

An alternative approach to somatic cell gene therapy

(human factor IX/retroviral vector/skin fibroblast/collagen implant)

DANIEL ST. LOUIS AND INDER M. VERMA*

Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92138

Communicated by Renato Dulbecco, December 18, 1987 (received for review November 17, 1987)

ABSTRACT Mouse primary skin fibroblasts were infected with a recombinant retrovirus containing human factor IX cDNA. Bulk infected cells capable of synthesizing and secreting biologically active human factor IX protein were embedded in collagen, and the implant was grafted under the epidermis. Sera from the transplanted mice contain human factor IX protein for at least 10–12 days. Loss of immunoreactive human factor IX protein in the mouse serum is not due to graft rejection. Instead, the mouse serum contains anti-human factor IX antibodies, which react with the protein. We suggest that retroviral-infected primary skin fibroblasts offer an alternative approach to somatic cell gene therapy.

The concept of human gene therapy involves the introduction of a functionally active gene into somatic cells of an affected individual to correct the defect. To ensure lifelong supply of the replaced gene product, it is essential to introduce and express the gene in cells that proliferate during the entire adult life of the recipient. Because pluripotent stem cells in bone marrow have both self-renewal capacity as well as ability to give rise to all hematopoietic lineages, they are a popular target for introducing functionally active genes (1–4). Recently, hepatocytes have been used as target cells for introducing functionally active genes (5, 6).

Murine retroviruses efficiently infect many fibroblast cell lines, including immortalized and normal human diploid fibroblasts (7–10) and human keratinocytes (11). This approach to gene transfer offers several advantages: (i) primary skin fibroblasts are readily accessible by skin biopsy and easily cultured and manipulated *in vitro*; (ii) efficient infection of primary cultures by retroviral vectors reduces the *in vitro* culturing period; (iii) use of fibroblasts as target cells for gene transfer overcomes inefficient gene expression by retroviral vectors in other target tissues, such as bone marrow; (iv) because skin fibroblasts reside in a highly vascularized compartment of the dermis, infected cells have direct access to the circulatory system; (v) use of syngeneic primary cultures obviates problems of graft rejection; and (vi) the implanted skin fibroblasts can be conveniently removed. We report the development and characterization of an alternative approach of gene product delivery using mouse skin fibroblasts.

MATERIALS AND METHODS

Construction and Infection by Recombinant Factor IX Retroviruses. The recombinant pAFFIXSVNeo is based on a retroviral construct pAFVXM generated by Kriegler *et al.* (12). A human factor IX cDNA was linked directly to the 5' long terminal repeat (LTR) by inserting a 1.6-kilobase (kb) *Bam*HI/*Hind*III fragment from the clone CVI between the *Bgl* II and *Hind*III sites of pAFVXM (13). The entire expression unit from the neomycin phosphotransferase expression

plasmid (pKoNeo) was excised by partial *Hind*III digestion and inserted into the *Hind*III site of the above factor IX viral construct (Fig. 1a). "Helper-free" recombinant ecotropic virus in Ψ 2 cells was generated as described (14, 15). The titers of recombinant retrovirus expressed from drug-resistant clones were done essentially as described (14).

Primary mouse embryo fibroblasts (MEF) were obtained from day-17 embryos of C57BL/6J mice (16). The BL/6 line is an immortalized skin cell line derived from x-ray irradiated skin fibroblasts obtained from C57BL/6J mice. The skin fibroblast cell line BL/6 and NIH 3T3 TK⁻ cells were infected with recombinant retroviruses from the cell line, Ψ FIXNeo 4, at a multiplicity-of-infection (moi) of 1–2 in the presence of Polybrene at 8 μ g/ml; MEF cells were infected at a moi of 5.

Implantation of Infected Mouse Fibroblasts in Mouse. Infected BL/6 and MEF cells were cultured in an artificial extracellular matrix composed of rat tail type I collagen (1 mg/ml; Sigma) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 5-cm dish (17, 18). The cells were cultured at 37°C for 3 days during which the collagen lattice contracted to a tissue-like structure (1/25th the area of the original gel). Once contracted, two artificial tissues containing $\approx 4 \times 10^6$ infected fibroblasts were grafted into the loose connective tissue of the dermis in the mid-back of a recipient C57BL/6 mouse. To ensure rapid vascularization of the grafted tissue, a 2-mm² piece of Gelfoam (Upjohn) containing 2 μ g of basic fibroblast growth factor was inserted into the loose connective tissue along with each graft. Serum samples were drawn at 2-day intervals and analyzed for the presence of human factor IX by ELISA.

