

acceptor for specific interaction. Possible acceptors within the basic domain include the side chain of glutamine and the peptide backbone (27), either of which may form H-bonds with one or both of the invariant G-C base pairs in the minimal Tfr38 binding domain. Another essential feature is likely to be recognition of a kinked structure (29) introduced by the pyrimidine bulge. Thus both sequence and structure of a limited TAR region contribute to specific recognition by the COOH-terminal domain of Tat.

The data presented here are consistent with and offer a basis for physical interpretation of a subset of the genetic data identifying elements of TAR or Tat that are required for transactivation in vivo. Our experiments delineate minimal protein and RNA elements that have pivotal roles in an interaction vital to the HIV-1 life cycle.

REFERENCES AND NOTES

1. H. Varmus, *Genes Dev.* **2**, 1055 (1988); B. R. Cullen and W. C. Greene, *Cell* **58**, 423 (1989).
2. A. D. Frankel *et al.*, *Science* **240**, 70 (1988).
3. M. Green and P. M. Loewenstein, *Cell* **55**, 1179 (1988).
4. M. Green, M. Ishino, P. M. Loewenstein, *ibid.* **58**, 215 (1989).
5. M. F. Laspias, A. P. Rice, M. B. Mathews, *ibid.* **59**, 283 (1989).
6. M. Braddock *et al.*, *ibid.* **58**, 269 (1989).
7. M. A. Muesing, D. H. Smith, D. J. Capon, *ibid.* **48**, 691 (1987).
8. A. Jakobovits *et al.*, *Mol. Cell. Biol.* **8**, 2555 (1988).
9. M. J. Selby, E. S. Bain, P. A. Luciw, B. M. Peterlin, *Genes Dev.* **3**, 3547 (1988); J. Hauber and B. R. Cullen, *J. Virol.* **62**, 673 (1988).
10. B. M. Peterlin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9734 (1986).
11. S. Feng and E. C. Holland, *Nature* **334**, 165 (1988).
12. J. A. Garcia *et al.*, *EMBO J.* **8**, 765 (1989).
13. B. Berkhout, R. H. Silverman, K.-T. Jeang, *Cell* **59**, 273 (1989).
14. C. Dingwall *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6925 (1989).
15. We constructed a gene that encodes a fusion protein containing the following sequences (NH₂- to COOH-terminal): (i) the cAMP binding domain (residues 1-134) from the *E. coli* CAP protein [H. Aiba, S. Fujimoto, N. Ozaki, *Nucleic Acids Res.* **10**, 1345 (1982); D. B. McKay, I. T. Weber, T. A. Steitz, *J. Biol. Chem.* **257**, 9518 (1982)]; (ii) a six-residue linker that can be cleaved by Xa or V8; and (iii) either residues 1-86 (full length) or 1-72 (first exon) of the HIV-1 Tat protein, BRU isolate [S. Wain-Hobson, P. Sonigo, O. Danos, S. Cole, M. Alison, *Cell* **40**, 9 (1985)]. The fusion proteins were expressed in *E. coli* strain BL21 (DE3) [F. W. Studier and B. A. Moffatt, *J. Mol. Biol.* **189**, 113 (1986)] with a T7 RNA polymerase-directed expression system analogous to that of A. H. Rosenberg *et al.* [*Gene* **56**, 125 (1987)].
