Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5173–5177, July 1990
Genetics

Phenotypic correction of factor IX deficiency in skin fibroblasts of hemophilic dogs

(molecular cloning/hemophilia B/retroviral vectors/endothelial cells/gene therapy)

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Contributed by K. M. Brinkhous, April 24, 1990

ABSTRACT **Primary skin fibroblasts from hemophilic dogs were transduced by recombinant retrovirus (LNCdF9L) containing a canine factor IX cDNA. High levels of biologically active canine factor IX (1.0 μg per 10^6 cells per 24 hr) were secreted in the medium. The level of factor IX produced increased substantially if the cells were stimulated by basic fibroblast growth factor during infection. Additionally, we also report that endothelial cells transduced by this virus can produce high levels of biologically active factor IX. We propose that skin fibroblasts and endothelial cells from hemophilia B dogs may serve as potential venues for the development and testing of models for treatment of hemophilia B by retrovirally mediated gene replacement therapy.**

Hemophilia B is a bleeding disorder that is caused by a partial or complete lack of clotting factor IX activity in the plasma (1). In its most severe form patients suffering from hemophilia B must receive factor IX replacement therapy on a regular basis to prevent bleeding (2). Despite constant medical attention and administration of factor IX concentrates, patients with severe factor IX deficiency often experience bleeding in the joints leading to joint deformity and crippling (1). In addition, complications resulting from contamination of factor IX concentrates by blood-borne pathogens, such as hepatitis B and human immunodeficiency virus, and high levels of factor IXa subject these patients to a continuous source of health risks and mental anxiety (3, 4). As with other genetic diseases, treatment of hemophilia B may one day be possible through the use of gene replacement therapies. One model approach to gene therapy that has been explored recently is the utilization of recombinant retroviruses carrying factor IX to transduce fibroblasts as a possible target cell type for gene transfer (5–7). When transplanted as allografts or xenografts in rodents, these fibroblasts produced substantial levels of factor IX detectable in the host plasma but only for short periods. The relatively short duration of factor IX production in the grafted animals was attributed at least in part to an immune response of the host against the foreign factor IX or, in the case of xenografts, to host rejection of the grafted cells. To test the feasibility of this approach it is imperative to utilize a totally homologous system that would avoid graft rejection and immune response against the recombinant factor IX. Additionally, utilization of a hemophilia B dog model system (8, 9) would allow testing of the efficacy and activity of the factor IX produced by recombinant retrovirus. Toward this goal we have isolated a cDNA clone coding for canine factor IX§ and have used this cDNA to construct recombinant factor IX retroviruses. We report here that a recombinant retrovirus expresses biologically active canine factor IX in skin fibroblasts obtained from a hemophilia B dog. Additionally, we have explored the use of endothelial cells as potential targets for gene transfer in the treatment of hemophilia B.

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MATERIALS AND METHODS

Construction and Isolation of a Canine Factor IX cDNA. A canine liver cDNA library was constructed using total cellular poly(A)$^+$ RNA isolated from mongrel dog liver. cDNA was prepared using a cDNA synthesis kit (Pharmacia) and ligated in the vector λZAP (Stratagene), which was packaged with Gigapack Plus (Stratagene) according to the manufacturer's instructions. The library was screened with a human factor IX cDNA probe (10) under conditions of moderate stringency [hybridization: 5× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate)/50% formamide/0.1% SDS/10 μg of salmon sperm DNA per ml at 37°C; wash: 1× SSC/0.1% SDS at 37°C].

Cells and Culture Conditions. Primary skin fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, except for the packaging cell lines GP+E86 (11) and ΨCrip (12), which were grown in medium supplemented with 10% bovine calf serum. Endothelial cells were grown in DMEM supplemented with 10% bovine calf serum and 2 ng of basic fibroblast growth factor (bFGF) per ml. Medium used during infections also contained 8 μg of Polybrene per ml and bFGF at concentrations indicated. For measurement of factor IX activity, cells were cultured in DMEM supplemented with 0.5 μg of vitamin K per ml (Sigma) in the absence of serum. Dog skin fibroblasts were isolated from small open biopsy specimens from the dermis of normal and hemophilia B dogs using standard protocols (13). Bovine corneal endothelial (ACE) cells were the generous gift of Andrew Baird.

