The Journal of Microbiology, December 1998, p.308-315

Vol. 36, No. 4

Studies on the Inhibition of HIV Replication with a Number of RRE Decoy Derivatives

Seong-Wook Lee

Department of Molecular Biology, Dankook University, Seoul 140-714, Korea

(Received September 30, 1998 / Accepted November 13, 1998)

RRE decoys are short RNA oligonucleotides corresponding to the HIV Rev response element (RRE) sequence, which protect cells from HIV replication by inhibiting the binding of the HIV regulatory protein Rev to the authentic HIV RRE region. Previously minimal RRE decoy containing the 13-nucleotide primary Rev binding domain of RRE was described to be a potent inhibitor of HIV in CEM cells. In this report, we analyzed and compared the ability of a series of RRE decoy derivatives to inhibit HIV replication in CEM cells to develop increasingly effective RRE decoy. Using an improved tRNA cassette to express high level of RRE transcripts in cells, we found that a variant form of stem-loop II (SLII) binding domain of wild type RRE termed RRE40 was more potent than any other RRE decoys previously developed or tested here and protected all cells most effectively from HIV. RRE40 was previously selected *in vitro* which binds to Rev protein 10-fold better than wild type RRE. CEM cells expressing RRE40 decoy RNAs were shown to be specifically deficient in supporting Rev function. This confirmed that RRE40 decoys inhibit HIV specifically by sequestering Rev binding to the authentic RRE target in HIV RNA and indicated that RRE40 RNA identified by using *in vitro* binding studies also binds Rev in cells. These observations have important implications for experiments involving optimization of clinical application of RNA decoy based gene therapy protocol against HIV.

Key words: Gene therapy, HIV-1, RNA decoy, RRE

Genetic modification of cells that leads to resistance against viral replication could be mediated by stable transfer and expression of genes which inhibit viral replication (1). A number of strategies based on the intracellular expression of inhibitor genes have been developed for inhibition of HIV-1. Broadly, one can distinguish between protein-based inhibitors, such as transdominant mutant viral proteins and intracellular antibodies against viral proteins, and RNA-based inhibitors which include antisense RNA, ribozyme, and RNA decoys (4). Compared to protein-based inhibitors, RNA-based inhibitors have more potential advantages because they are not likely to be immunogenic, may be easier to express at high levels, can be more specific, and therefore less likely to interfere with normal cellular functions. Although antisense RNA and ribozyme are highly specific and can be targeted to many regions of HIV genome or mRNA, their use could be potentially limited due to the propensity of HIV to generate escape mutants which will be resistant to inhibition.

RNA decoys are short RNA transcripts corresponding to critical regulatory sequences on HIV RNA termed the trans activation response (TAR) sequence and the Rev response element (RRE) sequence (8, 9, 19). The HIV encoded regulatory proteins Tat and Rev can activate HIV gene expression by binding to TAR and RRE, respectively (15). Therefore, expression of TAR or RRE decoys will inhibit HIV expression and replication by competing with authentic viral TAR or RRE sequences for binding to Tat or Rev. A potential advantage of RNA decoy in comparison to other RNA-based inhibitors is that the generation of variant strains may be less frequent since the RNA decoy target sequences are highly conserved and alteration in Tat or Rev that will inhibit binding to RNA decoy would also inhibit binding to their authentic RNA sequences on HIV RNA. We have previously shown that HIV replication was efficiently inhibited in the human CD 4+ T cell line CEM which were stably transduced with a retroviral or adeno-associated viral vector expressing TAR or RRE decoy RNA (8, 9, 18). Especially, we have developed a potent minimal RRE decoy consisting of only the minimal 13-nucleotide (nt) Rev binding domain (8). This primary site of Rev which is present at the base of stem-loop IIB was defined by using *in vitro* binding studies (6, 22). A potential advantage to using this short RRE decoy is that since no cellular factors binding to

(Tel) 2-709-2905; (Fax) 2-793-0176
(E-mail) swl0208@unitel.co.kr

this short sequence have been identified while cellular factors have been found to bind to other regions of the HIV RRE, it may be much safer than other inhibitors whose function may involve binding to physiological cellular factors (17). However, bulk of human peripheral blood CD4+ T cells transduced to express these developed RNA decoys have been protected from HIV-1 replication at low levels and only transiently (unpublished data). Thus, it will be necessary to continously improve the effectiveness of RRE decoy for the clinical application of RNA decoy-based gene therapy protocol to inhibit HIV.

