Journal of Virology, Apr. 1997, p. 3048–3053 Vol. 71, No. 4
0022-538X/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Multigene Antiviral Vectors Inhibit Diverse Human Immunodeficiency Virus Type 1 Clades

ALAIN GERVAIX, XINQIANG LI, GÜNTER KRAUS,† AND FLOSSIE WONG-STAAL*

Departments of Medicine and Biology, University of California, San Diego, California

Received 18 July 1996/Accepted 11 December 1996

The chronicity of infection by the human immunodeficiency virus (HIV) calls for therapeutic regimens that offer sustained antiviral effects, such as gene therapy. Recent studies have demonstrated that expression of HIV mutant transdominant proteins, RNA decoys, and ribozymes efficiently inhibited HIV replication. We have previously shown that an RNA decoy (stem-loop II of the Rev response element of HIV type 1 [HIV-1], named SL2) and a ribozyme (Rz) targeting the U5 region of the HIV-1 5′ long terminal repeat (LTR), combined in a fusion molecule, was more efficient in inhibiting HIV-1 replication than the ribozyme or the decoy alone. In this study, we expressed this fusion molecule in a retrovirus-based double-copy vector to obtain higher expression of this molecule. Furthermore, we inserted a sequence internally to drive expression of another fusion molecule with a ribozyme targeting the *env/rev* region linked to SL2 to obtain a triple-copy vector. These multigene antiviral vectors were subsequently transduced or transfected into human CD4$^+$ T cells (Molt-4). Results showed that the translocation of the SL2-Rz cassette from the 3′ to the 5′ LTR occurred in 80% of the transduced cells. The numbers of ribozyme RNA transcripts, estimated by competitive-quantitative reverse transcription (RT)-PCR, were 1.2 × 10^5, 1.2 × 10^4, and 1.5 × 10^3 copies per cell for the triple-copy, double-copy, and single-copy vectors, respectively. Cell challenge with multiple subtypes of HIV-1 (clades A to E) showed commensurate levels of virus inhibition for the three vectors. This study suggests that the combination of multiple anti-HIV genes, such as ribozymes and decoys, targeting multiple sites of HIV RNA and expressed at high levels are promising for the treatment of HIV-1 infection.

Gene therapy has recently emerged as a promising tool for the treatment of genetic diseases, cancer, and chronic infectious conditions such as AIDS (1, 2, 14, 17). Several strategies directly targeting human immunodeficiency virus (HIV) gene expression have been shown to be effective in inhibiting virus replication, and these include intracellular expression of transdominant mutant proteins (10, 11), RNA decoys (3, 19), and ribozymes (16, 27). Ribozymes are small catalytic RNA molecules that can be engineered to specifically bind an RNA target sequence by base pairing and subsequently cleave the target RNA, such as the HIV RNA. Theoretically, ribozymes can bind incoming viral RNA prior to reverse transcription (RT) and integration as well as de novo synthesized mRNA and genomic RNA in the latter stages of viral replication. Our previous studies have documented long-term expression and antiviral effects of anti-HIV type 1 (HIV-1) ribozymes in T-lymphocyte cell lines (24), in primary human peripheral blood lymphocytes (8), and in macrophages derived from ribozyme-transduced CD34$^+$ hematopoietic progenitor cells (28). However, because of the exquisite sequence specificity of ribozymes, the potential for virus mutation and escape is of concern. Our strategy is to combine ribozymes that target different sites on the viral genome and/or ribozyme with other antiviral genes. We have previously shown that an RNA decoy (stem-loop II of the Rev-responsive element [RRE] of HIV-1, named SL2) and a ribozyme (Rz) targeting the U5 region of the HIV-1 5′ long terminal repeat (LTR), combined in a fusion molecule, was more efficient in inhibiting HIV-1 replication than the ribozyme or the decoy alone (25). Combination of multiple strategies (multiple ribozymes and decoys) targeting different sites in the viral RNA at different steps of replication should further decrease the risk of viral escape and increase the antiviral potency, especially if high expression levels of the transgenes are obtained within the target cells.

In this study, we have developed retroviral vectors that achieve (i) high expression of the transgenes, (ii) different mechanisms of inhibition of HIV by using fusion molecules of ribozymes and of an RRE decoy, and (iii) multiple target sites for the ribozymes (U5 region of LTRs and *rev/env* region of HIV-1 RNA). Although we never detected the emergence of resistant virus mutants in vitro even in cells expressing a single ribozyme, we were able to demonstrate the greater antiviral potency for the multigene vectors. Furthermore, we have used phylogenetically diverse strains of HIV-1 (clades A to E) as challenge viruses and showed that the triple-copy vector efficiently inhibited replication of all virus clades tested, even though a single ribozyme or decoy-ribozyme vector allowed breakthrough of some clades. A double-copy vector displayed intermediate antiviral potency.

MATERIALS AND METHODS

Construction of recombinant retroviral vectors. pLNL-6, pMJT, pSL2U5, and pTCAG are all Moloney murine leukemia virus (MoMLV)-based vectors. pMJT is derived from pLNL-6 and carries the HIV-1 5′ leader sequence-specific ribozyme (U5Rz) driven by the internal human tRNA^{Val} promoter as previously described (26). pSL2U5 is a construct containing in the U3 region of the MoMLV 3′ LTR, a fusion molecule done by the sequence of the stem-loop II (SL2) of the HIV-1 RRE and the HIV-1 5′ leader sequence-specific ribozyme (U5Rz) driven by the internal human tRNA^{Val} promoter in antisense direction and derived from pSLMJT (25). To engineer pSL2U5, the SL2-U5Rz cassette of pSLMJT was amplified by PCR (5′-CTAGGCTGACTCCATCGAG-3′ sense primer; 5′-agagatctGCACTATACCAGACAAT-3′ antisense primer). Amplification was performed at 94, 48, and 72°C for 30 s at each temperature for 33 cycles. The purified product (198 bp) was then digested by *Mlu*I and *Bgl*II and inserted into another MoMLV-based vector (p1;19DCABw) just downstream of a tRNA^{Val} promoter cloned in its 3′ LTR. The pSL2U5 plasmid was further

* Corresponding author. Mailing address: Departments of Medicine and Biology, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0665. Phone: (619) 534-4852. Fax: (619) 534-7743. E-mail: fwongstaal@ucsd.edu.

