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26. We thank D. Denny for suggesting the sequence of the anchor polylinker. Supported by a Hulda Irene Duggan Investigator Award from the Arthritis Foundation (E.Y.L.), a Centennial Fellowship from the MRC of Canada (J.F.E.), the PEW Foundation (M.M.D.), and grants from the National Institute of Allergy and Infectious Disease (M.M.D.).

23 September 1988; accepted 2 December 1988

High-Level Recombinant Gene Expression in Rabbit Endothelial Cells Transduced by Retroviral Vectors

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By virtue of its immediate contact with the circulating blood, the endothelium provides an attractive target for retroviral vector transduction for the purpose of gene therapy. To see whether efficient gene transfer and expression was feasible, rabbit aortic endothelial cells were infected with three Moloney murine leukemia virus–derived retroviral vectors. Two of these vectors carry genes encoding products that are not secreted: N2, containing only the selectable marker gene *neo*^{R}, and SAX, containing both neo^R gene and an SV40-promoted adenosine deaminase (ADA) gene. The third vector, G2N, contains a secretory rat growth hormone (rGH) gene and an SV40-promoted neo^R gene. Infection with all three vectors resulted in expression of the respective genes. A high level of human ADA expression was observed in infected endothelial cell populations both before and after selection in G418. G2N-infected rabbit aortic endothelial cells that were grown on a synthetic vascular graft continued to secrete rGH into the culture medium. These studies suggest that endothelial cells may serve as vehicles for the introduction in vivo of functioning recombinant genes.

THE USE OF RETROVIRAL GENE transfer over the past decade has greatly facilitated the transfer of genetic material into mammalian cells. Retroviruses have a high efficiency of infection, stable integration, and expression in most cells (*1*). Nonimmortalized cells, which may be transfected at low efficiency (or not at all) by physical and chemical means, are very amenable to retroviral-mediated gene transfer. Although bone marrow hematopoietic progenitor cells have been frequently targeted for retroviral-mediated gene transfer, these cells suffer from drawbacks such as the variable ability to express certain genes in mouse bone marrow (*2, 3*) or inefficient gene transfer into hematopoietic progenitor cells in dog (*4*), primate (*5*), and human bone marrow (*6*). The requirement for a genetically engineered cell population capable of both long-term survival and stable expression has led to the consideration of other cell types such as fibroblasts (*7–10*), lymphocytes (*11, 12*), human keratinocytes (*13*), and hepatocytes (*14*) for use in gene therapy.

The endothelium, because of its contiguity with the bloodstream, is a particularly attractive target for the delivery of functional genes in vivo. The use of endothelium for gene transfer would permit secretion of a recombinant protein from genetically engineered endothelial cells directly into the blood. Alternatively, endothelial cells expressing a nonsecreted recombinant protein might be able to inactivate a toxic substance that is circulating in the blood.

To determine the efficacy of retroviral-mediated gene transfer while assessing the expression of genes encoding secreted and nonsecreted products, cultured rabbit aortic endothelial cells (RAEC) were infected with three recombinant retroviral vectors containing the neomycin resistance (neo^R) gene alone or in combination with either the adenosine deaminase (ADA) gene or the rat growth hormone (rGH) gene (Fig. 1).

Before infection, monolayers of RAEC (*15*) were characterized by morphology, uptake of diacetylated low density lipoprotein (diI-ac-LDL) (*16*), and by the presence of angiotensin-converting enzyme activity (4.1×10^5 molecules per cell) (*17*). Retroviral packaging lines were made that contained SAX, N2, or G2N (*18–21*). Vector-containing viral supernatants that exhibited titers of 5×10^5 colony-forming units per milliliter or greater were used for infection. After infection and selection in G418-containing

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medium, the morphology of RAEC was somewhat elongated, but the cells retained their endothelial phenotype in terms of angiotensin-converting enzyme activity and dil-ac-LDL uptake (*22*). The efficiency of infection was estimated to be 0.5 to 1%, as determined by the number of G418-resistant RAEC colonies that were present after infection with S3A supernatant. However, this most likely represents an underestimation of the true efficiency of infection because of the markedly diminished proliferation of RAEC when grown at clonal densities.