Analysis of Secreted Factor IX. Levels of antigenic factor IX were assayed by ELISA as described by Anson *et al.* (19). Biologically active human factor IX was immunoaffinity-purified using A7 antibody (19, 20). The amount of biologically active protein was determined by a one-step clotting assay using canine factor IX-deficient plasma (21). This assay is based on the ability of the sample to decrease the prolonged activated partial thromboplastin time of congenital factor IX-deficient plasma. Purified human factor IX was used as a control.

RESULTS

Transduction of Neomycin Resistance and Expression of Human Factor IX. The titers of helper-free Ψ FIXNeo virus produced in the various cell lines ranged from 3×10^5 to 7×10^5 G418-resistant colony forming units per ml when assayed on NIH 3T3 TK⁻ cells. As measured by ELISA, all virus-producing cell lines secreted essentially the same levels of factor IX into the culture media (≈ 200 ng/ml). All infected and drug-resistant cell lines were also found to secrete factor

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.

Abbreviations: MEF, mouse embryo fibroblasts; LTR, long terminal repeat; moi, multiplicity of infection.

*To whom reprint requests should be sent.

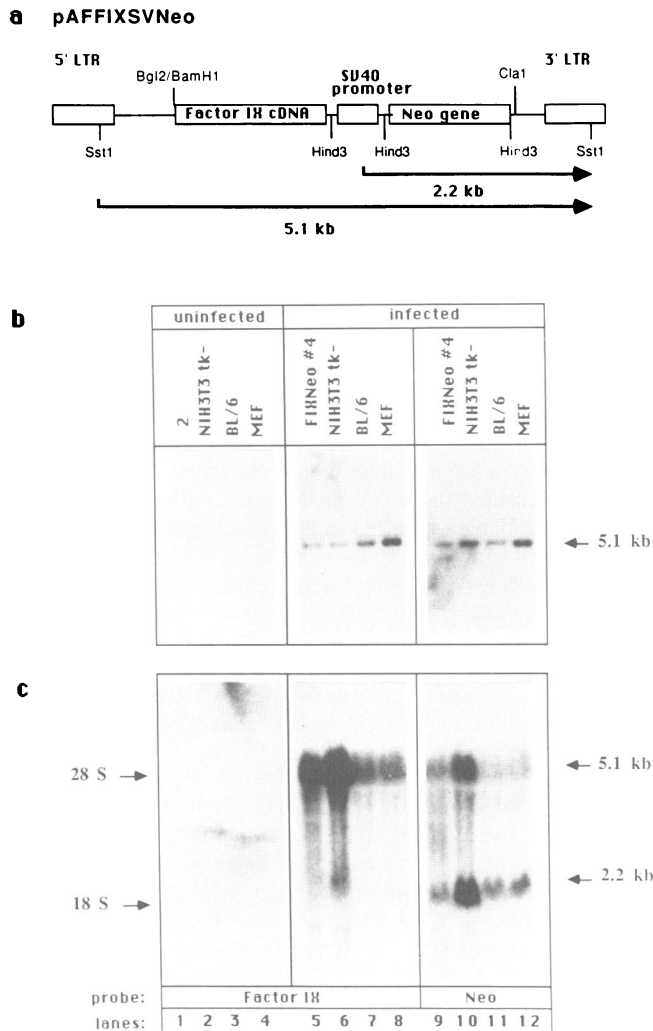


FIG. 1. Analysis of recombinant human factor IX retrovirus. (a) pAFFIXSVNeo. Arrows indicate transcripts that initiate at either the promoter in the 5' LTR or the simian virus 40 early promoter between the two LTRs and terminate at the polyadenylation signal in the 3' LTR. Bars indicate the putative initiation site of transcription. The restriction endonuclease cleavage sites *Sst* I, *Hind*III, *Bam*HI, *Bgl* II, and *Cla* I are diagnostic sites used during the construction of the vector or subsequent characterization of the provirus in the genome of infected cell lines. (b) Proviral DNA. Genomic DNA isolated from either uninfected or infected ΨFIXNeo 4, NIH 3T3 TK⁻, BL/6, MEF cells, and from the virus-producing cell line ΨFIXNeo 4 was digested with *Sst* I, fractionated by agarose gel electrophoresis, transferred onto a nitrocellulose membrane, and hybridized to either a nick-translated 1.6-kb factor IX cDNA probe (lanes 1-8) or 1.4-kb *Hind*III to *Bam*HI Neo DNA probe (lanes 9-12). Under these conditions of hybridization, human factor IX cDNA does not hybridize to mouse DNA. (c) RNA transcripts. Total RNA (10 μg) isolated from uninfected and infected cells and cell lines was subjected to RNA blot analysis.