In this study, we have developed very effective RNA decoys by analyzing and comparing the ability of various RRE decoy derivatives to protect CEM cells from HIV replication. Using a modified tRNA expression system to express high levels of RRE decoy derivatives in CEM cells, we found that a variant form of wild type RRE termed RRE 40 was more potent than any other RRE decoys previously developed or tested here. RRE40 RNA was previously selected and identified using *in vitro* selection technique called SELEX. It binds Rev protein 10-fold better than wild type RRE and contains SLII binding domain of RRE with several different sequences from wild type (2). This RNA was also shown to have increased Rev responsiveness in cells when inserted into the full length RRE in place of Rev binding domain (21). We found that CEM cells expressing RRE40 decoy RNAs exhibited diminished Rev function in cotransfection assay, indicating that RRE40 decoys function as HIV inhibitor by inhibiting Rev binding to its authentic RRE sequence in HIV RNA and confirming that RRE40 binds Rev in cells.

Materials and Methods

Cells and viruses

The HIV-1 virus strain used in this study was the ARV-2 isolate propagated in HUT78 cells (16) and provided by Dr. Cheng and Dr. Levy. CEM cell which was provided by Dr. Nara is a CD4+ human T-lymphocyte cell line which is highly susceptible to infection with HIV-1, including the ARV-2 isolate (14). CEM cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone, USA).

Construction of retroviral vectors and transduction of CEM cells

Oligonucleotides RREIIAB, mRRE A, mRRE B, RRE40, and mRRE40 were cloned between the *Sac*II and *Bam*HI site of the modified DCT vector previously developed (8, 9). This modified vector contains $tRNA_i^{met}$ gene (Δ3-2) which retains a functional 3' RNA processing signal and two stem loop structures (hpI and hpII) between $tRNA_i^{met}$ gene and transcription termination signal. In addition, these two stem loops were separated by short sequence, L. The oligonucleotides representing various forms of RRE sequences were inserted in the middle of the L sequence to generate DCT-RREIIAB, DCT-mRRE A, DCT-mRRE B, DCT-RRE40, and DCT-mRRE40, respectively. Therefore, in these vectors the oligonucleotide L is divided into LI and LII. Vector DNA was converted to corresponding virus as previously described (20). Briefly, 1 μg of plasmid DNA was electroporated into AM12 packaging cell line (13) using a gene pulser (BioRad, USA), and the transfected cells were selected with 0.7 mg/ml of G418. 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.

Infection of CEM cells with ARV-2 virus

2×10^5 CEM cells were infected 100~1,000 $TCID_{50}$ units of ARV-2 isolated from chronically infected HUT78 cells in a volume of 1 ml in the presence of 4 μg/ml of polybrene. Cells were washed once and resuspended in the original volume. Every 3-4 days, a sample of cells was withdrawn for analysis, and cells were passaged at a 1:4 dilution ratio in fresh RPMI 1640 with 10% fetal calf serum.

p24 antigen ELISA test

Viral protein concentration in the culture supernatant was determined by a p24 antigen specific ELISA kit from Dupont (Cat. No. NEK-060) according to the manufacturer's instructions.

CAT assay

Chloramphenicol acetyltransferase (CAT) assay were performed as previously described (7,8). Briefly, 0.3 ml of a 1.3×10^7 CEM cells per ml were transfected using electroporation with gene pulser supplied by BioRad (USA). All transfection mixtures contained 2.0 μg of a β-galactosidase expression vector as an internal control for both transfection efficiency and gene expression. Total DNA was adjusted to 10 μg with pCMV-IL2. 48 hrs after transfection, cell lysates were prepared and assayed for CAT activity as described previously (7, 8). The acetylated and unacetylated forms of [^{14}C]chloramphenicol were fractionated on a thin layer chromatography plate (Ba-

ker, USA) and shown by autoradiography. CAT activity was determined by the percentage conversion of unacetylated chloramphenicol into acetylated chloramphenicol after autoradiography.

