIII or antibodies are indicated by a minus sign. Mice that survived (+) or died (−) after tail-clipping are shown. Values represent mean ± SEM.

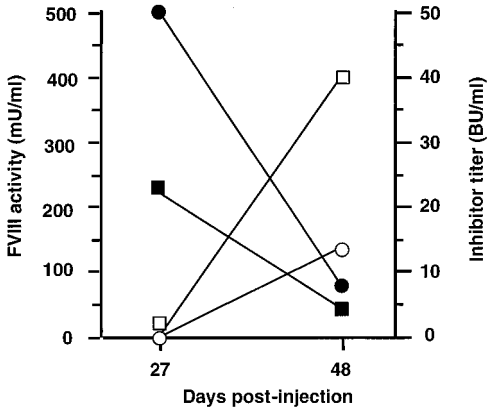

FIG. 2. Reciprocal correlation between FVIII activity and inhibitor titer in transient FVIII expressers. Functional FVIII expression, (●, ■) and inhibitory antibody titer (○, □) of mice 7 (circles) and 8 (squares) (see Table 1) were shown at different intervals postinjection of VSV_{293}-FVIII.

assays (7–350 Bethesda units/ml), continued to bleed after tail-clipping, and died within a few hours (Table 1 and Fig. 2). The FVIII activity from the transient expressers and the inhibitor titer was determined on days 27 and 48 postinjection (Fig. 2). In the absence of detectable human FVIII-specific inhibitory antibodies, FVIII expression is initially relatively high (200–500 milliunits of human FVIII/ml). Induction of relatively high-titer human FVIII-specific inhibitory antibodies (10–40 Bethesda units/ml) over time correlated with a concomitant decrease in functional human FVIII in the plasma. These kinetic data indicate that the induction of human FVIII-specific inhibitory antibodies is causally related to the decrease in FVIII expression in these transient expressers. Functional human FVIII or human FVIII inhibitory antibodies could not be detected in any of the FVIII-deficient control animals injected with PBS (Table 1), which all died after tail-clipping while control C57BL/6 mice survived.

To further confirm that the loss of FVIII expression in the negative mice is caused by immune mechanisms, concentrated VSV_{293}-F8 preparations were administered to neonatal FVIII-KO/SCID recipients. FVIII activity in the plasma of recipient FVIII-KO/SCID mice was measured 3–4 weeks postinjection. In contrast to the FVIII-deficient mice, in which only ≈50% of the mice expressed functional FVIII that correlated with the induction of inhibitory antibodies, all recipient FVIII-KO/SCID mice ($n = 6$) expressed functional human FVIII (90 ± 80 milliunits/ml, mean ± SEM).

To investigate whether the FVIII protein that co-concentrated with the vector particles could contribute to inhibitor formation while excluding potential interference from *de novo* expressed FVIII protein in transduced target cells, it was essential to prepare the viral vector preparation in conditions that prevented *FVIII* gene transfer. This was achieved by repressing VSV-G expression in the vector producer cells with tetracycline and by blocking vector infectivity with VSV-G-specific monoclonal antibodies (19). This treatment did not significantly affect FVIII activity in the concentrated vector preparation (data not shown). This combination approach completely blocked *FVIII* gene transfer and FVIII expression *in vitro* and *in vivo* (Fig. 3). Inhibitory antibodies to the co-precipitated FVIII protein in the vector preparations could not be detected in all recipient FVIII-deficient mice ($n = 9$). These data suggest that the presence of inhibitory antibodies in the nonexpressing mice is not caused by the co-precipitated human FVIII protein in the concentrated vector preparations but was caused by human FVIII expressed from transduced liver cells.

