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Comparison of *trans*-Dominant Inhibitory Mutant Human Immunodeficiency Virus Type 1 Genes Expressed by Retroviral Vectors in Human T Lymphocytes

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***trans*-Dominant inhibitory mutant versions of the human immunodeficiency virus type 1 (HIV-1) regulatory genes *tat* and *rev* have previously been described. We have constructed a series of retroviral vectors to transduce these genes and compare their inhibitory activities. The inhibitory activities were measured with transient transfection assays by using a reporter which expresses an HIV-1 *gag-Escherichia coli lacZ* fusion protein with strict dependence on coexpression of both *tat* and *rev*. Additionally, the vectors were packaged as amphotropic virions and used to stably transduce human CEM T lymphocytes. The transduced CEM cells were challenged with HIV-1, and the effects of the mutant HIV-1 genes were determined by measuring the levels of HIV-1 $p24^{gag}$ produced. A *tat* gene substituted at amino acid 41 (tat_{k41a}) retained partial *trans*-activating activity and lacked inhibitory activity. A *tat* gene with a premature stop codon at amino acid 54 (tat_{54ter}) showed moderate *trans*-dominant inhibition of the reporter plasmid but failed to significantly inhibit HIV-1 replication. The M10 *rev* mutant, with a 2-amino-acid substitution, showed strong *trans*-dominant inhibitory activity both in the reporter plasmid and in the HIV-1 infection assay. The greatest inhibition of HIV-1 growth was seen when M10 was expressed under the transcriptional control of a human cytomegalovirus promoter; slightly less inhibition was achieved when expression of M10 was controlled by the Moloney murine leukemia virus long terminal repeat, and minimal inhibition was seen when the HIV-1 long terminal repeat controlled the M10 gene. These results demonstrate the potential utility of retroviral vectors expressing *trans*-dominant inhibitory mutant HIV-1 genes for gene therapy approaches to AIDS.**

The potential application of gene therapy techniques to render cells resistant to viral infection has been described as "intracellular immunization" (2). Retroviral vectors have been produced which can transduce genes expressing products which inhibit human immunodeficiency virus type 1 (HIV-1) replication, such as antisense RNA, ribozymes, and TAR decoys (4, 10, 11, 28, 30, 31, 33). Another class of genes which has been demonstrated to inhibit HIV-1 are so-called *trans*-dominant mutant inhibitory genes (9). Because of the critical amplification roles played by *tat* and *rev* in the HIV-1 life cycle, *trans*-dominant inhibitory versions of these HIV-1 genes may potently inhibit HIV-1 replication (27). Additionally, expression of these mutant HIV-1 genes in hematopoietic stem cells and T lymphocytes may not interfere with normal cellular functions because they act at RNA sequences unique to HIV-1.

We have made a series of retroviral vectors containing genes encoding putative mutant *tat* and *rev* genes reported to possess *trans*-dominant inhibitory activities. To permit rapid and quantitative comparisons of their effects, we have constructed a reporter plasmid which expresses a fusion protein of HIV-1 *gag* and *Escherichia coli lacZ*, with strict dependence on coexpression of both *tat* and *rev*. Inhibitory effects of the *trans*-dominant HIV-1 genes may be determined by measuring reductions of the β-galactosidase activity produced by the reporter plasmid after electroporation into human T lymphocytes. Our results demonstrate that the *trans*-dominant *rev* gene M10 most effectively inhibited expression by the reporter plasmid in human T lymphocytes of the CEM cell line, especially when expressed under transcriptional control of the human cytomegalovirus (CMV) promoter. Parallel effects were observed on inhibition of replication of HIV-1 in CEM cells stably transduced by the *trans*-dominant gene retroviral vectors.

MATERIALS AND METHODS

Plasmids. The plasmid pSV2*tat*72, which expresses a synthetic *tat* gene under control of the simian virus 40 (SV40) early region promoter, and the HIV-1 genomic clone pNL43 were obtained from the AIDS Research and Reference Reagent Program (ARRRP, Rockville, Md.). pCMV*rev* and pCMV-M10, which express wild-type and *trans*-dominant inhibitory mutant versions, respectively, of the HIV-1 *rev* gene were obtained from Bryan Cullen, Duke University, Chapel Hill, N.C. (12). The retroviral vector plasmids pLXSN and pLNCX were obtained from A. D. Miller, Fred Hutchinson Cancer Center, Seattle, Wash. (18). pCMV-CAT was constructed by using the CAT (chloramphenicol acetyltransferase) gene from pSV2CAT (7) to replace the hygromycin-resistance gene from a CMV-hygro expression plasmid obtained from Steven Lupton, Immunex Corp., Seattle, Wash.

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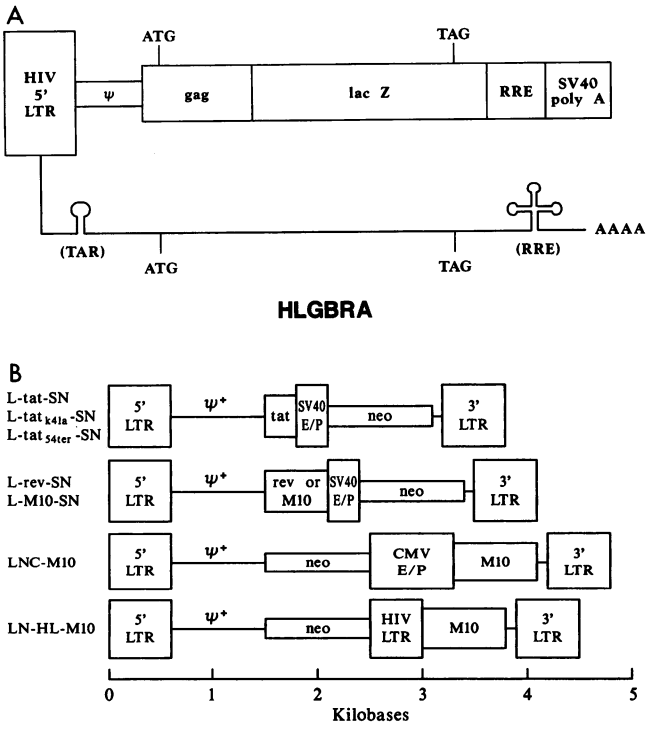

FIG. 1. Expression plasmids. (A) The reporter plasmid HLG BRA. The order of the elements from the 5′ end is as follows: the 5′ LTR, leader sequence, and *gag* region from HIV-1; the *E. coli lacZ* gene; the HIV-1 RREs; and the SV40 polyadenylation signal. The expected polyadenylated transcript is shown below the plasmid map and contains the HIV TAR and RRE domains flanking the coding region for a *gag-lacZ* fusion protein. (B) Retroviral vectors carrying wild-type and putative *trans*-dominant inhibitory mutant HIV-1 *tat* and *rev* genes. The vectors contain the 5′ and 3′ LTR and packaging (Psi [ψ^+]) region from Mo-MuLV; the bacterial dominant selectable marker neomycin phosphotransferase (*neo*) gene; and internal promoters from either SV40, CMV, or the HIV LTR.

