Inhibition of Human Immunodeficiency Virus Type 1 in Human T Cells by a Potent Rev Response Element Decoy Consisting of the 13-Nucleotide Minimal Rev-Binding Domain

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Intracellular immunization is an anti-viral gene therapy strategy based on the introduction of DNA templates into cells to stably express genetic elements which inhibit viral gene expression and replication. We have recently developed an intracellular immunization strategy for human immunodeficiency virus (HIV) infection that uses RNA decoys. RNA decoys are short RNA oligonucleotides corresponding to the HIV *trans* activation response element (TAR) or Rev response element (RRE) sequences, which function by inhibiting the binding of the HIV regulatory proteins Tat and Rev to the authentic HIV RNA TAR and RRE regions, respectively. In this report we describe the characterization of potent RRE decoys containing the minimal 13-nucleotide primary Rev binding domain of the RRE. Using an improved tRNA cassette to express high levels of RRE transcripts in CEM cells, we found that this new generation of minimal RRE decoys were more potent inhibitors of HIV in isolated cell lines than previously described TAR or RRE decoys. CEM cells expressing RRE decoys exhibited diminished Rev function in cotransfection assays, confirming the specificity of inhibition of HIV by RRE decoys and indicating that the 13-nucleotide minimal Rev binding domain defined by using *in vitro* binding studies also binds Rev *in vivo*. Significant differences in the degree of HIV inhibition between individual CEM cell lines transduced with RRE decoy vectors which were not due to sequence alterations in the tRNA-RRE DNA template, differences in RRE decoy expression level, or endogenous variations in the resistance of CEM clonal cell lines to HIV were observed. In order to evaluate the efficacy of RRE decoys in a more realistic fashion than by comparison of individual clonal cell lines, polyclonal populations of transduced CEM cells were infected with HIV. By using a novel flow cytometric method for quantitating intracellular p24 expression, one version of the RRE decoys tested in this study was found to be capable of durably protecting polyclonal populations of CEM cells from HIV.

The terms “pathogen-derived resistance” and “intracellular immunization” were coined to describe an experimental strategy designed to render cells resistant to human immunodeficiency virus (HIV) replication by introducing DNA templates (i.e., resistance genes) into HIV-susceptible cells that will inhibit the function of one or several essential viral genes and hence inhibit or prevent viral replication (3, 40, 41). Since HIV, the etiological agent of AIDS, infects hematopoietic cells, insertion of resistance genes into such cells could reduce the spread of virus in the patient.

A number of strategies for inhibiting HIV replication have been proposed and tested in tissue culture settings. Broadly, one can distinguish between protein-based inhibitors, such as transdominant mutant forms of viral protein, intracellularly expressed antibodies, and other substances, and RNA-based inhibitors, which include antisense RNA, ribozymes, and RNA decoys. (For a more complete discussion of various inhibition strategies, see references 17 and 53.) Compared with protein-based inhibitors, RNA-based inhibitors are not likely to be as immunogenic, they may be easier to express at high levels, and they could prove to be more specific and therefore less likely to interfere with normal cellular functions. Antisense RNA and

ribozymes are highly specific and can be targeted to many regions of the HIV genome. However, a potential limitation in their use stems from the propensity of HIV to generate variant strains which could be resistant to inhibition (i.e., escape mutants).

RNA decoys exploit unique regulatory circuits operating in the course of HIV replication. Tat and Rev are two key regulatory gene products which activate HIV gene expression by binding to specific regions of the nascent viral RNA, termed the *trans* activation response element (TAR) and the Rev response element (RRE), respectively (12, 37). The RNA decoy strategy is based on expression of short RNA transcripts corresponding to the TAR or the RRE which will compete for the binding of Tat or Rev to their physiological targets on the viral RNA. Unlike antisense RNA or ribozymes, RNA decoys may be less likely to be affected by HIV variability. The RRE and the TAR are highly conserved sequences, and mutations in Tat or Rev that will inhibit binding to TAR or RRE decoys may also inhibit binding to their physiological targets. A specific concern associated with the use of TAR or RRE decoys, however, is that cellular factors associate with these structures and consequently may be sequestered in cells over-expressing decoy RNAs (15, 16, 33, 38, 43, 44, 51). While we and others have previously shown that cells expressing TAR-containing transcripts confer varying degrees of resistance to HIV replication (18, 28, 45), attempts to dissociate TAR decoy function from cellular factor binding by introducing mutations in the loop sequence of TAR have been unsuccessful (46).

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The early gene product Rev, which controls the expression of the structural genes of HIV type 1 (HIV-1), exerts its function through binding to the RRE and facilitates the nuclear export of unspliced or partially spliced viral transcripts (12, 37). The RRE consists of about 234 nucleotides (nt) and can assume a highly complex secondary structure consisting of a central stem and five stem-loop structures. Initially it was shown that sequences within stem-loop II form the major Rev binding domain (9, 10, 13, 21, 23, 25, 32, 35). Subsequent fine mapping performed by using *in vitro* binding studies has pinpointed the primary binding site of Rev to a 13-nt region at the base of stem-loop IIB which forms a purine-rich "bubble," also termed stem-loop D (4, 20, 49). This region was also shown to bind Rev *in vivo* (48).

