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Gene therapy for hemophilia A: Production of therapeutic levels of human factor VIII *in vivo* in mice

(retroviral vectors)

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ABSTRACT **Continuous delivery of factor VIII (FVIII) protein in hemophiliacs by gene therapy will represent a major clinical advance over the current practice of infrequent administration of purified FVIII. Conceptually, retroviral vectors that can permanently insert the FVIII gene into the DNA of the host cell appear the most suitable vehicles for this specific purpose. However, most retroviral vector systems have shown a poor performance in the production of FVIII from primary cells *in vitro* and *in vivo*. Here we report the retroviral-mediated gene delivery of a B-domain-deleted human FVIII by using the MFG vector system. This vector permitted efficient transduction of the majority of the primary cells in culture without the use of a selectable marker. High levels of FVIII were produced by various transduced primary cells *in vitro*. Upon transplantation of primary fibroblasts into mice, therapeutic levels of FVIII in the circulation were obtained for >1 week. The capacity of primary cells to deliver the FVIII into the circulation was strongly dependent on the site of implantation. These results represent a major step forward in development of gene therapy for treating hemophilia A.**

Hemophilia A is an X chromosome-linked recessive genetic disorder, affecting 1 in 10,000 males, resulting in defective or deficient factor VIII (FVIII) molecules, which, in its severe form, is a life-threatening, crippling hemorrhagic disease (1).

Infusion of purified FVIII is currently the most widely used therapy (2–4). Recently, improved manufacturing procedures (5) and production of recombinant FVIII have reduced the threat of iatrogenic complications associated with earlier concentrates from plasma (6–8). Treatment in its present form is management of crisis rather than a long-term preventive measure. An alternative therapy that obviates the need for regular i.v. infusion would constitute a major advance clinically and economically. A long-term study in Sweden with regular infusion of FVIII started as early as age 2 helped to maintain plasma levels of 2–4 ng/ml; this stable level reduced the number of traumatic episodes and significantly improved the overall well-being of the patients (9). These studies suggest that continuous delivery of FVIII via gene therapy would provide significant therapeutic benefits. Therefore, hemophilia A is an obvious target for gene therapy (10–15).

Retroviruses are uniquely suitable as vectors for gene transfer. The viral integration machinery is very efficient, results in stable provirus with a predictable structure, and can infect a broad spectrum of cell types. However, in a number of studies utilizing various retroviral vectors and different forms of the human FVIII gene, only very low expression levels (1 milliunit to 0.1 unit per 10^6 cells per 24 hr) of human FVIII were obtained (11–15). FVIII cDNA has been shown to contain inhibitory sequences, which reduce the accumulation of vector RNAs (14). Very recently, human FVIII (B-domain-deleted form) production *in vivo* in mice from "transferrinfected" fibroblasts and myoblasts for up to 24 hr was reported (16).

We have used the highly efficient MFG retroviral vector system for transfer of FVIII cDNA into murine and human cells (primary and established cell lines). FVIII cDNA contains an open reading frame of 2351 amino acids encoding domains arranged in the order A_1–A_2–B–A_3–C_1–C_2. We utilized a FVIII cDNA clone lacking the B domain, as it is not required for pro-coagulant activity *in vitro* or *in vivo* (17–20). In contrast to published reports, we demonstrate high transduction efficiency and a high rate of FVIII production (11–15). We also demonstrate that FVIII-secreting cells transplanted into immune-deficient mice give rise to substantial levels of FVIII in the plasma.

MATERIALS AND METHODS

Isolation of Virus-Producing Cell Lines. ψ-CRE and ψ-CRIP packaging cell lines (21) and NIH 3T3 cells were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 4.5 g of glucose per liter, 10% calf serum, and antibiotics. To generate ψ-CRIP amphotropic virus-producing cell lines, MFG-FVIIIΔB and pSV_2neo plasmids were coelectroporated into the ψ-CRE packaging cell line. After G418 selection, virus from pooled colonies was used to infect the ψ-CRIP packaging cell line and the cells were cloned and analyzed for their transduction capacity.