16. Cells were lysed in 10 mM Tris, 700 mM NaCl, 2 mM EDTA, 10 mM dithiothreitol (DTT), and pH 8. Cleared lysates were treated with 0.5% polymin P. The supernatant was loaded on a cAMP-agarose column (Sigma), washed with the lysis buffer and 500 mM K₂HPO₄, 1 mM EDTA, 5 mM DTT, pH 7.5, and 10 mM Tris, 50 mM NaCl, 0.5 mM EDTA, 2 mM DTT, and pH 7.5. The fusion protein was eluted in the latter buffer containing 2 mM cAMP. Fractions containing the fusion protein were pooled and dialyzed against 10 mM Tris, 100 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM cAMP, pH 8 (Xa constructs), or pH 7.5 (V8 constructs). Fusion proteins were digested with the appropriate protease at 37°C for 2.5 hours (enzyme:substrate ratio 1:50 for Xa, 1:100 for V8); Xa digests require 2 mM CaCl₂.
17. Tfr14 was synthesized by the Yale University Medical School Protein and Nucleic Acid Chemistry Facility, purified by reversed-phase HPLC, and characterized by amino acid analysis and mass spectroscopy.
18. A. D. Frankel, S. Biancolana, D. Hudson, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7397 (1989).
19. U. Siebenlist and W. Gilbert, *ibid.* **77**, 122 (1980); D. A. Peattie and W. Herr, *ibid.* **78**, 2273 (1981); D. L. Thurlow, C. Ehresmann, B. Ehresmann, *Nucleic Acids Res.* **11**, 6787 (1983).
20. D. Peattie, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1760 (1979).
21. G. Myers, A. B. Rabson, S. F. Josephs, T. F. Smith, Eds., *Human Retroviruses and AIDS* (Los Alamos National Laboratory, Los Alamos, NM, 1989).
22. R. Gaynor, E. Soultanakis, M. Kuwabara, J. Garcia, D. S. Sigman, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4858 (1988); R. A. Marciniak, M. A. Garcia-Blanco, P. A. Sharp, *ibid.* **87**, 3624 (1990).
23. M. Emerman, M. Guyader, L. Montagnier, D. Baltimore, M. A. Muesing, *EMBO J.* **6**, 3755 (1987); S. K. Arya and R. L. Gallo, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9753 (1988).
24. J. Hauber, M. H. Malim, B. R. Cullen, *J. Virol.* **63**, 1181 (1989); S. Ruben *et al.*, *ibid.*, p. 1.
25. D. Lazinski *et al.*, *Cell* **59**, 207 (1989).
26. R. W. Warrant and S.-H. Kim, *Nature* **271**, 130 (1978).
27. M. A. Rould *et al.*, *Science* **246**, 1135 (1989).
28. N. C. Seeman, J. M. Rosenberg, A. Rich, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 804 (1976).
29. A. Bhattacharyya, A. I. H. Murchie, D. M. J. Lilley, *Nature* **343**, 484 (1990).
30. H.-N. Liu-Johnson, M. R. Gartenberg, D. M. Crothers, *Cell* **47**, 995 (1986).
31. RNAs were synthesized enzymatically with T7 polymerase [J. F. Milligan, D. R. Groebe, G. W. Witherell, O. C. Uhlenbeck, *Nucleic Acids Res.* **15**, 8783 (1987)]. RNA sequences are shown in Fig. 2, E and F.
32. We are indebted to M. R. Gartenberg for thoughtful and enthusiastic discussion. J. Qin and G. Sun provided technical assistance; T7 RNA polymerase was the generous gift of S. A. White. C.A. is supported by an Anna Fuller Fund Fellowship. This research was supported in part by NIH grants to T.A.S. (GM 39546) and D.M.C. (GM 21966).