Uninfected RAEC did not survive in tissue culture medium containing ≥0.2 mg of G418 per milliliter. RAEC infected with G2N, N2, or SAX vectors that survived selection with G418 were analyzed for neophosphotransferase (NPT) activity. Whereas no detectable NPT activity was present in uninfected RAEC, N2 and SAX-infected cells expressed a substantial amount of NPT activity as compared with mouse fibroblasts containing the SAX vector (Fig. 2A). These two vectors express neo^R from the Moloney murine leukemia virus (M-MuLV) long terminal repeat (LTR). NPT activity was also detected in G2N-infected endothelial cells containing the SV40-driven neo^R gene, but at a level somewhat lower than that of N2- and SAX-infected cells (Fig. 2B).

RAEC transduced with SAX were analyzed for human ADA expression by cellulose acetate gel electrophoresis, which separates the ADA isoenzymes of different species. A comparison of the human and rabbit isoenzyme band intensities in each lane indicates that G418-selected (Fig. 3A) RAEC infected with SAX expressed human ADA at a level fivefold as high, and unselected (Fig. 3B) twofold as high, as endogenous rabbit ADA.

Neither the neo^R nor the ADA proteins are secreted from cells. In order to see whether RAEC could secrete rGH, which contains a secretory signal sequence, cells were infected with the G2N vector and selected in G418-containing medium. Amounts of rGH in the medium were determined by radioimmunoassay. Whereas uninfected cells did not secrete detectable rGH into the culture medium, before selection with G418 RAEC infected with G2N secreted 179 ng per 10^6 cells in 24 hours. Selection of the infected cells in medium containing G418 resulted in a fivefold increase in rGH secretion (Table 1, part A).

One method of introducing endothelial cells into animals would be the implantation of pre-endothelialized vascular grafts within blood vessels. To find out whether cells grown on a vascular graft would continue to secrete rGH, RAEC infected with G2N and selected with G418 were seeded onto a 4-mm (inner diameter) Corvita vascular graft (*23*). The rGH continued to be secreted into

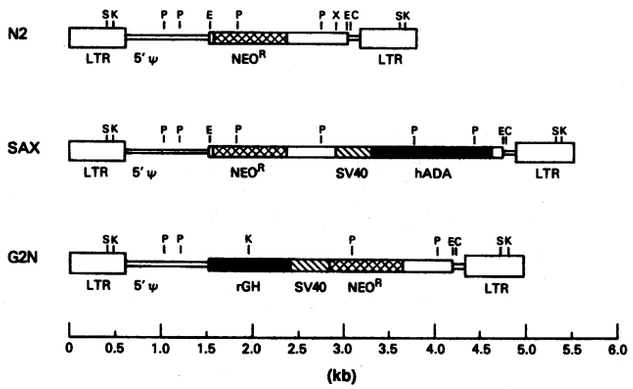

Fig. 1. Retroviral vectors. N2 (*31*) and SAX (*12*) construction has been described. In N2, a large portion of the M-MuLV coding sequence has been deleted and replaced by the neomycin resistance gene from the Tn*5* transposon (the hatched area is the coding sequence). SAX contains an SV40 early promoter–ADA cDNA fusion gene inserted into the Xho I site of N2. G2N was constructed by first inserting an 800-bp Xho I–Mae III fragment from pRGH-1 (*32*) into a Hinc II site in the N2-derived plasmid B2 (*33*). An 1800-bp Hind III–Xho I SV40-promoted *neo*^{R} fragment from the vector SV-N (*34*) was then placed into a Bam HI site location in a position 3′ to the rGH gene. The following regions are indicated: LTR, viral long terminal repeat; NEO^R, neomycin resistance gene (the hatched area is the coding sequence); SV40 early promoter; hADA, human ADA cDNA; rGH, rat growth hormone cDNA; 5′ splice donor site; ψ, viral packaging signal. Restriction sites: S, Sac I; K, Kpn I; E, Eco RI; P, Pst I; X, Xho I; C, Cla I.