Transduction and Clotting Factor Assays. The viral particles from individual ψ-CRIP cross-infected clones were used to infect NIH 3T3 and primary cells in the presence of DEAE-dextran (10 μg/ml). After 48 hr the cells were overlaid with DMEM with 10% heat-inactivated fetal bovine serum (FBS; 66°C, 30 min) and supernatants were assayed for FVIII activity by measuring the FVIII-dependent generation of factor Xa from factor X using a chromogenic assay (COA; Coatest FVIII, KabiVitrum, Stockholm) (22). A FVIII ELISA was performed by coating the wells with human FVIII light-chain- and heavy-chain-specific monoclonal antibodies (mAb) [anti-FVIII:C (human) from Boehringer Mannheim and American Diagnostica (Greenwich, CT), respectively]. The biotinylated mAb ESH-4 (American Diagnostica) was used as second antibody. In both assays pooled normal human plasma was used as standard (George King Biomedical, Overland Park, KS). The concentration of FVIII in this pool was assumed to be 200 ng/ml.

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Abbreviations: LTR, long terminal repeat; FBS, fetal bovine serum; FVIII, factor VIII; Mo-MLV, Moloney murine leukemia virus; COA, chromogenic assay; mAb, monoclonal antibody(ies); SCID, severe combined immunodeficiency; PTFE, poly(tetrafluoroethylene).

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Neo-Organ Formation and Implantation. Neo-organs were formed as described by Moullier *et al.* (23). Briefly, expanded poly(tetrafluoroethylene) (PTFE) fibers (W. L. Gore and Associates, Flagstaff, AZ) were coated with rat tail collagen type I (Collaborative Research). Coated fibers were arranged as a loose mesh at the bottom of a tissue culture dish. Genetically modified cells were harvested and resuspended at a final concentration of 50–75 $\times$ 10^6 cells per ml in a rat type I collagen solution (3 mg/ml) and growth factors. The mixture was plated on culture dishes with PTFE fibers and allowed to solidify at 37°C. One or 2 days later, two lattices each with $\approx$15 $\times$ 10^6 cells were surgically implanted into the peritoneal cavity of severe combined immunodeficiency (SCID) mice.

RESULTS

Vector Construction and Generation of Virus-Producing Cell Lines. A B-domain-deleted human factor VIII cDNA (amino acids 743–1648 deleted) was cloned into the MFG retroviral vector (24) (Fig. 1*a* and legend to Fig. 1). The source of the full-length FVIII sequence was plasmid pAGF8 obtained from Chiron (25). The B-domain deletion construct used in this study retained all relevant thrombin cleavage sites (26). This cDNA lacking any 3′ untranslated sequences was introduced into the MFG retroviral vector. The ATG of FVIII was placed at the same position as the MoMLV envelope ATG, and the second amino acid was changed from glutamine (CAA) to glutamic acid (GAA) to better fit the Kozak consensus sequence (27). Protein synthesis occurs from a singly spliced mRNA.

The ψ-CRIP amphotropic virus-producing cell lines were generated by cross-infection with ψ-CRE ecotropic virus (21). Of the 36 clones screened, 28 were positive for FVIII expression by COA test; 9 clones producing FVIII between 300 and 800 ng/24 hr per 10^6 cells were further characterized. Viral titers were estimated from the copy number in transduced 3T3 cells. Southern blot hybridization with DNA digested with *Asp*718 (Fig. 1*b*, lanes A–I) and other enzymes (data not shown) resulted in fragments of the expected size. This indicates that the provirus did not undergo rearrangements. The copy number varied from 0.5 to 1 per transduced cell, which