Results and Discussion

Construction of RRE decoy derivatives encoding retroviral vectors

To test the effectiveness of RRE decoy derivatives RNA to inhibit HIV-1, we constructed tRNA expression cassettes encoding several different forms of RRE sequences. Fig. 1 shows the structures of the RRE-derived sequences, tRNA expression cassette, and the retroviral vector used in this study. Fig. 1A (a) shows the secondary structure of the 234 nt full-length RRE and the location of the primary Rev binding domain, a stem-loop (SL) structure composed of 13 nucleotides (boxed sequences), referred to as a purine-rich bubble or as stem-loop D (6,22). Fig. 1A (b) shows RRE IIAB consisting of a 45 nt sequence encompassing stem-loops IIA and IIB of the RRE sequences, which was initially shown to be the major Rev binding domain (12). Expression of RRE IIAB via Δ3-5 tRNA transcription unit exerted a significant inhibitory effect on HIV-1 replication, albeit the extent of inhibition was less than that seen with TAR decoy (12). Fig. 1A(c) shows mRRE A which contains the 13 nt primary binding site for Rev found at the base of SLIIB of RRE forming the purine-rich "bubble" referred above (shaded box) and an artificial stem-loop (open box) that replaces the naturally occurring sequence in SLIIB of RRE (mRRE, m-minimal Rev binding domain). The artificial stem-loop sequence, 5'-CUUCGG-3', is frequently found in ribosomal RNA and is highly stable (3, 23). Using an improved tRNA cassette to express high levels of RRE transcripts, mRRE A was previously shown to be a potent inhibitor of HIV in T cell lines (8, 18). Fig. 1A(d) shows mRRE B, which contains the same 13 nt primary binding site (shaded box) and another artificial stem-loop (open box), 5'-UCUAACCUGA-3', which is much less stable than the artificial stem-loop in mRRE A. Fig. 1A(e) shows RRE40, a variant selected by *in vitro* genetic selection called SELEX for SL domain II of wild type RRE that binds to Rev 10 fold more tightly than wild type (2). Fig. 1A(f) shows mRRE40, a variant sequence of SLIID and IIB containing minimal but all the features required for the high affinity Rev binding of domain II, which is selected to bind Rev more tightly than wild type RRE (2). Fig. 1B shows the proposed chimeric tRNA-RRE transcripts. The various forms of RRE sequences shown in Fig. 1A were fused to the 3' end of a wild type $tRNA_i^{met}$ gene (Δ3-2) which retains a functional 3' RNA processing signal (indicated by arrow). To improve the stability of transcripts derived from this tRNA cassette, two stem loop structures (hpI and hpII) were added on both ends of the RRE sequences. In addition, these two stem loops were separated from the RRE oligonucleotide by short sequences, LI and LII, to minimize potential interference with Rev binding to the RRE sequence. These modification of tRNA structure resulted in a 5- to 20-fold intracellular increase in the expression of foreign RNA including TAR decoy relative to the unmodified tRNA cassette (9). Furthermore, the effectiveness to inhibit HIV replication has been improved by augmenting the intracellular expression level of TAR decoy via structural modification of tRNA expression cassette (9). The tRNA-RRE DNA constructs were inserted into the 3'LTR of the murine N2A retroviral vector as shown in Fig. 1C so that the tRNA-RRE templates would be duplicated and present at both LTRs of the proviral DNA in the infected cell line (5). The names of the various retroviral vectors are DCT-RREIIB, DCT-mRRE A, DCT-mRRE B, DCT-RRE40, and DCT-mRRE40. Control vectors containing tRNA without inserts are termed DCT.

Vector DNA was converted to corresponding virus and used to infect CEM cells. G418-resistant clones were individually isolated by limiting dilution and expanded to clonal cell lines for further analysis. CEM cells which were transduced with DCT-RRE derivatives were observed to express comparable RRE decoy RNAs using northern analysis as previously described (8, data not shown).

Protection of CEM cell lines expressing RRE decoy derivatives from HIV-1

Parental nontransduced CEM cells and CEM cell clones transduced with either control DCT vector or retroviral vectors encoding the various modified tRNA-RRE DNA templates were analyzed for their ability to suppress the replication of HIV-1. Cells were infected with 100~1,000 $TCID_{50}$ of the ARV-2 virus, and the spread of virus was measured by syncytia formation (Table 1). Patterns of syncytia formation in CEM cells following HIV infection was previously shown to directly correlate with the spread of virus in culture measured by a p24 antigen concentration (8). Therefore, syncytia formation will indicate HIV replication in tested CEM cells. The expression of tRNA-RRE transcripts in CEM cells had no measurable effect on their growth rate over an extended period time, and microscopic examination revealed no morphological differences between parental CEM cells and the clonal isolates expressing tRNA-RRE tran-