† Present address: Department of Microbiology and Immunology, University of Miami, Miami, FL 33101.

digested with *Cla*I and *Xba*I. This fragment containing the SL2-U5Rz cassette was then inserted in pSLOY-1 cut with the same enzymes. pSLOY-1 is another vector containing the sequences of the fusion molecule of SL2 and of a hairpin ribozyme which targets the *rev/env* coding region of HIV-1 RNA (HXB2; positions 8629 to 8644) (23, 25). This latter plasmid containing an SL2-U5Rz plus an SL2-RevRz was named pTCAG (see Fig. 1). Because the U3 region of the 3′ LTR of retroviruses is duplicated at the 5′ LTR during the step of retrotranscription, the pSL2U5- and pTCAG-based virions should integrate double and triple copies, respectively, of the SL2-Rz cassettes in the cells they infect.

Packaging cell lines and transduction. To generate retroviral particles, plasmid DNA were transfected in the amphotrophic packaging cell line PA317 by the calcium phosphate method as previously described (12). Briefly, subconfluent PA317 cultures were transfected with 15 μg each of calcium phosphate-precipitated pLNL-6, pMJT, pSL2U5, and pTCAG in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 13 μg of hypoxanthine per ml, 3.9 μg of thymidine per ml, 18 ng of aminopterin per ml, and 10% fetal bovine serum in 100-mm dishes and incubated at 37°C. After 48 h, the cells were washed three times with phosphate-buffered saline (pH 7.4, Ca^{2+} and Mg^{2+} free), split 1:10, and selected in G418 (600 μg/ml)-containing DMEM. The virus-containing supernatant of resistant cells, serially diluted, was titered on HT-1080 cells (ATCC CCL 121) by counting the number of colonies after selection by G418. Titers of 1.5×10^6 and 1.0×10^6 CFU/ml for pSL2U5 and pTCAG, respectively, were obtained, and used to infect human $CD4^+$ lymphocyte-derived Molt-4 clone 8 cells (Molt4/8). These cells were subsequently selected in G418 (600 μg/ml)-containing RPMI-1640 medium (GIBCO) supplemented with 1 mM sodium pyruvate, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum. pSL2U5 was also directly transfected in Molt4/8 by calcium phosphate precipitation in order to get only one copy of the pSL2U5 cassette per cell.

Molt4/8 cells stably transduced with pLNL-6, pMJT, pSL2U5, and pTCAG were designated mLNL-6, mMJT, mDC:SL2U5, and mTCAG, respectively. Transfected Molt4/8 by pSL2U5 was named mSC:SL2U5 (DC, TC, and SC, double, triple, and single copy, respectively). Clonal mDC:SL2U5 and mTCAG cell lines were obtained by two rounds of limited dilution cloning.

PCR analysis of transduced cell lines. Ribozyme-encoding sequences were analyzed by PCR. Genomic DNAs from transduced cell lines were extracted with the QIAamp Blood Kit (QUIAGEN) by following the instructions of the manufacturer and analyzed with the following primers (see Fig. 2): A (*1*), 5′-CCCC ACCCGTAGGTGGCAAGCT-3′; B (*Rib2*), 5′-TACCAGGTAATATACCAC-3′; C (*875*), 5′-AGATACAGAGCTAGTTAGCTAA-3′; D (*Rib4*), 5′-CACAC AACAAGAAGG-3′; E (*NL6H5*), 5′-CTAGGCTGACTCCATCGAG-3′; F (*Rib6*), 5′-TAGTTCCTAGAACCA-3′; *Neo 3*, 5′-GATCAAGAGACAGGATG AGG-3′; and *Neo 4*, 5′-CAGCCATGATGGATACTTTC-3′. Conditions for PCR were 94, 58, and 72°C for 1 min at each temperature for 33 cycles. Resulting amplified products were subjected to electrophoresis on a 1.5% low-melting-point agarose gel in Tris-acetate buffer (pH 7.2) and stained with ethidium bromide. Gel images were scanned and color inverted with Adobe Photoshop 3.0.

Ribozyme expression by RT-PCR. Total RNA was extracted from ribozyme-transduced cells or pLNL-6-transduced cells by the guanidine thiocyanate–phenol chloroform extraction method (4) and subsequently treated with DNase I (RQ1 Dnase; Promega). Two hundred and fifty nanograms of total RNA was added to an RT reaction mixture (10 mM Tris-HCl [pH 8.3], 90 mM KCl, 1 mM $MnCl_2$, 200 mM [each] dATP, dGTP, dCTP, and dTTP, 3 U of Tth DNA polymerase) by using a primer which is common for pMJT, pSL2U5, and pTCAG (*Rib2*; 5′-TACCAGGTAATATACCAC-3′) and incubated at 60°C for 20 min. PCR was carried out (94°C, 30 s; 54°C, 30 s; 72°C, 30 s; 33 cycles) with the addition of an opposing-strand primer *Rib4* (5′-CACACAACAAGAAGG-3′) for pLNL-6, pMJT, pSL2U5, and pTCAG or *Rib6* (5′-TAGTTCCTAGAACCA-3′) for the Rev ribozyme of pTCAG. Fifteen microliters of each PCR product was subjected to 3% gel electrophoresis and visualized under a UV lamp after staining with ethidium bromide. Gel image pictures were scanned and color inverted with Adobe Photoshop 3.0.