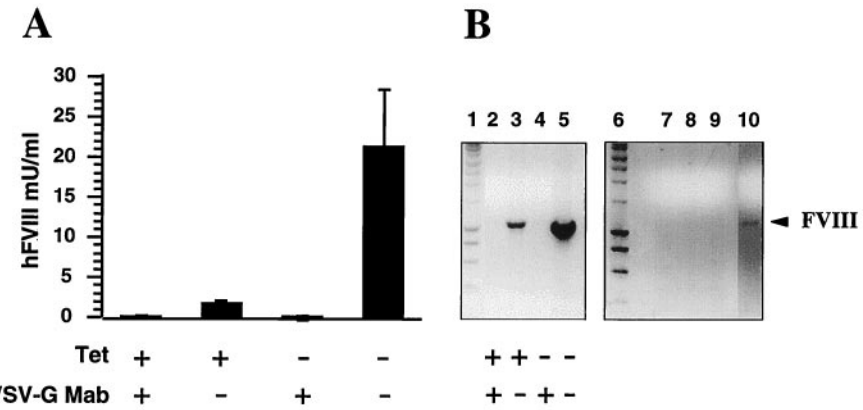

FIG. 3. Modulation of infectivity of VSV_{293}-FVIII. Viral vector producer cells were grown in the presence (+) or absence (−) of tetracycline (Tet) to repress or induce VSV-G expression, respectively, and were incubated with (+) or without (−) neutralizing VSV-G-specific mAb before transduction of COS-7 cells *in vitro* or injection in FVIII-deficient neonates. FVIII activity was determined in the 24-hr conditioned medium of the transduced COS-7 cells, and the relative transduction efficiencies were determined by FVIII-specific PCR (*B*, lanes 2–5). PCR also was performed on DNA from liver (lane 7), spleen (lane 8), and lungs (lane 9) of FVIII-deficient recipient mice injected with the same inactivated concentrated vector preparations. Positive control derived from FVIII-containing cells (lane 10) and molecular weight marker corresponding to the Smart Ladder (lanes 1 and 6) (Eurogentec, Liège, Belgium) are included, and *FVIII* (1.1 kb)-specific fragments are indicated.

Transduction Efficiency and *FVIII* mRNA Expression. PCR indicated that gene transfer occurred predominantly into liver, spleen, and lungs (Fig. 4A) whereas no vector-specific PCR fragment could be detected in testis, heart, brain, kidney, stomach, or intestine (Fig. 4*B*). The average vector copy number per diploid genomic equivalent in the liver corresponded to 0.1 (mouse 7), 0.5 (mouse 5), 0.6 (mouse 2), and 3.2 (mouse 1) and correlated strongly with differences in FVIII plasma levels (Fig. 1 and Table 1) (correlation coefficient: $r^2 = 0.99$) as well as *FVIII* mRNA (Fig. 6). PCR analysis revealed relatively low numbers of *FVIII*-transduced cells (<1%) in liver, spleen, and lungs in the nonexpressors (Fig. 5). Consistent with these relatively low numbers of transduced cells, no *FVIII* mRNA could be detected by RT-PCR (data not shown). *FVIII* mRNA expression could be detected by RT-PCR mainly in the liver (Fig. 6) of the long-term expresser mice whereas *FVIII* expression was undetectable in spleen and lungs. As expected, endogenous *β-actin* mRNA could be detected by RT-PCR in spleen, liver, and lungs of experimental and negative control animals (data not shown). The *FVIII*-specific RT-PCR product was not caused by contaminating residual genomic DNA because controls without RT were negative (Fig. 6).