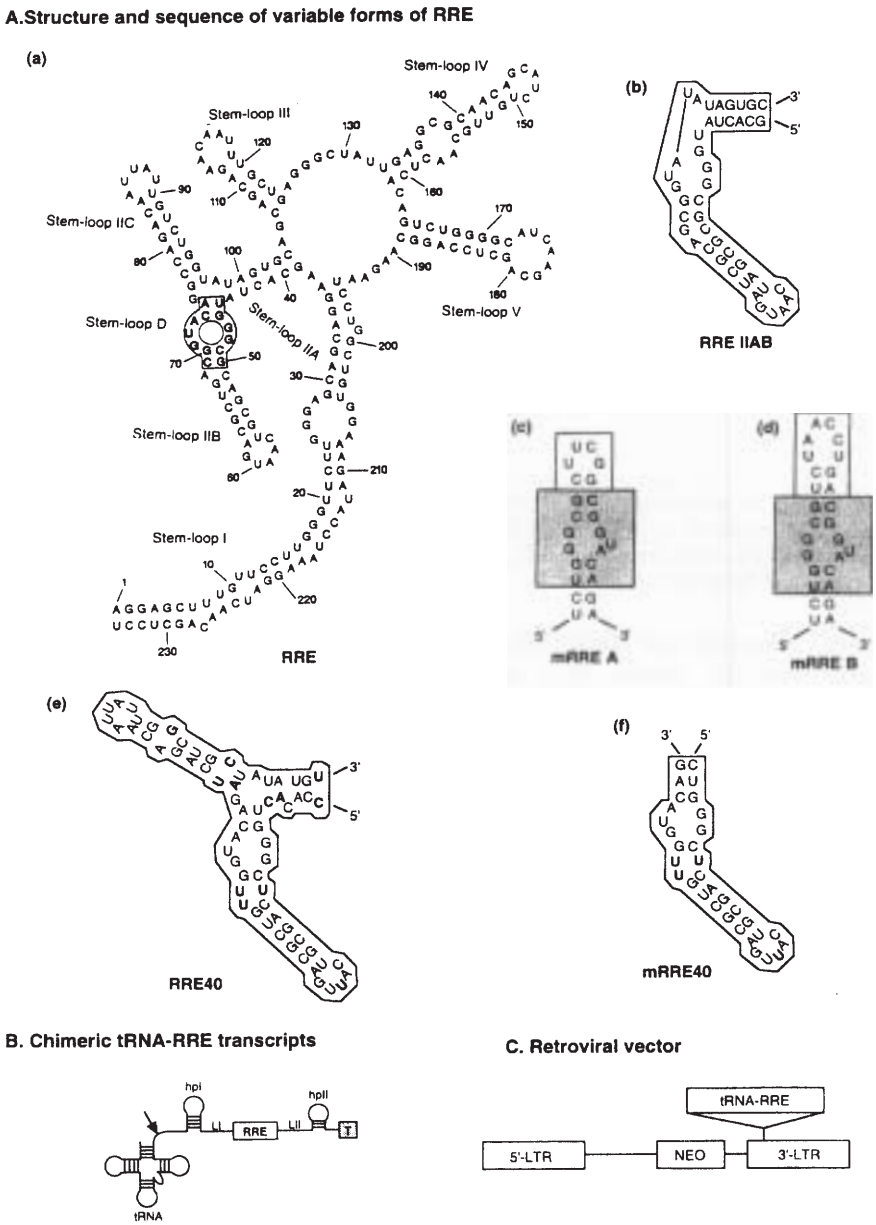

Fig. 1. Structure of chimeric tRNA, various forms of RRE decoy templates, and retroviral vectors. A. Structure and sequence of various forms of RRE. (a) The proposed secondary structure of the HIV Rev response element (RRE) shows a central stem and five stem-loop structures. The primary Rev binding site was mapped to a short region within stem loop (SL) II (boxed sequences) which forms a purine-rich "bubble", also known as stem-loop D. (b) The RRE-derived sequences used as decoy RNA (RREIIAB, open box) correspond to the sequences within SLIIA and SLIIB. (c) The RRE-derived sequences used as decoy RNA (mRRE A) correspond to the primary Rev binding domain (shaded box) and a short stem-loop structure (open box), which replaces the naturally occurring stem-loop in RRE. (d) The RRE-derived sequences used as decoy RNA (mRRE B) correspond to the primary Rev binding domain (shaded box) and a short stem-loop structure (open box) which replaces naturally occurring stem-loop of RRE. (e) The RRE-derived sequences used as decoy RNA (RRE40, open box) correspond to the sequences within the SLII domain with the variant sequences (bold letters) which were selected *in vitro* to bind Rev 10-fold more tightly than the wild type. (f) The RRE-derived sequences used as decoy RNA (mRRE40, open box) correspond to the sequences containing the primary Rev binding domain with the variant sequences (bold letters), which were shown to bind Rev more tightly than the wild type. B. The chimeric-various RRE decoy constructs consist of wild type human $tRNA_i^{met}$ gene and various RRE sequences inserted between the tRNA and the transcription termination signal (T). Two stem-loop structure (hpI and hpII) and additional short sequences (LI and LII) are present on both sides of the RRE sequence in the chimeric tRNA-various RRE transcript. C. The chimeric tRNA-variable RRE constructs were inserted into the polylinker sequence present in the 3'LTR of the N2A vector to generate retroviral vectors. In the target cells, the tRNA-RRE DNA template was duplicated and transferred to the 5'LTR.