The *tat*/*rev*-dependent *lacZ* expression reporter plasmid HLGBRA-BS. The reporter plasmid HLGBRA-BS contains (i) the 5′ long terminal repeat (LTR), leader sequence, and *gag* region from HIV-1, (ii) a synthetic linker to fuse the HIV-1 *gag* and *E. coli lacZ* genes and maintain the open reading frame, (iii) the *E. coli lacZ* gene, (iv) the HIV-1 *rev*-responsive element (RRE), (v) the SV40 polyadenylation signal, and (vi) the plasmid Bluescript (Stratagene, La Jolla, Calif.) (Fig. 1A). A 2.5-kb *Stu*I-*Apa*I fragment from the HIV-1 clone pNL43 was subcloned into Bluescript. This HIV-1 fragment contains approximately 500 bp of flanking cellular sequences, the 5′ LTR (including the TAR region), the 5′ untranslated leader (Psi) region, and 1,218 bases of the *gag* gene, encoding 406 of the normal 500 amino acids. A synthetic double-stranded oligonucleotide linker (upper strand, 5′-CTGCAG-3′; lower strand, 5′-GATCCTGCAG GGCC-3′) designed to fuse the HIV-1 *gag* and *E. coli lacZ* coding regions and maintain the translational reading frame was cloned to the *Apa*I site at the 3′ end of the HIV-1 fragment. The *Bam*HI-*Bam*HI fragment from the SP6-βgal plasmid (a gift from Connie Cepko, Harvard Medical School), which contains the entire *E. coli lacZ* gene except for the first 6 bp of the coding region, was cloned into the *Bam*HI site at the 3′ end of the synthetic linker. The region of the fusion consists of

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        |  ApaI  |  PstI  |  BamI
---G-G-G-C-C-C-T-G-C-A-G-G-A-T-C-C---
---C-C-C-G-G-G-A-C-G-T-C-C-T-A-G-G---
        |  |  |  |  |  |  |  |  |  |
          Ala   Val   Gln   Asp
gag amino acid 406  --linker--  lacZ amino acid 3.
```

The SV40 late region polyadenylation signal, isolated as a *Bam*HI-*Sal*I fragment from pSVL (Pharmacia Biotechnology Inc., Piscataway, N.J.), was cloned into the *Bam*HI site at the 3′ end of the *lacZ* gene. The RRE was isolated as a *Bam*HI-*Bgl*II fragment from plasmid RRE-pGEM (a gift from David Rekosh, State University of New York, Buffalo) and cloned into the *Bam*HI site between the 3′ end of the *lacZ* gene and the 5′ end of the SV40 polyadenylation signal fragment.

***tat* expression constructs.** The HIV-1 *tat* gene was removed from pSV2*tat*72 with *Hin*dIII and *Bam*HI and subcloned into pGEM7 (Promega Corp., Madison, Wis.). Because the *Bam*HI site lies at bp 181 within the coding region of the *tat* gene, this resulted in an incomplete *tat* gene missing the last 38 bp. The 3′ end of the *tat* coding region was restored by addition of a synthetic oligonucleotide which eliminated the *Bam*HI site at bp 181, while conserving the amino acid sequence, and added a new *Bam*HI site immediately after the termination codon.

The restored *tat* gene was cloned into the retroviral vector plasmid pLXSN to produce L-*tat*-SN, which expresses the wild-type *tat* gene under control of the Moloney murine leukemia virus (Mo-MuLV) LTR (Fig. 1B). To produce the intended *tat* mutants, the restored *tat* gene was subcloned into mp18 and subjected to site-directed mutagenesis to produce tat_{k41a}, in which codon 41 was changed from AAA (Lys) to GCA (Ala), and tat_{54ter}, in which codon 54 was changed from CAG (Gln) to TAG (termination codon). The sequences were verified by DNA sequence analysis. The mutagenized *tat* genes were cloned into pLXSN to produce vector plasmids L-tat_{k41a}-SN and L-tat_{54ter}-SN.

***rev* expression constructs.** The *rev* and M10 genes were isolated from pCMV*rev* and pCMV-M10 by polymerase chain reaction with primers which introduced an *Sal*I site at a position 18 bp 5′ to the start codon and a *Hin*dIII site 80 bp 3′ of the stop codon, and were subcloned into the Bluescript plasmid. The sequences of the subcloned genes were verified by DNA sequence analysis. *rev* and M10 were cloned into pLXSN, to produce the plasmids for L-*rev*-SN and L-M10-SN, respectively (Fig. 1B). The M10 gene was cloned into pLNCX to produce pLNC-M10, in which the M10 gene is under transcriptional control of the CMV promoter. The HIV-1 LTR was isolated as an *Xho*I-*Hin*dIII fragment from pU3R-III CAT (ARRRP) and inserted to replace the CMV promoter of pLNC-M10 to produce pLN-HL-M10, which places M10 under control of the HIV LTR promoter.