Construction of Factor IX Viruses and Virus-Producing Lines. Vectors shown in Fig. 2 were generated by insertion of a 1500-base-pair (bp) *Sal* I/*Bam*HI fragment containing the entire coding region of the canine cDNA clone dF9.28 into the *Xho* I/*Bam*HI sites of MLVneo (14) (LdF9NL) or as a *Hin*dIII fragment combined with the indicated cytomegalovirus (CMV) promoter into the *Hin*dIII site of LNL-XHC (15) (LNCdF9L, LN2CdF9L, LN9FdC2L). LN9Fd2CL contains the entire dF9.28 cDNA. The human CMV promoters (−1145 to +55 and −522 to +55) were a gift of J. A. Nelson (16). Helper-free recombinant amphotropic virus was generated in ΨCrip cells (12) by previously described methods (17). Virus producer clones were selected in G418, and final clones were chosen on the basis of high virus titer and unrearranged provirus as determined by Southern blot.

Abbreviations: bFGF, basic fibroblast growth factor; CMV, cytomegalovirus; APTT, activated partial thromboplastin time; LTR, long terminal repeat.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M33826).

Assay of Factor IX Antigen and Activity. Levels of factor IX antigen were assayed by ELISA using the anti-human factor IX monoclonal antibody FXC008 (18) as a primary antibody and polyclonal rabbit anti-human factor IX antibodies (Accurate Chemicals, Westbury, NY) as a secondary antibody. Bound rabbit antibodies were detected with horseradish peroxidase-linked goat anti-rabbit antibodies (HyClone) and developed with 3,3′,5,5′-tetramethylbenzidine (TMB) reagent (19). Biological activity of factor IX was determined by an activated partial thromboplastin time (APTT) assay using a Coagumate MLA Electra 700 automatic coagulation timer (Medical Laboratory Automation, Pleasantville, NY), factor IX-deficient plasma from hemophilia B dogs as a substrate (20), and Thrombosil I APTT reagent (Ortho Diagnostics). Pooled normal canine plasma was used as a factor IX standard (12.5 μg/ml) in the ELISA and activity assays. Purification and concentration of recombinant factor IX, where indicated, was performed by barium sulfate precipitation as described (21).

RESULTS

Isolation and Characterization of a Canine Factor IX cDNA. A cDNA clone (dF9.28) containing 3054 nucleotides was characterized by DNA sequence analysis of both strands (Fig. 1). It is composed of a coding region of 1356 nucleotides with 5′ and 3′ untranslated regions of 21 and 1677 nucleotides, respectively. Comparison of the amino acid sequence to its human counterpart (10) revealed a homology of 86%, but canine factor IX appears to be 10 amino acids shorter at the amino terminus, where the first 39 amino acids of the canine cDNA comprise a signal peptide. All sites of γ-carboxylation, β-hydroxylation, and N-linked glycosylation found in human factor IX are conserved in canine factor IX; however, canine factor IX appears to have an additional site of N-linked glycosylation located at Asn-297. Active site residues His-258, Asp-306, and Ser-402 as well as the amino acid residues Arg-Ala (185–186) and Arg-Val (217–218) at which peptide bond cleavage occurs during activation of factor IX in clotting are also conserved.

To test the fidelity of the cDNA, factor IX complementary RNAs were translated in a rabbit reticulocyte lysate translation system. The translation products could be immunoprecipitated using rabbit anti-human factor IX antiserum and, when analyzed by polyacrylamide gel electrophoresis, revealed a major protein band of M_r ≈47,000, which, as expected, migrated slightly ahead of the human factor IX. From these results we concluded that recombinant plasmid dF9.28 encoded full-length canine factor IX protein of proper antigenic specificity and molecular weight.

While this work was in progress, the isolation of a cDNA of canine factor IX was reported by Evans *et al.* (22). Comparison of the nucleotide sequences of the two cDNA clones revealed differences only within the untranslated regions, the major one being that dF9.28 has an additional poly(A) signal located about 400 nucleotides further 3′ from the end of the sequence published by Evans *et al.* (22). The coding region and the deduced amino acid sequences of the encoded proteins are identical.