IX into the culture media, albeit at different levels (see Fig. 2).

We determined the organization of the integrated recombinant retrovirus in the virus-producing cell line by Southern blot analysis of *Sst* I-digested (*Sst* I cleaves once in each LTR to generate a 5.1-kb DNA fragment (Fig. 1b) genomic DNA. All infected cells displayed a single band of the expected size of ≈5.1 kb, which hybridizes to both the factor IX cDNA and the Neo probe, therefore ruling out any detectable rearrangements. Furthermore, the size of this band in infected NIH 3T3 TK⁻, BL/6, and MEF cells is identical to that found in the virus-producing cell line ΨFIXNeo 4 (compare lanes 5 and 9 to other lanes).

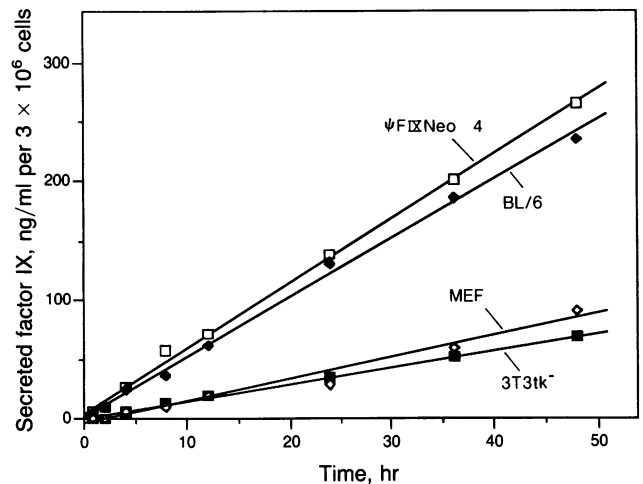


FIG. 2. Secretion of human factor IX. Rate of secretion of human factor IX by the virus-producing cell line ΨFIXNeo 4 (□) and by infected NIH 3T3 TK⁻ cells (■), BL/6 cells (◆), and MEF cells (◇). Cells were seeded at 3 × 10⁶ cells per 5-cm dish in 4 ml of medium. At each indicated time point 100 μl of medium was removed and assayed for human factor IX by enzyme-linked immunosorbent assay (ELISA) (19). The mouse anti-human monoclonal antibody, FXC008, generated by Bajaj *et al.* (22) was used as the primary antibody, whereas pooled normal human sera were used as a standard. Each time point was done in triplicate and thus represents an average amount of factor IX secreted over a 48-hr period. Curves were corrected for the slight increase in cell number over this period.

The RNA blot analysis of the RNA isolated from ΨFIXNeo 4, infected NIH 3T3 TK⁻, BL/6, and MEF is shown in Fig. 1c. When hybridized to factor IX probe, only one major transcript of the expected size of 5.1 kb, corresponding to full-length viral RNA, could be detected in the infected cells. Hybridization with Neo probe reveals an additional 2.2-kb transcript that is the predicted size of the mRNA species, the synthesis of which is initiated from the simian virus 40 early promoter and is terminated in the 3' LTR (Fig. 1c). Ratios of the steady-state levels of the 5.1-kb and the 2.2-kb transcripts varied within the different infected cell types. From results shown in Fig. 1, we conclude that the ΨFIXNeo recombinant retrovirus is properly integrated and expressed in the infected cells.

Secretion of Factor IX Protein. Because human factor IX is a secretory protein we wanted to verify whether it is secreted into the medium of the infected cells. Fig. 2 shows that both rate and extent of antigenic factor IX released into the medium is dependent on cell type rather than on relative amounts of the factor IX transcripts. For instance, steady-state levels of factor IX transcript in infected NIH 3T3 TK⁻ cells is much higher than in BL/6 cells (Fig. 1c); yet, the rate and amount of factor IX secreted in the latter cell type is much higher. Both the virus-producing cell line ΨFIXNeo 4 and infected skin fibroblast cell line BL/6 secreted antigenic factor IX at similar rates—≈5.7 ng per ml/hr for 3 × 10⁶ cells and 5.0 ng per ml/hr for 3 × 10⁶ cells, respectively. This rate was almost 3-fold higher than the rate of factor IX secretion seen for infected MEF (1.75 ng per ml/hr for 3 × 10⁶ cells) and infected NIH 3T3 cells (1.65 ng per ml/hr for 3 × 10⁶ cells). These results indicate that the rate of synthesis and/or secretion may be a property of the cell type, rather than the levels of expression.