Cells and HIV-1 virus. PE501 ecotropic packaging cells and PA317 amphotropic packaging cells were generously provided by A. Dusty Miller (17, 18). Packaging cells were grown in Dulbecco's modified Eagle's medium (high glucose) with 10% fetal calf serum. Human T lymphocytes of the CEM/CCRF line were obtained from the American Type Culture Collection, (Rockville, Md.) and maintained in RPMI with 10% fetal calf serum (R10). HIV-1 of the HTLV-III_B strain was obtained from the ARRRP as a cell-free, concentrated virus stock at 2.91×10^7 virus particles per ml.

HIV-1 of the NL43 strain was propagated in CEM cells and prepared by freeze-thaw lysis followed by low-speed centrifugation. The titer was measured by quadruplicate terminal dilution assay on 96-well plates containing CEM cells. Wells containing infected cells were identified by p24 antigen determination after 10 days, with a value of ≥100 pg/ml as positive. A titer of 3×10^6 tissue culture infective doses (TCID)/ml was obtained. All work with HIV-1 was performed under biologic containment level 2 conditions.

Electroporation of CEM cells for transient expression assays. For each sample, 2×10^7 CEM cells were suspended in 0.5 ml of R10, at room temperature, and placed into disposable electroporation cuvettes (0.4-cm gap width; Bio-Rad, Richmond, Calif.). Plasmid DNA samples were sterilized by filtration through 0.22-μm (pore size) Spin-X filters (Costar, Cambridge, Mass.). Transfection cocktails typically contained (i) the reporter plasmid HLGBRA (26 μg), (ii) plasmids expressing wild-type and/or mutant *tat* genes, (iii) plasmids expressing wild-type and/or mutant *rev* genes, and (iv) a plasmid expressing the bacterial CAT gene under control of the human CMV promoter (pCMV-CAT; 2 μg) to serve as an internal control for the efficiency of transfection. A total of 42 μg of the various plasmids was mixed and added to each electroporation sample. When the experimental design required less than 42 μg of the specific plasmids, the difference was achieved with the cloning plasmid Bluescript.

Electroporations were performed with the Bio-Rad Gene Pulser at 960 μF and 240 V. After electroporation, the cells were maintained in the cuvettes at room temperature for 10 min and then were transferred to flasks containing 20 ml of R10 medium. The cells were incubated at 37°C in 5% CO_2 for 24 h and then were collected and washed twice in Hank's buffered saline solution prior to lysis.

Measurement of β-galactosidase and CAT enzymatic activity. Lysates of the electroporated CEM cells were made by three cycles of freeze-thawing in 0.3 ml of phosphate-buffered saline. β-Galactosidase activity was measured with the spectrophotometric substrate *o*-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co., St. Louis, Mo.) essentially as described previously (20). CAT assays were performed on other portions of each cell lysate as described by Neumann et al. (21) and Eastman (5). In all reported experiments, the values for CAT activity differed by less than 10% among samples.

Immunoblotting. For Western immunoblot analysis, lysates were made from cells after electroporation by sonication in 50 mM potassium phosphate buffer (pH 6.5) with 0.25% Triton X-100. The samples were cleared of insoluble material by brief centrifugation at 12,000 × *g*, and the concentrations of protein in each sample were measured with the bicinchoninic acid assay reagent (Pierce, Rockford, Ill.). Supernatant protein (100 μg) was mixed with an equal volume of sample buffer (62.5 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, and 0.1% bromophenol blue) and boiled for 5 min. The samples were subjected to electrophoresis in an SDS-polyacrylamide gel (7% polyacrylamide) at 100 V for 1 h and transferred to a nitrocellulose membrane by electroblotting at 25 V overnight at 4°C. Immunoreactive *lacZ* proteins were detected by using rabbit polyclonal antibody to *lacZ* (Cappel Research Products, West Chester, Pa.), followed by alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad), and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate toluidinium colorimetric reagents as directed by the manufacturer. Psi2 murine fibroblasts and a clone of Psi2 containing the BAG *E. coli lacZ* expression vector were used as negative and positive controls, respectively, and were obtained from the American Type Culture Collection.

Establishment of vector-transduced T-cell lines. Clones of PA317 amphotropic packaging cells producing each retroviral vector at high titer were derived essentially as described previously (19). In brief, retroviral vector plasmid DNA was transfected by using DOTAP transfection reagent (Boehringer Mannheim, Indianapolis, Ind.) into the PE501 ecotropic packaging cell line. The transfected PE501 cells were selected in 0.5 mg of active G418 (Geneticin; Bethesda Research Laboratories, Bethesda, Md.) per ml. Cell culture supernatants from the G418-resistant PE501 cell pool were used to transduce PA317 amphotropic packaging cells. The transduced PA317 cells were cloned with G418, and individual clones were expanded and screened for the production of high titers of vector virus, assessed by transfer of neomycin resistance to 3T3 cells. A preestablished PA317 clone producing the parental LN vector at high titer (PA317-LNc11) was obtained from A. D. Miller.

CEM cells were transduced by cocultivation with the high-titer PA317 packaging cell clones. The PA317 cells were irradiated (4,000 rads) and plated at a density of 2×10^6 cells per 100-mm dish in R10 medium. The next day, 10^6 CEM cells were added to each plate, and the medium was then supplemented with Polybrene (Sigma) to a final concentration of 8 μg/ml. The CEM and PA317 cells were cocultivated for 48 h, and then the nonadherent CEM cells were collected and cultured in R10 with 0.5 mg of active G418 per ml until resistant cells were obtained (usually 2 to 3 weeks later).

HIV infection of vector-transduced CEM cells. CEM cells which had been transduced by the retroviral vectors and selected with G418 were assayed for resistance to HIV infection. CEM cells were pretreated by incubating 10^6 cells in 2 ml of R10 with Polybrene (8 μg/ml) for 2 h at 37°C. The cells were then pelleted, resuspended in 100 μl of HIV-1 diluted in R10, and incubated for 3 h at 37°C. CEM cells were infected with $HIV\text{-}1_{IIIB}$ at a low multiplicity of infection (MOI) of 7.28×10^4 virus particles per 10^6 cells or at a fivefold higher MOI. After exposure to HIV-1, the cells were washed once with R10, and 5×10^4 cells were plated in 2.5 ml of R10 and incubated at 37°C. Aliquots of medium were removed from the cultures at 2- to 3-day intervals after infection and assayed for HIV replication by measurements of HIV-1 p24 antigen production by using an enzyme-linked immunosorbent assay (ELISA) kit (Coulter Corp., Hialeah, Fla.).