QC RT-PCR. Total RNA from mSC:SL2U5, mDC:SL2U5, and mTCAG was extracted by the guanidine thiocyanate-phenol chloroform extraction method (4) and subsequently treated with DNase I (RQ1 Dnase; Promega). For quantitative-competitive (QC) RT-PCR, in vitro-transcribed RNA of the U5 ribozyme with a tetraloop substitution (5′-ACA, CAA, CAA, GAA, GGC, AAC, CAG, AGA, AAC, ACA, CG G, ACU, UCG, GUC, CGU, GGU, AUA, UUA, CCU, GGU, A-3′) was used as a competitor RNA as previously described (25). Briefly, total cellular RNA (250 ng) and the competitor RNA, diluted serially to obtain samples from 5 fg to 50 pg, were added to the RT reaction mixture containing 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM $MnCl_2$, 200 mM (each) dATP, dGTP, dCTP, and dTTP, 50 pmol of *Rib2*, and 3 U of Tth DNA polymerase (Promega), final volume, 16 μl. After the RT reaction at 60°C for 20 min, 34 μl of PCR mixture, containing 50 pmol of the primer *Rib4* for mSC:SL2U5 and mDC:SL2U5 or 50 pmol of *Rib4* and 50 pmoles of *Rib6* for pTCAG, and an additional 200 mM (each) dATP, dGTP, dCTP, and dTTP, the PCR was carried out (94°C, 30 s; 54°C, 30 s; 72°C, 30 s; 33 cycles). Fifteen microliters of each PCR product was subjected to electrophoresis on agarose gel (4% low melting point). The expected sizes of the PCR product were 61 and 53 bp for the competitor RNA and the test RNA, respectively. After staining with ethidium bromide, gel images were scanned and color inverted with Adobe Photoshop 3.0.

Challenge of transduced cells with HIV-1 subtypes A to E. Titered stocks of HIV-1 subtypes A, B, C, D, and E, classified according to their envelope (*env*) coding sequence, were kindly supplied by D. Ho, the Aaron Diamond AIDS Research Center, New York, N.Y. The transduced cells mLNL-6, mMJT, mSC:SL2U5, mDC:SL2U5, and mTCAG were incubated under rotation overnight at 37°C with each of these HIV strains at an input multiplicity of infection (MOI) of 0.05 (50% tissue culture infective dose, $200/4 \times 10^3$ cells) in the presence of Polybrene (4 μg/ml) in a total volume of 0.8 ml. The cells were then washed twice with PBS and resuspended in 1 ml of RPMI-1640 medium supplemented with 10% fetal calf serum. From day 6 postinfection, cells were split every 4 to 5 days 1:5 with medium to adjust to a cell concentration of approximately 10^5/ml.

Infection of the cell lines was monitored by HIV-1 p24 antigen levels, determined by antigen capture enzyme-linked immunosorbent assay (ELISA) (Coulter) every 3 to 4 days for 27 days.

RESULTS

Characterization of stable Molt4/8 cells transduced with SL2U5 and TCAG vectors. The structure of the retroviral vectors are shown in Fig. 1 (black lines). The U5 ribozyme and the SL2-ribozyme fusion molecules were all inserted downstream of an internal human $tRNA^{Val}$ promoter (PolIII) in an antisense direction. Insertion of the transcription unit into the 3′ LTR U3 region of the retroviral plasmid will result in the translocation of these cassettes to the proviral 5′ LTR (gray lines) during RT in transduced cells. This double-copy retroviral vector design was first described by Hantzopoulos et al. (6) and should give rise to increased copy number of the transcriptional cassette in transduced cells. However, a recent study suggested that rearrangement and deletion frequently occur in retrovirus-based double-copy vectors (7). In order to analyze the proviral LTR structure, DNA-PCR analyses specific for the 5′ and 3′ LTRs were performed (Fig. 2). To better understand the results in Fig. 2b, we followed the strategy depicted in Fig. 2a. To distinguish between PCR products of the duplicated sequences of the 5′ and 3′ LTR, specific primers for the Rz sequence-encoded cassette and for sequences outside of the LTRs were used. As a control for the DNA preparation and the PCR, genomic DNA was also amplified by using *neo*-specific primers (Fig. 2b, lanes 3, 7, and 11). Results in Fig. 2b showed that the SL2-Rz cassette had been effectively copied from the 3′ to the 5′ LTR in transduced mDC:SL2U5 and mTCAG. Examination of the 5′ LTR revealed a PCR product of the predicted length, either using a specific primer for ribozyme (*B:Rib2*) and a primer (*C:875*) outside of the LTR (lanes 6 and 10) or using a primer binding to the first 22 bases of the LTR (*A:1*) and the same primer outside of the LTR (*C:875*) (lanes 5 and 9). These latter bands showed a difference in size of 220 bp compared to mSC:SL2U5 (lane 1) and correspond to the $tRNA^{Val}$ promoter SL2-Rz cassette in the U3 region of the 5′ LTR. Examination of the 3′ LTR shows a band of bigger size for mTCAG (lane 12) than for mSC:SL2U5 (lane 4) and mDC:SL2U5 (lane 8) because of the presence of the SL2-RevRz cassette. Specific amplification of this cassette by using the primer D (*NL6H5*) and a specific primer for RevRz (*Rib6*) revealed a band of the predicted size (175 bp) (data not shown). We also examined the 5′ LTR of clonal population of transduced cells obtained by two rounds of limited dilution. From 10 different clonal populations, 8 were found positive for the SL2-Rz cassette by using the specific primers *A:1* and *C:875* (Fig. 3) or the primers *B:Rib2* and *C:875* (data not shown) for mTCAG and mDC:SL2U5. These results indicate that with our vector the translocation of the transcriptional cassette from the 3′ to the 5′ LTR occurs at a high rate (80%) after viral retrotranscription. Clones of mTCAG and mDC:SL2U5 containing the SL2-Rz cassettes in the U3 region of 5′ LTR were selected, grown, and used for further experiments.

