

A Rev-inducible mutant *gag* gene stably transferred into T lymphocytes: An approach to gene therapy against human immunodeficiency virus type 1 infection

(simian immunodeficiency virus/retrovirus inhibition/Gag polypeptide)

JASON A. SMYTHE*†, DAISY SUN*, MICHAEL THOMSON*, PHILIP D. MARKHAM‡, MARVIN S. REITZ, JR.*,
ROBERT C. GALLO*, AND JULIANNA LISZIEWICZ*§

*Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and †Advanced BioScience Laboratories, Inc., Kensington, MD 20895

Contributed by Robert C. Gallo, January 3, 1994

ABSTRACT One strategy for somatic gene therapy for human immunodeficiency virus type 1 (HIV-1) infection is based on the regulated expression of dominant negative mutants of the HIV-1 *gag* gene. To limit expression of the mutant Gag polypeptide to HIV-1-infected cells, we have constructed a replication-defective retroviral vector that contains a Rev-responsive element. By using this construct we have obviated problems that can be associated with constitutive expression of an exogenous gene, an important step toward developing a human therapy. In uncloned T lymphocytes infected (transduced) with this retroviral construct, HIV-1 replication was inhibited by 94% with a concomitant decrease in the cytopathic effects of the virus. In addition, simian immunodeficiency virus (SIV) replication was also shown to be significantly inhibited, suggesting that this mutant Gag protein may have antiviral efficacy against a broad range of primate lentiviruses and that an SIV/macaque model can be used for further *in vivo* studies. These results have important implications in assessing the potential of somatic gene therapy in the treatment of HIV-1 infection.

The potential application of somatic gene therapy to the treatment of human disease is currently the focus of intensive investigation (1, 2). Somatic gene therapy involves the stable integration of genetic elements into cells to complement, replace, or even inhibit the function of an existing gene. Proponents of gene therapy have suggested that this new modality may be applicable to a number of human diseases, including acquired immunodeficiency syndrome (AIDS), for which there are currently no effective chemotherapeutic or vaccine therapies (3).

Human immunodeficiency virus type 1 (HIV-1), the etiological agent of AIDS, is a complex retrovirus that infects thymus-derived T lymphocytes and cells of the monocyte-macrophage lineages (4–8). In addition to *gag*, *pol*, and *env* genes, common to all retroviruses, HIV-1 also contains two regulatory genes, *tat* and *rev*, and four accessory genes, *nef*, *vpr*, *vpu*, and *vif* (9–14). Although the functions of some of these genes remain poorly understood, the complexity of their interactions in the events of infection, integration, and HIV-1 replication has suggested a number of targets for gene therapy.

A number of different strategies for a potential gene therapy against HIV-1 infection, including the use of RNA decoys, antisense sequences, and transdominant mutant proteins have already been described (15–22). The objective of our study was to inhibit HIV-1 replication in T lymphocytes by controlled expression of a dominant negative mutant of

the HIV-1 *gag* gene. Given the complex processing of the Gag polypeptide during assembly of the viral capsid, dominant negative mutants of Gag have been recognized as potentially potent inhibitors of HIV-1 replication (21). To ensure high efficiency gene transfer to the target cells, we chose to use a replication-defective retroviral vector. Although similar vectors have already been approved for human experimentation, they are limited in that they are designed to function as constitutive expression vectors. An important consideration in our strategy was to have regulated expression of the mutant Gag polypeptide. By limiting expression of the mutant *gag* gene to HIV-1-infected cells it should be possible to avoid problems, such as toxicity, dysregulation of cellular function, activation of cytotoxic T lymphocytes, and down-regulation of transcription, that may otherwise be associated with constitutive expression of the foreign protein. To ensure regulated expression required that we substantially modify the existing retroviral vectors, and we have achieved this goal by utilizing a viral regulatory element that controls HIV-1 RNA processing.