Construction and Comparison of Canine Factor IX Viruses. Previous attempts to express factor IX using retroviral vectors, which relied on factor IX cDNAs placed under the transcriptional control of the Moloney leukemia virus long terminal repeat (LTR), achieved levels of human factor IX synthesis likely to be insufficient for treatment of hemophilia B in large animals (6). In an attempt to obtain higher levels of synthesis we constructed a series of recombinant retroviral vectors (Fig. 2) in which expression of the factor IX cDNA was driven either by the retroviral 5′ LTR or by an internal human CMV immediate-early gene promoter (16). A com-

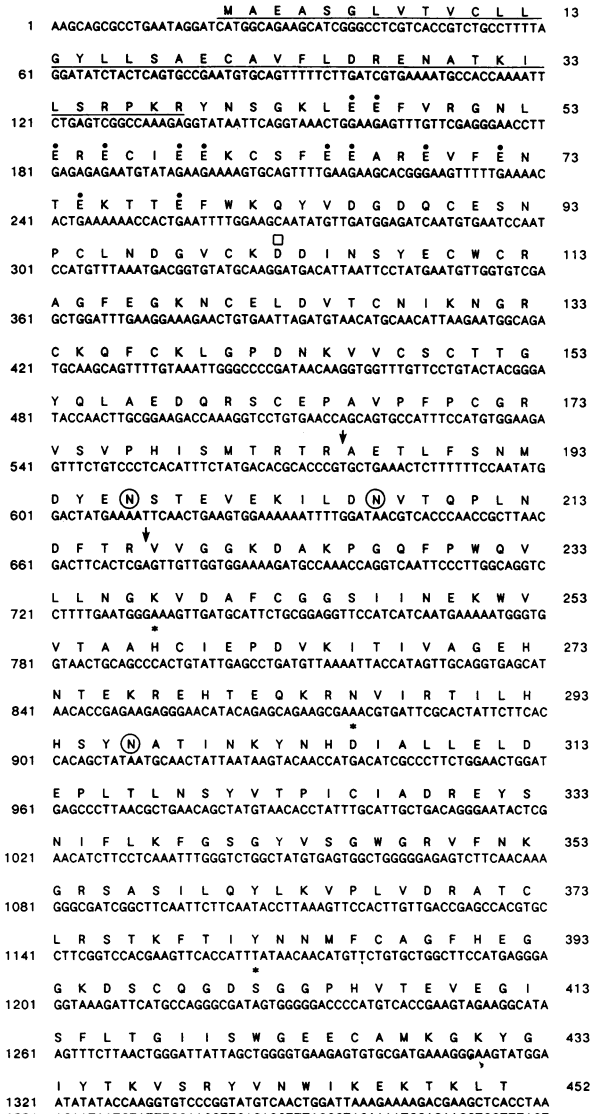

FIG. 1. Sequence of canine factor IX cDNA clone dF9.28 and the deduced amino acid sequence. Numbering of the nucleotide sequence is on the left and that of the amino acid sequence is on the right. Amino acids 1–39 represent the signal sequence and the mature protein begins at amino acid 40 and terminates at amino acid 452. Vertical arrows indicate the peptide bonds cleaved during activation in clotting. Posttranslational modifications are marked (●, γ-carboxyglutamyl residues; □, β-hyroxyaspartyl residues; and ○, Asn-linked carbohydrate residues). Active site amino acids His-258, Asp-306, and Ser-401 are marked (*). Potential poly(A) signals (boxed) are located at nucleotides 2653 and 3033.