Secreted Human Factor IX Protein Is Biologically Active. The primary translation product of factor IX gene undergoes extensive post-translational modification, which include addition of sialic carbohydrates (23, 24), vitamin K-dependent conversion of glutamic acid residues to γ-carboxy/glutamic

acid, (25) and β -hydroxylation of aspartic acid residue 64 (26). The γ -carboxylation of factor IX is essential for clotting activity, and this modification generally occurs in the liver, the primary source of factor IX synthesis in the body. We took two different approaches to assess biological activity of human factor IX secreted from cells in culture: (i) the infected mouse embryo fibroblasts were cultured in factor IX-deficient canine serum obtained from hemophiliac dogs, supplemented with epidermal growth factor (10 ng/ml) and vitamin K (25 ng/ml). Media harvested after 48-hr incubation was monitored for activity by a one-step assay (21). Conditioned media from MEF cells contained biologically active human factor IX at 210 ng/ml, which is similar to the levels seen with ELISA assays (Fig. 4). (ii) Because BL/6 cells did not attach to the tissue culture dish in canine sera, we had to resort to a different approach. Infected BL/6 cells were grown in 10% fetal calf serum supplemented with vitamin K (25 ng/ml), and the media harvested after 48-hr incubation was applied to an immunoaffinity column containing human factor IX monoclonal antibody A-7 (19, 20). This monoclonal antibody recognizes the calcium-binding domain of human factor IX protein, thus discriminating between carboxyl-lacking factor IX and biologically active γ -carboxyl human factor IX. One-hundred and sixty millili-

ters of the media obtained from BL/6 cells containing 32 μ g of antigenic human factor IX (determined by ELISA) was passed through the column. Nearly 3.5 μ g of the biologically active material was recovered from the column. This represents over 10% of the total antigenic factor IX in the starting sample. No biologically active factor IX could be identified from uninfected MEF or BL/6 cells. Despite lack of information on the extent of γ -carboxylation or other post-translational modifications, we conclude that the infected cells used for subsequent implantation studies synthesize biologically active human factor IX.

Detection of Human Factor IX in Mice Grafted with Infected Fibroblasts. Infected MEF cells and BL/6 cells were cultured in an artificial extracellular matrix, composed of collagen, before grafting. We chose a tumor cell line, BL/6, in addition to MEF because it has an advantage in growth and vascularization and thus would increase our chances of detecting secreted factor IX in the sera. Attachment of the cells to the collagen resulted in a three-dimensional array of cells stacked on top of one another. After the primary fibroblast cells (MEF) or the tumor cell line BL/6 contracted in the collagen gel, the cells were grafted onto the loose connective tissue of the mid-back dermis of a recipient syngenic C57BL/6 mouse (Fig. 3A). Fig. 3B shows that the inserted implants were extensively vascularized by day 14. A similar extent of vascularization was also detected in 28-day implants (data not shown).

The serum levels of the human clotting factor were measured in engrafted mice by ELISA over a 34-day period. Fig. 4 shows that the average levels of human factor IX in three mice progressively increased from 20 ng/ml at day 2 to a peak of 97 ng/ml 7 days after grafting the BL/6 cells into the mice. The four mice grafted with the infected MEF fibroblasts showed a similar pattern of increase, in which an average peak of 25 ng/ml of factor IX was detected at day 9. This rise was followed by a rapid decline to near nondetectable levels of serum human factor IX at day 16 in both the BL/6 and MEF grafts. A minor peak of factor IX was seen at day 20 in mice with either graft, which was followed by loss of any detectable factor IX antigen. In parallel experiments, 10^7 infected BL/6 or MEF cells were injected directly into the peritoneal cavity of the recipient C57BL/6 mice. Serum levels of human factor IX in the injected