MATERIALS AND METHODS

Plasmid Construction. The 248-base-pair (bp) *Hind*III–*Bam*HI restriction fragment, corresponding to the HIV-1_{HXB2} Rev-responsive element (RRE), was isolated from plasmid pRRE (23) and cloned between the *Hind*III and *Bcl*I restriction enzyme sites of pLXSN (24) by standard techniques (25). This construct was designated as pLXSRN. The mutant HIV-1 *gag* gene sequence was derived from the plasmid VI-ΔE-dhfr (a gift from D. Trono, Salk Institute, La Jolla, CA), which contains HIV-1_{HXB2} proviral DNA modified with an in-frame deletion (21). The deleted sequence (from the *Hind*III site at nucleotide 1084 to the *Xmn*I site at nucleotide 1275) overlaps the p17/p24 protease cleavage site in the Gag precursor polypeptide. By using the *Bgl*II fragment from plasmid VI-ΔE-dhfr we further modified the mutant HIV-1 *gag* gene by deleting the 3' sequence corresponding to the p6 polypeptide. The *Bgl*II fragment was cloned into the single *Bam*HI site in pLXSRN, generating pLGSRN. The cloning procedure introduced a stop codon after the *gag* gene sequence (Fig. 1*b*).

Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; MoMLV, Moloney murine leukemia virus; RRE, Rev-responsive element; SIV, simian immunodeficiency virus.

†Present address: The R. W. Johnson Pharmaceutical Research Institute-Sydney, 74 McLachlan Avenue, Rushcutters Bay, New South Wales 2011 Australia.

§To whom reprint requests should be addressed at: Laboratory of Tumor Cell Biology, Building 37, Room 6A09, National Cancer Institute, 9000 Rockville Pike, Bethesda, MD 20892.

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.