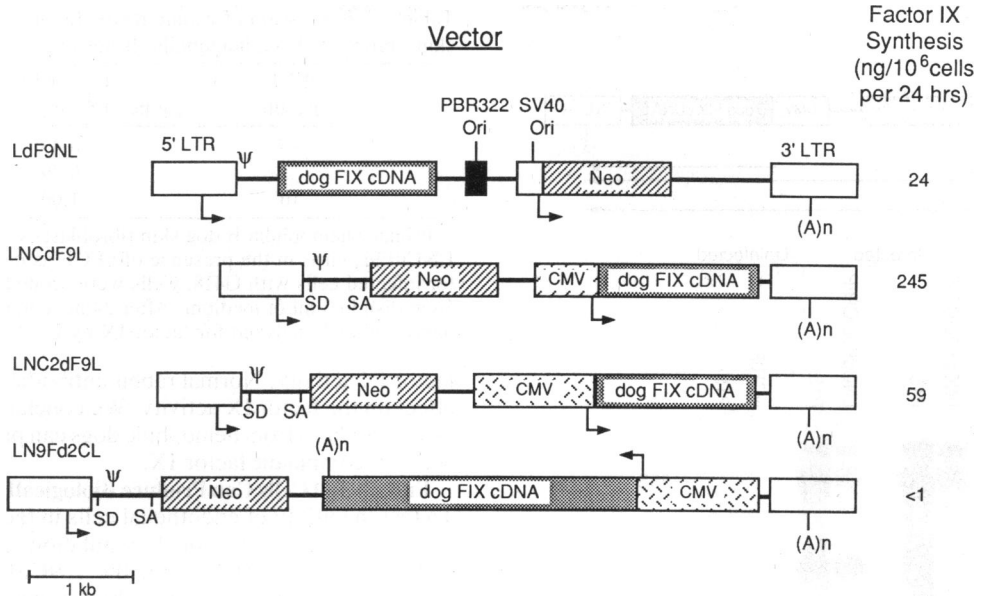

FIG. 2. Structure of retroviral vectors containing the canine factor IX cDNA and analysis of factor IX production by infected NIH 3T3 cells. Vector designation refers to order of genetic elements: L, LTR; N, neo; dF9, canine factor IX (FIX) cDNA; C, CMV immediate-early gene promoter (−522 to +55); 2C, CMV promoter (−1145 to +55). SD, splice donor; SA, splice acceptor; (A)n, poly(A) signal; SV40, simian virus 40; kb, kilobases. Ψ indicates packaging signal and arrows indicate transcription initiation sites and orientation relative to the viral transcription. Factor IX virus produced in transiently transfected GP+E86 cells was utilized to infect TK-NIH 3T3 cells. G418-resistant cells were pooled and analyzed for canine factor IX secretion. Cells were seeded at 2×10^6 cells per 5-cm dish in 3 ml of medium. Conditioned medium was collected after 24 hr and analyzed for canine factor IX by ELISA. The mouse anti-human monoclonal antibody FXC008 (18) was used as the primary antibody, and pooled normal canine sera were used as a standard.

parison of the efficacy of these vectors to direct the synthesis of factor IX in NIH 3T3 cells transduced with viruses generated by transient transfection of the viral plasmid DNA into the packaging cell line GP+E86 is shown in Fig. 2. The construct, LNCdF9L, which contained a 577-bp fragment (−522 to +55) of the CMV immediate-early gene promoter, was by far the most efficient, producing 245 ng per 10^6 cells per 24 hr. The factor IX cDNA driven by the viral LTR promoter, LdF9NL, produced only 10% as much factor IX, and the construct containing a larger CMV promoter fragment (−1145 to +55) produced intermediate levels. On the other hand, in LN9Fd2CL, where the cDNA was transcribed by the same promoter in an orientation opposite to that of viral transcription, no factor IX synthesis was detectable. Based on these results we concluded that the construct LNCdF9L was the most efficient for expression of factor IX. Recombinant virus-producing clones were generated in the amphotropic packaging cell line ΨCrip (12), and several clones (13/41) with titers of $>10^5$ colony-forming units/ml have been isolated. All clones were helper virus free.

RNA isolated from LNCdF9L/Crip and infected hemophilic dog skin fibroblasts (P15) was analyzed by Northern blot analysis to determine whether cells from hemophilic B dogs would produce transcripts of the appropriate size (Fig. 3*A*). When hybridized with a Neo probe, the two major viral transcripts of the expected size of 5.2 kb and 4.7 kb, corresponding to the full-length viral genomic RNA and the spliced transcript, respectively, could be detected in the infected cells (Fig. 3*A*, lanes 1 and 2). Hybridization with a factor IX probe revealed an additional 2.1-kb transcript that is the expected size of the mRNA specie initiated from the internal CMV promoter and terminating in the 3′ LTR (Fig. 3*A*, lanes 3 and 4). Uninfected ΨCrip and P15 cells expressed no Neo or factor IX homologous RNAs (Fig. 3*A*, lanes 5 and 6). From these results we conclude that the LNCdF9L virus is properly expressed in the infected cells.