We have previously shown that expression of a 45-nt transcript encompassing stem-loops IIA and IIB in CEM cells exerts a significant inhibitory effect on HIV-1 replication, albeit the extent of inhibition was less than that seen with TAR decoys (27). Bevec et al. have shown that expression of multiple copies of the HIV RRE was highly effective in preventing HIV replication in CEM cells (6). In this report, we describe the characterization of highly potent RRE decoys containing only the 13-nt minimal Rev binding domain. A potentially important advantage to using this short RRE decoy is that while cellular factors which bind to other regions of the HIV RRE (44, 51) have been described, no cellular factors which bind to this short sequence have been identified. Consequently, this decoy may be safer than alternative inhibitors used in HIV intracellular immunization strategies, whose function may involve binding to physiologic cellular factors.

Using a modified tRNA expression system to express high levels of RRE decoy transcripts in CEM cells, we found that this new generation of RRE decoys are more potent than the previously described RRE and TAR decoys and that they can withstand extensive viremia in the culture. We also describe a highly informative flow cytometric method for measuring HIV expression and replication *in situ* in order to eliminate some of the uncertainties encountered when determining the efficacy of intracellular immunization strategies in the case of polyclonal populations by measuring extracellular p24 concentration.

MATERIALS AND METHODS

Cells and viruses. The HIV-1 strain used in this study was the ARV-2 isolate propagated in HUT 78 cells (39) and provided by C. Cheng and J. Levy. CEM SS which was provided by P. Nara is a CD4⁺ human T-lymphocyte cell line which is highly susceptible to infection with HIV-1, including the ARV-2 isolate (34). CEM SS was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone).

Construction of retrovirus vectors and transduction of CEM cells. The Moloney murine leukemia virus-based N2 retrovirus vector (2), which contains the bacterial neomycin resistance gene (*neo*), was modified by the insertion of a 52-bp polylinker (containing the unique restriction sites 5'-*Apa*I-*Bgl*II-*Sna*BI-*Sac*II-*Mlu*I-3') into the *Nhe*I restriction site present in the U3 region of the 3' long terminal repeat (LTR). This polylinker-modified N2 vector was designated N2A (19). A 500-bp DNA fragment containing the Δ 3-2 mutant tRNA^{Met} gene (1) was excised from plasmid pH2D with the restriction enzymes *Stu*I and *Bam*HI and cloned into the *Sna*BI site in the 3' LTR of the N2A vector. An oligonucleotide encoding a *Bam*HI site and a polymerase III transcription termination signal (Ter) was inserted downstream of the tRNA, between the *Sac*II and *Mlu*I sites of the polylinker. This vector was called DCT. An oligonucleotide encoding a *Sac*II site and a stem-loop structure

(hpI) was cloned between *Sac*II and *Bam*HI. A second oligonucleotide, harboring another stem-loop structure (hpII), was cloned into the *Bam*HI site, and a third oligonucleotide (L), encoding *Sac*II and *Bam*HI sites in the middle of the oligonucleotide, was inserted between the *Sac*II and *Bam*HI sites. An oligonucleotide encoding the minimal RRE sequence (mRRE) shown in Fig. 1A was cloned between the *Sac*II and *Bam*HI sites of the modified DCT vector described above to generate DNA construct DCT-RRE4. Thus, in the vector DCT-RRE4 the oligonucleotide L was split into LI and LII as shown in Fig. 1B. To construct the DCT-RRE5 vector, which lacks the 5' stem-loop (hpI) and LI sequences, a 3-kbp *Sac*II-*Nde*I DNA fragment containing the LII, hpII, and polymerase III termination sequences was cloned into *Sac*II and *Nde*I sites of the DCT vector, and the mRRE oligonucleotide was cloned into the *Sac*II and *Bam*HI sites of the resulting vector. As shown in Fig. 1B, the resulting DNA construct, DCT-RRE5, lacked both hpI and LI.

The sequences of the oligonucleotides described above were as follows (the polymerase III termination signal and the restriction sites referred to in the text are in bold letters): Ter, 5'-GGTGGATCCGGT^{TTTTT}TCGCA-3'; hpI, 5'-TTAAGCTTGCCTAACTGTGCAAGCTTAAGCGGTACCGCGGAGG; hpII, 5'-GATCCAAGCTTGTACTTGGTCAGGA; L, 5'-GAACTCGAGACCGCGGTACGTAGGATCCAGGCCTAGAAGGCCT; and mRRE, 5'-GTTCTGGGCGCTTCGGCGGTACAGACCGCGGTACGTAG.

The correct sequence of the chimeric tRNA DNA templates was verified by sequencing. Vector DNA was converted to corresponding virus by established procedures (47). Briefly, 1 μ g of plasmid DNA was electroporated into the packaging cell line AM12 by using a Bio-Rad gene pulser, and productively transduced cells were selected with G418 (0.7 mg/ml). G418-resistant colonies were pooled and used in subsequent experiments. Vector-containing virus was used to infect CEM cells, and clonally infected cell lines were isolated by G418 selection and limiting dilution. Uncoloned CEM populations harboring a retrovirus vector were obtained by G418 selection without limiting dilution.

Infection of CEM cells with ARV-2 virus. CEM cells (2×10^5) were infected in a 1-ml volume in the presence of 4 μ g of Polybrene per ml for 2 h with 100 to 1,000 50% tissue culture infective dose units of ARV-2 virus obtained from chronically infected HUT 78 cells. Cells were washed once and resuspended in the original volume. Every 3 to 4 days, a sample of cells and supernatant was withdrawn for analysis and cells were passaged at a 1:4 dilution ratio in fresh RPMI 1640-10% fetal calf serum.