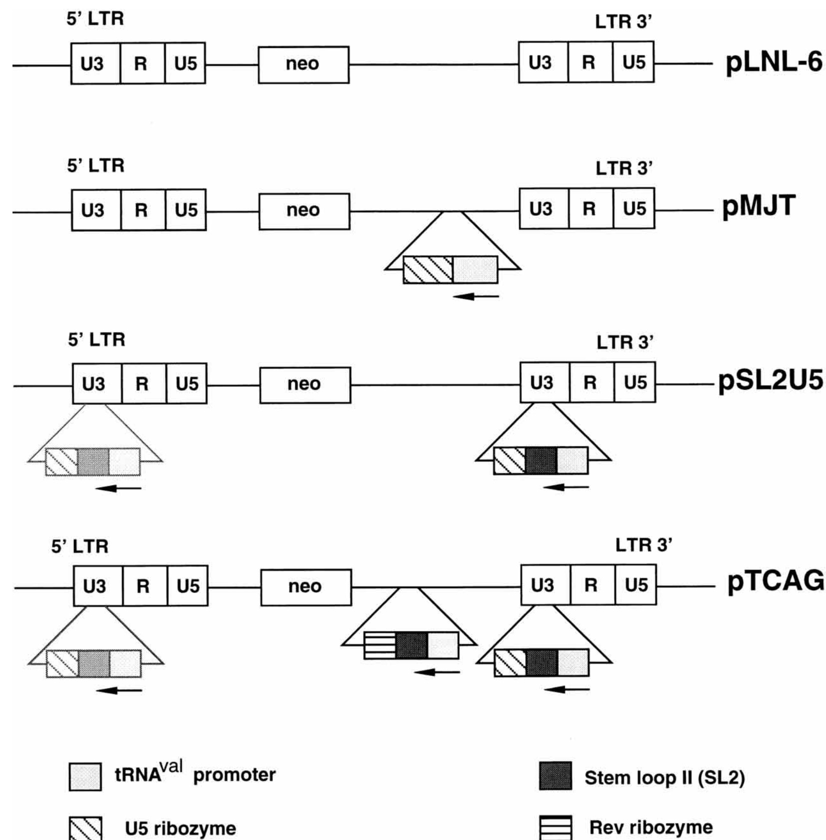

FIG. 1. Structure of the retroviral vectors. pLNL-6 is the parental MoMLV-based retroviral vector. pMJT contains a ribozyme sequence targeting the U5 region of HIV-1 LTR (positions +111/112 from the cap site). pSL2U5 contains the anti-U5 ribozyme and the stem-loop II sequence (SL2) of HIV-1 RRE in the U3 region of the 3′ MoMLV-LTR. Because the U3 region of the 3′ LTR of retroviruses is duplicated at the 5′ LTR during the step of retrotranscription, we expect that the SL2-Rz cassette would also be present at the 5′ LTR after transduction of the target cells (gray bars) and RT. Such a vector is named a double-copy vector. pTCAG is similar to pSL2U5 but contains an additional internal cassette expressing SL2 fused to a ribozyme which targets the *rev/env* coding region of HIV-1 RNA (positions 8629 to 8644 of HXB2). All of these cassettes are driven by a tRNA^{Val} promoter in an antisense direction (arrow).

Expression of the SL2-ribozyme cassette in transduced Molt4/8. After showing by PCR the presence of the SL2-Rz cassettes in transduced Molt4/8, we specifically addressed the question of expression of ribozymes by the tRNA^{Val} promoter using RT-PCR with a primer specific for all hairpin ribozymes (*Rib2*) and another primer specific either for the U5 ribozyme (*Rib4*) or for the Rev ribozyme (*Rib6*) (Fig. 4). The amplified products (expected size, 53 nucleotides) were detected only when the PCR was carried out after retrotranscription. When RT-PCR was carried out with a 5′ primer for SL2 and a 3′ primer for Rz (*Rib2*) we got an amplified product of 120 bp, thus showing that both Rz and RRE decoy sequences were appended after transcription (data not shown). Similar results were obtained with mMJT as previously described (24). Since mDC:SL2U5 and mTCAG carry a double-copy vector (DC: SL2U5) and a triple-copy vector (DC:SL2U5–SL2-RevRz), we might expect the number of copies of the SL2-ribozyme transcripts to be higher in these cell lines than in the single-copy vector-containing cell line mSC:SL2U5. To answer this question, we performed a QC RT-PCR (Fig. 5). The competitor RNA was serially diluted from 50 pg to 5 fg and added to the RT mixture containing 250 ng of total cellular RNA. The amount of competitor RNA that results in equal signal intensities for the amplified product of competitor and test RNA was estimated to be between 10 and 30 fg for the single-copy vector mSC:SL2U5 (lanes 4 and 5), at 50 fg for the double-copy vector mDC:SL2U5 (lane 9), and at 500 fg for the triple-copy TCAG (lane 13). Since the amount of total cellular RNA is generally assessed at 1 mg of RNA/10^5 cells, we estimated that each mSC:SL2U5, mDC:SL2U5, and mTCAG cell was expressing approximately 1.5×10^3, 1.2×10^4, and 1.2×10^5 copies of SL2-Rz, respectively. The estimation for mSC:SL2U5 is comparable to that reported previously (25). Similar results were obtained when comparing a bulk population of cells or the clones we selected. Ribozyme expression persisted over 6 months without any difference in signal intensity of the amplified product over this period (data not shown). The constitutive levels of SL2-Rz expression had no apparent deleterious effect on proliferation of the transduced cells compared with parental Molt4/8 and were indistinguishable with respect to viability with passage every 3 to 5 days for over 6 months.