Table 1. Result of challenging tRNA-RRE transcript expressing CEM cell clones with HIV-1[a]

Cells	days PI					Cells	days PI				
	d10	d13	d18	d24	d28		d10	d13	d18	d24	d28
CEM	+w	+	+++	++++	++++	DCT-mRREB-1	+	+++	++++	++++	++++
						-2	?	?	++	+++	++++
DCT -1	+	++	+++	++++	++++	-3	?	+	+++	+++	++++
-2	?	+	+	+++	++++	-4	?	?	+	++	+++
DCT-RREIIAB-1	+	+	+	++	++++	DCT-RRE40-1	?	+w	+w	+w	+w
-2	+	+	+	++	+++	-2	?	-	+	+	+
-3	+	++	++	+++	++++	-3	?	+	+	+	+
-4	?	+	+	++	+++	-4	-	+	-	?	+
DCT-mRREA-1	-	?	+	+++	+++	DCT-mRRE40-1	-	-	?	++	++
-2	-	-	-	+	+	-2	-	+	+	+	+
-3	-	+w	++	+++	++	-3	-	-	?	+w	++
-4	-	-	-	-	?	-4	-	?	+w	+	+

[a] Patterns of HIV-1 replication in individual cells were determined by degree of syncytia formation in 10^6 cells at different days post-infection (PI). The numbers under vectors indicate individual CEM cell clones. (-; no syncytia, ?; indeterminate syncytia, +w; less than 1% in 10^5 cells form syncytia, +; 1-10% form syncytia, ++; 10-15% form syncytia, +++; 25-50% form syncytia, ++++; more than 50% form syncytia.)

scripts (data not shown). The parental CEM cells and two clones transduced with the control DCT vector manifested similar patterns of syncytia formation. CEM clones transduced with the DCT-RREIIAB or DCT-mRRE B vector did not seem to have resistance against HIV-1 replication as compared with the control cells. In contrast, CEM cells transduced with the DCT-mRRE A or DCT-mRRE40 vector showed more inhibition of syncytia formation than control cells or the clonal cells transduced with the DCT-RREIIAB or DCT-mRRE B vectors. However, as previously reported (8), we observed some heterogeneity in the degree of resistance to HIV-1 among CEM clones in spite of comparable expression of RNA among clones transduced with the same vector. In contrast to mRRE B decoy whose expression showed no inhibition of HIV-1 and contains only 13 nt primary binding site for Rev and an unstable stem-loop, mRRE A decoy whose expression slightly inhibited HIV-1 replication contains both the same 13 nt primary binding site and an artificial stable stem-loop. This observation implies the importance of stable structural maintenance and native configuration of RRE decoy for its function as an inhibitor of HIV-1. We have previously observed a similar correlation between stable secondary structure and effectiveness of RNA decoys in inhibiting HIV-1 replication (8, 9). CEM clones transduced with the DCT-RRE40 vector exhibited most efficient inhibition of syncytia formation. Especially all the cells transduced with this vector were most durably protected against HIV-1, a result not seen in other clones transduced with other vectors. In conclusion, in the challenge experiments of clonal cell lines with HIV-1, RRE40 decoy was found to be more effective in inhibiting HIV-1 than natural RRE decoys containing the Rev binding domain. RRE40 is a variant selected by *in vitro* genetic selection of SL domain II of wild type RRE that binds to Rev 10-fold more tightly than wild type (2). This indicates that increment in the binding affinity of *in vitro* selected RRE derivatives to Rev protein can also improve the effectiveness of RRE decoy RNA to inhibit HIV-1 replication in cells.