PCR analysis of genomic DNA from CEM cells. The sequence of the 5' primer was 5'-CTTGGCAGAACAGCAGAGTGG-3', corresponding to a sequence within the tRNA^{Met} gene. The sequence of the 3' primer was 5'-ACAGAGACAA CACAGAACGAT-3', which is complementary to a vector sequence in the *gag* region of the Moloney murine leukemia virus vector backbone. For PCR analysis, genomic DNA was extracted by using an Iso Quick kit (Microprobe Corp.). A PCR using 100 ng of genomic DNA was performed in a DNA thermal cycler (Perkin-Elmer) as follows: cycle 1, 5 min at 95°C (denaturation), 1 min at 64°C (annealing), and 1 min at 72°C (extension); cycles 2 through 30: identical, except that the denaturation time was reduced to 1 min and a 5-min final extension at 72°C was added. Following cycle 30, PCR products were analyzed by agarose gel electrophoresis. PCR DNA products were purified from the agarose gel by using NACS columns (Bethesda Research Laboratories). Purified PCR DNA products (5 fmol) were sequenced with the fmol DNA

A. Rev response element (RRE)

B. Chimeric tRNA-mRRE transcripts

C. DCT-RRE retroviral vectors

FIG. 1. Structures of chimeric tRNA-mRRE decoy templates and retrovirus vectors. (A) The proposed secondary structure of the HIV RRE shows the central stem and four stem-loop structures. The primary Rev binding site was mapped to a short region within stem-loop II (boxed sequences), and it forms a purine-rich bubble, also known as stem-loop D. The RRE-derived sequences used as decoy RNA (mRRE) correspond to the primary Rev binding domain (shaded box) and a short stem-loop structure, 5'-CUUCGG-3' (open box), that replaces the naturally occurring stem-loop in RRE. (B) The chimeric tRNA-mRRE decoy constructs consist of a wild-type human tRNA^{Met} gene and the mRRE sequence inserted between the tRNA and the transcription termination signal (T). Two stem-loop structures (hpI and hpII) and additional short sequences (LI and LII) are present on both sides of the mRRE sequence (RRE4). In RRE5 the hpI and LI sequences are absent. The arrows point to the site where processing should take place. (C) The chimeric tRNA-mRRE constructs, RRE4 and RRE5, were inserted into the polylinker sequence present in the 3' LTR of the N2A vector to generate the vectors DCT-RRE4 and DCT-RRE5, respectively. In the target cell, the tRNA-mRRE DNA template is duplicated and transferred to the 5' LTR.

sequencing system from Promega by using an additional PCR in the presence of deoxy/dideoxy nucleoside triphosphate mixtures.

RNA blot analysis. Intracellular expression of RRE decoy transcripts in vector-transduced cells was determined as previously described (47). Briefly, total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method (8). Low-molecular-weight RNA was fractionated on a 6% polyacrylamide-urea gel, transferred to a nylon membrane (Biotrans; ICN), and hybridized with ^{32}P -labeled probes, as previously described. The hybridization probes used were either a ^{32}P -labeled tRNA^{Met} gene-specific probe (a 550-bp DNA fragment excised with restriction enzyme *Nhe*I from plasmid DCT and generated with an oligolabeling kit from Pharmacia) or a ^{32}P -end-labeled mRRE-specific oligonucleotide, 5'-GATCCTACGTACCGCGGTCTGTACCGCCGAAGCGCCCAGAACGC-3', generated with terminal transferase (Boehringer Mannheim).

CAT assays. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (22). Briefly, 0.3 ml of a sample containing 1.3×10^7 viable CEM cells per ml was electroporated with a Bio-Rad gene pulser. All transfection mixtures also contained 2.0 μg of a β -galactosidase expression vector (pCMV- β -gal) as an internal control for both transfection efficiency and gene expression. Total DNA was adjusted to 10 μg with pCMV-IL2. At 48 h after transfection, cell lysates were prepared by freeze-thawing and were assayed for CAT enzyme activity as described previously (22). For quantitation, the acetylated and unacetylated forms of [^{14}C]chloramphenicol were cut out of the thin-layer chromatography plate and radioactivity was analyzed in a scintillation counter. The values were expressed as percentages of the acetylated form.

Intracellular p24 measurement. Cells (10^6) were washed twice in phosphate-buffered saline (PBS) and then fixed on ice for 2 min in 0.25% paraformaldehyde. Cells were then washed twice with PBS and resuspended in 195 μl of digitonin (0.5 mg/ml; Sigma) with 5 μl of a fluorescein isothiocyanate-conjugated anti-p24 antibody (KC-57; Coulter). Cells were held on ice for 30 min and then washed twice with a 10-fold volume of 5% Tween 20 (Sigma). Stained cells were then resuspended in 0.05% paraformaldehyde and analyzed on either a FACScan apparatus (Becton Dickinson) or a Coulter Counter by collecting 10,000 events and gating on live cells defined by fetal calf serum/SSC ($1 \times \text{SSC}$ is 0.15 M NaCl plus 0.015 M sodium citrate) parameters. Results were displayed as frequency distribution histograms of log fluorescence. Irrelevant fluorescein isothiocyanate-conjugated isotypic control monoclonal antibodies were used in each experiment as negative controls. Cursors to define p24-positive cells were set so that <1% of the isotypic control stained cells were positive.