Virus derived from pNL43 was used for challenge of each transduced cell line at a high MOI of 0.1 50% TCIDs per cell. Viable cells (6×10^4) were exposed to 6×10^3 50% TCIDs of NL43 for 1 h at 37°C, washed three times, and then suspended in 1.25 ml of R10 medium. A total of 250 μl of each culture was removed for the day 0 time point. The remaining 1.0 ml of each culture was incubated in a 48-well plate. A total of 250 μl of each culture (cells and supernatant) was removed at days 1, 3, and 6 for the p24 assay (Abbott Laboratories, North Chicago, Ill.). R10 medium (250 μl) was added after each sampling.

RESULTS

Reporter plasmid HLGBRA for quantitating inhibition of HIV-1 *tat* or *rev* function. The plasmid HLGBRA was constructed to provide a convenient reporter to measure the effects of genes designed to inhibit HIV replication (Fig. 1A). Numerous investigators have used the β-galactosidase activity of the *E. coli lacZ* gene as a readily quantified reporter

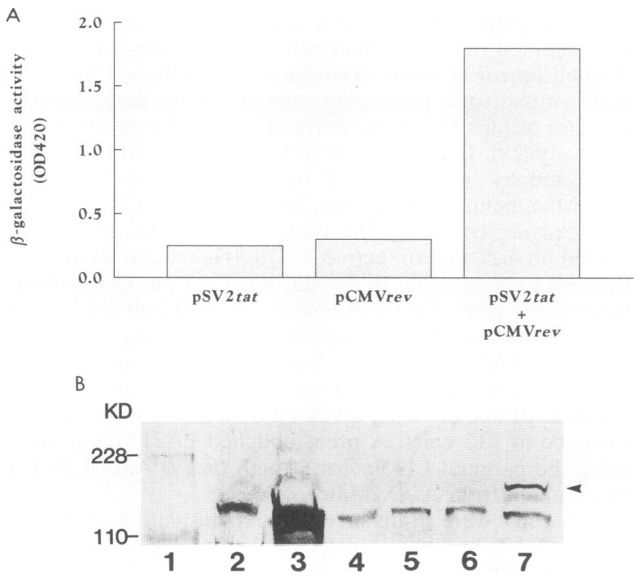

FIG. 2. Dependence on coexpression of *tat* and *rev* for β-galactosidase expression by HLGBRA. (A) β-Galactosidase enzymatic assay. CEM cells were electroporated as described in Materials and Methods with the HLGBRA reporter and 10 μg of either pSV2*tat*72, pCMV*rev*, or both. Cell lysates were made after 24 h, and the β-galactosidase activities were measured by spectrophotometry. OD420, optical density at 420 nm. (B) Western blot analysis with anti-β-galactosidase antibody. CEM cells were electroporated, as described above, and lysates were prepared for Western blot analysis with an anti-β-galactosidase antibody. Lanes: 1, molecular mass standards; 2, Psi2 murine fibroblasts; 3, BAG-Psi2 cells expressing *E. coli lacZ* protein; 4 through 7, CEM cell lysates transfected with control Bluescript plasmid (lane 4), transfected with HLGBRA and pSV2*tat*72 (lane 5), transfected with HLGBRA and pCMV*rev* (lane 6), and transfected with HLGBRA, pSV2*tat*72, and pCMV*rev* (lane 7). The arrowhead indicates the position of the new immunoreactive protein (lane 7) with an estimated molecular mass of 165 kDa.

which can be measured with spectrophotometric, histochemical, and fluorescence-activated cell sorting techniques. In HLGBRA, expression of the *gag-lacZ* fusion gene is under the transcriptional control of the HIV LTR and would be expected to be strongly dependent on the stimulatory effects of the HIV-1 transcriptional *trans*-activator protein *tat* (1). The HIV-1 TAR sequences, present at the 5′ end of the HLGBRA transcript, provide the site for *tat* action. Additionally, the presence of the *cis*-repressive sequences in the *gag* coding region would be expected to confer dependence for the REV protein in *trans* and the RRE in *cis* for expression of the *gag-lacZ* fusion gene (15, 26, 29). Therefore, a DNA fragment containing the RRE from HIV-1 was included in the construct, placed between the end of the *lacZ* coding sequence and the SV40 polyadenylation signal.

Expression of β-galactosidase activity by HLGBRA is, as predicted, strictly dependent on coexpression of both *tat* and *rev* (Fig. 2A). When CEM T cells are transfected with the HLGBRA reporter plasmid plus either a *tat* or a *rev* expression plasmid, only background levels of β-galactosidase activity are seen. However, when both the *tat* and the *rev* expression plasmids are cotransfected with the HLGBRA plasmid, β-galactosidase is readily detected.

Western blot analysis was performed to confirm the production of the *lacZ-gag* fusion gene product. CEM T cells were electroporated with the HLGBRA reporter in combination with either a *tat* or a *rev* expression plasmid or both. Cell lysates were made and analyzed for the presence of a protein which immunologically cross-reacts with antibodies to β-galactosidase. An immunoreactive protein of the molecular weight predicted from the fusion of the HIV *gag* and *E. coli lacZ* genes was seen only when both *tat* and *rev* plasmids were cotransfected (Fig. 2B, lane 7). The requirement for *tat* and *rev* expression implies that there should be a decreased level of β-galactosidase activity produced by HLGBRA if the cells coexpress genes which interfere with either *tat* or *rev* functions (e.g., functioning *trans*-dominant inhibitory mutant HIV genes).