Challenge of transduced Molt4/8 with HIV-1 subtypes A to E. An important issue of gene therapy for HIV-1 infection is to consider the genetic variability of the virus and to find targets conserved among most of the biologically and geographically diverse isolates. In this study, we examined the ability of the different vectors to inhibit replication of five HIV-1 clades: clade A, mostly found in Central Africa; clade B, mostly found in the United States, Europe, South America, and Thailand; clade C, found in Southern Africa and India; clade D, found in Central Africa; and clade E, found in Thailand and India. An input MOI of 0.05 of the different titered virus stocks was used

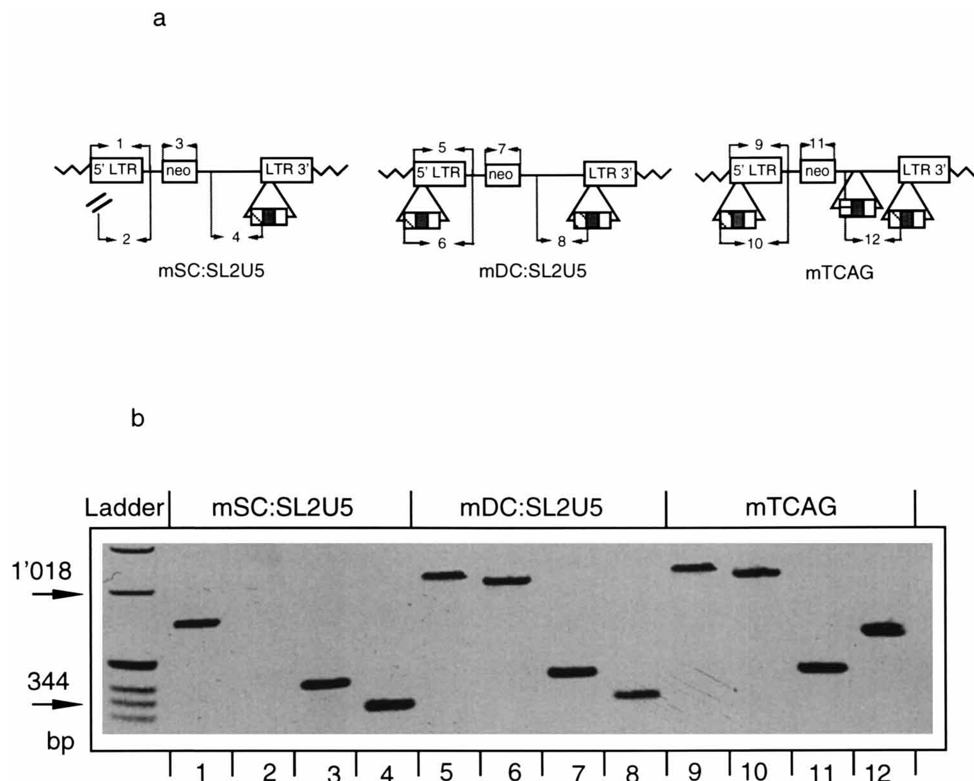

FIG. 2. PCR analysis of retroviral vectors from genomic DNA of transduced Molt4/8. To ensure that the fusion RNA (SL2-Rz) has been duplicated after retrotranscription, we analyzed the 5′ LTR of mDC:SL2U5 and mTCAG by PCR analysis and compared it to mSC:SL2U5 in which no step of retrotranscription has occurred (transfection). (a) Strategy used and the expected PCR fragments (not to scale). The numbers 1 to 12 refer to the lanes in panel b. (b) PCR results. A primer binding downstream of the 5′ LTR (*C:875*) was used with a primer specific for the U5 ribozyme (*Rib2*) (lanes 2, 6, and 10) or with a primer binding the first 22 nucleotides of the LTRs (*A1*) (lanes 1, 5, and 9). As an internal control of the DNA preparation, amplification of the *neo* gene was also performed (lanes 3, 7, and 11). Analysis of the 3′ LTR was performed using a primer upstream of the 3′ LTR (*D:NL6H5*) and a specific primer for the U5 ribozyme (*Rib4*) (lanes 4, 8, and 12).

to challenge mMJT, mSC:SL2U5, and clonal populations of mDC:SL2U5 and mTCAG. About 2.0 to 2.5 × 10^4 CD4 receptors are known to be expressed at the surface of this cell line. Production of p24 antigen was monitored every 3 to 4 days for 27 days (Fig. 6). The overall results showed that while virus replication was inhibited in all Rz- or SL2-Rz-expressing cell lines compared to the cells transduced with the parental vector LNL-6 for all HIV-1 clades tested, the degree of inhibition directly correlated with the number of therapeutic genes and/or levels of transgene expression. Inhibition was obtained in mTCAG > mDC:SL2U5 > mSC:SL2U5 > mMJT for all clades tested.

DISCUSSION

Gene therapy for HIV infection has progressed from preclinical studies to phase 1 clinical evaluation. Woffendin et al. (22) have conducted the first study, reinfusing $CD4^+$ cells of HIV^+ subjects after ex vivo transfection with a gene expressing a transdominant mutant Rev protein (Rev M10) and showed that these genetically altered cells exhibited a survival advantage compared to $CD4^+$ cells transfected with a control vector. These first results encourage further efforts to develop gene therapy for clinical application. Because of the constant selection and generation of HIV genotypes with altered epitopes (quasispecies) and biologic properties that contribute to resis-

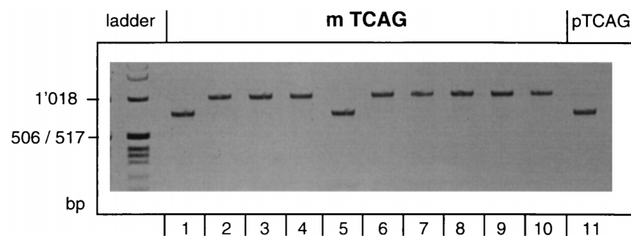

FIG. 3. Analysis of the 5′ LTR of clonal populations of mTCAG. Ten clones of mTCAG were obtained by two rounds of limited dilution. Examination of the proviral 5′ LTR was from genomic DNA by using the set of primers *A1* and *C(875)* (lanes 1 to 10). The plasmid pTCAG was used as control (lane 11).

