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

OSAMU YAMADA,¹ GÜNTER KRAUS,¹ LEO LUZNIK,² MANG YU,^{1†} AND FLOSSIE WONG-STAAL^{1*}

*Departments of Medicine and Biology, University of California, San Diego, La Jolla, California 92093-0665,¹ and
Department of Medicine, University of Arizona Health Sciences, Tucson, Arizona 85724²*

Received 8 August 1995/Accepted 8 December 1995

We have previously shown that hairpin ribozymes targeting the human immunodeficiency virus (HIV) genome can effectively inhibit virus replication in a variety of primary and cultured hematopoietic cells. To further increase antiviral potency and minimize the chance of viral resistance, we have now cloned the stem-loop II sequences of the HIV type 1 Rev response element into ribozyme transcription cassettes. Fusion RNA molecules were shown to function both as RNA decoys and ribozymes. Stable Molt-4/8 cell lines expressing fusion RNA of stem-loop II and a ribozyme directed at the HIV type 1 U5 sequence (MSLMJT) or its disabled counterpart (MSLdMJT) were generated. The expression of fusion RNA was persistent for at least 6 months without apparent cytotoxicity. When virus inhibition was examined after the cocultivation of transduced cells with chronically infected Jurkat cells, much greater protection was observed in MSLMJT cells than in MSLdMJT or MMJT (expressing only the ribozyme) cells. Furthermore, to specifically compare the ribozyme activities in various transduced cells, we determined the quantitative levels of proviral DNA in the first round of virus replication (7 h after infection with HXB2). By competitive PCR, the proviral DNA levels in MSLMJT and MMJT cells were found to be reduced to 1/7 and 1/3, respectively, compared with those in MSLdMJT and MdMJT cells. These results suggest not only that the greater inhibition afforded by this fusion RNA was due to its function both as decoy and ribozyme but also that the ribozyme activity may be facilitated.

The life cycle of human immunodeficiency virus (HIV) provides many attractive steps for potential intervention by gene therapy, including transdominant mutant core and envelope genes to interfere with virus entry, Tat response element (TAR) decoys to inhibit transcription and *trans* activation, and Rev response element (RRE) decoys or transdominant Rev mutants to inhibit RNA processing (33). We have focused on the use of ribozymes as therapeutic genes because of the following potential advantages (24, 25). (i) As RNA molecules, they are not likely to induce host immunity that eliminates transduced cells; (ii) although they resemble antisense molecules in their sequence-specific recognition of target RNA, their ability to cleave target RNA catalytically renders them more efficient; (iii) they can potentially cleave both afferent and efferent viral RNAs and therefore can inhibit both preintegration and postintegration steps of the virus replication cycle. We have previously shown that T-cell lines (30) and primary lymphocytes (15) transduced with retroviral vectors expressing an anti-HIV hairpin ribozyme are resistant to exogenous infection with diverse strains of HIV type 1 (HIV-1). Furthermore, macrophages derived from primary CD34⁺ hematopoietic stem/progenitor cells were also resistant to challenge with a macrophage tropic strain of HIV-1 (34). However, one potential pitfall of the ribozyme approach is the propensity for escape mutants to emerge, since a single nucleotide change at a critical position of the target sequence could lead to resistance to ribozyme cleavage (5, 13, 29). In addition to focusing on highly conserved regions as targets, we also ex-

plored the design of vectors combining the ribozyme gene with other anti-HIV gene(s).

A significant inhibitory effect on HIV replication has been shown in human T cells expressing an RNA decoy corresponding to the major Rev binding site (stem-loop II [SL II]) within the HIV-1 RRE (16, 17). An RRE decoy may be less likely to be affected by HIV variability because mutations in Rev that abolish binding to the decoy may also affect binding to its physiological target (16). Hence, we inserted the SL II sequence into ribozyme transcription cassettes to express SL II-ribozyme fusion molecules. If such a fusion molecule maintains the antiviral effects of both components, we hope to preserve the ability of the vector to suppress both virus infection and virus expression from infected cells. In the present work, we compared cells expressing SL II linked to either a functional or nonfunctional ribozyme with those expressing the ribozyme alone. The results demonstrated that an SL II-ribozyme molecule was most effective in inhibiting virus replication, that it functions both as decoy and ribozyme, and that the ribozyme activity may be enhanced.