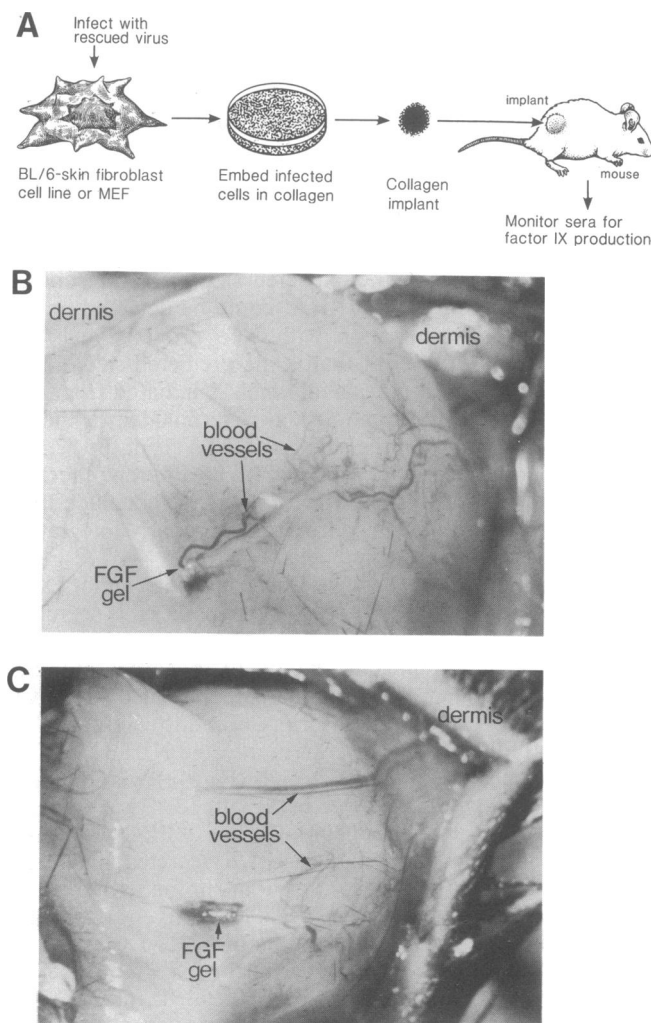


FIG. 3. Embedding and implantation. (A) Schematic representation of the protocol used to generate and graft the collagen implants into the loose connective tissue of the skin of the mouse model system. (B and C) View of both MEF (B) and BL/6 (C) collagen implants at day 14. The grafts (white area) and the high degree of vascularization are clearly visible. The implant is 0.75 cm in diameter. FGF, fibroblast growth factor.

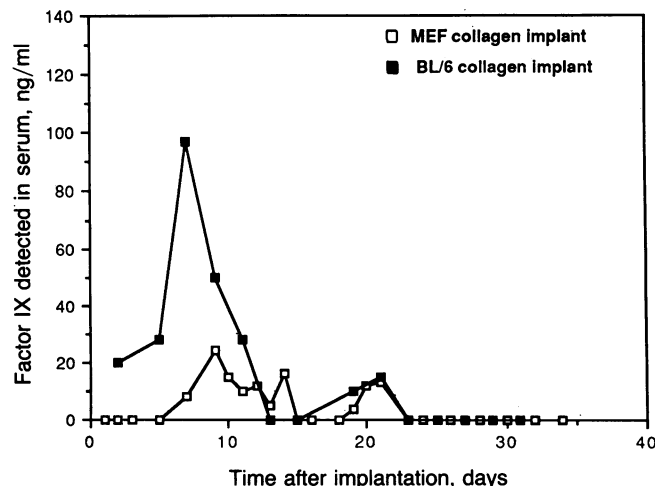


FIG. 4. Factor IX in human sera. Average (four mice for MEF; three mice for BL/6) amount of human factor IX detected in the sera of mice that received two collagen implants containing $\approx 4 \times 10^6$ cells each. Sera was drawn from each animal at the indicated times. Levels of circulating human factor IX were determined by ELISA; levels of factor IX fluctuated 2- to 3-fold between experiments.

Table 1. Amount of antigenic factor IX secreted from cells explanted from grafts

Days after implantation	Collagen explants, ng/ml	
	BL/6	MEF
14	180	40
21	210	27
28	150	11

Tissue explanted from the grafts at times indicated after implantation were cultured *in vitro*. When cells were confluent, medium was replaced; after 48 hr, levels of factor IX secreted into the culture were assayed by ELISA.

animals followed a similar profile as that seen with the grafts (data not shown).