2 April 1990; accepted 25 June 1990

Site-Specific Gene Expression in Vivo by Direct Gene Transfer into the Arterial Wall

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A recombinant β -galactosidase gene has been expressed in a specific arterial segment in vivo by direct infection with a murine amphotropic retroviral vector or by DNA transfection with the use of liposomes. Several cell types in the vessel wall were transduced, including endothelial and vascular smooth muscle cells. After retroviral infection, a recombinant reporter gene was expressed for at least 5 months, and no helper virus was detected. Recombinant gene expression achieved by direct retroviral infection or liposome-mediated DNA transfection was limited to the site of infection and was absent from liver, lung, kidney, and spleen. These results demonstrate that site-specific gene expression can be achieved by direct gene transfer in vivo and could be applied to the treatment of such human diseases as atherosclerosis or cancer.

DESPITE RECENT ADVANCES IN THE understanding of eukaryotic gene structure and regulation, a major obstacle to the therapeutic management of human disease remains the site-specific expression of genes in vivo. Although tissue-specific gene expression can be determined by enhancer or other cis-acting regulatory

elements, this expression might also be achieved through the delivery of vectors to specific anatomic sites in vivo. We have previously demonstrated that a recombinant gene can be expressed in the vasculature by means of genetically modified endothelial cells implanted at specific sites on the arterial wall (1). Because these studies required that

syngeneic cell lines be established before genetic modification, adaptation to the treatment of human disease remained cumbersome. We now report that a recombinant gene can be efficiently expressed at a specific site in vivo by direct introduction of genetic material at the time of catheterization.

The retroviral vector (2) was derived from the Moloney murine leukemia virus and utilized the promoter from the chicken β -actin gene to express β -galactosidase mRNA. Viral particles from the supernatant of transfected Ψ CRIP retrovirus packaging cells (3) were concentrated by centrifugation (10^4 to 10^6 particles per milliliter) and instilled into Yucatan or outbred pig iliofemoral arteries. These animals provide an experimental model for atherosclerosis when fed a high-fat diet (4). After anesthesia and surgical exposure, a catheter was inserted as

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*This work resulted from an equal contribution by the first two authors.

previously described (1), and the proximal and distal balloons of the catheter were inflated, creating a protected space into which β -galactosidase-transducing retrovirus was introduced. Polybrene was included

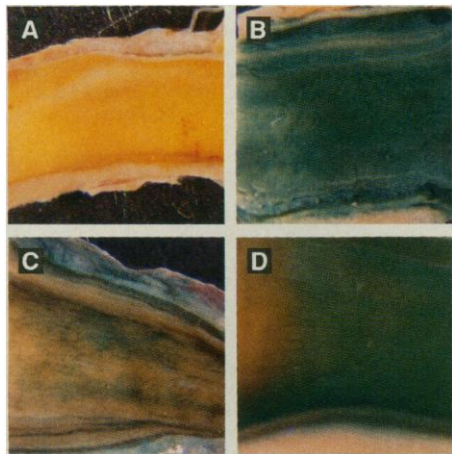


Fig. 1. Analysis of the arterial wall infected with β -galactosidase-transducing retrovirus in vivo. β -Galactosidase activity was documented by histochemical staining in a segment of (A) normal control artery that was sham-infected or segments infected directly with the replication-defective β -galactosidase retroviral vector and analyzed after (B) 8 weeks or (C) 21 weeks, and (D) segments transduced by liposome transfection after 4 days. The β -galactosidase-transducing Moloney murine leukemia virus vector, prepared from Ψ CRIP cells (3), was used to generate viral particles, which were filtered and concentrated by centrifugation as previously described (14). Female Yucatan or outbred pigs were anesthetized with pentobarbital and underwent sterile surgical exposure of the iliofemoral arteries. To introduce the retrovirus, we inserted a double balloon catheter (C. R. Bard, Inc.) into the iliofemoral artery. Both balloons were inflated, and the segment was irrigated with heparinized saline. In some instances, the arterial segment was partially denuded by inflation and passage of the proximal balloon, but was not required for infection. The viral supernatant was instilled for 30 min in the central space of the catheter, with polybrene (8 μ g/ml) added after introduction of the virus. The catheter was removed, and antegrade blood flow was restored. The vessel segments were excised 10 days to 21 weeks later. A portion of the artery was placed in 1.25% glutaraldehyde for 15 min followed by histochemical staining as described (1, 5). Liposomes containing β -galactosidase were prepared by combining 30 μ g of DNA from a β -galactosidase expression vector (14) and 100 μ l of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) (Bethesda Research Laboratories) in serum-free medium. Anesthesia, surgery, and catheter placement were performed in five outbred pigs as described above. The arterial segment was rinsed with minimum essential medium (opti-MEM, Bethesda Research Laboratories), and the liposomes were instilled through the catheter into the arterial central space for 30 min. Blood flow was restored after removal of the catheter. Arterial segments were removed 4 to 42 days later. Segments were fixed in 1.25% glutaraldehyde for 15 min followed by incubation in X-gal chromagen (1, 5). (Magnification, $\times 18$.)

after instillation of the virus to improve the efficiency of infection.