To confirm that the expression of RRE40 decoy RNAs in cells can effectively inhibit HIV-1 replication, the spread of virus in cells transduced with the DCT-RRE40 vector was measured and compared to

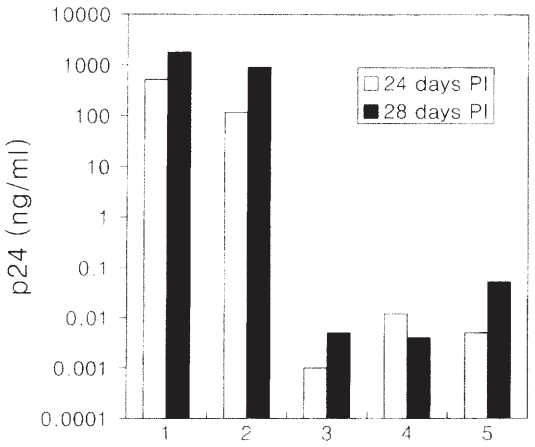

Fig. 2. HIV-1 secretion from CEM cells transduced with retroviral vectors encoding RRE40 decoy RNA. Two CEM clones transduced with DCT (DCT-1 (lane 1) and -2 (lane 2)) and three CEM clones transduced with DCT-RRE40 (DCT-RRE40-1 (lane 3), -2 (lane 4) and -3 (lane 5)) were infected with HIV-1, and the secretion of virus into the media 24 and 28 days post infection was determined by a p24 ELISA test.

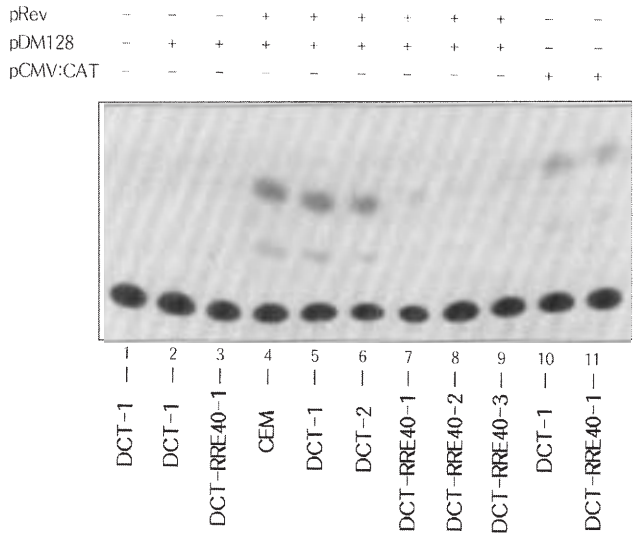

Fig. 3. CAT activity in CEM cells transfected with CAT expression plasmids. Parental CEM cells or CEM clones transduced with the DCT or the DCT-RRE40 vector were transfected with no DNA (lane 1) or a CAT expression vector containing RRE (pDM128) alone (lanes 2 and 3) or together with a Rev expression vector (pRev) (lanes 4-9). As control, cells were transfected with CMV-derived CAT expression vector (pCMV:CAT) (lanes 10 and 11). CAT activity was determined as described in Materials and Methods. The numbers under the vectors indicate individual CEM cell clones.

those with the control DCT vector by a p24 ELISA assay of culture supernatant at 24 and 28 days post-infection (Fig. 2). As shown in lanes 1 and 2 of Fig. 2, two CEM cells transduced with the control DCT vector supported the replication of HIV. However as shown in the lanes 3-5 of Fig. 2, p24 antigen release was very limited and reduced 10^4-10^5 fold in the case of all CEM cell clones expressing RRE40 decoy RNA. Consequently, RRE40 decoy RNA is a very potent antiviral agent against HIV-1.

Inhibition of Rev function in cells expressing RRE decoy derivatives

To clarify whether RRE40 decoys function by inhibiting Rev, we assayed Rev function in CEM cells expressing RRE40 in a transient transfection system using a reporter plasmid, pDM128, and a Rev encoding plasmid, pRev, as shown in Fig. 3. pDM 128 has a single intron containing both the HIV-1 RRE sequence and the bacterial chloramphenicol acetyltransferase (CAT) coding sequences (7). Since this intron is efficiently removed by splicing in the absence of Rev, cells transfected with pDM128 will express only trace amount of CAT activity. As shown in the lanes 2 and 3, transfection of pDM128 without pRev into cells resulted in very low level of CAT expression. Rev enhances accumulation of unspliced and singly spliced HIV RNA in the cytoplasm of HIV infected cells and diminishes accumulation of multiple spliced viral RNA species produced early in viral infection. Thus, cotransfection of pDM128 with pRev will permit unspliced transcripts to enter the cytoplasm, increasing CAT activity many folds. As shown in the lanes 4-6 of Fig. 3, cotransfection of CEM cell and two CEM clones stably transduced with the control vector DCT with pDM128 and pRev resulted in a 15-20 fold increase in CAT activity. In contrast, if Rev function is inhibited, in RRE40 decoy expressing CEM cells cotransfected with pDM128 and pRev, splicing will be efficient, resulting in a decrease in CAT activity. As shown in the lanes 7-9 of Fig. 3, Rev dependent stimulation of CAT activity increased only 2-5 fold in three CEM clones stably transduced with DCT-RRE40. This decrease in the CAT activity was not due to nonspecific inhibition of CAT activity in the cell extract since Rev-independent CAT expression from a standard CMV promoter driven CAT expression plasmid was efficient in all cell lines tested (lane 10 and 11 of Fig. 3). Together with the fact that no inhibition of Rev dependent stimulation of CAT activity was seen in CEM clones expressing TAR decoy RNA (8 and data not shown), these observations strongly suggest that the inhibition of CAT activity in CEM clones harboring DCT-RRE40 vector is the result of specific interaction between the vector transcribed RRE40 decoy RNA and the transfected Rev gene product. In conclusion, cotransfection experiments demonstrated that cells expressing RRE40 decoy RNA were specifically deficient in supporting Rev function, indicating that these RRE decoy derivatives inhibited HIV-1 replication in cells specifically by sequestering Rev from binding to authentic RRE target in HIV RNA. In addition, these experiments confirm that the *in vitro* selected Rev binding SLII domain of the RRE, which was previously systematically selected by *in vitro* binding assays (2), also binds Rev in cells.