RESULTS

Construction of minimal RRE (mRRE) decoy encoding retrovirus vectors. Figure 1 shows the structures of the RRE-derived sequences, the tRNA expression cassette, and the retrovirus vector used in this study. Figure 1A shows the secondary structure of the 234-nt RRE and the location of the primary Rev binding domain, which assumes a stem-loop structure composed of 13 nt, referred to as a purine-rich bubble or as stem-loop D (4, 20, 49). The RRE decoy RNA used in this study contained the purine-rich bubble (shaded boxes) and an artificial stem-loop (open boxes) that replaced the naturally occurring sequence in stem-loop IIB of RRE (mRRE, minimal rev binding domain). The artificial stem-loop

sequence, 5'-CUUCGG-3', is frequently found in rRNA and is highly stable (7, 50). In preliminary studies involving challenge of clonal cell lines with HIV, minimal RRE decoys containing the artificial stem-loop were found to be more effective than minimal RRE decoys containing the authentic RRE IIB loop in inhibiting HIV (data not shown). In all subsequent studies, only RRE decoys containing the artificial stem-loop were evaluated. Oligonucleotides containing mRRE and additional sequences (see below; Fig. 1B) were fused to the 3' end of a human tRNA^{Met} gene. In previous studies we have used a derivative of a wild-type tRNA^{Met} gene ($\Delta 3-5$) from which 18 nt encompassing the 3' RNA processing signal was deleted (27, 45, 47). Hence the tRNA decoy RNAs were expressed in the cell as fusion transcripts. In this study, we fused the mRRE oligonucleotides to the 3' end of a wild-type human tRNA^{Met} gene ($\Delta 3-2$) retaining the 3' RNA processing sequence. Since it was expected that sequences fused to a wild-type tRNA would be processed and released (Fig. 1B, arrow), stem-loop structures (hpI and hpII) were added on either one or both sides of the RRE sequence in an attempt to stabilize the processed transcript (RRE4 or RRE5). To minimize potential interference with Rev binding, the stem-loops were separated from the mRRE insert by short sequences (LI and LII). We found that transcripts were expressed from this tRNA cassette at levels 3- to 10-fold higher than those at which they were expressed from the previously described $\Delta 3-5$ cassette (unpublished data).

The tRNA-mRRE DNA constructs were inserted into the 3' LTR of the murine N2A retrovirus vector so that in the infected cell line, the tRNA-mRRE templates would have been duplicated and present at both LTRs of the proviral DNA as shown in Fig. 1C (19); hence, the name of the vector is DCT-RRE (DC is a prefix for double copy; T indicates the tRNA expression cassette). Control vectors containing a tRNA without an insert were termed DCT. The important feature of the double-copy vectors is that the second copy of the tRNA-mRRE template present in the 5' LTR was placed outside the viral transcription unit. This has previously been shown to facilitate the expression of retrovirally carried genes, including chimeric tRNA genes (19, 47). Vector DNA was converted to corresponding virus and used to infect CEM cells. Either G418-resistant colonies were pooled or individual clones were isolated by limiting dilution and expanded to clonal cell lines for further analysis.

Proviral structure of mRRE decoy-encoding vectors in CEM cells. The presence of extensive secondary structure in the tRNA-mRRE region of the vector transcripts as shown in Fig. 1B raised the concern that reverse transcription might be hindered and that rearranged virus would be generated at a high frequency. Of particular concern was an observation that in vitro, reverse transcriptase cannot transcribe through stable stem-loops containing the 5'-CUUCGG-3' loop sequence, which is present in mRRE4 (50) (Fig. 1A). To test whether the DCT-RRE4 vectors can faithfully reverse transcribe their genetic content, and to determine the proportion of rearranged virus generated in this process, we analyzed the structures of the proviruses present in clonally isolated transduced CEM cells by PCR. DNA from 8 of 10 CEM clones transduced with DCT-RRE4 virus gave rise to the PCR product of the predicted size, indicating that about 80% of the viruses generated from DCT-RRE4 were unrearranged and faithfully transferred their genetic contents to the target cells (data not shown). This degree of in vivo fidelity is typical for Moloney murine leukemia virus reverse transcriptase, indicating that the secondary structure of the tRNA-mRRE region does not significantly hinder generation of proviral DNA.

FIG. 2. RNA blot analysis of RRE decoy transcripts expressed in CEM cells. Total RNA was isolated from three CEM clones transduced with the DCT-RRE4 vector, two CEM clones transduced with the control DCT vector, and untransduced CEM cells. RNA was subjected to electrophoresis in polyacrylamide-urea gels, blotted to nylon paper, and hybridized with a ^{32}P -labeled mRRE-specific probe (A) or a human tRNA_{Met} -specific probe (B). Bands corresponding to the putative chimeric tRNA-mRRE transcript and the processed mRRE transcript are indicated.

Expression of mRRE decoy transcripts in CEM cell lines.