Arterial segments were analyzed for the presence of β -galactosidase activity at different times after infection in ten animals. Incubation of infected tissue with the X-gal chromagen (5) revealed visible areas of blue coloration, indicative of β -galactosidase activity in cells infected by the retrovirus. β -Galactosidase activity was absent from sham-infected control segments (Fig. 1A), but was evident at all times after infection with virus, ranging from 10 days to 21 weeks (Fig. 1, B and C, and Table 1).

Optimal expression was observed between 2 and 3 months after infection (Fig. 1B). Site-specific gene transfer was also achieved by transfecting arterial segments in vivo with the use of liposomes containing a β -galactosidase expression vector plasmid. Arterial segments analyzed by X-gal staining revealed extensive blue coloration after 4 days (Fig. 1D) and persisted for at least 6 weeks after transfection (6).

Microscopic examination of vessels transduced by either technique revealed β -galactosidase activity in all layers of the arterial wall, including the intima, media, and ad-

Table 1. Direct infection of arterial wall with β -galactosidase-transducing retrovirus. Yucatan or outbred pig iliofemoral arteries were infected with the indicated titers of β -galactosidase-producing virus and removed from 10 days to 21 weeks after infection. Tissue staining of random sections from lung, liver, kidney, and femoral artery distal to the site of infection was assessed after incubation with X-gal chromagen. The presence of replication-competent helper virus or reverse transcriptase was assayed with either platelet-poor plasma or supernatant from unstimulated or stimulated peripheral blood lymphocytes in tissue culture (16). Intensity of arterial staining (column 3) was judged by visual inspection, with segments from Fig. 1B representative of +++ and Fig. 1C of +. Abbreviations: NT, not tested; PBL, peripheral blood lymphocytes; U, unstimulated; S, stimulated; -, negative.

Pig	Duration (days)	Arterial staining	Viral titer ($\times 10^5$)	Tissue staining	Helper virus assay			Reverse transcriptase	
					Plasma	PBL		U	S
						U	S		
Control	10	-	0	-	-	-	-	-	-
1	10	+++	1.5	NT	-	NT	NT	NT	NT
2	11	+++	2.6	NT	-	NT	NT	NT	NT
3	11	+++	0.1	NT	-	NT	NT	NT	NT
4	10	+++	0.4	-	-	NT	NT	NT	NT
5	56	+++	2.8	-	-	-	-	-	-
6	91	++	3.0	-	-	-	-	-	-
7	91	++	8.0	-	-	-	-	-	-
8	112	+	2.5	-	-	-	-	-	-
9	119	++	1.4	-	-	-	-	-	-
10	147	+	1.3	-	-	-	-	-	-

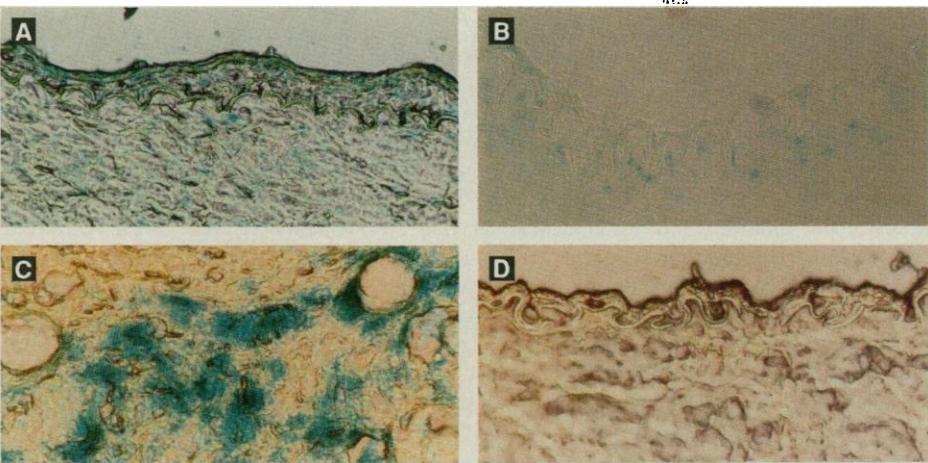


Fig. 2. Microscopic analysis of β -galactosidase-expressing cells in arterial segments. Microscopic sections were analyzed by histochemical staining for β -galactosidase activity in the intimal and medial layers of arteries removed at (A) 10 days after retroviral infection (B) 4 days after liposome transfection, in (C) the adventitial layer 10 days after retroviral infection, or in (D) control sham-infected arteries 10 days after infection. Ten-micrometer frozen sections (A, C, and D) were incubated in 1.25% glutaraldehyde and stained with X-gal chromagen as described (1, 5). Arterial segments fixed in 1.25% glutaraldehyde and stained with X-gal chromagen were embedded in glycol methacrylate and sectioned at 7- μ m thickness (B). (Magnification: $\times 250$.)