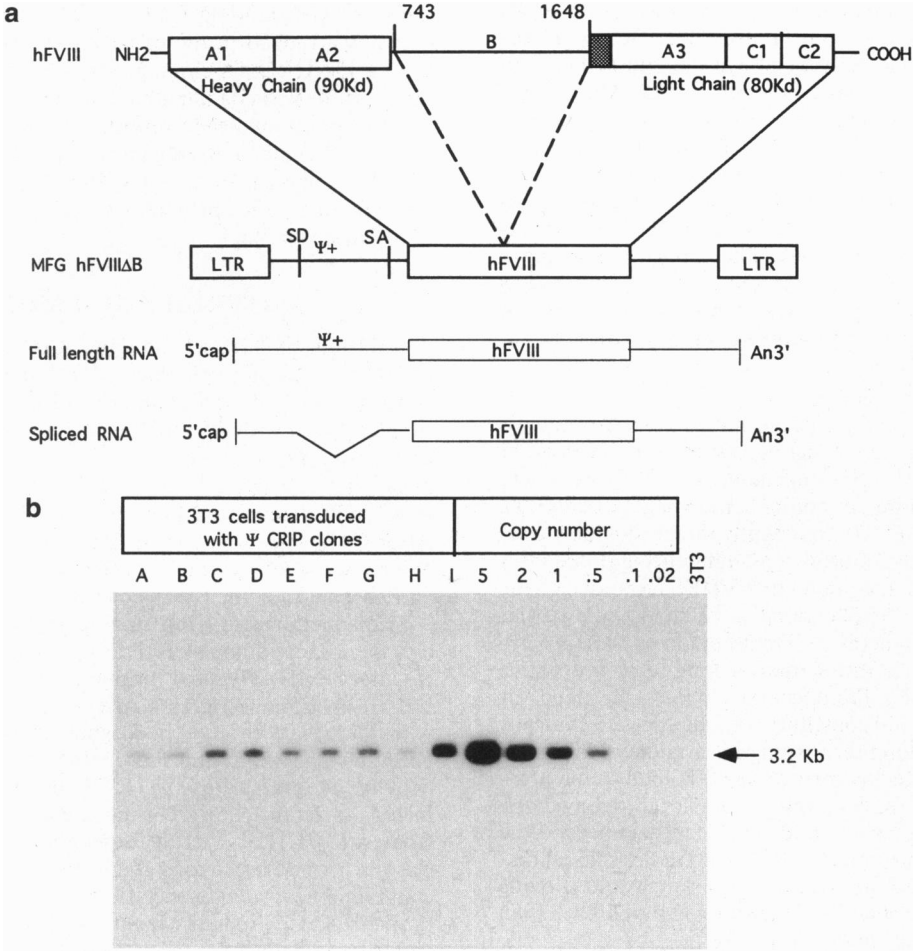

FIG. 1. (*a*) Human FVIII protein domain structure and human FVIII (B-domain-deleted) retroviral vector. The MFG-FVIIIΔB retroviral vector consists of the following sequences inserted between the *Hin*dIII and *Eco*RI sites of pBR322: (*i*) 396 bp of 5′ murine chromosomal DNA (5′ flanking sequences), (*ii*) an entire Moloney murine leukemia virus (Mo-MLV) 5′ long terminal repeat (LTR) and adjacent sequences up until the *Nar* I site at nucleotide 1035 (a 6-bp *Sma* I linker was inserted at nucleotide 624), (*iii*) sequences containing the viral 3′ splice acceptor (SA) extending from nucleotide 5401 to nucleotide 5780 (the A nucleotide of *Nla* III site has been changed to C as to create an *Nco* I site at the end of this fragment), (*iv*) cDNA of human FVIII (B-domain-deleted), (*v*) Mo-MLV proviral sequences extending from the *Cla* I site at nucleotide 7674 to the end of the 3′ Mo-MLV LTR, (*vi*) 695 bp of 3′ murine chromosomal DNA (3′ flanking sequences). The domain structure (A1–A2–B–A3–C1–C2) of FVIII protein is indicated. The structure of the integrated recombinant provirus (MFG-FVIIIΔB), together with viral transcript, is depicted. SD, splice donor site; ψ+, extended retrovirus packaging signal; A_n, poly(A) tract. (*b*) Southern blot analysis of genomic DNA extracted from virally infected 3T3 cells. Equal amounts of DNA (10 μg) from 3T3 cells infected with ψ-CRIP FVIII clones (lanes A–I) were digested to completion with *Asp*718 and fractionated on a 1% agarose gel. Following transfer onto a nylon filter, hybridization with a ^{32}P-labeled FVIII probe was performed. Copy number standards were reconstituted with plasmid DNA cleaved with *Asp*718 and mixed with the same amount of genomic DNA isolated from naive 3T3 cells.