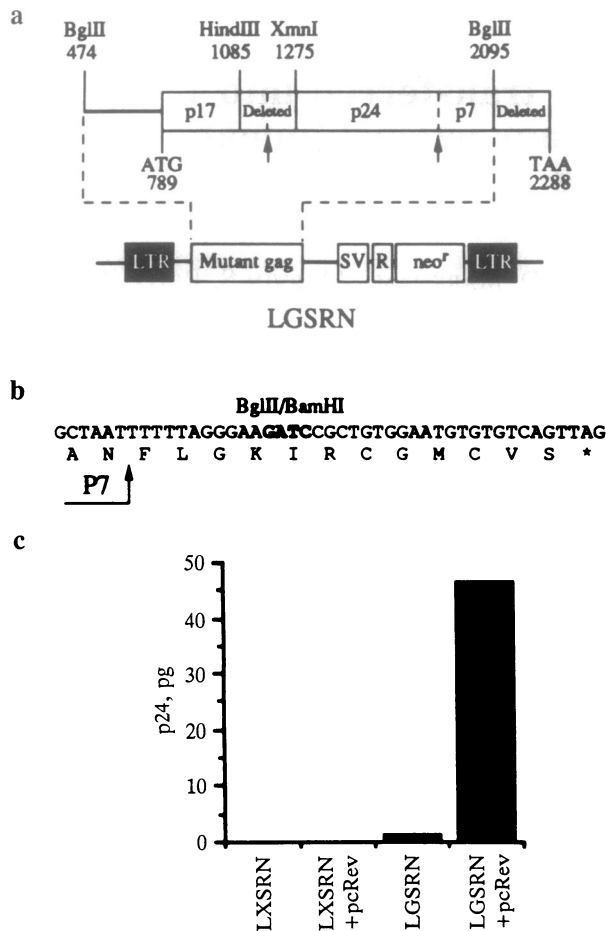


FIG. 1. Construction of pLGSRN and Rev-dependent expression of the mutant *gag* gene. (a) The *Bgl* II fragment containing the mutant HIV-1 *gag* gene was isolated from the plasmid VI- Δ E-dhfr, in which sequences corresponding to the p17/p24 protease cleavage site had been deleted. Arrows indicate the HIV-1 protease cleavage sites. The mutant gene was cloned into pLXSRN, a modified pLXSN vector that contained a 248-bp HIV-1 RRE (R), between the simian virus 40 early promoter (SV) and the *neo* gene sequence (*neo*^r). The final construct was designated pLGSRN. LTR, long terminal repeat. (b) Ligation of the *Bgl* II fragment (which lacks the sequence corresponding to the HIV-1 p6 polypeptide) into the *Bam*HI site of pLXSRN resulted in the addition of 7 amino acids of vector origin to the carboxyl end of the p7 polypeptide, and a stop codon. (c) Expression of the mutant HIV-1 *gag* gene in pLGSRN was tested by transient transfection of HeLa cells. The constitutive level of expression of the mutant Gag protein was very low (at the limits of detection by ELISA), and expression was enhanced >40-fold when the cells were cotransfected with pcRev. Vector controls were negative for p24.

Transient Transfection of HeLa Cells. HeLa cells were maintained at 37°C in a 5% CO₂ incubator in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells (2×10^5) were plated in 3 ml of medium and transfected 24 hr later (26) with 5 mg of either pLXSRN or pLGSRN and cotransfected with 5 mg of a Rev expression plasmid, pcRev (27), or 5 mg of a control plasmid (pMAMNeo) as indicated. After 48 hr the cells were harvested, lysed in sample diluent, and assayed for p24 antigen by ELISA (DuPont/NEN) according to the manufacturer's protocol.

Amphotropic Virus Production and Stable Transduction of CEM SS Cells. The GP+*env*Am12 retroviral packaging cell line (28) was used to generate the amphotropic LGSRN virus. GP+*env*Am12 cells were maintained at 37°C in a 5% CO₂ incubator in DMEM supplemented with 10% fetal bovine

serum. Cells (1×10^6) either were transfected (26) with 25 mg of pLGSRN or cotransfected with 5 mg of pLGSRN and 20 mg of pcRev to generate the LGSRN^{Rev+} and LGSRN^{Rev-} producer cells, respectively. CEM SS cells (29) were then cocultured (5×10^5 cells) with either the LGSRN^{Rev+} or LGSRN^{Rev-} producer cells in RPMI 1640 supplemented with 10% fetal bovine serum for 3 days at 37°C in a 5% CO₂ incubator. Then G418 sulfate (GIBCO) at 1000 μ g/ml (active weight) was added to each culture for 2 weeks of selection. The CEM SS cells were then harvested and maintained in RPMI 1640 supplemented with 10% fetal bovine serum and G418-sulfate at 400 μ g/ml. Genomic DNA was isolated from transduced or control CEM SS cells (25), and polymerase chain reaction (PCR) was performed with a GeneAmp kit (Perkin-Elmer/Cetus) and *neo* gene-specific primers (30) to generate the 415-bp fragment.

Infection of Transduced and Control CEM SS Cells with HIV-1. LGSRN^{Rev+}-transduced, LGSRN^{Rev-}-transduced, or control CEM SS cells (2×10^6) were incubated at 37°C for 3 hr with 1 ml of culture supernatant (cell free) from MOLT-3 cells that were chronically infected with HIV-1_{IIIIB}. The cells were then washed three times in serum-free RPMI 1640, suspended in 15 ml of RPMI 1640 supplemented with 10% fetal bovine serum, and maintained at 37°C in a 5% CO₂ incubator. Supernatant samples (1 ml) were collected daily and replaced with fresh medium. The collected samples were tested for the presence of HIV-1 p24 antigen by ELISA (DuPont/NEN).

Infection of Transduced CEM SS Cells with Simian Immunodeficiency Virus (SIV). LGSRN^{Rev+}- or LGSRN^{Rev-}-transduced CEM SS cells (1×10^6) were infected with SIV-1_{mac251} at a low multiplicity of infection [0.5 median infectious dose (ID₅₀)] for 2 hr at 37°C. The cells were then washed, resuspended in RPMI 1640 with 10% fetal bovine serum, and maintained at 37°C in a 5% CO₂ incubator. Supernatant samples were collected daily and tested for the presence of SIV p27 antigen by ELISA (Coulter).