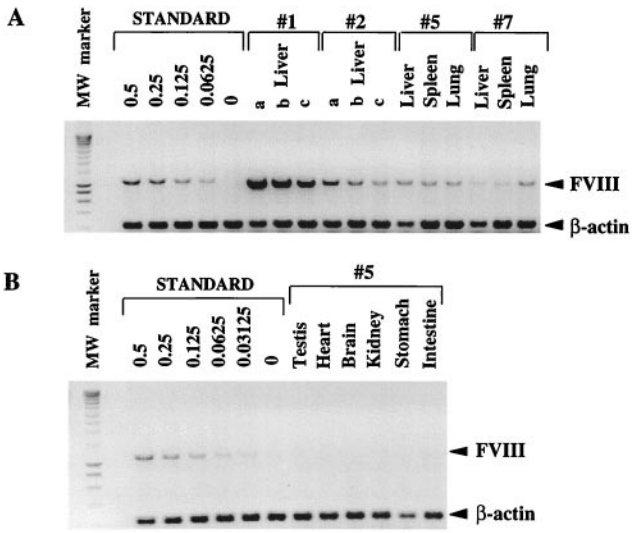

FIG. 4. Analysis of gene transfer efficiency by quantitative PCR in liver, spleen, and lungs (*A*) and in testis, heart, brain, kidney, stomach, and intestine (*B*). The average vector copy number per diploid genomic equivalent was determined by comparison with a serially diluted linear standard (correlation coefficient $r^2 = 0.98$) ranging from 0.5 to 0 (negative control) copies per diploid genomic equivalent. Organs from individual mice were indicated (1, 2, 5, and 7 from Fig. 1 and Table 1). Mice 1 and 2 were not killed, but a liver biopsy was taken instead, and decreasing amounts of target DNA were used as template: 200 ng (a), 100 ng (b), and 50 ng (c). A constant amount of total DNA (200 ng) was maintained in the standard and in the liver samples from mice 1 and 2 by adding spleen DNA from a FVIII-deficient mouse. *FVIII* (1.1 kb) or control *β-actin* (0.2 kb)-specific fragments are indicated. The molecular weight (MW) marker corresponds to the Smart Ladder.

DISCUSSION

The present study shows that intravenous injection of high-titer FVIII retroviral vectors into newborn FVIII-deficient mice leads to efficient stable gene transfer and secretion of the FVIII protein into the circulation, correcting the hemophilia A phenotype. The use of newborn recipient mice and of high-titer VSV-G pseudotyped vectors may have been essential to achieve these high levels of gene transfer and FVIII expression. This is consistent with previous reports showing that increasing

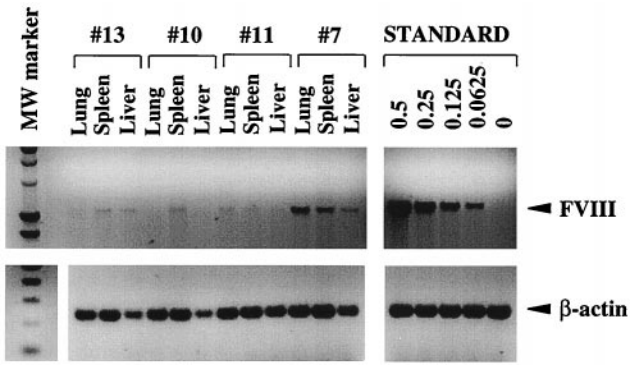

FIG. 5. Analysis of gene transfer efficiency by quantitative PCR in liver, spleen, and lungs of nonexpressor (10, 11, and 13, from Table 1) and transient expresser (7) mice. Bands corresponding to the amplified *FVIII* or control *β-actin*-specific fragments are indicated (1.1 and 0.2 kb, respectively). The same standard was used as described in the legend of Fig. 4. The molecular weight (MW) marker corresponds to the Smart Ladder for the FVIII-PCR and the 1-kb ladder for *β*-actin.

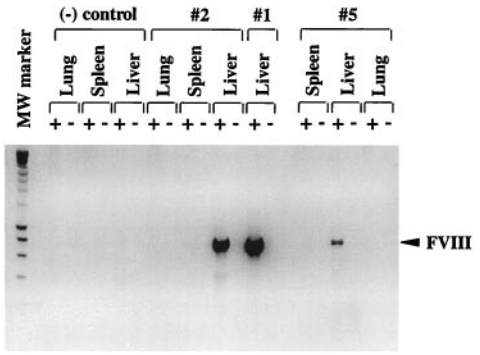

FIG. 6. Expression analysis of *FVIII* mRNA by RT-PCR in transduced organs derived from either FVIII-expressor mice (1, 2, and 5 from Fig. 1 and Table 1) or a PBS-injected FVIII-deficient mouse as negative (−) control. RNA samples with (+) or without (−) RT as controls were shown to exclude genomic DNA amplification, and *FVIII* -specific RT-PCR products are indicated (0.7 kb).

retroviral titer and VSV-G pseudotyping correlates with enhanced hepatic transduction (20–22).

In the present study, viral titers exceeding 10^9 cfu/ml could be achieved, which represents a significant, 10^6-fold increase in viral titer compared with nonpseudotyped, nonconcentrated first-generation FVIII retroviral vectors (4, 5, 7). These high titers could be attributed to the use of a strong *CMV* promoter used to drive the *gag* and *pol* genes in the 293GPG cells, improved vector design by using introns, and vector concentration. Additional advantages of using these pseudotypes for gene therapy include their increased resistance to human complement and a reduced likelihood to generate replication competent retroviruses (11).