Explanted Grafts Make Factor IX. The decline in serum levels of antigenic human factor IX in animals that were either grafted or injected i.p. was not associated with necrosis of cells in the grafts. BL/6 cells in the collagen matrix grew as an aggressive tumor at the site of the graft; the tumor continued to grow until animals were sacrificed at day 32. Mice with grafts containing infected MEF were visibly vascularized upon gross inspection until day 28; however, by day 120 the extent of vascularization was reduced, but the implant was still viable (data not shown). Additionally, cells explanted at various times during the course of the experi-

ment produced factor IX when grown in culture (Table 1). The explanted BL/6 cells grew well in culture and secreted antigenic factor IX at levels similar to that before grafting. The MEF cells explanted from the grafts at days 14 and 21 grew well in culture, but produced slightly lower levels of factor IX. Cells explanted at day 28 did not grow well, and the low level of factor IX secreted from these cells is, perhaps, a consequence of this poor growth.

Detection of Serum Anti-Factor IX Antibodies. To further investigate the decline of serum levels of human factor IX we reasoned that the recipient animal mounted an immunological response against the highly immunogenic human factor IX protein. To test whether mice bearing grafts with infected BL/6 or MEF cells are generating anti-factor IX IgG antibodies, pooled serum samples were used to probe immunologic blots containing purified human factor IX protein (Fig. 5). The levels of anti-human factor IX IgG antibodies were not detectable in mice with MEF grafts at day 7 to day 21. Slightly higher levels of serum antibodies were detected in mice with BL/6 grafts during this period—presumably because they are releasing more factor IX. Maximum levels of anti-human factor IX antibodies were detected at day 28 in mice with either graft. The mice with BL/6 grafts exhibited the highest level of xenoantibodies. Pooled serum drawn from mice 28 days after i.p. injection with infected MEF also showed anti-factor IX IgG antibodies, albeit at much lower levels. Naive animals, which have not been exposed to infected BL/6 or MEF cells, do not make anti-human factor IX antibodies. These observations would suggest that human factor IX is continuously produced in grafted mice but is not detectable due to a large pool of mouse anti-human factor IX antibodies.

DISCUSSION

We present the development and characterization of a different approach of gene product delivery into an animal model system. The BL/6 cells and MEF cells infected with a helper-free recombinant retroviral vector containing the human clotting factor IX cDNA secrete partially biologically active clotting factor at a rate 10-fold higher than seen with another retroviral vector containing the human clotting factor cDNA (19). In addition, we have demonstrated that these genetically modified cells can be reintroduced into the loose connective tissue of the dermis of a syngeneic mouse. Grafts are quickly vascularized in the presence of angiogenic factor, fibroblast growth factor, and remain vascularized for at least 28 days. Grafts containing the BL/6 cells grow as aggressive tumors over this period, while the size of the grafts containing MEF cells does not increase over the same period. The clotting factor secreted from the infected cells in the graft is accessible to the circulatory compartment and can easily be detected in serum of the recipient. Functional status of the infected cells in the grafts can be measured by monitoring serum levels of human factor IX or by the ability of explanted cells to continue secreting the human protein. However, C57BL/6 mice recognize the human blood clotting factor as foreign and thus mount a strong humoral immune response against it. Although a humoral response against factor IX clearly exists, there does not appear to be a major cell-mediated response against the cells in the grafts. The cells in the graft are still viable after 28 days of implantation and continue to synthesize factor IX protein.

Recently two groups have used mouse fibroblasts to introduce and express foreign genes in mice (28, 29). One group implanted mice with DNA-transfected cell line and showed that the recipient mice made the gene product (growth hormone) and maintained the graft only if the mice are immunosuppressed (28). The other group using retroviral vectors containing α_1 -antitrypsin gene generated a cell line