RESULTS

Expression of the Mutant *gag* Gene in pLGSRN Is Rev-Dependent. A replication-defective retrovirus based on pLXSN (24) was developed as a delivery vector for the mutant *gag* gene (Fig. 1a). We modified the pLXSN vector by introducing an HIV-1 RRE into the Moloney murine leukemia virus (MoMLV) 5' transcriptional unit and then cloned the mutant *gag* gene downstream of the MoMLV 5' LTR. The mutant HIV-1 *gag* gene sequence that we used was derived from HIV-1_{HXB2} proviral DNA that had an in-frame deletion overlapping the p17/p24 protease cleavage site in the Gag precursor polypeptide (21). We further modified the mutant HIV-1 *gag* gene by deleting the 3' sequence corresponding to the p6 polypeptide. By cloning the mutant *gag* gene sequence into pLXSRN, a stop codon was introduced after the sequence corresponding to the Gag p7 polypeptide (Fig. 1b). The pLGSRN construct was tested for regulated expression of the mutant HIV-1 Gag protein in a transient-transfection assay in HeLa cells. pLGSRN DNA was cotransfected into HeLa cells with a Rev expression plasmid, pcRev, and 48 hr later cellular lysates were prepared and analyzed for the presence of mutant Gag protein by a quantitative p24 antigen ELISA. The mutant HIV-1 Gag protein was expressed at significant levels (an \approx 40-fold enhancement) only in the presence of Rev, demonstrating the desired Rev-dependent regulation (Fig. 1c).

Stable Transduction of T-Cell Lines with pLGSRN. To ensure high-efficiency gene transfer to the target T cells, virus was generated from pLGSRN by transient transfection of the amphotropic packaging cell line GP+*env*Am12. Since the HIV-1 Rev protein acts posttranscriptionally to stabilize

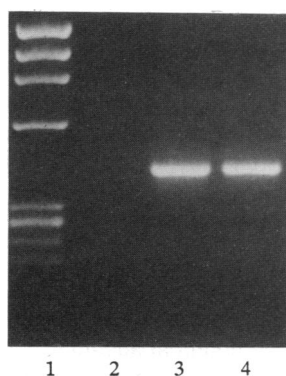


FIG. 2. Detection of integrated LGSRN retroviral genomes in transduced and G418-selected T cells. CEM SS cells were transduced with LGSRN^{Rev+} or LGSRN^{Rev-} and maintained in culture under G418 selection. PCR was performed on genomic DNA samples isolated from CEM SS cells (lane 2), LGSRN^{Rev-}-transduced cells (lane 3), and LGSRN^{Rev+}-transduced cells (lane 4). Primers corresponding to sequences within the *neo* gene were used to generate the 415 bp fragment. Molecular size markers (lane 1) were *Hae*III fragments of phage ϕ X174 DNA.

and increase transport of all RRE-containing mRNAs (31–35), it seemed that Rev might be necessary for efficient packaging of the full-length pLGSRN RNA. We tested this hypothesis by cotransfecting pLGSRN DNA into the amphotropic packaging cell line with or without a Rev expression plasmid (pcRev), to generate producer cells. Supernatants from the amphotropic producer cells were filtered and then used to infect (transduce) a human T-cell line, CEM SS. After transduction the CEM SS cells were subjected to G418 selection without single-cell cloning. After G418 selection, DNA samples were isolated from cells that had been infected with LGSRN that had been packaged in the presence of pcRev (designated LGSRN^{Rev+}) and cells that had been infected with LGSRN that was packaged in the absence of pcRev (designated LGSRN^{Rev-}). The presence of retroviral genomes in the transduced CEM SS cells was confirmed by

PCR analysis (Fig. 2). To ensure that any inhibition of HIV-1 replication was not due to changes in surface expression of CD4 (the dominant receptor for HIV-1) resulting from the transduction and G418 selection of the T-cell lines, a flow microfluorimetric analysis was performed. This study confirmed that there were no detectable differences in surface expression of CD4 between the transduced T-cell lines and the CEM SS control cells.