Retroviral vectors for transduction of *trans*-dominant inhibitory HIV genes. We used the HLGBRA reporter system to compare the inhibitory effects of several mutant HIV genes which have been reported to have *trans*-dominant inhibitory activity. Green et al. (8) reported that a synthetic *tat* protein containing an alanine residue substituting for lysine 41 produced *trans*-dominant inhibition of *tat*-directed expression by an HIV LTR CAT plasmid. Pearson et al. (23) described a different *trans*-dominant inhibitory *tat* mutant truncated by a premature stop codon at amino acid 54. The truncation is within a region rich in basic amino acids which comprises a putative activation domain. In transient transfection assays with HeLa cells, an expression plasmid with the truncated *tat* gene inhibited expression from a cotransfected HIV LTR-CAT plasmid. Malim et al. (12, 16) identified a mutant of the HIV-1 *rev* gene, named M10, containing substitutions at amino acids 78 (L→D) and 79 (E→L), which displayed strong *trans*-dominant inhibitory features in transient transfection assays with COS cells.

These *trans*-dominant inhibitory mutant HIV-1 genes may potentially be applied for clinical gene therapy of HIV infection by transduction of the hematopoietic stem cells or T lymphocytes of patients. It is therefore important to define the relative efficacy of these genes in inhibiting HIV-1 replication. We have made a series of retroviral vectors which carry genes encoding each of these *tat* or *rev* mutant genes to compare their relative inhibitory activities (Fig. 1B). The retroviral vectors were used as transient transfection expression plasmids by electroporation into human T cells or were packaged as amphotropic vectors and used to stably transduce the genes into T cells.

Analysis of putative *trans*-dominant inhibitory mutant HIV-1 genes. The first series of experiments compared the inhibitory effects of each of the putative *trans*-dominant inhibitory genes in transient transfection assays. The effects of the inhibitory genes were quantitated by measuring decreases in the β-galactosidase expression from the HLGBRA reporter plasmid. Human T lymphocytes of the CEM cell line were electroporated with a mixture of plasmids, including (i) the HLGBRA reporter, (ii) plasmids encoding wild-type *tat* and/or *rev* proteins, (iii) plasmids encoding the putative *trans*-dominant HIV-1 genes, and (iv) pCMV-CAT to serve as an internal control for the efficiency of electroporation. Twenty-four to 36 h after electroporation, the T cells were collected and lysates were made for measurement of β-galactosidase and CAT activity.

We first compared vector plasmids made with the pLXSN vector, which places each of the mutant HIV genes under expression control of the Mo-MuLV LTR (L-tat_{k41a}-SN, L-tat_{54ter}-SN, and L-M10-SN; Fig. 1B). As seen previously, cells transfected with HLGBRA and both the wild-type *tat* (pSV2*tat*) and *rev* (pCMV*rev*) plasmids showed strong β-galactosidase expression (Fig. 3A). The L-tat_{k41a}-SN plasmid

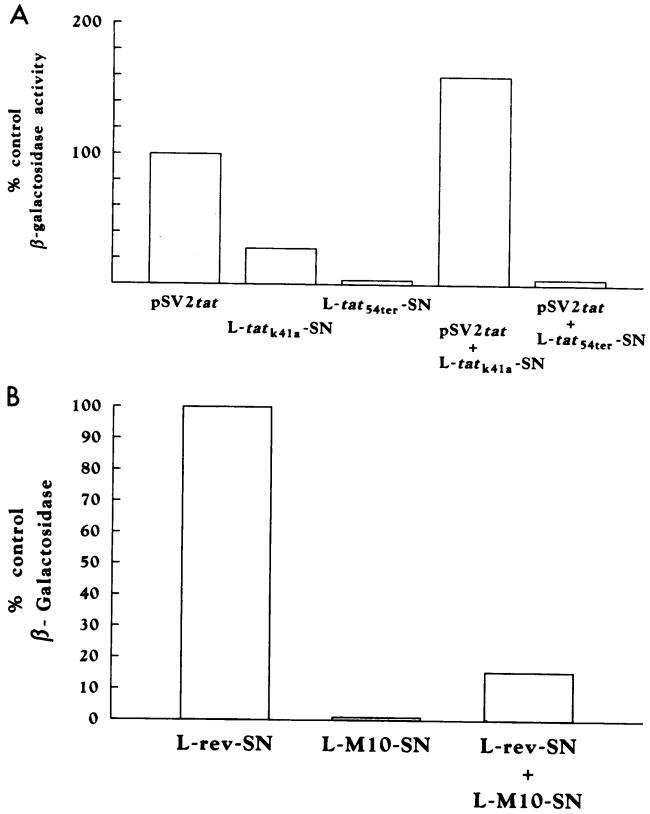

FIG. 3. Effects of transiently-transfected mutant *tat* and *rev* genes on expression by HLGBRA. (A) Analysis of putative *trans*-dominant inhibitory *tat* genes. CEM cells were transfected with HLGBRA, pCMV*rev*, and 10 μg of either pSV2*tat*72, L-tat_{k41a}-SN or L-tat_{54ter}-SN, or combinations of pSV2*tat*72 and either L-tat_{k41a}-SN or L-tat_{54ter}-SN. Cell lysates were made after 24 h, and the β-galactosidase activities were determined by spectrophotometry. The activity produced by the pSV2*tat*72 control alone was set as 100%, and the other values were calculated as percentages of the control. (B) Analysis of a putative *trans*-dominant inhibitory *rev* gene. CEM cells were electroporated with HLGBRA, pSV2*tat*72, and 10 μg of either L-*rev*-SN, L-M10-SN, or a combination of both. Cell lysates were made after 24 h and the β-galactosidase activities were measured by spectrophotometry. The activity produced by the L-*rev*-SN control alone was set as 100%, and the other values were calculated as percentages of the control.

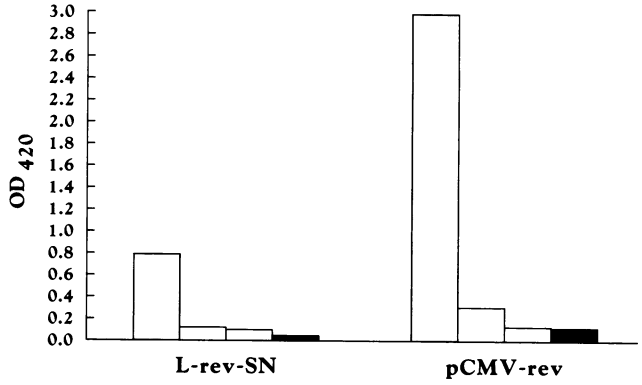

FIG. 4. Comparison of transiently transfected M10 vectors on expression by HLGBRA. CEM cells were transfected with HLGBRA, pSV2*tat*72, one of the plasmids expressing wild-type *rev* (L-*rev*-SN or pCMV*rev*), and either no M10 plasmid (stippled bars), L-M10-SN (open bars), LNC-M10 (shaded bars), or LN-HL-M10 (solid bars). Cell lysates were made after 24 h, and the β-galactosidase activities were measured by spectrophotometry. OD_{420}, optical density at 420 nm.