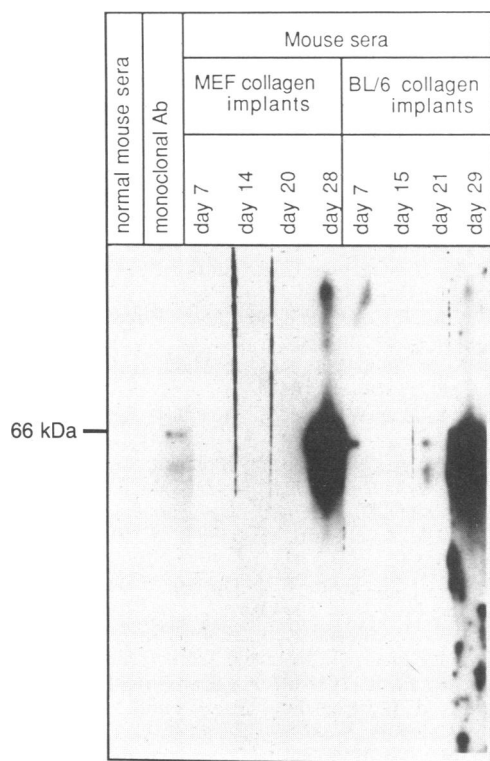


FIG. 5. Detection of mouse anti-human factor IX antibodies in mice grafted with collagen implants. Purified human factor IX was subjected to PAGE under denaturing conditions and then transferred onto nitrocellulose as described (27). The nitrocellulose strips were treated with blocking solution for 2 hr followed by 1:100 dilution of naive normal mouse serum (lane 1); 1:100 dilution mouse monoclonal anti-factor IX antibody FXC008 (lane 2); 1:100 dilution of serum from mouse harboring grafts containing infected MEF cells drawn at day 7, day 14, day 20, and day 28 (lanes 3–6); 1:100 dilution of serum from mouse harboring grafts containing infected BL/6 cells drawn at day 7, day 15, day 21, and day 29 (lanes 7–10). After overnight incubation at 37°C the strips were washed, incubated with ¹²⁵I-labeled goat anti-mouse IgG antibody, and then subjected to autoradiography as described (31).

and then transplanted them into the peritoneal cavity of nude mice (29). In both cases cell lines were generated that would potentially be tumorigenic in mice. Neither study addresses the issue of cell maintenance in grafted mice without the use of harsh immunosuppressive agents. Moreover, because of their potential tumorigenicity it is not advisable to generate syngenic human diploid cell lines and use them for introducing foreign genes. The method we have described obviates the need for cell lines and instead essentially uses the cells of recipient mice, minimizing the possibility of rejection. Culturing the cells in collagen matrix circumvents the problem of necrosis that would ensue following s.c. injection (30). The high efficiency of retroviral infection and expression in fibroblasts (>80%) simplifies the overall endeavor of introduction of foreign genes. Clinical examples for this type of treatment would include hemophilia, endocrine deficiency, α_1 -antitrypsin, etc. Even though the data presented here was obtained from mouse embryo fibroblasts, we wish to add that we have extended these observations by infecting adult hemophilic dog fibroblasts with factor IX retrovirus.

Further advances toward application of this approach would require the following: (i) increased levels of factor IX proteins. In normal individuals levels of factor IX protein are $\approx 5 \mu\text{g/ml}$ of plasma. Although the levels reported here are lower by several orders of magnitude, we note that individuals containing $0.5 \mu\text{g}$ of biologically active factor IX per ml in plasma do not show the symptoms of hemophilia. The low levels of factor IX can be increased either by making improved vectors capable of generating large amounts of factor IX proteins or, alternatively, by grafting more cells. According to the data presented here, we can generate up to 25 ng of factor IX per hr from an implant containing 4×10^6 cells (Fig. 4). In larger animals multiple grafts of up to 10^8 cells can be easily implanted, increasing the levels of factor IX protein to that required to alleviate the deficiency. (ii) Improved culturing conditions—culturing infected cells in a defined medium (without fetal bovine serum) and improved technology for reconstitution of living skin would also increase the efficiency of the system (30). Moreover, improved surgical skills may ensure that the implant would lay flat in the dermal compartment of the mouse skin to allow more uniform vascular development and hence improve cell viability during the brief period required for vascularization. Although the extent of cell viability has not yet been determined in grafts containing MEF cells, experiments in rats have shown that transplanted fibroblasts persist for at least 13 months (30).

In conclusion, we have shown that skin fibroblasts can be used as a viable mode of introduction and expression of foreign genes in animals. The process of manipulation of genetically engineered fibroblasts appears to be both less complex and cumbersome than the widely accepted use of bone marrow transplantation for somatic cell gene therapy.

We are grateful to Drs. G. G. Brownlee, D. Anson, P. Bajaj, K. Smith, S. Maki, and A. Baird for reagents. We are particularly indebted to Drs. K. Brinkhous and D. McCord for performing clotting assays. We thank Dr. J. Rhinewald, Dr. D. Anson, and B. Malone for their interest and P. McClintock for preparing the text. D.S.L. was supported by a postdoctoral fellowship from the Na-

tional Cancer Institute, Canada. This work was supported by grants from the National Institutes of Health to I.M.V.