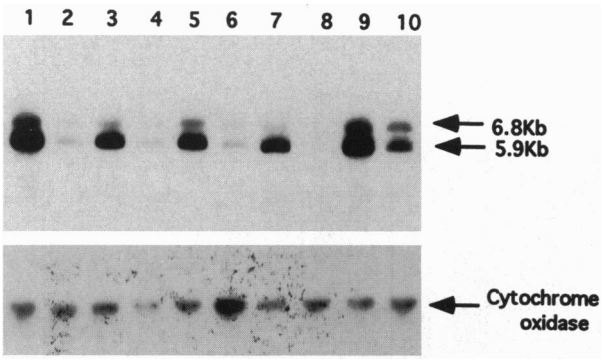

FIG. 2. Northern blot analysis on cytoplasmic RNA isolated from virally infected 3T3 cells and the corresponding parental cell lines. Cytoplasmic RNA (200 ng) from parental cell or virally infected cell lines was separated on a formaldehyde gel and blotted onto a nylon membrane. Hybridization was performed with a ^{32}P-labeled FVIII probe and filters were exposed to x-ray films for 14 hr. The same blot was stripped and reprobed with cytochrome oxidase to ensure equal loading of RNA. Lanes 1, 3, 5, 7, and 9, RNA isolated from ψ-CRIP clones; lanes 2, 4, 6, 8, and 10, RNA isolated from 3T3 cells transduced with the corresponding clones.

indicates that with one round of infection 50–100% of the cells can be transduced.

Northern blot analysis of 200 ng of total RNA hybridized with a FVIII-specific probe revealed the expected transcripts (Fig. 2). The 6.8-kb and 5.9-kb bands represent the full-length viral RNA and the spliced mRNA, respectively. Retroviral stocks were tested for the presence of helper virus and appeared free from replication competent retrovirus (21).

FVIII Protein Is Efficiently Expressed by Various Cell Types. One amphotropic producer clone, XF2, was used to determine the ability of this vector to transduce different cell types and to study the bioactivity of expressed FVIII by a COA.

The cell lines transduced included mouse NIH 3T3 cell line, C2C12 (a mouse myoblast line), primary human skin fibroblasts, primary human myoblasts, and primary human sinusoidal endothelial cells. The results of these assays show efficient secretion of functional protein from all transduced cell lines (Table 1). To confirm that the observed activity was due to the presence of FVIII, samples were incubated with polyclonal antibody against FVIII prior to the activity assay. This reduced the coagulating activity to undetectable levels (data not shown). The ELISA and COA test results show that in the presence of heat-inactivated serum, ≈50–70% of the FVIII secreted *in vitro* is biologically active (Table 1).

Characterization of FVIII Protein Produced in Different Cell Types. To investigate the nature of FVIII secreted from the cells, Western blot analysis was performed with the heavy chain-specific mAb C5 (28). The protein appears stable in the presence of DMEM with 10% heat-inactivated serum, while in the absence of heat-inactivated serum cleavage of the protein to smaller molecular mass forms can be detected (data not shown). At least three bands were detected with mAb C5 (Fig. 3). The bands represent the full-length B-domain-deleted FVIII (≈175 kDa) and the processed heavy chain (≈100 kDa and ≈50 kDa). The C5 mAb reacted nonspecifically with serum albumin, resulting in a strong signal at 69 kDa (Fig. 3). The recombinant FVIII synthesized from full-length cDNA consists of a heterogenously sized heavy chain (90–200 kDa) paired via a metal ion bridge with the 80-kDa light chain (Fig. 3) (29). The intactness of the thrombin cleavage sites was demonstrated by performing Western blot analysis with two mAb (28) that react to either the heavy (C5) or light chain (C2) (data not shown).

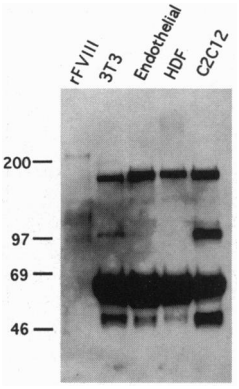

FIG. 3. SDS/PAGE and Western blotting of the culture supernatants from transduced cells. Aliquots containing 1 ng of FVIII protein (measured by COA) collected in DMEM with 10% heat-inactivated serum were loaded on to a SDS/PAGE gel and the protein was transferred to nitrocellulose for Western blotting. The mAb C5 with specificity to the heavy chain (epitope 351–361) was used (26) for detection. Molecular size markers are indicated in kDa. rFVIII, recombinant FVIII; HDF, human dermal fibroblasts.