Transduction of Adult Skin Fibroblasts from Normal and Hemophilic B Dogs. Because factor IX is a secreted protein, we wanted to verify whether it is secreted into the medium of infected skin fibroblasts from the hemophilic dogs similarly to cells from the wild-type dogs. Therefore the accumulation of canine factor IX in conditioned medium from LNCdF9L virus-infected adult dog skin fibroblasts was determined. Fig. 3*B* shows that secretion of canine factor IX from infected adult skin fibroblasts is similar whether the cells originate from wild-type or hemophilia B dogs. Furthermore, levels of dog factor IX accumulate to high levels (0.6–0.9 μg per 10^6 cells). Uninfected cells produced no detectable factor IX protein.

Retroviral transduction occurs only in actively growing cells. Because adult skin fibroblasts grow relatively slowly under standard culture conditions, we stimulated the cells with a mitogen, bFGF (23), during infection. When cells were exposed to LNCdF9L virus in the presence of bFGF, higher levels of factor IX production were observed (Table 1). In several experiments, the rate of secretion of factor IX from these cells ranged from about 0.4 to 1.0 μg per 10^6 cells per 24 hr and correlated directly with the concentration of bFGF in the medium during infection. Since in all cases infected cells were selected for G418 resistance, this result perhaps reflects the increased efficiency of viral transduction in actively replicating cells.

Factor IX from Hemophilic Skin Fibroblasts Is Biologically Active. To assess the biological activity of recombinant canine factor IX produced in LNCdF9L infected hemophilic dog skin fibroblasts, conditioned medium was collected and tested for its ability to complement pooled factor IX-deficient plasma from hemophilia B dogs in an APTT clotting assay. Conditioned medium from uninfected fibroblasts cultured in the absence of serum contained high levels of a background procoagulant activity despite the absence of detectable factor IX protein. This activity could not be inhibited by either the anti-human factor IX antibodies or the monoclonal antibody FXC008. To remove the procoagulant activity, the conditioned medium from the LNCdF9L infected cells was purified and concentrated by barium sulfate precipitation. Conditioned