ventia (Fig. 2, A through C), but not in control arteries (Fig. 2D). To determine which cell types within the vessel wall were infected by the retroviral vector, we performed immunohistochemical analyses. The identity of smooth muscle cells was confirmed by treating with a mouse monoclonal antibody specific for smooth muscle (anti-actin; Sigma), and then with a horseradish peroxidase-coupled antibody against mouse immunoglobulin G (IgG). When incubated with peroxidase substrate, areas of blue coloration representing β -galactosidase activity in the intima and media were counterstained red (Fig. 3A, see arrows), confirming the presence of smooth muscle actin in the β -galactosidase-expressing cells. When the anti-actin was omitted, peroxidase staining was absent (6). Thus, vascular smooth muscle cells represent a cellular target of retrovirus infection in the arterial wall.

Endothelial cells within the intimal layer were identified by their ability to endocytose fluorescent acetylated low density lipoprotein (AcLDL), in contrast to fibroblasts, vascular smooth muscle, and other mesenchymal cells, which do not express functional AcLDL receptors (7). Cells were identified on the luminal surface of the artery, which displayed both β -galactosidase activity (Fig. 3B) and AcLDL uptake (Fig. 3C), demonstrating that endothelial cells were also a target of retroviral infection. No evidence of inflammation was detected in these vessel segments (Fig. 2, A through C, and Fig. 3, A and B). In some instances, hyperplasia was observed in the intimal layer of both infected and sham-infected arteries (Fig. 2A) (8). This type of hyperplasia is commonly seen in catheter-induced arterial injury (9), and these replicating cells could represent a target for viral infection. Microscopic examination of arteries transfected with liposomes containing DNA also revealed β -galactosidase activity in endothelial and vascular smooth muscle layers (Fig. 2B). These data suggest that both methods of gene transfer allow expression of recombinant genes in endothelial, vascular smooth muscle, and other cell types.

In order to ensure the safety of site-specific gene expression by direct retroviral infection in vivo, it is essential that the generation of replication-competent helper virus be negligible and that the sites of infection be controlled in vivo. The presence of replication-competent helper virus in infected animals was assayed in several ways. Serum from each minipig was tested for the presence of virus at the time of instillation and analysis. The retroviral vector was not detected in the serum after the initial retroviral infection or during analysis of arterial segments in all animals, up to 5 months after