1. Miller, A. D., Eckner, R. J., Jolly, D. J., Friedmann, T. & Verma, I. M. (1984) *Science* **225**, 630–632.
2. Williams, D. A., Lemischka, I. R., Nathans, D. G. & Mulligan, R. C. (1984) *Nature (London)* **310**, 476–480.
3. Keller, G., Paige, C., Gilboa, E. & Wagner, E. F. (1985) *Nature (London)* **318**, 149–154.
4. Dick, J. E., Magli, M. C., Huszar, D. H., Phillips, R. A. & Bernstein, A. (1985) *Cell* **42**, 71–79.
5. Ledley, F. D., Darlington, G. J., Hahn, T. & Woo, S. L. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5335–5339.
6. Wolfe, J. A., Yee, J.-K., Skelly, H. F., Moores, J. C., Respass, J. G., Friedmann, T. & Leffert, H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3344–3348.
7. Ledley, F. D., Grenett, H. E., McGinnis-Shelnutt, M. & Woo, S. L. C. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 409–413.
8. Sorge, J., Kuhl, W., West, C. & Beutler, E. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 906–909.
9. Palmer, T. D., Hock, R. A., Osborne, W. R. A. & Miller, A. D. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1055–1059.
10. Garver, R. I., Jr., Chytil, A., Courtney, M. & Crystal, R. G. (1987) *Science* **237**, 762–764.
11. Morgan, J. R., Barrandon, Y., Green, H. & Mulligan, R. C. (1987) *Science* **237**, 1476–1479.
12. Kriegler, M., Perez, C. F., Hardy, C. & Botchan, M. (1984) *Cell* **38**, 483–491.
13. Anson, D. S., Choo, K. H., Rees, D. J. G., Gianelli, F., Goad, K., Huddleston, J. A. & Brownlee, G. G. (1984) *EMBO J.* **3**, 1053–1060.
14. Miller, A. D. & Buttimore, C. (1986) *Mol. Cell. Biol.* **6**, 2895–2902.
15. Mann, R., Mulligan, R. C. & Baltimore, D. (1983) *Cell* **33**, 153–159.
16. Todaro, G. J. & Green, H. J. (1963) *J. Cell Biol.* **17**, 299–313.
17. Elsdale, T. & Bard, J. (1972) *J. Cell Biol.* **54**, 626–637.
18. Bell, E., Ivarsson, B. & Merrill, C. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1274–1278.
19. Anson, D. S., Hock, R. A., Austen, D., Smith, K. J., Brownlee, G. G., Verma, I. M. & Miller, A. D. (1987) *Mol. Biol. Med.* **4**, 11–20.
20. Smith, K. J., Singaraju, C. & Smith, L. F. (1987) *Am. J. Clin. Pathol.* **87**, 370–376.
21. Goldsmith, J. C., Chung, K. S. & Roberts, H. R. (1978) *Thromb. Res.* **12**, 497–502.
22. Bajaj, S. P., Rapaport, S. I. & Maki, S. L. (1985) *J. Biol. Chem.* **260**, 11574–11580.
23. Chavin, S. I. & Weidner, S. M. (1984) *J. Biol. Chem.* **259**, 3387–3390.
24. Fournel, M. A., Newgren, J., Madanat, M. & Pancham, N. (1985) *Thromb. Haemostasis* **54**, 147 (abstr.).
25. Suttie, J. W. (1980) *CRC Crit. Rev. Biochem.* **8**, 191–223.
26. Ferlund, P. & Stenflo, J. (1983) *J. Biol. Chem.* **258**, 12509–12512.
27. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
28. Selden, R. F., Skoskiewicz, M. J., Howie, K. B., Russell, P. S. & Goodman, H. M. (1987) *Science* **236**, 714–718.
29. Garver, R. I., Jr., Chytil, A., Karlsson, S., Fells, G. A., Brantley, M. L., Courtney, M., Kantoff, P. W., Nienhuis, A. W., Anderson, W. F. & Crystal, R. G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1050–1054.
30. Bell, S., Sher, S., Hall, B., Merrill, C., Rosen, S., Chamson, A., Asselineau, D., Dubertret, L., Coulomb, B., Capiere, C., Nusgens, B. & Neveue, Y. (1983) *J. Invest. Dermatol.* **81**, 25–103.
31. Glenney, J. (1986) *J. Anal. Biochem.* **156**, 315–319.