Recently it was shown that doublets of heavy and light chains were the predominant forms of B-domain-deleted FVIII made in Chinese hamster ovary cells (30). Only a small amount of single-chain FVIII was observed, possibly as a result of cleavage of arginine 1648 (31). The B-domain-deleted form we constructed lacks this residue.

Clearance of Recombinant FVIII from Plasma. To study the FVIII release into the circulation and its subsequent degradation *in vivo*, we measured the clearance of recombinant human FVIII (Recombinate, Baxter Health Care, Mundelein, IL) from mouse plasma following i.v., i.p., s.c., and i.m. injection. The results shown in Fig. 4 demonstrate that the clearance time of human FVIII in mice is shorter than expected on the basis of the known half-life of human FVIII in man (≈12 hr) (32). Similar results were obtained with purified FVIII from human plasma (Hemofil-M, Baxter Health Care) and with another recombinant human FVIII (Kogenate, Miles).

Surprisingly, we were unable to detect FVIII in plasma of mice injected either i.m. or s.c.; only after i.v. or i.p. injection were significant levels of FVIII detected. The uptake via the i.p. route was ≈20% that of the i.v. route. The absence of FVIII in circulation after i.m. and s.c. injections may be due to the susceptibility of FVIII protein to proteolysis, a feature that would decrease the amount reaching the circulation, especially in view of the short half-life of the protein in mice. Earlier drug delivery studies have shown that i.p. delivery is relatively efficient, probably as a result of the extensive vascularization of tissues lining the peritoneal cavity (33).

Table 1. FVIII secretion from virus-transduced cells

Transduced cell type	FVIII activity,* μg per 1×10^6 cells per 24 hr	Copy number	Activity/antigen ratio
NIH 3T3 cells	0.5	1.5	50
C2C12 myoblast cell line	0.6–1.0	5	55
Human myoblasts	0.8–1.0	2	60
Human sinusoidal endothelial cells	1.6–2.0	4	50–60
Human skin fibroblasts	0.8–1.2	2	50–70

*The target cells were transduced in the presence of DEAE-dextran (10 μg/ml). Confluent cultures were fed with fresh medium (DMEM/10% heat-inactivated FBS), 24-hr culture supernatants were collected, and FVIII protein was assayed for antigen and activity by ELISA and COA, respectively.

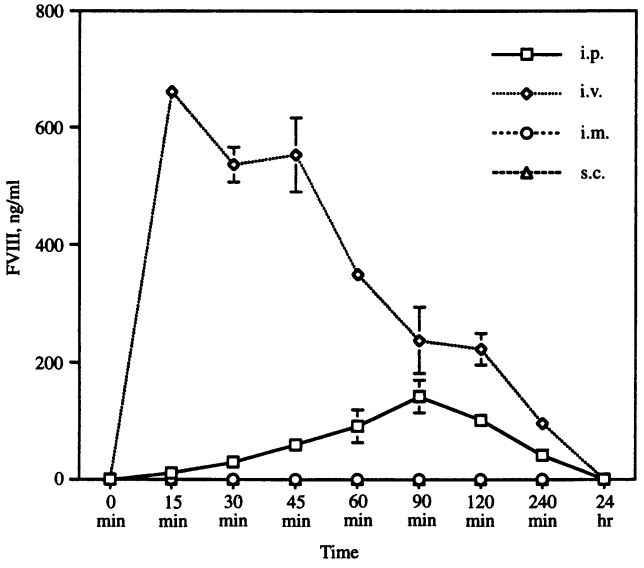

FIG. 4. Clearance of recombinant human FVIII from mouse plasma. Recombinant human FVIII (Recombinate, Baxter) was injected by various routes (100 units/kg of body weight) and plasma samples were collected at the indicated time points. At all time points samples from at least three animals were analyzed for FVIII by ELISA as described in the legend to Table 2. Values are expressed as average ± SD.