Even though HIV replication was efficiently inhibited in selected human CD4+ T cell line transduced with retroviral vectors expressing RNA decoys (8, 9, 19), HIV replication was inhibited at low levels and only transiently in bulk of human peripheral blood CD4+ T cells transduced to express RNA decoys (unpublished data). Therefore, several approaches to improve the effectiveness of RNA decoys to inhibit HIV replication will be necessary for clinical application of gene therapy protocols to HIV infection. One such approach is to improve the gene transfer efficiency by vector development. Another approach is to increase the intracellular concentration of RNA decoys. We have previously shown that the ef-

fectiveness of TAR decoys could be improved by augmenting the expression level of TAR decoys via structural modification of tRNA expression cassette (9). A potent Pol II transcription unit, we have recently developed, could be also used to increase the intracellular expression level of RNA decoys (11). This unit is based on highly expressed TBN transcript which is a spliced RNA product utilizing cryptic splice signals present in the normally spliced DHFR mRNAs and accumulating exceedingly high levels comparable to the levels reached by the highly abundant endogenous actin mRNA, or MoMLV RNA expressed in chronically infected cells. Another approach which has been studied here is to develop increasingly effective RNA decoys. In this study, RRE40 decoy RNA, which was previously selected *in vitro* to bind Rev protein 10-fold much better than wild type SLII binding domain of RRE (2), was found to be a more potent inhibitor against HIV-1 replication than mRRE A decoy RNA which was previously developed (8). *In vitro* selection methods called SELEX can be employed to isolate short RNA molecules from RNA libraries that bind to many types of proteins, including non-RNA binding proteins, with high affinity and specificity (10, 24). Results from studies here suggest that *in vitro* selection of RNA ligands which bind specifically and avidly HIV structural or regulatory proteins could be useful for the development of HIV inhibitors. Stable expression of such RNA ligands would render cells resistant to HIV replication.

To evaluate the efficacy of RRE40 decoys in clinical setting, polyclonal peripheral blood CD4+ T cells will be transduced with vectors expressing RRE40 decoy RNA and then challenged with HIV. In addition, the effectiveness of RRE40 decoy RNA as HIV inhibitor will need to be compared with other potentially useful HIV resistance mechanisms that have recently been described (4). These studies will have important implications for experiments involving the development of clinically relevant gene therapy protocols.

Acknowledgments

We deeply thank Dr. Eli Gilboa and Dr. Clay Smith in Duke University Medical Center for their kind supply of facilities and valuable discussions and Humilidad Gallardo for her excellent technical assistance throughout these studies.

The present research was conducted by the research fund of Dankook University.