Inhibition of HIV-1 Replication in pLGSRN-Transduced T Cells. The LGSRN^{Rev+}- and LGSRN^{Rev-}-transduced T cells were then challenged with an infectious dose of HIV-1_{IIIB}. By 5 days after challenge both the control and the LGSRN^{Rev-}-transduced T cells showed increasing levels of HIV-1 replication when monitored by p24 ELISA and reverse transcriptase assays (Fig. 3 *Left*). In contrast, HIV-1 replication in the LGSRN^{Rev+}-transduced T cells was significantly lower. By day 8 after challenge, HIV-1 replication in the LGSRN^{Rev+}-transduced T cells was inhibited by $\approx 94\%$ when compared with controls, indicating that the modified *gag* gene was acting as a dominant negative mutant, in agreement with the results of Trono *et al.* (21). We were intrigued by the observation that there was no significant inhibition of HIV-1 replication in the LGSRN^{Rev-}-transduced T cells. Although this is consistent with our hypothesis that the HIV-1 Rev protein is necessary for packaging the LGSRN genome, further studies will be needed to confirm this conclusion and elucidate the mechanism.

The effects of inhibiting HIV-1 replication could also be observed microscopically. There was a concomitant reduction in syncytium formation and cell death in the LGSRN^{Rev+}-transduced T-cell cultures after HIV-1 infection when compared with controls (Fig. 3 *Right*), demonstrating that the mutant *gag* gene protects cells from the cytopathic effects of the virus, a desirable characteristic for an anti-HIV therapy.

Inhibition of SIV Replication in pLGSRN-Transduced T Cells. We were interested in determining whether the product of the mutant HIV-1 *gag* gene could also inhibit the replication of distantly related retroviruses. In addition to providing information about the efficacy of the dominant negative mutant, it would be extremely useful to know whether this