Total RNA isolated from three CEM clonal cell lines transduced with DCT-RRE4 and two CEM clones transduced with DCT virus was subjected to electrophoresis in 6% polyacrylamide-urea gels, transferred to nylon paper, and hybridized with an mRRE-specific probe (Fig. 2A) or a tRNA-specific probe (Fig. 2B). The CEM clones transduced with the DCT-RRE4 vector expressed two transcripts which hybridized to an mRRE-derived oligonucleotide probe. The larger species corresponded to the calculated 238-nt unprocessed tRNA-mRRE fusion transcript, and the shorter species corresponded to the 166-nt processed transcript. In spite of the presence of a wild-type tRNA and a 3' processing signal, processing of the chimeric tRNA was incomplete, as was observed previously (1). Comparable levels of RRE-containing transcripts were expressed in the three clones that were analyzed (Fig. 2). The abundant, low-molecular-weight RNAs detected with a tRNA-specific probe on all CEM clones (Fig. 2B) represent the primary and mature tRNA species expressed from the 12 copies of tRNA_{Met} genes present in the human genome (42). Minor species detected with the tRNA probe may represent partially processed tRNA or aberrant tRNA-like transcripts expressed from tRNA pseudogenes. As would be expected, hybridization with a tRNA probe has detected only the larger RRE RNA transcript and not the shorter transcript.

In subsequent RNA blot analysis, all eight CEM clones that were shown to harbor an intact provirus, as indicated by the PCR analysis, expressed the two RRE transcripts shown in Fig. 2 and no significant variations in the steady-state levels of RRE transcripts were apparent. No RRE-specific transcripts were detected in two CEM clones that were transduced with DCT-RRE4 virus but did not harbor an intact virus (data not

shown). Expression of the chimeric tRNA-mRRE transcripts in CEM cells had no measurable effect on the growth rate or morphology of these cells in comparison with the parental CEM cells (data not shown).

Inhibition of HIV replication in CEM cell lines expressing RRE4 decoy RNA transcripts. CEM clones transduced with either DCT-RRE4 or control DCT vectors were analyzed for their ability to support the replication of HIV-1. Cells were infected with 100 to 1,000 50% tissue culture infective dose units, and the spread of virus in culture was measured by a p24 enzyme-linked immunosorbent assay (ELISA) of the culture supernatant (Fig. 3). Parental CEM cells (data not shown) and two clones transduced with the control DCT vector supported the replication of HIV, as shown by the initial increase in immunoprecipitable p24 antigen concentration, which peaked at about day 23. The subsequent decrease in p24 antigen concentration reflects the cytopathic effect of HIV replication on CEM cells. Logarithmic display of p24 antigen measurements reveals significant differences in the rate of HIV replication among the three clones which harbored the DCT-RRE4 vector and expressed comparable levels of decoy RNA (Fig. 2). In clone 4-2, HIV replication was highly inhibited (over 10^6 -fold); in clone 4-3, inhibition was intermediate (10^4 -fold); and in clone 4-1, inhibition was moderate (10^2 - to 10^3 -fold). Overall, among the 10 CEM clones transduced with DCT-RRE4 and analyzed for HIV replication, the 2 clones which were shown to lack an intact provirus and which did not express RRE decoy RNA were fully permissive for HIV replication, a high degree of HIV inhibition was seen in 3 clones, intermediate inhibition was seen in 2 clones, and 3 clones exhibited only moderate inhibition. The reason for the differences among the CEM clones expressing RRE4 RNA is

FIG. 3. Time course of virus secretion in CEM cells infected with HIV-1. CEM clones transduced with DCT or DCT-RRE4 were infected with HIV-1, and the level of secretion of virus into the medium was determined by a p24 ELISA. Symbols: ●, DCT-1; ▲, DCT-2; ○, DCT-RRE4-1; □, DCT-RRE4-2; △, DCT-RRE4-3.

unclear, particularly because all were shown to express comparable levels of RRE transcripts, and sequencing of the PCR-amplified products revealed no alterations in the original sequence (data not shown). In addition, we have subcloned two CEM clones, clones 4-1 and 4-2, and all 10 subclones from each original clone were found to express RRE transcripts at comparable levels (data not shown).

Inhibition of Rev function in cells expressing mRRE decoys. Rev enhances the accumulation of unspliced and singly spliced HIV RNA in the cytoplasm of HIV-infected cells and diminishes accumulation of the multiply spliced viral RNA species produced early in viral infection. Consequently, in CEM cells expressing RRE decoys, if Rev function is inhibited, multiply spliced HIV RNA species should predominate in the cytoplasm. Analysis of HIV RNA by RNA blotting 4 days after HIV challenge revealed that the steady-state levels of all HIV RNAs were significantly reduced in CEM cells expressing RRE decoy RNA, including the multiply spliced RNA species (data not shown). While it appeared that the accumulation of the unspliced and singly spliced RNA species was more severely affected, the extent of overall inhibition made a quantitative analysis difficult. A similar effect was seen in previous studies of CEM cells expressing a 45-nt RRE decoy (27).