Human FVIII expression ranged between 20 and 1,250% of normal human FVIII levels, and expression was sustained for at least 14 months in recipients that did not develop inhibitory antibodies, which represent the highest stable levels of FVIII reported after gene therapy. Efficient gene transfer occurred in the active reticuloendothelial organs, including liver, spleen, and lungs, with predominant *FVIII* mRNA expression in the liver. This corroborates previous observations that the Moloney murine leukemia virus/long terminal repeat, used to drive the *FVIII* gene, is a strong promoter in liver cells (20, 21, 23).

In previous studies relying on the use of FVIII-retroviral vectors or nonviral *ex vivo* gene therapy approaches, *in vivo* FVIII expression was either completely absent (6, 24, 25) or transient (9, 26) and lower than the current FVIII levels. This could be ascribed to promoter inactivation, high cell mortality, or posttranscriptional inhibition of FVIII production, which was not observed in the present study. The high levels of FVIII also compared favorably with previous results showing that intraportal injection of retroviral vectors expressing canine factor IX into partially hepatectomized hemophilia B dogs led to 0.1% of physiologic factor IX levels (21). Adenoviral vectors were shown to express transient therapeutic levels of human FVIII or factor IX in mice or dogs (1), possibly because of residual expression of adenoviral antigens leading to hepatotoxicity and/or vector-specific immune rejection (1, 27). The present results further support the use of other viral vectors devoid of viral genes, including adeno-associated viral vectors and helper-dependent "gutless" adenoviral vectors, for gene therapy of hemophilia (28–30).

Long-term correction of hemophilia has been achieved in ≈50% of the FVIII-deficient animals whereas the other half was not corrected. This differential response is attributable to a specific immune mechanism because the latter mice developed inhibitory antibodies to human FVIII whereas none of the corrected mice did. Furthermore, in the transient FVIII expressing mice, induction of antibodies was causally related to the decrease in FVIII expression. Finally, in the absence of a specific immune response in FVIII-KO/SCID mice, human FVIII expression could be detected in all recipient animals. The differential response is reminiscent of the lack of therapeutic efficacy in 10–20% of the hemophilia A patients that develop inhibitory antibodies to FVIII after protein replacement therapy. The higher proportion of nonexpressors in FVIII-deficient mice could be attributable to the inherently higher immunogenicity of xenogenic human FVIII in nonhuman species. Because there is a lower level of *FVIII* gene transfer in the transient and nonexpressor recipient mice compared with the long-term expressers, a mechanism whereby transduced cells were eliminated by a cellular immune response cannot be ruled out. Given the strong correlation between the presence of inhibitory antibodies and the low number of transduced cells in the nonexpressor mice, it is possible that a mechanism of antibody-dependent cellular cytotoxicity might have contributed to the immune rejection of transduced cells.

The possibility that overexpression of FVIII in ectopic tissues may have contributed to increased inhibitor formation in the hemophilic mice is unlikely because *FVIII* mRNA was only detectable in the liver. It cannot be excluded that *in vivo* expression of the B domain-deleted form of FVIII itself may increase the risk of inhibitor formation. However, it has previously been shown by using a mouse model of neonatal tolerance induction that full-length FVIII and its B domain-deleted counterpart are immunologically indistinguishable (31), although this may still vary among different B-domain deleted versions (32). Despite the presence of FVIII in the concentrated vector preparations, no inhibitory antibodies could be detected when *FVIII* gene transfer was prevented. This could be explained by the relatively low amounts of human FVIII in the vector preparation, its relatively short half-life in mice (25), and/or the relatively immature status of the neonatal immune system. These data suggest that the presence of inhibitory antibodies in the nonexpressing mice is attributable to *de novo* human FVIII expression from transduced liver cells and does not result from the coprecipitated human FVIII protein in the concentrated vector preparations.