supported a moderate level of β-galactosidase expression (27.6% of control), suggesting that this amino acid substitution of the *tat* protein only partially inactivates the *trans*-activation function. In contrast, the L-tat_{54ter}-SN plasmid failed to support expression by HLGBRA (2.3% of control).

A similar analysis of the *rev* expression plasmids was performed (Fig. 3B). Cotransfection of a plasmid expressing the wild-type *rev* protein (L-*rev*-SN) plus the pSV2*tat*72 plasmid produced high levels of β-galactosidase activity. Substitution of the wild-type *rev* expression plasmid with the L-M10-SN expression plasmid resulted in less than 1% of the control level of β-galactosidase expression, demonstrating that the M10 mutant lacks normal *rev* function.

To test the *trans*-dominant inhibitory activity of the mutant HIV-1 plasmids, they were cotransfected with plasmids expressing their wild-type counterparts (Fig. 3). Both the truncated *tat* expression plasmid (L-tat_{54ter}-SN) and the mutant *rev* expression plasmid (L-M10-SN) strongly suppressed *trans*-activation by the wild-type genes, demonstrating their *trans*-dominant inhibitory activity. In contrast, the L-tat_{k41a} plasmid actually increased the *trans*-activation produced by the wild-type *tat* plasmid, failing to support the contention that it is a *trans*-dominant inhibitory mutant gene.

Comparison of inhibitory effects of vectors expressing M10 under control of the Mo-MuLV LTR, the CMV promoter, and the HIV LTR. Preliminary experiments suggested that the CMV promoter produced higher levels of gene expression than the Mo-MuLV LTR in CEM cells. High-level expression of an inhibitory gene is likely to produce more consistent suppression of HIV replication and would be a desirable goal for gene therapy. Therefore, we made vectors containing the M10 *trans*-dominant *rev* mutant under transcriptional control of either the Mo-MuLV LTR (L-M10-SN) or the CMV promoter (LNC-M10) to compare their relative inhibitory activities (Fig. 1B). A vector was also made in which the M10 gene was controlled by the HIV LTR (LN-HL-M10), which would be expected to be transactivated by *tat* expression to produce high levels of transcription. Such an inducible vector may be desirable, in that it would conditionally express the M10 gene only upon entry of HIV-1 into a transduced cell.

We determined the abilities of each of the M10 vector plasmids to produce *trans*-dominant inhibition of expression by HLGBRA (Fig. 4). The plasmid pSV2*tat*72 was cotransfected to produce *tat*, and wild-type *rev* was expressed under control of either the Mo-MuLV LTR (L-*rev*-SN) or the CMV promoter (pCMV*rev*) (Fig. 4). Comparing the two control transfections performed without a plasmid containing M10, it can be seen that pCMV*rev* produced almost fourfold higher β-galactosidase activity than did an equal amount of the L-*rev*-SN.

Expression by HLGBRA driven by L-*rev*-SN was completely inhibited by each of the plasmids expressing the *trans*-dominant M10 gene under control of either the Mo-MuLV LTR, the CMV promoter, or the HIV LTR. In contrast, when the wild-type *rev* was controlled by the CMV promoter (pCMV*rev*), LNC-M10 and LN-HL-M10 inhibited β-galactosidase expression by HLGBRA more effectively than L-M10-SN did. These studies indicate that the CMV

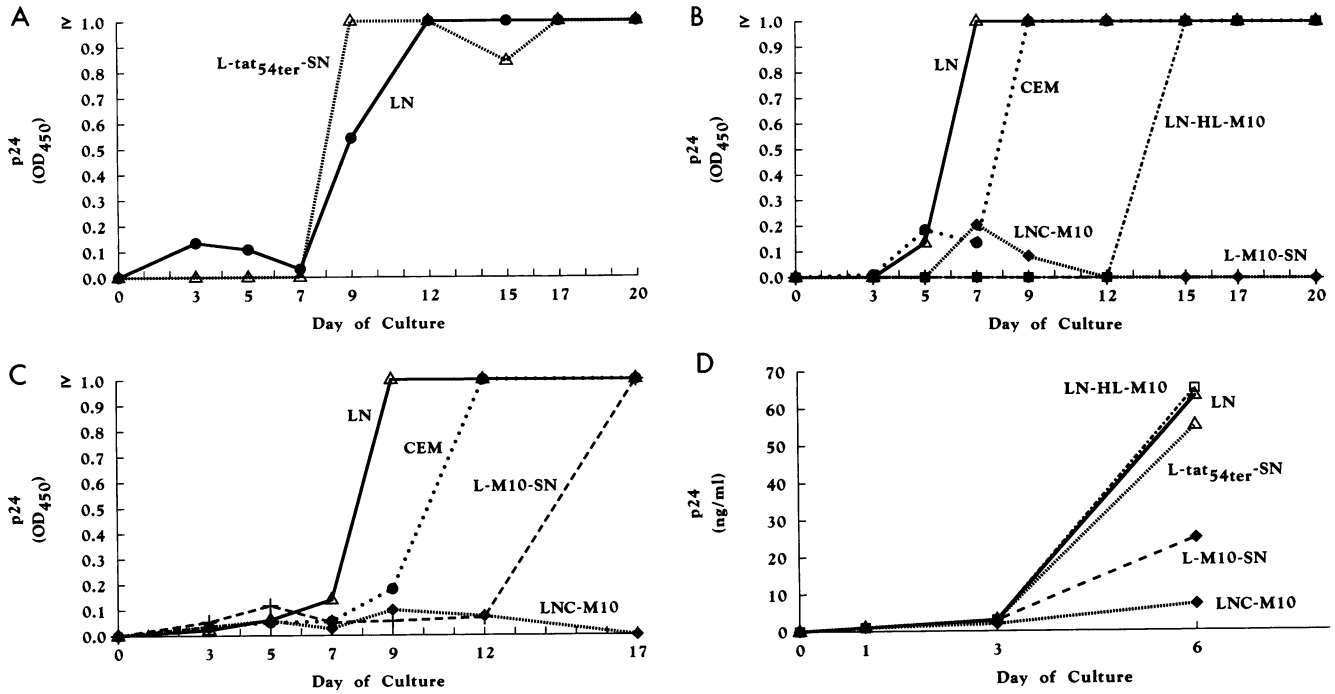

FIG. 5. Effects of stably transduced mutant *tat* and *rev* genes on infection of CEM human T lymphocytes by HIV-1. (A) Analysis of cells transduced by L-tat_{54ter}-SN. CEM cells transduced by either the control LN vector (●) or the L-tat_{54ter}-SN vector (△) and selected with G418 were challenged by inoculation with HIV-1_{IIIB}. The culture supernatants were sampled at the indicated times and the levels of $p24^{gag}$ were measured by ELISA. (B) Analysis of cells transduced by M10 retroviral vectors. Nontransduced CEM cells (●) or CEM cells transduced by LN (△), L-M10-SN (+), LNC-M10 (◆), or LN-HL-M10 (□) and selected with G418 were challenged by inoculation with HIV-1_{IIIB}. The culture supernatants were sampled at the indicated times and the levels of $p24^{gag}$ were measured by ELISA. (C) Analysis of cells transduced by L-M10-SN or LNC-M10 by challenge with a higher inoculum of HIV-1. CEM cells transduced with vectors as indicated above were challenged with a fivefold higher inoculum of HIV-1, and then serial ELISAs were performed to quantitate the levels of $p24^{gag}$. (D) Analysis of cells challenged with pNL43-derived HIV-1 at high MOI. CEM cells transduced with vectors as indicated above were challenged with NL43 with 0.1 50% TCID per cell, and serial ELISAs were performed to quantitate the levels of $p24^{gag}$.