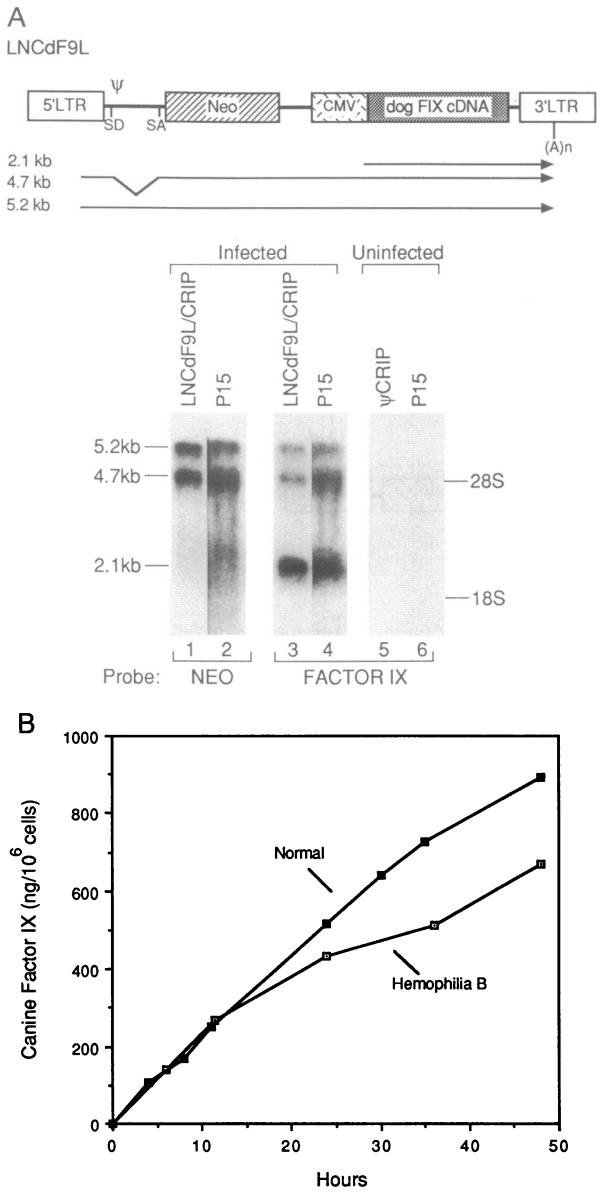

FIG. 3. Analysis of LNCdF9L RNA and secretion of canine factor IX by LNCdF9L infected adult dog skin fibroblasts. (*A*) Total cytoplasmic RNA (10 μg) isolated from uninfected and infected primary cells and cell lines was subjected to Northern blot analysis. RNA were fractionated on formaldehyde/agarose gels, transferred onto a nylon membrane, and probed to either a nick-translated 1.5-kb canine factor IX cDNA probe or 1.4-kb *Hin*dIII to *Bam*HI Neo DNA probe. The expected sizes of the transcripts are indicated. (*B*) Fibroblasts explanted from skin biopsy specimen of a normal adult dog (filled symbols) and a factor IX-deficient dog (P15) (open symbols) were infected with LNCdF9L virus and selected for G418 resistance. Cells were seeded at 2×10^6 cells per 5-cm dish in 4 ml of medium. At each indicated time point 100 μl of medium was removed and assayed for canine factor IX as described in the legend to Fig. 2. Each time point was done in duplicate and curves were corrected for the slight increase in cell number over this period.

medium from infected hemophilic dog skin fibroblasts containing 9.2 μg of canine factor IX protein (determined by ELISA) was precipitated with barium sulfate. Nearly all of the factor IX antigen was brought down with the barium sulfate. The eluted fibroblast factor IX had a specific activity of 96% when compared to a standard of pooled normal canine plasma (Table 2). Furthermore, pretreatment with either rabbit anti-human factor IX antibodies or the monoclonal antibody FXC008 inhibited 92% and 85%, respectively, of the purified factor IX activity. Normal rabbit antibodies had no inhibitory effect on the factor IX activity. We conclude that the infected skin fibroblasts from hemophilic dogs can produce biologically active recombinant factor IX.

Table 1. Expression of canine factor IX in LNCdF9L transduced skin fibroblasts from hemophilia B dogs in the presence of bFGF

bFGF, ng/ml	Factor IX, μg per 10^6 cells per 24 hr
—	0.43
2	0.79
10	1.00

Primary hemophilia B dog skin fibroblasts were infected with the LNCdF9L virus in the presence of bFGF as indicated and selected for infected cells with G418. Cells were seeded at 2×10^6 cells per 5-cm dish in 3 ml of medium. After 24 hr, conditioned medium was harvested and analyzed for factor IX by ELISA.

Endothelial Cells Can Produce Biologically Active Factor IX. To test the utility of endothelial cells in factor IX production we have infected a clonal population of bovine corneal endothelial cells (ACE) with the LNCdF9L virus and assessed their ability to produce biologically active factor IX. Uninfected ACE cells produced no factor IX activity nor any related factor IX antigens. Additionally, unlike the skin fibroblasts, the uninfected endothelial cells produced no procoagulant activity that interfered in the APTT assay. ACE cells transduced with the LNCdF9L virus and selected with G418 sulfate produced high levels of canine factor IX (1.0 μg per 10^6 cells per 24 hr). Analysis of the ACE factor IX to complement canine factor IX-deficient plasma in an APTT assay showed that it had a specific activity of 83% when compared to a standard of normal pooled canine plasma (Table 2). This activity was completely quenched by preincubation with rabbit anti-human factor IX antibodies but was uninhibited by normal rabbit serum. Because the endothelial cells are able to produce biologically active factor IX they can serve as a potential target for gene transfer in the treatment of hemophilia B.