MATERIALS AND METHODS

Construction of chimeric SL II RRE-ribozyme vectors. pMJT and pOY-1 are Moloney murine leukemia virus vectors carrying either the HIV-1 5' leader sequence-specific ribozyme (anti-U5 ribozyme) or the HIV-1 Rev sequence-specific ribozyme (anti-Rev ribozyme), respectively, driven by the internal human tRNA^{Val} promoter (29, 34). pMJT is a construct containing the disabled form of the anti-U5 ribozyme, with the CGU at positions 24 to 26 replaced by AAA (22). The SL II sequence of RRE in HIV-1_{MN} (nucleotides 7824 to 7889) was amplified with the primer pair 5' SL2 (5'-ag aga tct GCA CTA TGG GCG CAG C-3') and 3' rcSL2 (5'-cg gga tcc GCA CTA TAC CAG ACA AT-3'). The PCR product was digested with *Bam*HI-*Bgl*II and then ligated with *Bam*HI-digested pMJT. After transformation with this plasmid in *Escherichia coli* DH5 α , a clone in which SL II was linked to the ribozyme sequence in the same orientation was

* Corresponding author.

† Present address: Immusol, Inc., San Diego, CA 92121.

obtained by screening. The ribozyme sequence in this plasmid, designated pSLMJT, was replaced with that of the disabled ribozyme or anti-Rev ribozyme at *Bam*HI-*Mlu*I to generate pSLdMJT or pSLOY-1, respectively.

Generation of stable cell lines. Molt-4/8 cells were transfected with parental vector DNA, pMJT, pOY-1, pMJT, pSLMJT, pSLOY-1, and pSLdMJT by the liposome-mediated method with DOTAP (Boehringer Mannheim). Transfected cells were selected by growth in G418 (GIBCO)-supplemented media, as described previously (29). Resistant Molt-4/8 cells were designated MLNL6, MMJT, MOY-1, MdMJT, MSLMJT, MSLOY-1, and MSLdMJT, respectively.

HIV-1 SF2 infection of MOY-1 and MSLOY-1 cells. G418-selected MOY-1, MSLOY-1, and parental Molt-4/8 cells were incubated with infectious SF2 at an input multiplicity of infection (MOI) of 0.01 for 2 h and washed twice. These cells were cultured at an initial concentration of 10^5 cells per ml in RPMI 1640 medium supplemented with 10% fetal calf serum. On days 5 and 8 after infection, infected cells were split 1:5 with medium to adjust to a cell concentration of approximately 2×10^5 cells per ml. Culture supernatants were collected on days 3, 5, 8, and 11 after infection, and the level of HIV-1 p24 antigen was determined by HIV-1 antigen capture enzyme-linked immunosorbent assay (ELISA; Coulter).

Cocultivation of stable cell lines with HXB2-infected Jurkat cells. Jurkat cells chronically infected with HIV-1_{HXB2} were washed twice with RPMI 1640 medium. Cells (100 or 1,000) were suspended in 1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum and containing 10^5 cells each of the stably transduced cell lines. On day 4 after infection, cells were split 1:5 with medium every 3 days thereafter. Culture supernatants were used for the measurement of p24 antigen by HIV antigen capture ELISA (Coulter).