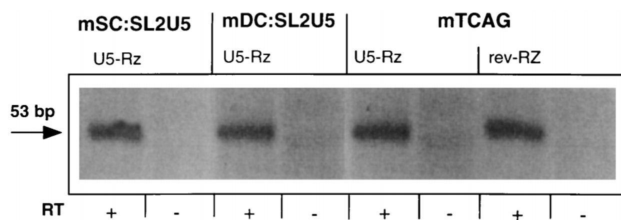

FIG. 4. Expression of ribozyme in transduced Molt4/8. The expression of ribozyme RNA was examined by PCR combined with (+) or without (−) RT. For U5-Rz the set of specific primers used was *Rib2* and *Rib4*, and for Rev-Rz the set of primers was *Rib2* and *Rib6* (refer to Materials and Methods for the exact sequence of these primers). Ten microliters of the PCR products was loaded on 3% low-melting-point agarose and stained with ethidium bromide.

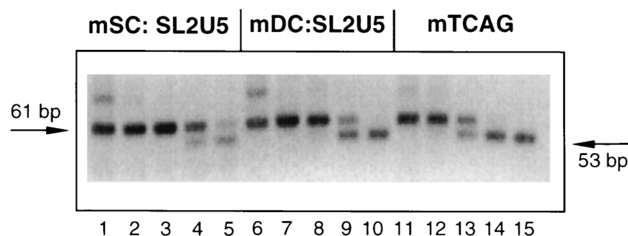

FIG. 5. QC RT-PCR. Two hundred and fifty nanograms of total cellular RNA extracted from mSC:SL2U5 (lanes 1 to 5), mDC:SL2U5 (lanes 6 to 10), and mTCAG (lanes 11 to 15) was subjected to RT-PCR amplification in the presence of known amounts (lanes 1 to 5, 6 to 10, 11 to 15, 50 pg, 5 pg, 500 fg, 50 fg, and 5 fg, respectively) of competitor RNA (61 bp). Ten microliters of each PCR product was loaded on a 4% low-melting-point agarose gel and stained with ethidium bromide. The image of the gel was scanned and color inverted with Adobe Photoshop 3.0. The amount of competitor RNA that results in equal signal intensity for the amplified product of competitor and test RNA was estimated to be between 10 and 30 fg for the single-copy vector mSC:SL2U5 (lanes 4 and 5), at 50 fg for the double-copy vector mDC:SL2U5 (lane 9), and at 500 fg for the triple-copy TCAG (lane 13).

tance to immunologic or chemotherapeutic interventions, the targets for gene therapy should be multiple and used in combination to decrease the risk of viral escape. In this study, we combined in the same vector different mechanisms of HIV inhibition (ribozymes and decoy) targeting multiple vital substrates of HIV (two sites on the RNA and Rev protein). To obtain higher expression of the transgenes, we inserted our cassette into the 3′ LTR of a retroviral vector to get two copies after integration in the host cells as described by Sullenger et al. (20). In contrast to a recent study in which genetic rearrangements impaired the function of a double-copy vector (7), we found a high efficiency of translocation of the transcriptional cassette from the 3′ to the 5′ LTR after viral retrotranscription. Examination of individual cell clones by PCR with specific primers for the U5 ribozyme revealed that about 80% of transduced cells carried the transgene in both LTRs. A likely explanation for the difference in our results and that of Junker et al. (7) is that in our construct, no direct repeat sequence flanking the transcriptional cassette is present, since genetic rearrangements are more likely to occur during retrotranscription among repeated homologous sequences (15). To further investigate the expression of the transcriptional cassettes in triple-, double-, and single-copy vectors, we performed a QC RT-PCR. Similar to a previous study, about 1,500 copies per cells were estimated in cells containing only one copy of the SL2-Rz cassette (25), whereas 1.2×10^4 and 1.2×10^5 copies per cell were calculated in double-copy and triple-copy transduced cells, respectively. The disproportionate increase in the number of transcripts in the double- and triple-copy vector-expressing cells is likely due to the close proximity of the PolIII promoters which might enhance the activity of the polymerase. Thompson et al. also showed that modifications of the secondary structure of tRNA-ribozyme transcripts can improve accumulation and activity of ribozymes up to 100 times (21). The predicted secondary structure energy of the $tRNA^{Val}$ SL2-RZ transcript is −120 and −72 kcal/mol for the same molecule without the SL2 sequence. Addition of the SL2 sequence at the 5′ end of the ribozyme is then likely to improve the general molecule stability and to allow a longer half-life