To clarify whether minimal RRE decoys function by inhibiting Rev, we assayed Rev function in CEM cells expressing RRE4 in a transient transfection system using the reporter plasmid pDM128 and a Rev-encoding plasmid, pRev. pDM128 has a single intron containing both the HIV-1 RRE sequence and the bacterial CAT coding sequences (22, 23). Since this intron is efficiently removed by splicing, in the absence of Rev, cells transfected with pDM128 will express only trace amounts of CAT activity. Cotransfection of pDM128 with pRev permits unspliced transcripts to enter the cytoplasm, increasing CAT activity manifold. Figure 4A demonstrates that transfection of the CAT reporter plasmid pDM128 without pRev into CEM cells resulted in low levels of CAT expression. Cotransfection of CEM cells and two CEM clones stably transduced with the control vector DCT with pDM128 and pRev resulted in a 14- to 19-fold increase in CAT activity. In contrast, Rev-dependent stimulation of CAT activity was increased only three to fivefold in three CEM clones stably transduced with DCT-RRE4 and

FIG. 4. CAT activity in CEM cells transfected with CAT expression plasmids. CEM cells or CEM clones transduced with the DCT, the DCT-TAR, or the DCT-RRE4 vector were transfected with a CAT expression vector, alone or together with a Rev expression plasmid. CAT activity was determined as described in Materials and Methods. (A) pDM128 has a single intron containing both the HIV-1 RRE and the bacterial CAT gene (22). pREV is an HIV-1 Rev expression plasmid (31). (B) pRxRRE:CAT is analogous to pDM128, except that the HIV-1 RRE was replaced by the HTLV-1 RRE (kindly provided by Bryan Cullen). pRex is an HTLV-1 Rex expression plasmid (36).

expressing RRE4 RNA. The specificity of inhibition of Rev activity by RRE4 is indicated by the following observations. First, no inhibition of Rev-dependent stimulation of CAT activity was seen in CEM clones expressing TAR decoy RNA (Fig. 4A). Second, Rev-independent CAT expression from a standard cytomegalovirus promoter-driven CAT expression plasmid was efficient in all cell lines tested, with no endogenous intracolon variation (data not shown). Third, human T-cell leukemia virus type 1 (HTLV-1) Rex-dependent stimulation of CAT activity was not inhibited in CEM clones expressing RRE4 RNA (Fig. 4B). HTLV-1 Rex, while functionally interchangeable with HIV-1 Rev, binds to the HIV-1 RRE at a different site than Rev (11). Collectively, these observations strongly argue that the inhibition of CAT activity in CEM clones harboring the DCT-RRE4 vector is the result of a specific interaction between the vector-transcribed RRE4 decoy RNA and the transfected *rev* gene product.

Inhibition of HIV in polyclonal populations of CEM cells transduced with mRRE decoy-encoding vectors. In clinical applications of intracellular immunizations using RRE decoys,

FIG. 5. Virus secretion in CEM cells transduced with DCT-RRE vectors. CEM cells and CEM cells transduced with the control DCT vector or DCT-RRE vectors were infected with HIV-1, and the level of secretion of virus into the medium at the peak of virus production in the control cells (20 days postinfection) was determined by a p24 ELISA. Bulk, vector-transduced G418-selected polyclonal population of CEM cells; DCT-RRE4-2, a G418-selected CEM clonal isolate transduced with the DCT-RRE4 vector described in Fig. 2 and 3.

primary hematopoietic cells, including peripheral blood CD4⁺ T cells and pluripotent stem cells, are transduced and transplanted. Since significant heterogeneity was observed in the resistance of clonal isolates of CEM cells to HIV (Fig. 3), it is likely that significant variation in the degree of HIV resistance will occur in vivo as well. Consequently, in addition to demonstrating the effectiveness of RRE decoys in homogeneous transduced clonal cell lines, it is important to determine whether mRRE decoys will protect polyclonal populations of cells from HIV infection. Measurements of secreted p24 antigen in polyclonal populations of CEM cells, as opposed to clonal isolates (Fig. 3), have shown that only a modest reduction in virus replication occurred. p24 secretion was effectively inhibited in the CEM clone 4-2, but only a twofold reduction in p24 secretion was seen in DCT-RRE4-transduced cultures at day 20, when HIV replication is at its maximum, and up to a fivefold reduction in p24 antigen secretion was seen in cultures transduced with the DCT-RRE5 vector (Fig. 5). DCT-RRE5 is a modified version of the RRE decoy which differs from RRE4 by the absence of the 5' stem-loop (hpl) and LI sequences (Fig. 1B). Analysis of the RRE4 RNA secondary structure by using a computerized algorithm (MULFOLD) indicated that the RRE4 Rev binding domain is inaccessible in a large proportion of thermodynamically stable secondary structure configurations (Fig. 6 and Table 1), thereby limiting its efficacy. In contrast, consistent with the observation shown in Fig. 5 and below, RNA secondary structure analysis of RRE5 predicted that its Rev binding domain would be more likely to assume

A. RRE4 ($\Delta G = -96.1$ Kcal/mole)

B. RRE5 ($\Delta G = -68.0$ Kcal/mole)

FIG. 6. Secondary RNA structure analysis of DCT-RRE4 and DCT-RRE5. The most stable secondary RNA structures for unprocessed RRE4 transcript (A) and DCT-RRE5 (B) were determined by using the MULFOLD program (24). Thick lines and bold letters denote the positions of the mRRE sequences (Fig. 1A). The boxed shaded area shows that in the predicted secondary RNA structure of DCT-RRE5 the primary Rev binding domain assumes the purine-rich bubble configuration. 1 cal = 4.184 J.

the native configuration of the purine-rich bubble found in the HIV RRE than would that of RRE4 (Fig. 6 and Table 1). As with the RRE4 decoy vector-transduced cells, 80% of the G418-resistant CEM clones transduced with the DCT-RRE5 vector expressed the RRE decoy transcripts, and expression of RRE4 and RRE5 decoy RNA in uncloned CEM populations was comparable (data not shown).