Transplantation of Transduced Cell Lines into SCID Mice. Cell-mediated delivery of FVIII into the circulation was first determined via transplantation of transduced cell lines. We transplanted 3×10^7 FVIII-transduced C2C12 cells or 3T3 cells (*in vitro* production rate, 1 and 0.5 μg per 1×10^6 cells per 24 hr, respectively) into the peritoneal cavity of SCID mice. The plasma levels of human FVIII ranged from 50–80 ng/ml on day 1 to 600 ng/ml on day 21 in C2C12 transplanted animals and slightly lower in 3T3 transplanted mice. We monitored these animals until they showed signs of tumor growth (day 25–30), at which point they were sacrificed.

To determine whether FVIII can reach the circulation from the transduced cells implanted into muscle, we used C2C12 cells doubly transduced with retroviral vectors encoding the human FVIII and canine factor IX (K9F9) proteins. The *in vitro* levels of K9F9 and human FVIII expressed by these cells were determined, and the cells were injected i.m. into SCID mice. i.m. injection of doubly transduced C2C12 cells leads to significant levels of K9F9 but not of human FVIII (Table 2).

Table 2. Human FVIII and K9F9 ELISA values in plasma of SCID mice

Delivery	FVIII, ng/ml	K9F9, ng/ml
i.m. injection		
Day 2	ND	215
Day 7	ND	197
Day 10	5	235
i.p. implant		
Day 2	30	248
Day 8	26	192
Day 15	38	295

SCID mice implanted with C2C12 myoblast cell line doubly transduced with human FVIII and K9F9 viruses. The *in vitro* rate of production of FVIII by COA was 1 μg per 1×10^6 cells per 24 hr and of K9F9 by ELISA was 700–800 ng per 1×10^6 cells per 24 hr. C2C12 myoblast cell line doubly transduced with FVIII and K9F9 viruses was grown up and implanted as neo-organs i.p. (two implants of 15×10^6 cells each) or 1×10^7 cells suspended in 200 μl of phosphate-buffered saline and injected i.m. at multiple sites. Three animals were used in each group and the average values are represented. ND, none detected.

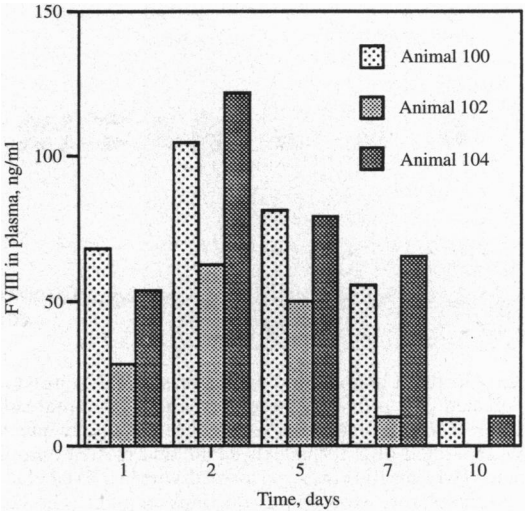

FIG. 5. *In vivo* secretion of human FVIII in plasma of mice. FVIII ELISA in ng/ml of plasma in SCID mice with collagen-embedded transduced human dermal fibroblasts implanted i.p. as neo-organs.

When the same cells were transplanted into the i.p. cavity, expression of both factors was detected in the plasma (Table 2). This is in agreement with results obtained after i.m. injection of purified recombinant FVIII.

Transplantation of Primary Fibroblasts into SCID Mice. In view of the tumorigenic properties of established cell lines, we explored the use of transduced primary human fibroblasts by embedding in collagen matrix with PTFE fibers as a backbone for the cell lattices. Significant levels of FVIII were detectable until day 10 (Fig. 5). Implantation of $25–30 \times 10^6$ primary cells (dermal fibroblasts) initially gave rise to very high levels of FVIII in the plasma. Levels dropped by day 6, and by day 13 FVIII was usually undetectable.