promoter and HIV LTR are more active than the Mo-MuLV LTR in CEM T lymphocytes in expressing the M10 gene in the context of a transient transfection assay.

Inhibition of HIV-1 replication in CEM cells stably transduced with the *trans*-dominant inhibitory mutant gene vectors. The retroviral vectors were packaged as amphotropic viruses with the PA317 cell line and used to stably transduce CEM cells. The CEM cells were grown with G418 to select for populations which were uniformly transduced. Control CEM cells were transduced by the LN vector, which carries only the *neo* gene, and subjected to selection with G418. Transient transfection of these cells with the HLGBRA reporter plasmid showed no inhibition by L-tat_{k41a}-SN and strong inhibition by L-tat_{54ter}-SN and each of the M10 vectors (data not shown), confirming that the mutant genes are expressed.

The abilities of the stably transduced mutant HIV-1 gene vectors to inhibit HIV-1 replication in CEM cells were determined. The vector-transduced CEM cells were inoculated with the HIV_{IIIB} strain of HIV-1 at a low MOI which had been predetermined to produce detectable viral growth over 1 to 2 weeks. HIV-1 replication was monitored by using an ELISA to measure the amount of $p24^{gag}$ protein released into the culture supernatant. The results shown are typical of at least three separate experiments.

In the control cells, either parental, nontransduced CEM cells or CEM cells transduced with the LN vector and selected with G418, the level of p24 typically increased by the seventh or ninth day after addition of HIV-1 and remained elevated thereafter (Fig. 5 and Table 1). CEM cells transduced by L-tat_{k41a}-SN showed a pattern of HIV-1 growth similar to that of the control cells, consistent with the lack of inhibition by this mutant *tat* gene seen in the transient transfection assays (data not shown). Surprisingly, CEM cells transduced with L-tat_{54ter}-SN also were not protected from HIV-1 infection, which replicated in these cultures at the same rate as in the control cells (Fig. 5A). A lack of inhibitory effects on HIV-1 replication by stably transduced L-tat_{54ter}-SN was seen in four separate studies with three separate pools of transduced CEM cells, including the same cells which showed decreased expression of the HLGBRA plasmid in the transient transfection assay.

LN-HL-M10-transduced CEM cells showed a slightly delayed outgrowth of HIV-1, but by day 15 postinoculation, a high level of p24 was present (Fig. 5B). In contrast, CEM cells stably transduced by either the L-M10-SN or the LNC-M10 vector showed no viral growth through the 20 days of the cultures.

To discriminate the relative potencies of inhibitory activities of these latter vectors, the transduced CEM cells were challenged with a fivefold higher inoculum of HIV-1 virus (Fig. 5C). In three separate experiments, late HIV-1 viral growth was seen in the CEM cells containing the L-M10-SN vector, but no virus was detected from cells transduced by

TABLE 1. Effects of stably transduced mutant *tat* and *rev* genes on infection of CEM human T lymphocytes by HIV-1

Vector	Day of HIV-1 outgrowth[a] at:						
	1× virus				5× virus		
None	15		7	9	9		7
LN	15	13	7	9	9	10	7
L-tat_{k41a}-SN	15	10					
L-tat_{54ter}-SN	15	10					
L-M10-SN		>20	>17	>20	17	16	9
LNC-M10		>20	>17	>20	>17	>20	>20
LN-HL-M10			9	15			5

[a] Outgrowth was determined by an ELISA measurement of $p24^{gag}$ protein levels exceeding an optical density of 0.2. at 450 nm.

LNC-M10 (Table 1). These results again suggest that the CMV promoter is more effective for expressing inhibitory levels of M10 in these human T cells than is the Mo-MuLV LTR.