In view of the observed heterogeneity among RRE decoy vector-transduced CEM cells, it was not unexpected that ELISA measurements of secreted p24 antigen concentrations would not show more than two to fivefold differences between experimental and control populations, because p24 antigen would be secreted both by the unprotected cells carrying

TABLE 1. The five most stable secondary RNA structure configurations of RRE decoy RNA^a

RRE decoy	Primary transcript		Processed transcript	
	Configuration of Rev binding domain	Stability (kcal/mol) ^b	Configuration of Rev binding domain	Stability (kcal/mol)
RRE4	Sequestered	-96.1	Sequestered	-73.0
	Sequestered	-95.9	Free	-72.4
	Sequestered	-95.6	Sequestered	-71.5
	Free	-95.5	Sequestered	-70.7
	Sequestered	-94.8	Free	-70.4
RRE5	Free	-68.0	Free	-44.9
	Free	-67.8	Sequestered	-43.9
	Sequestered	-67.0	Free	-42.2
	Free	-66.7	Free	-41.2
	Free	-65.7	Free	-39.8

^a Configurations were determined by using the MULFOLD RNA secondary structure program (24).^b 1 cal = 4.184 J.

rearranged proviruses and by the poorly protected cells. In addition, secreted p24 represents an average measure of HIV replication, and it does not permit the distinction between infected cells and uninfected cells or partially resistant cells generating small to intermediate amounts of progeny virus. We therefore developed a more informative assay to detect intracellular p24 expression within individual cells by using immunofluorescence staining and flow cytometry. This assay permits the simultaneous determination of the fraction of infected cells in the population and the amount of p24 expressed within individual cells. Results of a representative flow cytometric analysis of intracellular p24 expression in CEM cells performed 20 days after infection with HIV are depicted in Fig. 7. At this time, virtually 100% of the uncloned CEM cells transduced with the control vector DCT expressed high levels of intracellular p24. In contrast, almost no evidence of HIV infection was noted at this time in CEM clone 4-2 cells expressing RRE-4 decoy RNA, except for the observation that 2 to 5% of the cells expressed variable levels of intracellular p24 (Fig. 7A). In the case of uncloned CEM cells transduced with the DCT-RRE4 vector, virtually all cells became infected with HIV by this time and expressed intracellular p24 (Fig. 7B). The diminished fluorescence intensity of DCT-RRE4-transduced cells relative to that of controls, however, indicates that the expression of p24 was significantly inhibited at this time point. A similar pattern of intracellular p24 expression was seen in uncloned populations of CEM cells transduced with DCT-RREIIAB (Fig. 7C), a previously described RRE decoy (27). In the case of uncloned CEM cells transduced with the DCT-RRE5 vector, while approximately 25% of the cells expressed low to moderate levels of p24 at day 20, the majority of the cells expressed very low or undetectable levels of p24 (Fig. 7D), indicating either that these cells had not been infected by HIV or more likely that the cells had been infected but p24 expression was effectively inhibited.

In order to measure the durability of the protective effect of RRE decoys in a polyclonal transduced population, fluorescence-activated cell sorter (FACS) analysis for intracellular p24 expression was performed weekly until 40 days following infection with HIV. The percentages of cells expressing intracellular p24 at successive time points following infection with HIV are shown in Fig. 8. The majority of parental CEM cells and CEM cells transduced with the control DCT vector became p24 positive within 20 to 30 days, and chronically infected p24-expressing cells subsequently persisted for the duration of the culture. In the case of uncloned CEM cells

transduced with the DCT-RRE4 vector or with the previously described DCT-RREIIAB vector (27), despite inhibition of p24 expression at early time points (i.e., at day 20), HIV infection proceeded with only a moderate lag compared with that in control cultures, and eventually most of the cells became chronically infected and expressed high levels of p24 similar to those expressed by control cells. Apparently, the fraction of CEM cells expressing RRE4 or RREIIAB decoy RNA which were highly resistant to HIV on a clonal level (Fig. 3) were unable to withstand infection by virus generated in the cultures of polyclonal cells. In contrast, the uncloned CEM population transduced with the DCT-RRE5 vector remained essentially p24 negative following HIV infection, except for a brief period around day 20 when 25% of the culture became transiently p24 antigen positive. This minor subpopulation most likely consisted of cells containing rearranged provirus or cells that were only partially protected from HIV, as seen in the analysis of isolated clonal cell lines. At subsequent time points, cells expressing intracellular p24 were not observed. Expression of RRE5 but not RRE4 decoy RNA was also able to inhibit virus replication in polyclonal CEM populations challenged with two other laboratory-adapted strains of HIV, IIB and NL4-3 (data not shown). Challenge with natural HIV isolates was not attempted, since CEM cells, like the majority of immortalized cell lines, are not susceptible to replication of non-culture-adapted natural HIV isolates.

DISCUSSION

Minimal RRE decoys derived from the 13-nt primary Rev binding domain of the RRE were developed as a strategy for intracellular inhibition of HIV because previous studies indicated that use of these decoys may be safer than alternative inhibition strategies. In contrast to TAR decoys and larger RRE decoys (6, 18, 27, 28, 45), the 13-nt primary Rev binding sequence of the RRE is not known to bind and sequester cellular factors which could be important in normal physiologic processes. In addition, RRE decoys may be effective against a wide range of HIV isolates because of the high degree of sequence conservation in RRE stem-loop D among virus isolates (26). Also, mutations in Rev that will prevent its binding to the RRE decoy RNA will also prevent its binding to the authentic RRE sequences in the viral genome, and they should be deleterious to the virus. Consequently, for viable escape mutants to develop, two mutations, one in Rev and a second compensatory mutation in RRE, would be necessary.