We also studied the fate of cells in the implants after expression had ceased. Animals were sacrificed, and implants that were moderately vascularized were removed. Cells were dissociated with collagenase and cultured *in vitro*. After 2 weeks, the COA showed that the cells expressed significant levels of FVIII protein (0.2–0.5 μg per 10^6 cells per 24 hr). Although it was impossible to carefully quantitate the number of cells recovered from the implants, there were significantly fewer cells than the number originally transplanted, and, therefore, cell death might have contributed to the decline in FVIII expression *in vivo*. However, reverse transcription PCR analysis performed on RNA isolated immediately after removal of implants failed to detect FVIII message, whereas control mRNAs were readily detectable, as were FVIII DNA sequences. This indicates that FVIII mRNA was no longer present. This is in line with published reports of extinction of the LTR-driven RNA expression in transplanted fibroblasts (34, 35). Whether this is due to transcriptional impairment or reduced mRNA stability is presently unclear.

DISCUSSION

Development of somatic gene therapy for hemophilia A has been a frustration to investigators in the field. A recent report by Lynch *et al.* (14) concluded that, although they could generate FVIII-expressing viruses, the titers were very low as was the production of FVIII from transduced cells. They attributed this mainly to the low levels of RNA present in FVIII-expressing clones. They also concluded that a particular 1.2-kb fragment of FVIII cDNA inhibited RNA accumulation.

We have obtained numerous virus-producing clones generating high-titer FVIII virus (28 of 36 clones expressed functional FVIII). The levels of genomic and spliced mRNAs are

significantly higher than reported earlier (Fig. 2). The full-length B-domain-deleted protein retains the functional thrombin cleavage sites and was readily detected by Western blot analysis in the supernatant of FVIII transduced cells. Use of the MFG vector, which utilizes authentic viral splicing signals and lacks a selectable marker gene, has been crucial in obtaining high-titer FVIII viral stocks (36–38). Use of the specific B-domain-deleted form of FVIII may also have contributed to our success. Importantly, we do see abundant levels of full-length FVIII message in the amphotropic packaging cell lines (Fig. 2).

High levels of bioactive FVIII were expressed by various cell types. The presence of ≈50% active protein in the culture supernatants may be due to sensitivity of the protein to proteases under *in vitro* conditions. This might not be a problem when the cells are implanted in a vascularized environment, as FVIII can be immediately taken up and bound to von Willebrand factor in the circulation. The combination of high virus titers and high expression levels obviated the need for selection of clones. Consequently, populations of primary cells can be used for transduction without the requirement for long-term propagation *in vitro*.

Our study shows that the site of transplantation is critical for the delivery of FVIII. Efficient delivery of FVIII was achieved by direct i.v. or i.p. injections but not after i.m. or s.c. injection. This may be due to the susceptibility of FVIII protein to proteases in the extracellular space of muscle and skin or to specific characteristics of the FVIII protein itself. These factors may be more significant in mice, in which the half-life of FVIII is relatively short (1 hr vs. 12 hr in man) (32).

The levels of FVIII achieved in mouse plasma were close to the normal values found in man, in spite of the short half-life of FVIII in mouse plasma. We achieved expression from implanted cells for 7–10 days. A number of factors might have contributed to the ultimate cessation of expression, such as limited cell survival and promoter shutoff. A recent report by Moullier *et al.* (23) demonstrates that the use of the phosphoglycerate kinase promoter can lead to long-term expression *in vivo* in fibroblasts. This indicates that the shutoff is not inherently associated with transduced, primary transplants and can therefore likely be overcome by vector modifications.

In conclusion, we have demonstrated here that gene therapy can result in therapeutic levels of FVIII in the circulation. Assuming that all of the FVIII expressed is biologically active (≈50–100 ng/ml), we achieved expression levels that are 10-fold higher than required for correction of hemophilia, even though the half-life of human FVIII in mice is only 1 hr. In humans 10–20 ng/ml is therapeutically effective, and it should be possible to achieve this level with $\approx 1 \times 10^8$ implanted cells, which brings gene therapy for this disease within reach, once the necessary vector modifications have been made to significantly prolong the expression of FVIII by implanted cells. Subsequently, gene therapy protocols for hemophilic dogs, an animal model that closely mimics the human condition, can be attempted.

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