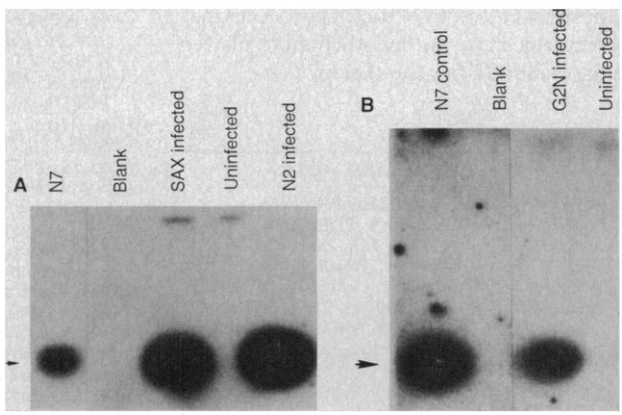

Fig. 2. In situ neophosphotransferase assay. Freeze-thawed cell lysates were assayed by nondenaturing SDS–polyacrylamide gel electrophoresis as described (*31*). The region of NPT activity in the gel was determined by the catalyzed transfer of a ^{32}P-labeled γ phosphate from adenosine triphosphate to a kanamycin substrate in an agar overlay, followed by filter transfer and autoradiography. The region of NPT activity expressed by the neo^R gene is indicated by the arrow. (**A**) The lanes contain the following: N7, 2 × 10^5 cells of the N2-transfected, G418-selected PA317 packaging line (*19*) served as a positive indicator of NPT expression; SAX infected, 5 × 10^5 SAX-infected, G418-selected RAEC; uninfected, 2 × 10^5 uninfected RAEC served as a negative control; N2 infected, 2 × 10^5 N2-infected, G418-selected RAEC. (**B**) N7 control, 2 × 10^5 N7 cells; G2N infected, 2 × 10^5 G2N-infected, G418-selected RAEC; uninfected, 2 × 10^5 uninfected RAEC.

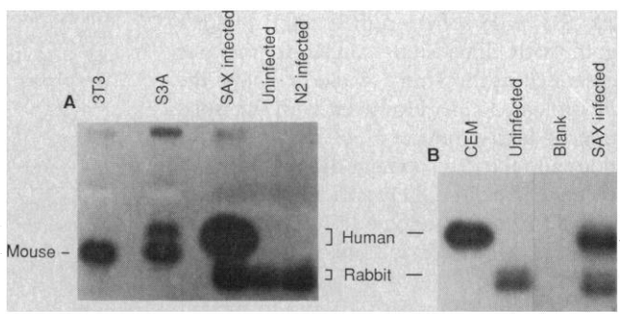

Fig. 3. ADA cellulose acetate gel electrophoresis. The procedure is a modification of the in situ enzyme assay described by Lim *et al.* (*3*). We loaded 1 μl of a 10-μl freeze-thaw cell lysate in buffer [10 m*M* tris-HCl (*p*H 7.5) and 1 m*M* EDTA] onto a cellulose acetate plate in a Titan III electrophoretic apparatus (Helena Industries). After separation, plates were stained with substrate in a 0.5% agar overlay. The locations of human, rabbit, and mouse ADA enzyme activities are indicated. (**A**) Each lane contains 2 × 10^4 cell-equivalents of the following: 3T3, NIH 3T3 mouse fibroblasts; N2 infected and SAX infected, G418-selected RAEC infected with the respective vectors; uninfected, uninfected RAEC. (**B**) The lanes contain the following: CEM, 1 × 10^4 cell-equivalents of an immortalized human T lymphocyte line called CEM that expresses only the human ADA isoenzyme; uninfected, 2 × 10^4 cell-equivalents of uninfected RAEC; SAX infected, 2 × 10^4 cell-equivalents of unselected SAX-infected RAEC.