The cause of the heterogeneous antibody response and FVIII levels among the different recipients is not clear. Because the FVIII-deficient mouse is not an inbred strain, genetic differences may have contributed to this variability. Alternatively, high levels of FVIII may have been required to induce immune tolerance to FVIII in the FVIII-deficient mice and to prevent induction of inhibitory antibodies. This would be reminiscent of tolerance induction by repeatedly injecting FVIII proteins at high concentrations in hemophilia A patients (33). However, expression of a xenoprotein in neonatal mice is quite different from giving a species-specific protein to a subject with a mature immune system. In particular, neonatal mice can become tolerant of soluble xenoantigens because neonatal splenic B-cells are tolerance susceptible for several days after birth, during which B-cell clones may be expanding (34). The proportion of tolerance-susceptible splenic B-cells decreases to <10% after the first week after birth (34). It is indeed possible that exposure of neonatal FVIII-deficient animals to sufficiently high levels of human FVIII protein after *FVIII* gene transfer resulted in the induction of tolerance to human FVIII, at least in some of the recipients. This hypothesis would be consistent with the observed correlation between high *FVIII* gene transfer and expression and lack of inhibitory antibodies in these mice and with the induction of tolerance of FVIII in neonatal mice receiving high doses of human FVIII (31). Conversely, when gene transfer efficiency was low, FVIII expression may have been below a critical threshold during the neonatal phase to induce tolerance, resulting in the induction of inhibitory antibodies. The potential immunogenicity of human FVIII in some of the recipient FVIII-deficient mice is

consistent with previous reports indicating that repeated administration of human FVIII protein in adult FVIII-deficient mice led to the induction of inhibitory antibodies (35).

The efficient hepatic gene transfer in neonatal mice could be attributable primarily to the rapid growth of mouse hepatocytes and/or targeting to hepatic stem cells (36, 37). Hepatocyte turnover rate is higher in newborn versus adult animals. The hepatocytes are highly differentiated, but their division potential indicates that many of them have stem cell-like properties by virtue of their extensive self-renewing capacity and regenerative potential, which is more pronounced in younger than in older animals. However, hepatocytes can only give rise to new hepatocytes whereas bile duct-derived oval cells can give rise to both biliary cells and hepatocytes and appear to consist of hepatic precursor cells. These oval cells represent the progeny of facultative pluripotential stem cells located in the biliary tree and share some antigenic determinants with hematopoietic cells. The relative abundance of these putative hepatic progenitor cells in neonates and their relative proximity to the vasculature may have facilitated retroviral transduction (38).

Our findings suggest that *in vivo* gene therapy with high-titer retroviral vectors may hold promise as a method for treating hemophilia or other diseases, including familial hypercholesterolemia, lysosomal storage disorders, and hepatocellular carcinoma, provided that a sufficiently large number of proliferating hepatocytes can be transduced. Liver weight increases gradually up until adolescence, with the highest relative increase in liver weight (100%) during the first year. Assuming that a fraction of the liver is dividing at any given time in humans, repeated injections of high-titer VSV-G pseudotyped vectors may transduce sufficient target cells and may lead to therapeutic FVIII levels (>10 milliunits/ml). These levels are 500- to 1,000-fold lower than the levels obtained in the highest expresser mice. Alternatively, retroviral transduction may be enhanced by inducing hepatocyte proliferation with specific growth factors (39) or by using lentiviral vectors (40).