As an additional measure of the inhibitory activity of the *trans*-dominant mutant gene vectors, the stably transduced cells were challenged with a different strain of HIV-1. Virus derived from pNL43 was used to infect the transduced cells at an MOI of 0.1 50% TCID per cell (Fig. 5D). Viral outgrowth was more rapid with this inoculum, but the results confirmed those obtained with HIV_{IIIB}. The truncated *tat* vector, L-tat_{54ter}-SN, and the M10 vector controlled by the HIV LTR, LN-HL-M10, did not inhibit proliferation of pNL43. L-M10-SN partially inhibited HIV-1 production and LNC-M10 markedly suppressed viral growth over the time of observation.

DISCUSSION

Gene therapy for AIDS represents a novel approach which may potentially confer resistance to HIV-1 infection by mechanisms which are complementary to current pharmacologic approaches. By preventing production of viral RNA and proteins, viral replication and spread may be suppressed. It is possible that cells which express HIV-1 resistance genes may have a selective survival advantage over normal cells and thereby increase in frequency as nonresistant cells are eliminated.

Among the various gene products which have been reported to inhibit HIV-1 in cell culture models, the inhibitory effects of antisense RNA and catalytic ribozymes have been partial or transient (10, 11, 25, 30, 33). Sullenger et al. (31) have shown strong, persistent inhibition by using vectors producing so-called TAR decoys, which are RNA molecules from the *tat* binding region. Our data presented here represent a direct comparison between several putative *trans*-dominant mutant inhibitory HIV-1 genes. Of the three genes we examined, the M10 mutant of *rev* demonstrated the highest level of efficacy. Retroviral vectors expressing M10 consistently inhibited both the surrogate reporter expression plasmid in the transient transfection assay and HIV-1 replication when stably transduced into CEM cells.

In contrast to the excellent results with the *rev* mutant M10, the *tat* gene containing an amino acid substitution (alanine for lysine at amino acid 41) did not display any *trans*-dominant inhibitory activity and, in fact, produced mild *trans*-activation, as has been reported previously with a transient transfection system (32). The truncated *tat* mutant (with a termination codon at amino acid 54) showed significant *trans*-dominant inhibitory activity in the transient transfection assays with the reporter plasmid. However, T cells stably transduced by the L-tat_{54ter}-SN vector were not resistant to HIV-1 replication. Because *tat* acts on the HIV-1 LTR in a positive feedback loop to increase its own expression, a *trans*-dominant *tat* inhibitor may be readily overcome by the wild-type *tat* expressed by the virus. Although these results have only been demonstrated with this specific *trans*-dominant *tat* mutant, they suggest that genetic inhibitors of HIV-1 targeted against the *tat* gene may not be effective at completely suppressing viral replication.

Our comparison suggests that the activity of the *rev* gene may constitute a more useful target. Production of HIV-1 virion proteins shows strict dependence on *rev* expression above a critical threshold level (13, 24). *rev* negatively regulates its own expression from HIV-1, so that leakage of a small amount of wild-type *rev* activity should not trigger a positive feedback loop (6). The lack of positive feedback by *rev* on its own expression may allow complete suppression of HIV-1 replication to be achieved by only partially reducing wild-type *rev* activity to a level below its threshold.

The effectiveness of the M10 *trans*-dominant inhibitory mutant gene allows comparisons between vector constructs with different promoters to be made. Vectors in which M10 is under transcriptional control of either the Mo-MuLV LTR or the human CMV promoter produced strong inhibition of reporter expression and HIV-1 replication at the lower dose of HIV-1. Our comparison of these two constructs in response to higher inocula of HIV-1 showed that the LNC-M10 vector produced more complete and persistent inhibition of HIV-1 than did the L-M10-SN vector. However, we have found that vectors derived from LNCX are typically produced in titers that are 10 to 50 times lower than those derived from LXSN, associated with lower levels of the full-length genomic transcript from LNCX derivatives (unpublished observation). Because efficient transduction of primary hematopoietic cells requires high-vector titers, L-M10-SN may prove to be more useful for clinical purposes. The lower intrinsic inhibitory activity of L-M10-SN may still be sufficient to protect T lymphocytes from productive infection in vivo. The most relevant comparison between these vector constructs will be in primary human T lymphocytes and in vivo, such as with murine gene transfer in bone marrow transplantation models, examining expression in mature T lymphocytes produced from hematopoietic stem cells.

Notably, the vector in which M10 was controlled by the HIV LTR showed disparate results between the transient transfection assay and the HIV-1 challenge of stably transduced cells. Although LN-HL-M10 produced strong inhibition of expression by the reporter plasmid, it failed to significantly block HIV replication. This dichotomy may reflect that either the kinetics of induction of M10 expression

by the *tat* protein produced from the HIV-1 virus occurs too late to effectively inhibit viral replication or that there is poor induction of expression by the HIV-1 LTR promoter in the vector when it is stably incorporated into the cell chromosomes. The discrepancy between the results in the transient reporter plasmid assays and the HIV infection assays with both the truncated *tat* vector and the HIV LTR-controlled M10 vector underscores the need to perform full virologic analysis for accurate assessments of genes to inhibit HIV-1 replication. While transient assays appear to identify vectors which would fail to inhibit HIV-1, they did not guarantee success.

Two recent publications similarly determined that the M10 gene, transduced by retroviral vectors, produces effective blockade of HIV-1 in CEM cells (3, 14). The studies by Bevec et al. (3) and Malim et al. (14) used selected clones of transduced cells, which may lead to bias because of clonal-specific levels of expression. Our results with polyclonal pools of transduced cells better reflect the heterogeneity of expression levels that would be expected in a clinical gene therapy situation and, therefore, strengthen the evidence that the M10 is an effective gene for inhibiting HIV-1 replication.

It will now be necessary to determine whether these effects can be generalized with regard to protection against more clinically relevant strains of HIV-1 in primary human T lymphocytes and monocytic cells. It will also be essential to assess the effects of the toxicity of this gene on normal cellular functions. It is known that *tat* and *rev* do interact with normal cellular proteins, so their constitutive expression in developing cells of the hematopoietic system may interfere with normal differentiation. Current retrovirus mediated gene transfer techniques allow efficient transduction of primary human bone marrow progenitor cells (22), which can then be examined in long-term bone marrow culture for adverse effects of these mutant HIV-1 genes on hematopoiesis.

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