References

1. **Baltimore, D.** 1988. Intracellular immunization. *Nature* **335**, 395-396.
2. **Bartel, D.P., M.L. Zapp, M.R. Green, and J.W. Szostak.** 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base-pairs in viral RNA. *Cell* **67**, 529-536.
3. **Cheong, C., G. Varani, and J. Tinoco.** 1990. Solution structure of an unusually stable RNA hairpin. 5'GGAC (UUCG)GUCC. *Nature* **346**, 680-682.
4. **Gilboa, E. and C. Smith.** 1994. Gene therapy for infectious diseases: the AIDS model. *Trends in Genetics* **10**, 139-144.
5. **Hantzopoulos, P., B. Sullenger, G. Ungers, and E. Gilboa.** 1989. Improved gene expression upon transfer of the adenosine deaminase minigene outside the transcriptional unit of a retroviral vector. *Proc. Natl. Acad. Sci. USA* **86**, 3519-3523.
6. **Heaphy, S., J. Finch, M. Gait, J. Karn, and M. Singh.** 1991. Human immunodeficiency virus type I regulator of virion expression, rev, forms nucleoprotein filaments after binding to a purine-rich "bubble" located within the rev-responsive region of viral mRNAs. *Proc. Natl. Acad. Sci. USA* **88**, 7366-7370.
7. **Hope, T., X. Huang, D. McDonald, and T. Parslow.** 1990. Steroid receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif. *Proc. Natl. Acad. Sci. USA* **87**, 7787-7791.
8. **Lee, S.-W., H.F. Gallardo, E. Gilboa, and C. Smith.** 1994. 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. *J. Virol.* **68**, 8254-8264.
9. **Lee, S.-W., H.F. Gallardo, O. Gasper, C. Smith, and E. Gilboa.** 1995. Inhibition of HIV-1 in CEM cells by a potent TAR decoy. *Gene Ther.* **2**, 377-384.
10. **Lee, S.-W., B.A. Sullenger.** 1997. Isolation of a nuclease-resistant decoy RNA that can protect human acetylcholine receptors from myasthenic antibodies. *Nature Biotechnol.* **15**, 41-45.
11. **Lee, S-W, and E. Gilboa.** 1998. High level accumulation of an aberrantly spliced human DHFR RNA species. *Mol. Cells* **8**, 189-200.
12. **Lee, T., B. Sullenger, H. Gallardo, G. Ungers, and E. Gilboa.** 1992. Overexpression of RRE-derived sequences inhibits HIV-1 replication. *New Biol.* **4**, 66-74.
13. **Markowitz, D., S. Goff, and A. Bank.** 1988. Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* **167**, 400-406.
14. **Nara, P. and P. Fischinger.** 1988. Quantitative infectivity assay for HIV-1 and -2. *Nature* **332**, 469-470.
15. **Rosen, C. and G. Pavlakis.** 1990. Tat and rev: positive regulators of HIV gene expression. *AIDS* **4**, 499-509.
16. **Sanchez-Pescador, R., M. Power, P. Barr, K. Steimer, M. Stempien, S. Brown-Shimer, W. Gee, A. Renard, A. Randolph, J. Levy, D. Dina, and P. Luciw.** 1985. Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). *Science* **227**, 484-492.
17. **Shukla, R., P. Kimmel, and A. Kumar.** 1994. Human immunodeficiency virus type 1 Rev-responsive element RNA binds to host cell-specific proteins. *J. Virol.* **68**, 2224-2229.

18. **Smith, C., S.-W. Lee, E. Wong, H. Gallardo, K. Page, O. Gasper, J. Lebkowski, and E. Gilboa.** 1996. Transient protection of human T-cells from human immunodeficiency virus type 1 infection by transduction with adeno-associated viral vectors which express RNA decoys. *Antiviral Rese.* **32**, 99-115.
19. **Sullenger, B., H. Gallardo, G. Ungers, and E. Gilboa.** 1990. Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. *Cell* **63**, 601-608.
20. **Sullenger, B.A., T.C. Lee, C.A. Smith, G.E. Ungers, and E. Gilboa.** 1990. Expression of chimeric tRNA-driven antisense transcripts renders NIH 3T3 cells highly resistant to moloney murine leukemia virus replication. *Mol. Cell. Biol.* **10**, 6512-6523.
21. **Symensma, T.L., L. Giver, M. Zapp, G.B. Takle, and A.D. Ellington.** 1996. RNA aptamers selected to bind human immunodeficiency virus type I Rev *in vitro* are Rev responsive *in vivo*. *J. Virol.* **70**, 179-187.
22. **Tiley, L., M. Malim, H. Tewary, P. Stockley, and B. Cullen.** 1992. Identification of a high affinity RNA-binding site for the human immunodeficiency virus type 1 Rev protein. *Proc. Natl. Acad. Sci. USA* **89**, 758-762.
23. **Tuerk, C., P. Gauss, C. Thermes, D. Groebe, M. Gayle, N. Guild, G. Stormo, Y. Aubenton-Carafa, O. Uhlenbeck, J. Tinoco, I. Brody, and L. Gold.** 1988. CUUCGG hairpins: extraordinarily stable RNA secondary structures associated with various biochemical processes. *Proc. Natl. Acad. Sci. USA* **85**, 1364-1368.
24. **Tuerk, C., and L. Gold.** 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* **249**, 505-510.