DISCUSSION

Our laboratory has been developing retroviral vectors for gene transfer in cells that can be used for somatic cell gene therapy. Toward this goal we have developed vectors for efficient transduction in hematopoietic stem cells (24) and skin fibroblasts (6). We have previously shown that mouse skin fibroblasts infected with a recombinant retrovirus expressing human factor IX following implantation in mice produce and secrete human factor IX in the plasma (6). Since a hemophilia B dog model system is available, we have elected to investigate the potential use of adult skin fibroblasts from hemophilic B dogs to correct the genetic defect leading to factor IX deficiency. We had two principal aims in this study: (*i*) to ascertain that transduction of normal factor IX gene in skin fibroblasts from hemophilia B dogs will

Table 2. Biological activity of recombinant canine factor IX from hemophilia B dog skin fibroblasts and bovine endothelial cells

Cell type	Factor IX, μg per 10^6 cells per 24 hr	Specific activity
Fibroblast	1.0	0.96
Endothelial	1.0	0.83

Factor IX produced by hemophilia B dog skin fibroblasts and bovine corneal endothelial cells infected with LNCdF9L virus was assayed for functional activity. Factor IX protein in the samples was determined by ELISA, and activity is expressed as a ratio of the activities of the recombinant factor IX determined by a standard of pooled normal canine plasma and the quantity of recombinant factor IX protein determined by ELISA.

produce and secrete biologically active factor IX protein and (*ii*) to master manipulation of skin fibroblasts from affected dogs before attempting implantation in animals. The results of our study clearly indicate that skin fibroblasts from adult hemophilic B dogs can serve as an appropriate model for gene therapy studies. First, skin fibroblasts can be easily obtained from the affected dogs from small skin biopsies, often without the necessity of administration of factor IX supplements or frozen normal plasma. Second, these cells are easily maintained, grown to large numbers in culture, and can be readily infected by retroviruses before returning them to the host animal from which they originated. Finally, when transduced by an appropriate vector, these cells can produce substantial quantities of biologically active factor IX. The amount of factor IX produced *in vitro* by LNCdF9L virus-infected dog skin fibroblasts is of the same order of magnitude as observed by Palmer *et al.* (7) for the infection of human fibroblasts with a similar virus coding for human factor IX. Thus the specific activity of the canine factor IX produced by the dog skin fibroblasts is similar to the recombinant human factor IX produced by mouse embryo fibroblasts and human diploid fibroblasts (6, 7). We also deduce from these results that the biochemical pathways responsible for the proper posttranslational modification of factor IX function properly in the hemophilia B dog.

Recently published reports have shown the feasibility of utilizing endothelial cells as a venue for gene transfer by retroviral vectors (25, 26). Hemophilia B would appear to be an ideal candidate for treatment by this approach because the recombinant factor IX produced by the endothelial cells transplanted to blood vessel walls would have immediate access to the blood. However, in order to consider the utilization of endothelial cells for the treatment of hemophilia B it must be shown that they are capable of producing biologically active recombinant factor IX. The results presented here demonstrate that endothelial cells are able to produce substantial amounts of biologically active factor IX and thus should be considered as a potential source of readily available and genetically modifiable cells for treatment of this disease.

In determining the utility of skin fibroblasts and endothelial cells as appropriate targets for gene therapy, there remain several issues to be addressed: (*i*) whether the transplanted cells survive for long periods following transplantation, (*ii*) whether the genetically modified cells continue to produce factor IX at levels similar to those observed in cells *in vitro*, and lastly (*iii*) whether the factor IX produced by the cells *in vivo* will be biologically active and alleviate the symptoms of the disease. These questions can be addressed by experiments in the hemophilic B dogs as well as in immune compromised, athymic nude mice.

We are most grateful to M. Vogt and R. Scharfmann for helpful discussions, to S. Maki and J. Nelson for the generous gifts of FXC008 monoclonal antibodies and CMV promoters, respectively, and to A. Baird for the ACE cells and bFGF. We also thank M. Heeb, A. Grubber, and J. Griffin for assistance in utilizing the Coagumate and setting up the factor IX bioassay. J.H.A. was supported by an EMBO long term fellowship. This work was supported by Grants R1HL37072A (to I.M.V.), HL-01648-42 (to K.M.B.), and HL-2639-08 from the National Institutes of Health.

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