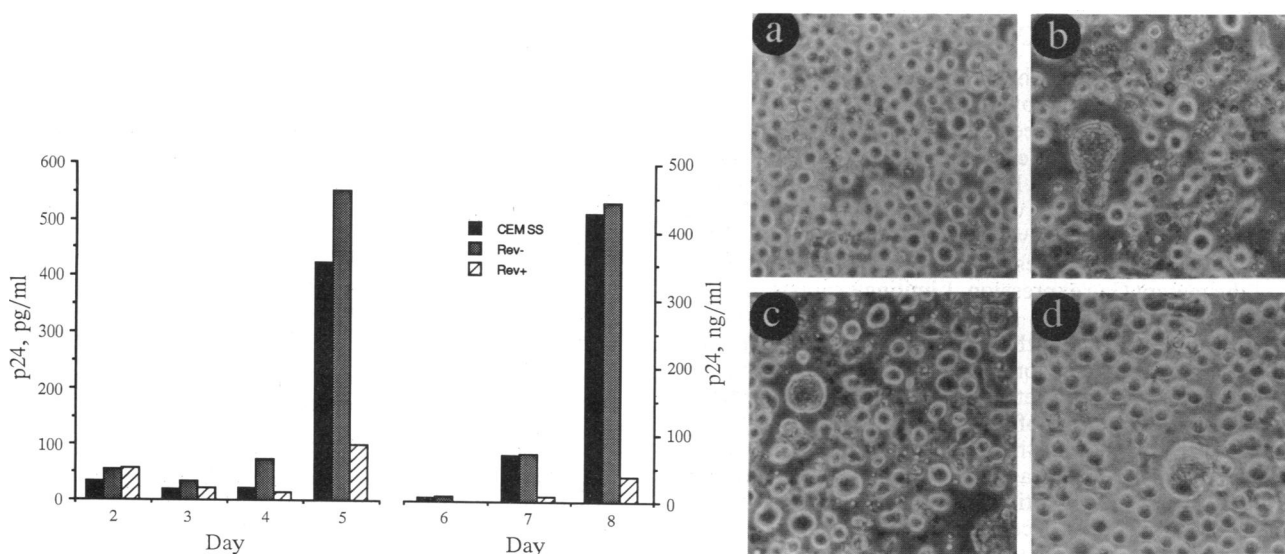


FIG. 3. Inhibition of HIV-1 replication is demonstrated by decreased p24 release and increased cell viability *in vitro*. (*Left*) CEM SS cells (black bars), LGSRN^{Rev-}-transduced cells (gray bars), and LGSRN^{Rev+}-transduced cells (hatched bars) were infected with HIV-1_{IIIB} and analyzed through day 8 by p24 ELISA. Inhibition of HIV-1 replication was evident by day 5 and was maintained through day 8, reaching 90% in the LGSRN^{Rev+}-transduced cells. [Note difference in scale of left (days 2–5) and right (days 6–8) ordinates.] Comparable results were obtained when supernatant samples were assayed for reverse transcriptase (data not shown). (*Right*) LGSRN^{Rev+}-transduced T cells exhibited minimal cell death and syncytium formation 9 days after infection with HIV-1_{IIIB} when compared with the LGSRN^{Rev-}-transduced and CEM SS cells. (a) Uninfected CEM SS cells. (b) CEM SS cells infected with HIV-1_{IIIB}. (c) LGSRN^{Rev-}-transduced cells infected with HIV-1_{IIIB}. (d) LGSRN^{Rev+}-transduced cells infected with HIV-1_{IIIB}. ($\times 270$.)

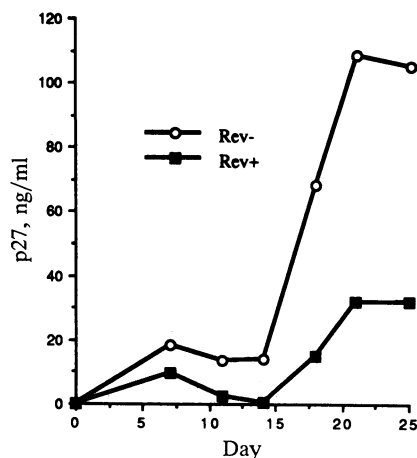


FIG. 4. Inhibition of SIV replication in LGSRN^{Rev+}-transduced T cells. LGSRN^{Rev+}-transduced (■) and LGSRN^{Rev-}-transduced (○) cells were infected with SIV_{mac251} and supernatant samples were tested by ELISA for SIV p27 antigen. There was a significant reduction in p27 release from the SIV-infected LGSRN^{Rev+}-transduced T-cells throughout the 25-day experiment, indicating inhibition of viral replication. Inhibition of SIV replication reached a maximum of ≈70% by day 21.

antiviral gene therapy could be tested in an animal model. Therefore, a parallel series of experiments were performed to evaluate the inhibitory effects of the mutant HIV-1 *gag* gene on SIV replication. LGSRN^{Rev+}- and LGSRN^{Rev-}-transduced T cells were infected with SIV_{mac251} and monitored by quantitative p27 antigen ELISA. In contrast to HIV-1, infection with SIV_{mac251} did not result in syncytium formation in the cultures. Therefore, the SIV-infected T cells could be monitored over a longer time course than could the HIV-1-infected T cells. SIV replication was significantly inhibited (by up to 70%) in the LGSRN^{Rev+}-transduced T cells when compared with controls (Fig. 4), indicating that the product of the mutant HIV-1 *gag* gene can dominantly interfere with the function of the SIV Gag protein.

DISCUSSION

In HIV-1-infected T lymphocytes, expression of the Gag polypeptide is regulated by a complex series of events involving interactions between the HIV-1 Rev protein and the cis-acting RRE (31–35). We have exploited this regulatory mechanism in developing a Rev-regulated retroviral expression vector. An HIV-1 RRE was introduced into pLXSN, and then the mutant *gag* gene was inserted downstream of the MoMLV 5' LTR, which resulted in Rev-dependent regulation of the mutant Gag expression. Limiting expression of the mutant Gag polypeptide to HIV-1-infected cells should obviate problems that may otherwise be associated with constitutive expression of the foreign protein, and we believe this to be the first time that a retroviral vector that is capable of Rev-regulated expression of a protein has been described.