FIG. 7. FACS analysis of p24 antigen expression in HIV-infected CEM cells. CEM cells were infected with HIV-1 and analyzed by flow cytometry for p24 antigen expression at 20 days postinfection as described in Materials and Methods. In each histogram, the left-hand curve represents uninfected control CEM cells, the right-hand curve represents HIV-infected CEM cells, and the shaded curve represents the experimental sample. (A) Clone DCT-RRE4-2; (B through D) a G418-selected, uncloned population of CEM cells transduced with DCT-RRE4 (B), DCT-RREIIB (C), or DCT-RRE5 (D). FITC, fluorescein isothiocyanate.

The frequency of such a double event is expected to be very low.

We have observed that the minimal RRE decoys described in this study are highly effective in protecting CEM cells from HIV replication and are significantly more potent than previously described TAR and RRE decoys (references 27 and 45 and data not shown) (Fig. 8). The increased potency of minimal RRE decoys relative to a previously described RRE decoy (27) appears to be due to a series of modifications introduced into the tRNA-RRE decoy sequence which were designed to facilitate the processing of the chimeric transcript into free, stable RRE decoy oligonucleotides (26a). Processing of the chimeric tRNA-RRE transcript was achieved by introducing a wild-type 3' tRNA processing sequence into the decoy molecule. Modifications designed to impart stability to the RRE decoy included the addition of sequences which would

form hairpins on either one or both sides of the RRE decoy and the replacement of the authentic IIB stem-loop of the RRE with the stable 5'-CUUCGG-3' stem-loop found in rRNA. However, since processing was incomplete (Fig. 2), it remains unclear whether inhibition of HIV was mediated primarily by the processed RNA decoy transcript, the unprocessed RNA decoy transcript, or both.

Cotransfection experiments (Fig. 4) demonstrated that cells expressing RRE4 decoy transcripts were specifically deficient in supporting Rev function, indicating that these RRE decoys inhibited HIV specifically by sequestering Rev from binding to the authentic RRE target in HIV RNA. These experiments indicate that the modifications introduced into the RRE decoy to impart processing and stability did not interfere with Rev binding, and more importantly, they confirm that the minimal

FIG. 8. Time course of HIV infection of CEM cells measured by flow cytometry. CEM cells were infected with HIV-1, and aliquots were analyzed for intracellular p24 expression as described in Materials and Methods and the legend to Fig. 7. Similar results were observed in three separate experiments. Values shown are for CEM cells (■) and for G418-selected, uncloned CEM cells transduced with the control DCT vector (●), the DCT-RREIAB vector (△), the DCT-RRE4 vector (□), or the DCT-RRE5 vector (○).

Rev binding domain of the RRE, which was previously identified by *in vitro* binding assays, also binds Rev *in vivo*.

We observed that significant heterogeneity in the degree of resistance to HIV existed among CEM clonal isolates transduced with RRE decoy vectors. While a portion of this heterogeneity was found to be due to cells containing rearranged proviruses, wide variations in HIV resistance that were not due to sequence alterations in the tRNA-mRRE DNA template, differential expression of RRE decoys, or inherent intracellular heterogeneity were also observed. Of note, no spontaneous resistance to HIV replication, which could have accounted for this phenomenon, has been observed in numerous experiments involving infection of CEM cells or clonal derivatives with HIV-1. Other investigators have also observed a similar heterogeneity among clones transduced with a Rev transdominant inhibitor (5, 30) or RRE decoy (6). Studies to elucidate the molecular, cellular, or viral basis for this phenomenon are ongoing.

Heterogeneity among clonal isolates of CEM cells transduced with HIV resistance genes, including cells harboring nonfunctional rearranged vectors, has important implications for clinical applications of gene therapy approaches to HIV infection, because it suggests that even in populations of cells transduced at high efficiency, a fraction of the genetically modified cells will still be susceptible to HIV expression and replication. This implies that an intracellular immunization protocol will be effective only if the highly protected cells will be able to withstand infection by HIV generated from the unprotected or poorly protected transduced cells in addition to host cells which are producing virus. To address this issue in an *ex vivo* culture setting, we tested the inhibitory potential of RRE decoys in uncloned populations of CEM cells by using an *in situ* immunofluorescence staining assay for p24 expression. The advantage of this assay is that it permits evaluation of HIV infection and expression within individual cells and subpopulations. By using flow cytometric analysis, subpopulations of cells transduced with DCT-RRE5, but not DCT-RRE4, were determined to be capable of durably withstanding

infection by virus generated in the culture (Fig. 7 and 8). The absence of p24 expression in DCT-RRE5-transduced cells was reproducibly noted for the duration of 40-day cultures, and it occurred under conditions in which control cells expressed microgram quantities of secreted p24. These observations attest to the potency of RRE5 under stringent conditions in polyclonal and heterogeneous populations of transduced T cells.

It is not clear from these studies how RRE5 decoys compare with other potentially useful HIV resistance genes that have recently been described (14, 29, 52). This will have to be determined in future comparative studies. Finally, the evaluation of inhibition strategies with cultures of immortalized T-cell lines challenged with laboratory-adapted HIV strains is only a first step in the development of clinically relevant gene therapy protocols.

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