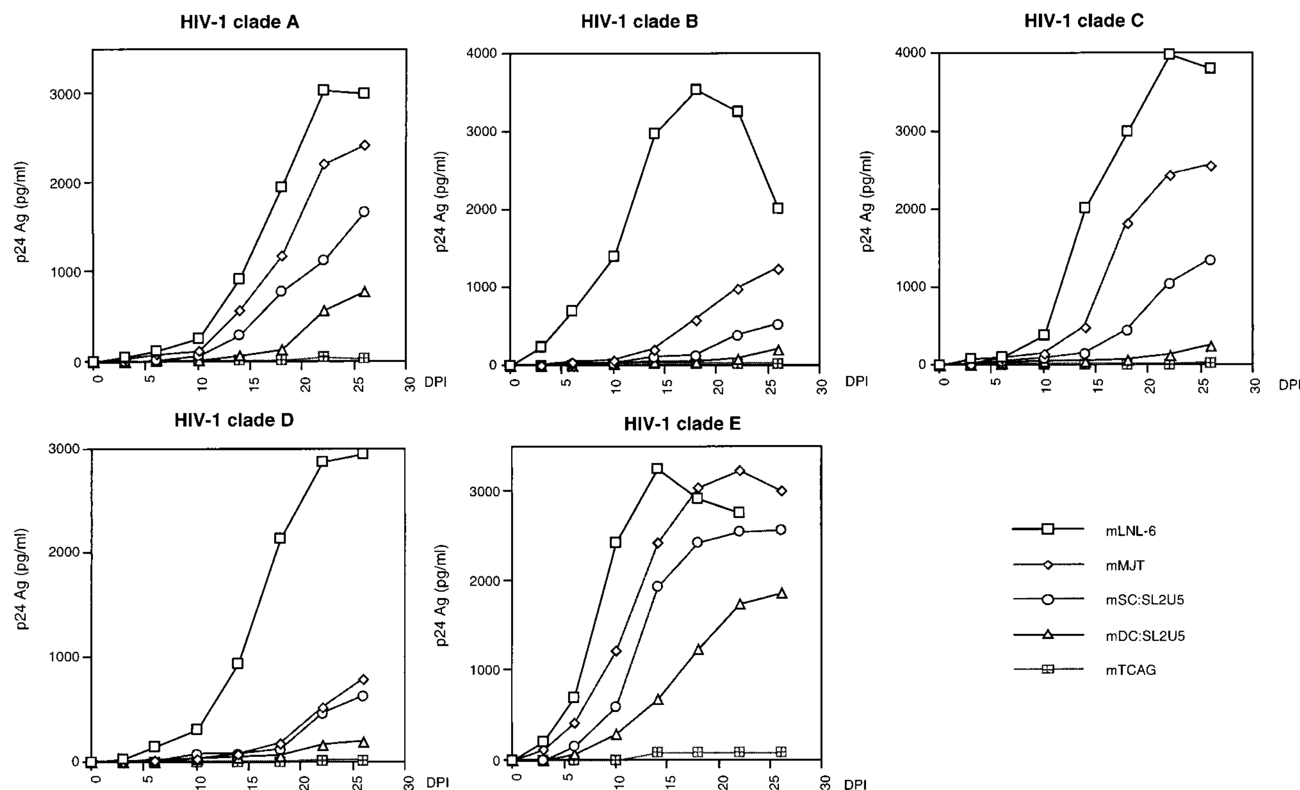

FIG. 6. Inhibition of expression of p24 antigen from different subtypes (A to E) of HIV-1. mLNL-6 (□), mMJT (◇), mSC:SL2U5 (○), mDC:SL2U5 (△), and mTCAG (⊞) were challenged with HIV-1, clades A to E, at an input MOI of 0.05. Small aliquots of the culture cell supernatant were collected every 3 to 4 days and the level of p24 antigen was determined by HIV-1 antigen capture ELISA (Coulter). These experiments have been repeated twice with similar results. DPI, days postinfection.

within the cell. The overall goal of these multiple gene vectors is to effectively inhibit long-term viral replication of geographically and phylogenetically diverse strains of HIV. HIV-1 isolates obtained worldwide have been grouped into clades, based on their envelope-coding sequences. At present, the majority of HIV-1 isolates fall into one major group subdivided into eight clades, A to H. In addition, a few vastly distant isolates are assigned to group O (for outliers). A recent study suggested that different clades may have different target cell tropism and therefore potentially different routes of transmission (18). For example, clades C and E infect Langerhans' cells found in the epithelium and may be more efficiently transmitted through heterosexual contact, while clade B is more T-cell tropic and is transmitted primarily through blood or blood products. Our results in Fig. 6 showed that over 4 weeks, cells transduced with the triple-copy vector were completely inhibited for the replication of HIV clades A to E, whereas cells transduced by all the other vectors were only partly inhibited. In view of the target sequence specificity of ribozymes, we compared the consensus sequences of HIV-1 clades A, B, C, D, and E at the target sites for the U5 and Rev ribozymes (13a). Interestingly, the target site for the U5 ribozyme is very well conserved among clades A, B, and D and not available for clades C and E. However, clade A has 2 nucleotides at the 3′ end of the binding site that do not match with the U5 ribozyme sequence that may destabilize the ribozyme/substrate complex. This may explain why mMJT is only marginally resistant to a clade A HIV-1 (Fig. 6). Greater inhibition was seen in mSC:SL2U5, confirming our previous results of the synergistic action of the ribozyme and the decoy (25). The mDC:SL2U5 cells resisted HIV infection better than mSC:SL2U5 for all clades tested. These cells expressed the same SL2-U5Rz cassette but at a 10-fold higher level in mDC:SL2U5 than in mSC:SL2U5. The TCAG vector, which exerted the greatest antiviral effects, expressed an SL2-RevRz cassette in addition to the SL2-U5Rz cassette. The cleavage site of RevRz is conserved among HIV-1 clades A, B, and C but not in clades D and E. Therefore, its greater potency for all clades is probably due to the high number of copies of the transgenes expressed, thus enhancing the activity of the U5Rz as well as the decoy effect, which is less susceptible to divergence of the HIV genome. In conclusion, these data show that continuous improvement of vector construction to achieve high levels of expression of a combination of antiviral genes can effectively inhibit a wide spectrum of HIV-1 clades and potentially eliminate the possibility of virus escape. These considerations are essential for the application of gene therapy to HIV-infected patients.

ACKNOWLEDGMENTS

We thank D. Ho for kindly providing the titered HIV clades A to E.
This work was supported in part by the NIH SPIRAT award and NIH grant DK49618 to F.W.-S. and the Center for AIDS Research award to UCSD. A.G. is supported by a Swiss National Science Foundation fellow training grant.