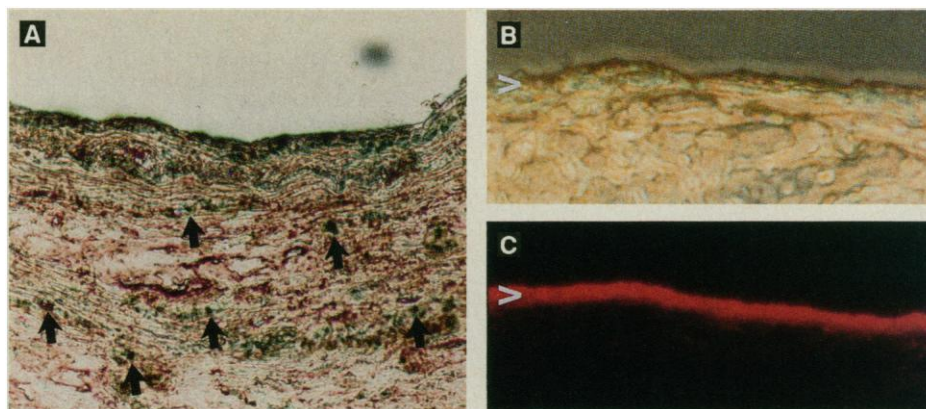


Fig. 3. Cell-specific localization of β -galactosidase activity in infected arteries. Vascular smooth muscle cells were identified by immunohistochemistry with (A) primary antibody anti-actin; the second antibody was coupled to peroxidase. Endothelial cells in sequential sections of artery were analyzed for expression of (B) β -galactosidase and (C) fluorescent AcLDL uptake in the intima. Black arrows indicate representative cells that coexpress β -galactosidase and smooth muscle actin (A), and white arrows indicate endothelial cell layer (B and C). To identify vascular smooth muscle cells, we incubated glycol methacrylate-embedded sections from previously fixed, X-gal chromagen-stained arteries with monoclonal anti-actin (Sigma) (15 μ g/ml) in the presence of 1% horse serum. Peroxidase-coupled horse antibody to mouse IgG (Vector Laboratories) (4 μ g/ml) was used as the second antibody, and peroxidase staining was developed with 3-amino-9-ethylcarbazole (AEC) (Sigma) according to standard procedures (15). In order to identify endothelial cells, arterial segments excised 10 days after retroviral infection were incubated with fluorescent AcLDL (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (Biomedical Technologies) (10 μ g/ml) for 4 hours at 37°C and frozen for sectioning. Ten-micrometer sections were fixed in 1.5% paraformaldehyde for 5 min and visualized by phase-contrast or fluorescent microscopy. A contiguous section of the same arterial segment was fixed in 1.25% glutaraldehyde for 5 min, rinsed in phosphate-buffered saline, and stained with X-gal chromagen. Because these sections represent adjacent portions of the same cell, the same endothelial cells could be identified as displaying both recombinant β -galactosidase activity and AcLDL fluorescence. [Magnification: (A) $\times 250$, (B and C) $\times 400$.]

infection (Table 1). In addition, reverse transcriptase and helper virus activity were absent from culture supernatants of unstimulated or mitogen-stimulated lymphocytes from six pigs examined (Table 1). Examination of β -galactosidase activity elsewhere in the same vessel and in organs not directly exposed to the retrovirus revealed no X-gal staining. In addition, no β -galactosidase was observed in gross or random microscopic sections from the liver, lung, or kidney, except for cells with endogenous activity (Table 1). Because splenic tissue contained extensive endogenous β -galactosidase activity, it could not be evaluated by the X-gal chromagen; however, no evidence of provirus was found by means of the polymerase chain reaction (PCR) (10). In the case of liposome transfection, β -galactosidase activity was not detected in other tissues with PCR (10), provided that liposomes did not pass into the systemic circulation after catheterization.

These studies demonstrate that a recombinant gene can be delivered to a specific site by direct gene transfer in vivo by means of infection with a retroviral vector or DNA transfection with liposomes. A major advantage of these approaches is the ease of gene delivery and its widespread applicability. In several earlier studies, endothelial cells were used as a vector to deliver a recombinant

gene (1, 2, 11). Although this method was effective, it required that cells syngeneic to the recipient animal be prepared and transduced, which took several weeks to prepare. Direct retroviral infection and liposome transfection allow the introduction of recombinant genes into any site accessible to a catheter without advanced preparation.

Because this approach minimizes potential complications, it may prove beneficial in the treatment of a variety of inherited and acquired diseases. In cardiovascular disease, for example, common complications of percutaneous transluminal coronary angioplasty (PTCA) include acute closure and restenosis at the site of disruption of the atherosclerotic plaque, produced by thrombus formation and vascular smooth muscle cell proliferation, respectively (9, 12). Recombinant genes that exert an antithrombotic effect or inhibit smooth muscle cell proliferation could be delivered directly to the PTCA site in order to prevent thrombosis or restenosis. Cell-specific expression of such genes might be further refined through the use of tissue-specific enhancer elements in the retroviral vector. Site-specific gene expression might also prove useful in the treatment of malignancy. The expression of recombinant cytokines in malignant cells, for example, has been shown to provide an

antitumor effect (13). Infection of the tumor vasculature with retroviral vectors that express immunologic activators could provoke a local response, which might enhance tumor rejection.