Table 1. (A) Rat growth hormone production by retroviral vector-transduced rabbit endothelial cells. Two confluent T75 flasks containing either G418-selected or -unselected G2N-infected RAEC were fed with Ryan Red medium (Medium 199 containing 5% pretested bovine calf serum, 5% pretested fetal bovine serum, and $10^{-5}M$ thymidine) (*29*) 24 hours before an aliquot was collected for the rGH radioimmunoassay (*30*). Results are the means ± SD. (B) G418-selected G2N-infected RAEC were harvested with a rubber policeman from two confluent T75 flasks and seeded onto a 4 mm (inner diameter) by 10 cm Corvita graft by filling the lumen with cells suspended in Ryan Red medium and clamping the ends. The graft was rotated for 24 hours for even cell distribution, and the clamps were removed. Twelve days later the graft was sectioned, and a 3.6-cm segment (4.5 cm^2) was cultivated in Ryan Red medium. Aliquots for rGH determination were obtained at the indicated times after seeding. Cell density was determined by trypsinizing and counting cells on the graft segment on day 32. ND, not detectable.

Sample	rGH production (ng/10^6 cells per 24 hours)
(A) Cells on tissue culture plastic (3×10^4 cells per square centimeter)	
Uninfected cells	ND
Unselected cells	179 ± 16
G418-selected cells	849 ± 10
(B) Cells on graft (6×10^4 cells per square centimeter)	
Day 13	930
Day 32	1060

the tissue culture medium at a rate of ~1000 ng per 10^6 cells per day for at least 4 weeks after the graft was seeded (Table 1, part B).

These studies demonstrate that cultured rabbit endothelial cells are readily infected with retroviral vectors and efficiently express recombinant genes under the transcriptional regulation of both the M-MuLV LTR and the SV40 promoter. Moreover, these cells can secrete proteins when the appropriate signal peptide is present. Previous studies with implants in animals of genetically engineered fibroblasts have demonstrated that significant levels of secreted recombinant proteins—including rGH (*10*), human growth hormone (*24*), human α-1-antitrypsin inhibitor (7), and human Factor IX (*9*)—can be achieved in vivo. If we extrapolate to the adult rabbit the data for the secretion and metabolic clearance rate of rGH in the rat (*25*), rGH secretion in an animal with a graft with dimensions similar to the one described in this study would be below the physiologic range. However, with the isolation of high-producer rGH clonal populations and the development of more effective vectors, a rate of rGH secretion approaching the normal physiologic range might be achieved. Moreover, in spite of the relatively short half-life of growth hormone in the bloodstream ($t_{1/2}$ of 5 to 10 min in the rat, 20 min in humans), human growth hormone is effective in the treatment of hypopituitary dwarfism even when administered at weekly intervals (*26*). Consequently, it may not be necessary to sustain a high level of growth hormone in the blood to bring about a physiologic effect.

Endothelial cells in particular provide an attractive system for gene transfer in vivo where their location in immediate contact with the circulating blood facilitates both implant survival and delivery of the recombinant gene product. Although endothelial cells would be useful for the therapeutic delivery of many gene products, in certain instances (for example, insulin) such an approach may be complicated by the need for stricter control of secretion, which would require the use of gene promoters that can be regulated.

Normally, endothelial cells exist in different vascular environments ranging from the major arteries to the microcirculation. Genetically engineered endothelial cells could be placed in either milieu in a recipient, either by first seeding the cells on vascular grafts (*27*) or in a collagen matrix. For example, an angiogenesis factor applied to collagen sponges will promote the formation of a vascular bed necessary for both the survival and the continued function of cell implants (*28*). Both of these approaches may eventually allow the endothelium to play an important role in gene therapy.

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22 July 1988; accepted 27 October 1988