Although expression of the dominant negative Gag polypeptide was consistently associated with potent antiviral activity (up to 94% inhibition of HIV-1 replication in the LGSRN^{Rev+}-transduced T cells) there was a residual level of HIV-1 replication observed in the cell cultures. There are several possible explanations for this observation. One is that the low-level expression of the mutant Gag polypeptide was insufficient to completely block HIV-1 replication, resulting in a significantly reduced, but persistent, virion release. Another explanation could involve the Rev-dependent regulation of the LGSRN vector. Since we have established that Rev is essential for expression of the mutant *gag* gene, some HIV-1 gene expression would be required to produce Rev.

This may result in the release of infectious HIV-1 particles before the dominant negative mutant Gag polypeptide can inhibit further replication. A third explanation is that HIV-1 was replicating in transduced T cells in which the mutant Gag polypeptide was not being expressed. This may result, for example, from rearrangements or deletions in LGSRN during or after integration.

Significant inhibition of SIV_{mac251} replication (up to 70%) was also observed in the LGSRN^{Rev+}-transduced T cells. This indicated that the product of the mutant HIV-1 *gag* gene could also dominantly interfere with the function of the SIV Gag protein. This inhibition of SIV replication clearly demonstrates one of the potential advantages of a gene-therapy strategy based on the use of a dominant negative mutant *gag* gene. If the expressed product of a mutant HIV-1 *gag* gene can be used to inhibit SIV replication it should, therefore, inhibit the replication of other primate lentiviruses such as HIV-2 and a broad range of HIV-1 variants. In addition, these results would suggest that this strategy may obviate the potential of the virus to generate escape mutants, a major problem with many chemotherapeutic approaches. Moreover, inhibition of SIV replication in LGSRN^{Rev+}-transduced T cells suggests the possibility of using a primate animal model to test the antiviral effects of this type of dominant negative Gag mutant *in vivo*.

There are at least two additional advantages in our approach to a gene therapy strategy for HIV-1 infection. The first is that we can demonstrate up to 94% inhibition of HIV-1 replication in the transduced T cells without resorting to selective single-cell cloning. If a protocol for gene therapy were to be successful in treating HIV-1 infection in humans, single-cell cloning of transduced T lymphocytes would not be feasible because of the limited proliferative capacity of these cells. The second advantage in our approach is in having Rev-dependent regulated expression of the transdominant mutant gene. Regulated expression would probably be essential if the success of gene therapy for HIV-1 infection ultimately requires the treatment of hematopoietic progenitor and stem cells (3).

The potential application of somatic gene therapy to the clinical treatment of human disease is one of the most exciting recent developments in biomedical research (1, 2). We have shown that Rev-regulated expression of a mutant HIV-1 *gag* gene can inhibit HIV-1 (and SIV) replication in T cells, with concomitant reduction in the cytopathic effects of the virus and an increase in T-cell survival. These results highlight the potential advantages of using dominant negative mutants in a gene therapy for the treatment of HIV-1 infection and suggest an appropriate animal model for further *in vivo* studies.

We thank P. Lusso and R. Crowley for assistance with flow microfluorimetry, S. Arya for helpful advice and discussions, and L. Anderson for assistance with the manuscript. J.A.S. was supported by a fellowship from the Irvington Institute for Medical Research (New York) and a National Health and Medical Research Council C. J. Martin Fellowship (Australia). M.T. is supported by a fellowship from the Fondo de Investigacion Sanitaria, Spain.