REFERENCES AND NOTES

- E. G. Nabel, G. Plautz, F. M. Boyce, J. C. Stanley, G. J. Nabel, *Science* **244**, 1342 (1989).
- J. M. Wilson *et al.*, *ibid.*, p. 1344.
- O. Danos and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6460 (1988).
- J. S. Reitman, R. W. Mahley, D. L. Fry, *Atherosclerosis* **43**, 119 (1982).
- A. M. Dannenberg and M. Suga, in *Methods for Studying Mononuclear Phagocytes*, D. O. Adams, P. J. Edelson, H. S. Koren, Eds. (Academic Press, New York, 1981), pp. 375-395.
- E. G. Nabel, G. Plautz, G. J. Nabel, unpublished observations.
- T. J. C. VanBerkel, J. F. Nagelkerke, J. K. Kruijt, *FEBS Lett.* **132**, 61 (1981); J. M. Wilson, D. E. Johnston, D. M. Jefferson, R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4421 (1988); T. Kodama *et al.*, *Nature* **343**, 531 (1990); L. Rohrer, M. Freeman, T. Kodama, P. Penman, M. Krieger, *ibid.*, p. 570.
- Animals were prepared for surgery, and the catheter was inserted as described in the legend to Fig. 1. Medium containing polybrene was instilled for 30 min. Examination of the artery at 4 weeks revealed no β -galactosidase activity but hyperplasia in the intimal layer.
- P. M. Steele *et al.*, *Circ. Res.* **57**, 105 (1985); D. P. Faxon *et al.*, *Arteriosclerosis* **4**, 189 (1984); W. McBride, R. A. Large, L. D. Hillis, *N. Engl. J. Med.* **318**, 1734 (1988).
- Detection of β -galactosidase was performed by means of the PCR with primers for β -galactosidase as follows: sense: TGG AGC GCC GAA ATC CCG AAT CTC TAT CGT; antisense: TAG CCA GCG CGG ATC ATC GGT CAG ACG ATT.
- D. A. Dichek *et al.*, *Circulation* **80**, 1347 (1989).
- J. R. Wilentz *et al.*, *ibid.* **75**, 636 (1987); L. Schwartz *et al.*, *N. Engl. J. Med.* **318**, 1714 (1988).
- R. I. Tepper, P. K. Pattegal, P. Leder, *Cell* **57**, 503 (1989); E. R. Fearon *et al.*, *ibid.* **60**, 397 (1990).
- J. Price, D. Turner, C. Cepko, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 156 (1987).
- R. J. Mayer and J. H. Walker, in *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London, 1987), pp. 263-275.
- Lymphocytes isolated from blood by buoyant density centrifugation were incubated in RPMI 1640 containing 10% fetal bovine serum, penicillin, streptomycin, and 5×10^{-5} M 2-mercaptoethanol alone (unstimulated) or with phorbol myristate acetate (PMA) (10 ng/ml) or PMA and phytohemagglutinin (PHA) (2 μ g/ml) (stimulated). After filtration, 200- μ l aliquots of supernatant were added to 3T3 cells containing a β -galactosidase (β -gal-at-gag) provirus (3T3-BAG) (14) in the presence of polybrene (8 μ g/ml). 3T3-BAG cells were passaged up to four times, and filtered supernatant was tested for its ability to infect an appropriate target cell and express β -galactosidase. Presence of virus was assessed by infecting 3T3 cells at 10% confluence and staining with X-gal chromagen when cells had reached confluence and was estimated to be sensitive to <10 infectious particles per milliliter. No colonies were detected in normal pig plasma or lymphocytes, nor in any experimental animals, whereas supernatant from a helper-producing Ψ -AM subline [R. Mann, R. C. Mulligan, D. Baltimore, *Cell* **33**, 153 (1983); R. D. Cone and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6349 (1984)] shown previously to produce replication-competent helper virus, produced colonies too numerous to count. Reverse transcriptase was tested in 500 μ l of supernatant from lymphocytes maintained as described above. Supernatants were concentrated by centrifugation at 13,000 rpm in a microfuge at 4°C for 60 min and resuspended in 50 μ l of a reaction mixture containing polyadenylic acid (5 μ g/ml) (Pharmacia), oligo(dT) primer (14 nucleotides) (1.57 μ g/ml), 15 mM tris-Cl (pH 7.8), 7.5 mM KCl, 2 mM dithiothreitol, 5 mM MgCl₂, 5 mM MnCl₂, 0.05% NP-40 and 25 nM [³²P]dTTP (deoxythymidine triphosphate) (Amersham, 400 Ci/mM). Samples were incubated at 37°C for 90 min, and 10 μ l was placed onto Whatman DE81 paper. Filters were washed five times with 2 $\times$ SSC (saline sodium citrate) for 5 min per wash, twice with 95% ethanol for 2 min and quantitated in a scintillation counter. Duplicate samples of supernatant from unstimulated, PMA-, or PMA- and PHA-stimulated lymphocytes at 24, 48, or 96 hours after culture showed no difference when normal uninfected pigs were compared to infected experimental animals and were not significantly above background (<100 cpm). Helper virus-positive Ψ -AM cell line supernatant and 0.2 unit of recombinant Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) yielded values significantly above background, 4000 to 5000 or 6000 to 7000 cpm, respectively. Sensitivity of the reverse transcriptase assay was estimated to be 0.003 unit.
- We thank A. McDermott for secretarial assistance, and F. Collins, J. Leiden, and J. Wilson for helpful discussions. G. Plautz is a recipient of a National Research Service Award (GM-13457). This work was supported in part by funds from the Department of Internal Medicine, University of Michigan Medical Center, and grants from NIH [AI 29179 (G.J.N.) and DK 42706 (E.G.N.)].