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1. Connelly, S. & Kaleko, M. (1997) *Thromb. Haemostasis* **78,** 31–36.
2. Chuah, M. K., Collen, D. & VandenDriessche, T. (1998) *Crit. Rev. Oncol. Hematol.* **28,** 153–171.
3. Koster, T., Blann, A. D., Briet, E., Vandenbroucke, J. P. & Roosendael, F. R. (1995) *Lancet* **345,** 152–155.
4. Chuah, M. K., VandenDriessche, T. & Morgan, R. A. (1995) *Hum. Gene Ther.* **6,** 1363–1377.
5. Hoeben, R. C., van der Jagt, R. C., Schoute, F., van Tilburg, N. H., Verbeet, M. P., Briet, E., van Ormondt, H. & van der Eb, A. J. (1990) *J. Biol. Chem*. **265,** 7318–7323.
6. Hoeben, R. C., Einerhand, M. P., Briet, E., van Ormondt, H., Valerio, D. & van der Eb, A. J. (1992) *Thromb. Haemostasis* **67,** 341–345.
7. Israel, D. I. & Kaufman, R. J. (1990) *Blood* **75,** 1074–1080.
8. Lynch, C. M., Israel, D. I., Kaufman, R. J. & Miller, A. D. (1993) *Hum. Gene Ther.* **4,** 259–272.
9. Dwarki, V. J., Belloni, P., Nijjar, T., Smith, J., Couto, L., Rabier, M., Clift, S., Berns, A. & Cohen, L. K. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1023–1027.
10. Chuah, M. K., Brems, H., Vanslembrouck, V., Collen, D. & VandenDriessche, T. (1998) *Hum. Gene Ther*. **9,** 353–365.
11. Ory, D. S., Neugeboren, B. A. & Mulligan, R. C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 11400–11406.
12. Miller, A. D. & Rosman, G. J. (1989) *Biotechniques* **7,** 980–982.
13. Bowles, N. E., Eisensmith, R. C., Mohuiddin, R., Pyron, M. & Woo, S. L. (1996) *Hum. Gene Ther*. **7,** 1735–1742.
14. Bi, L., Lawler, A. M., Antonarakis, S. E., High, K. A., Gearhart, J. D. & Kazazian, H. H., Jr. (1995) *Nat. Genet*. **10,** 119–121.
15. Bosma, M., Schuler, W. & Bosma, G. (1988) *Curr. Top. Microbiol. Immunol.* **137,** 197–202.
16. 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.
17. Gilles, J. G., Arnout, J., Vermylen, J. & Saint Remy, J. M. (1993) *Blood* **82,** 2452–2461.
18. Kasper, C. K., Aledort, L., Aronson, D., Counts, R., Edson, J. R., van Eys, J., Fratantoni, J., Green, D., Hampton, J., Hilgartner, M., *et al.* (1975) *Thromb. Diath. Haemorrh*. **34,** 612.
19. Lyles, D. S. & Lefrancois, L. (1982) *Virology* **121,** 157–167.
20. Rettinger, S. D., Kennedy, S. C., Wu, X., Saylors, R. L., Hafenrichter, D. G., Flye, M. W. & Ponder, K. P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1460–1464.
21. 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. *et al.* (1993) *Science* **262,** 117–119.
22. Yee, J. K., Miyanohara, A., LaPorte, P., Bouic, K., Burns, J. C. & Friedmann, T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9564–9568.
23. Miyanohara, A., Yee, J. K., Bouic, K., LaPorte, P. & Friedmann, T. (1995) *Gene Ther*. **2,** 138–142.
24. Evans, G. L. & Morgan, R. A. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5734–5739.
25. Hoeben, R. C., Fallaux, F. J., Van Tilburg, N. H., Cramer, S. J., Van Ormondt, H., Briet, E. & Van Der Eb, A. J. (1993) *Hum. Gene Ther*. **4,** 179–186.
26. Zatloukal, K., Cotten, M., Berger, M., Schmidt, W., Wagner, E. & Birnstiel, M. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5148–5152.
27. Kafri, T., Morgan, D., Krahl, T., Sarvetnick, N., Sherman, L. & Verma, I. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 11377–11382.
28. Schiedner, G., Morral, N., Parks, R. J., Wu, Y., Koopmans, S. C., Langston, C., Graham, F. L., Beaudet, A. L. & Kochanek, S. (1998) *Nat. Genet*. **18,** 180–183.
29. 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.
30. 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.
31. Pittman, D. D., Alderman, E. M., Tomkinson, K. N., Wang, J. H., Giles, A. R. & Kaufman, R. J. (1993) *Blood* **81,** 2925–2935.
32. Esmon, P. C., Kuo, H. S. & Fournel, M. A. (1990) *Blood* **76,** 1593–1600.
33. Kreuz, W., Becker, S., Lenz, E., Martinez Saguer, I., Escuriola Ettingshausen, C., Funk, M., Ehrenforth, S., Auerswald, G. & Kornhuber, B. (1995) *Semin. Thromb. Hemostasis* **21,** 382–389.
34. Klinman, N. R. (1996) *Cell* **5,** 189–195.
35. Qian, J., Borovok, M., Bi, L., Kazazian, H. H. & Hoyer, L. W. (1999) *Thromb. Haemostasis* **81,** 240–244.
36. Alison, M. (1998) *Curr. Opin. Cell Biol.* **10,** 710–715.
37. Fausto, N. (1997) *Am. J. Pathol.* **151,** 1187–1189.
38. Tian, Y. W., Smith, P. G. & Yeoh, G. C. (1997) *Histochem. Cell. Biol.* **107,** 243–250.
39. Kosai, K. I., Finegold, M. J., Thi-Huynh, B. T., Tewson, M., Ou, C. N., Bowles, N., Woo, S. L. C., Schwall, R. H. & Darlington, G. J. (1998) *Hum. Gene Ther.* **9,** 1293–1301.
40. Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F. H., Verma, I. M. & Trono, D. (1996) *Science* **272,** 263–267.