1. Anderson, W. F. (1992) *Science* **256**, 808–813.
2. Miller, A. D. (1992) *Nature (London)* **357**, 455–460.
3. Baltimore, D. (1988) *Nature (London)* **335**, 395–396.
4. Barre-Sinoussi, F., Chermann, J., Rey, F., Nugeyre, M., Chamaret, S., Gruest, J., Dautet, C., Axler-Blin, C., Veinot-Brun, F., Rouzioux, C., Rosenbaum, W. & Montagnier, L. (1983) *Science* **220**, 868–870.
5. Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. (1984) *Science* **224**, 497–500.
6. Gallo, R. C., Salahuddin, S. Z., Popovic, M., Shearer, G., Kaplan, M., Hayes, B., Palker, T., Redfield, R., Oleske, J., Safai, B., White, G., Foster, P. & Markham, P. (1984) *Science* **224**, 500–502.
7. Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J.,

- Geutard, D., Hercend, T., Gluckman, J. & Montagnier, L. (1984) *Nature (London)* **312**, 767–768.
8. Gartner, S., Markovits, P., Markovitz, D., Kaplan, M., Gallo, R. C. & Popovic, M. (1986) *Science* **233**, 215–219.
 9. Arya, S., Guo, C., Josephs, S. & Wong-Staal, F. (1985) *Science* **229**, 69–73.
 10. Sodroski, J., Patarca, R., Rosen, C., Wong-Staal, F. & Haseltine, W. (1985) *Science* **229**, 74–77.
 11. Fisher, A., Feinberg, M., Joseph, S., Harper, M., Marsella, L., Reyes, G., Gonda, M., Aldovini, A., Debouk, C., Gallo, R. C. & Wong-Staal, F. (1986) *Nature (London)* **320**, 367–371.
 12. Meusing, M., Smith, D., Cabradilla, C., Benton, C., Lasky, L. & Capon, D. (1985) *Nature (London)* **313**, 450–458.
 13. Garrett, E., Tiley, L. & Cullen, B. (1991) *J. Virol.* **65**, 1653–1657.
 14. Strebler, K., Klimkait, T. & Martin, M. (1988) *Science* **241**, 532–534.
 15. Sullinger, B., Gallardo, H., Ungers, G. & Gilboa, E. (1990) *Cell* **63**, 601–608.
 16. Lisziewicz, J., Rappaport, J. & Dhar, R. (1991) *New Biol.* **3**, 82–89.
 17. Lisziewicz, J., Sun, D., Smythe, J., Lusso, P., Lori, F., Louie, A., Markham, P., Rossi, J., Reitz, M. & Gallo, R. C. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8000–8004.
 18. Chatterjee, S., Johnson, P. & Won, K. (1992) *Science* **258**, 1485–1488.
 19. Malim, M., Bohnlein, S., Hauber, J. & Cullen, B. (1989) *Cell* **58**, 205–214.
 20. Green, M., Ishino, M. & Lowenstein, P. (1989) *Cell* **58**, 215–223.
 21. Trono, D., Feinberg, M. & Baltimore, D. (1989) *Cell* **59**, 113–120.
 22. Freed, E., Delwart, E., Buchschacher, G. & Panganiban, A. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 70–74.
 23. Daefler, S., Klotman, M. & Wong-Staal, F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4571–4575.
 24. Miller, A. & Rosman, G. (1989) *BioTechniques* **7**, 980–990.
 25. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
 26. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745–2752.
 27. Cullen, B. (1986) *Cell* **46**, 973–982.
 28. Markowitz, D., Goff, S. & Banks, A. (1988) *Virology* **167**, 400–406.
 29. Nara, P. & Fischinger, P. (1988) *Nature (London)* **332**, 469–470.
 30. Ferrari, G., Rossini, S., Giavazzi, R., Maggioni, D., Nobili, N., Soldati, M., Ungers, G., Mavilio, F., Gilboa, E. & Bordignon, C. (1991) *Science* **251**, 1363–1366.
 31. Malim, M., Hauber, J., Le, S.-Y., Maizel, J. & Cullen, B. (1989) *Nature (London)* **338**, 254–257.
 32. Zapp, M. & Green, M. (1989) *Nature (London)* **342**, 714–716.
 33. Malim, M., Tiley, L., McCarn, D., Rusche, J., Hauber, J. & Cullen, B. (1990) *Cell* **60**, 675–683.
 34. Malim, M. & Cullen, B. (1991) *Cell* **65**, 241–248.
 35. Schwartz, S., Felber, B. & Pavlakis, G. (1992) *J. Virol.* **66**, 150–159.