11 April 1990; accepted 29 June 1990

5-Methylcytosine as an Endogenous Mutagen in the Human LDL Receptor and p53 Genes

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Direct genomic sequencing revealed that cytosine residues known to have undergone a germ-line mutation in the low density lipoprotein receptor gene or somatic mutations in the p53 tumor suppressor gene were methylated in all normal human tissues analyzed. Thus, these mutations should be scored as transitions from 5-methylcytosine to thymine rather than from cytosine to thymine. Methylated cytosines occur exclusively at CpG dinucleotides, which, although markedly underrepresented in human DNA, are sites for more than 30 percent of all known disease-related point mutations. Thus, 5-methylcytosine functions as an endogenous mutagen and carcinogen in humans, in that methylation seems to increase the potential for mutation at cytosine residues at least by a factor of 10.

LESS THAN 1% OF THE BASES OF human DNA are 5-methylcytosine. Methyl groups that occur on both C residues in the double-stranded palindrome CpG account for more than 90% of the

methylated C residues (1). Although the CpG sequence is underrepresented by a factor of five in the vertebrate genome (2), it is the site of a disproportionately high number of human germ-line point mutations. Estimates suggest that 35% of point mutations causing human genetic disorders have occurred at CpG dinucleotides (CpGs), and over 90% of these were transitions from C to T or corresponding G-to-A transitions (3). CpGs may also be overrepresented

among sites of somatic mutation in tumor suppressor genes, such as the p53 or retinoblastoma genes, in which about 40% of reported point mutations are localized to CpGs (4, 5).

Methylation of CpGs in normal tissues might increase the probability of mutations at such sites because of the ability of 5-methylcytosine to undergo deamination, resulting in a thymine (6). Because of the inverse relation between the presence of 5-methylcytosine and gene expression (7), active genes might be expected to be unmethylated. Despite this inverse relation often observed between methylation and gene expression, many genes (such as HPRT and PGK) are expressed with methylation in their coding sequences (8). We therefore used ligation-mediated polymerase chain reaction (PCR) genomic sequencing (9) to analyze directly CpGs that have undergone either a germ-line mutation in the low density lipoprotein (LDL) receptor or somatic mutations in the p53 tumor suppressor genes (Fig. 1). These CpGs were all methylated in several human tissues obtained from five individuals.

We first obtained an overall indication of the methylation status of the LDL receptor and p53 genes by digesting DNA obtained from sperm or white blood cells (WBC)

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