REFERENCES

1. **Bordignon, C., L. D. Notarangelo, N. Nobili, G. Ferrari, G. Casorati, P. Panina, E. Mazzolari, D. Maggioni, C. Rossi, P. Servida, A. G. Ugazio, and F. Mavilio.** 1995. Gene therapy in peripheral blood lymphocytes and bone marrow for ADA-immunodeficient patients. Science **270:**470–475.
2. **Bridges, S. H., and N. Sarver.** 1995. Gene therapy and immune restoration for HIV disease. Lancet **345:**427–475.
3. **Chang, H. K., R. Gendelman, J. Lisziewicz, R. C. Gallo, and B. Ensoli.** 1994. Block of HIV-1 infection by a combination of antisense tat RNA and TAR decoys: a strategy for control of HIV-1. Gene Ther. **1:**208–216.
4. **Chomczynski, P., and N. Sacchi.** 1987. Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Ann. Biochem. **162:**156–159.
5. **Dougherty, J. P., and H. M. Temin.** 1988. Determination of the rate of base-pair substitution and insertion mutations in retrovirus replication. J. Virol. **62:**2817–2822.
6. **Hantzopoulos, P. A., B. A. Sullenger, G. E. Ungers, and E. Gilboa.** 1989. Improved gene expression upon transfer of the adenosine transaminase minigene outside the transcriptional unit of a retroviral vector. Proc. Natl. Acad. Sci. USA **86:**3519–3523.
7. **Junker, U., E. Boehnlein, and G. Veres.** 1995. Genetic instability of a MoMLV-based antisense double-copy retroviral vector designed for HIV-1 gene therapy. Gene Ther. **2:**639–646.
8. **Leavitt, M. C., M. Yu, O. Yamada, G. Kraus, D. Looney, E. Poeschla, and F. Wong-Staal.** 1994. Transfer of an anti-HIV-1 ribozyme gene into primary human lymphocytes. Hum. Gene Ther. **5:**1115–1120.
9. **Lee, T. C., B. A. Sullenger, H. F. Gallardo, G. E. Ungers, and E. Gilboa.** 1992. Overexpression of RRE-derived sequences inhibit HIV-1 replication in CEM cells. New Biol. **4:**66–74.
10. **Liu, J., C. Woffendin, Z. H. Yang, and G. J. Nabel.** 1994. Regulated expression of a dominant negative form of Rev improves resistance to HIV replication in T cells. Gene Ther. **1:**32–37.
11. **Malim, M. H., S. Boenhlein, J. Hauber, and B. R. Cullen.** 1989. Functional dissection of the HIV-1 Rev trans-activator-derivation of a trans-dominant repressor of Rev function. Cell **58:**205–214.
12. **Maniatis, T., E. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Miller, A. D.** 1992. Human gene therapy comes of age. Nature **357:**455–460.
13a. **Myers, G., B. Korber, S. Wain-Hobson, K.-T. Jeang, L. E. Henderson, and G. N. Pavlakis.** 1994. Human retroviruses and AIDS. Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, N.Mex.
14. **Ojwang, J. O., A. Hampel, D. L. Looney, F. Wong-Staal, and J. Rappaport.** 1992. Inhibition of human immunodeficiency virus type 1 expression by a hairpin ribozyme. Proc. Natl. Acad. Sci. USA **89:**10802–10806.
15. **Rhode, B. W., M. Emerman, and H. M. Termin.** 1987. Instability of large direct repeats in retrovirus vectors. J. Virol. **61:**925–927.
16. **Sarver, N., E. M. Cantin, P. S. Chang, J. A. Zaia, P. A. Ladne, D. A. Stephens, and J. J. Rossi.** 1990. Ribozyme as potential anti-HIV-1 therapeutic agents. Science **247:**1222–1225.
17. **Snyder, D. S., Y. Wu, J. L. Wang, et al.** 1993. Ribozyme-mediated inhibition of bcr-abl gene expression in a Philadelphia chromosome-positive cell line. Blood **82:**600–605.
18. **Soto-Ramirez, L. E., B. Renjifo, M. F. McLane, R. Marlink, C. O'Hara, R. Sutthent, C. Wasi, P. Vithayasai, V. Vithayasai, C. Apichartpiyakul, P. Auewarakul, V. Pena Cruz, D. S. Chui, R. Osathanondh, K. Mayer, T. H. Lee, and M. Essex.** 1996. HIV-1 Langerhans' cell tropism associated with heterosexual transmission of HIV. Science **271:**1291–1293.
19. **Sullenger, B. A., H. F. Gallardo, G. E. Ungers, and E. Gilboa.** 1990. Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. Cell **63:**601–603.
20. **Sullenger, B. A., H. F. Gallardo, G. E. Ungers, and E. Gilboa.** 1991. Analysis of trans-acting response decoy RNA-mediated inhibition of human immunodeficiency virus type 1 transactivation. J. Virol. **65:**6811–6816.
21. **Thompson, J. D., D. F. Ayers, T. A. Malmstrom, T. L. McKenzie, L. Ganousis, B. M. Chowrira, L. Couture, and D. T. Stinchcomb.** 1995. Improved accumulation and activity of ribozymes expressed from a tRNA-based RNA polymerase III promoter. Nucleic Acids Res. **23:**2259–2268.
22. **Woffendin, C., U. Ranga, Z. Y. Yang, and G. Nabel.** 1996. Expression of a protective gene prolongs survival of T-cells in human immunodeficiency virus-infected patients. Proc. Natl. Acad. Sci. USA **93:**2889–2894.
23. **Yamada, O., G. Kraus, M. C. Leavitt, M. Yu, and F. Wong-Staal.** 1994. Activity and cleavage site specificity of an anti-HIV-1 hairpin ribozyme in human T cells. Virology **205:**121–126.
24. **Yamada, O., M. Yu, J. K. Yee, G. Kraus, D. Looney, and F. Wong-Staal.** 1994. Intracellular immunization of human T cells with a hairpin ribozyme against human immunodeficiency virus type 1. Gene Ther. **1:**38–45.
25. **Yamada, O., G. Kraus, L. Luznik, M. Yu, and F. Wong-Staal.** 1996. A chimeric human immunodeficiency virus type 1 (HIV-1) minimal Rev response element-ribozyme molecule exhibits dual antiviral function and inhibits cell-cell transmission of HIV-1. J. Virol. **70:**1596–1601.
26. **Yu, M., J. A. Ojwang, O. Yamada, A. Hampel, J. Rappaport, D. Looney, and F. Wong-Staal.** 1993. A hairpin ribozyme inhibits expression of diverse strains of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **90:**6340–6344.
27. **Yu, M., E. Poeschla, and F. Wong-Staal.** 1994. Progress towards gene therapy for HIV infection. Gene Ther. **1:**13–26.
28. **Yu, M., M. C. Leavitt, M. Maruyama, D. Young, A. D. Ho, and F. Wong-Staal.** 1995. Intracellular immunization of human fetal cord blood stem/progenitor cells with a ribozyme against human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **92:**699–703.