QC-RT-PCR. Total cellular RNA was extracted from ribozyme-transduced cells or parental Molt-4/8 cells by the guanidine thiocyanate-phenol-chloroform extraction method (4) and subsequently treated with DNase I (QI DNase; Promega) as previously described (30). For quantitative competitive reverse transcription-PCR (QC-RT-PCR), in vitro-transcribed RNA of the anti-U5 ribozyme with a tetraloop substitution (5'-ACA CAA CAA GAA GGC AAC CAG AGA AAC ACA CGG ACU UCG GUC CGU GGU AUA UUA CCU GGU A-3') was used as competitor RNA. Total cellular RNA (0.5 mg) and competitive RNA diluted 10-fold serially (from 10 fg to 10 pg) were added to the RT reaction mixture (final volume, 16 μ l) containing 10 mM Tris-HCl (pH 8.3); 90 mM KCl; 1 mM MnCl₂; 200 mM (each) dATP, dGTP, dCTP, and dTTP; 50 pmol of Rib 2; and 3 U of *Tth* DNA polymerase (Promega). After the RT reaction at 60°C for 20 min, 34 μ l of PCR buffer containing 25 mM Tris-HCl, 1.1 mM EGTA, 200 mM KCl, 3.75 mM MgCl₂, 50 pmol of Rib 4 (30), and 200 mM (each) dATP, dGTP, dCTP, and dTTP was added to each tube, and PCR was carried out (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; 30 cycles). Ten microliters of each PCR product was subjected to 5% low-melting-point agarose gel electrophoresis. The expected sizes of the amplified products were 61 and 52 bp for competitor RNA and test RNA, respectively. The gel images after ethidium bromide staining were scanned with a Twain Scan Duo 600 (Mustek) and Color it version 3.0 and analyzed by using NIH image version 1.54 and a Macintosh computer.

QC-PCR. Cells (10^6 each of MMJT, MdMJT, MSLMJT, and MSLdMJT) were suspended in 1 ml of a DNase-treated HIV-1_{HXB2} preparation ($10^{5.25}$ 50% tissue culture infective doses per ml) in a 1.5-ml tube. Infected cells were incubated for 7 h at 37°C and washed two times with RPMI 1640 medium. Five hundred microliters of lysis buffer containing 50 mM Tris, 40 mM KCl, 1 mM dithiothreitol, 6 mM MgCl₂, 0.45% Nonidet P-40, and 200 mg of proteinase K per ml was added to each tube and incubated for 2 h at 50°C. Cell lysates were heated for 10 min in boiling water and used as template DNA for QC-PCR. In QC-PCR, a 5' primer, ³²P-end-labeled SK29 (corresponding to nucleotides 501 to 518 in the long terminal repeat [LTR]), and a 3' primer, SK30 (corresponding to nucleotides 605 to 589 in the LTR) (23), were used. Competitor DNA was prepared as follows. PCR was carried out with HXB2 DNA as the template and with a 5' primer, X + 5' LTR, which has 18 random bases (X sequences) flanking the 5' end of the HXB2 LTR (nucleotides 516 to 534) (5'-gat agc ggg tag cta gat GCT TAA GCC TCA ATA AAG C-3') and a 3' primer, SK 30. The PCR product was reamplified with a 5' primer, SK29 + X, which contains X sequences immediately 3' of the region corresponding to SK29 (5'-ACT AGT GAA CCC ACT GCT gat agc ggg tag cta gat g-3') and a 3' primer, SK 30. The reamplified product was cloned into pUC19 at the *Sma*I site, and the resultant plasmid (pUC SK29+X/SK30) was used as competitor DNA. Twenty-five microliters of cell lysate and 5 μ l each of different concentrations (10^3 to 10^5 copies in 5 μ l) of the competitor DNA preparation were added to each 0.5-ml tube containing the reaction mixture (total volume, 50 μ l). The composition of the reaction mixture for PCR was 50 mM Tris-HCl (pH 8.3); 3 mM MgCl₂; 40 mM KCl; 1 mM dithiothreitol; 200 mM (each) dATP, dGTP, dCTP, and dTTP; and 2.5 pmol of SK29 (5×10^3 to 7.5×10^5 cpm). The conditions of amplification were 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s for 25 cycles. *Taq* polymerase (1.25 U; Promega) was added to each reaction tube after the first denaturation step (95°C for 30 s). The expected sizes of the amplified products were 105 and 123 bp for the test PCR product and competitor DNA product, respectively. After PCR, 3 μ l of each PCR product was loaded onto an 8% polyacrylamide gel and autoradiographed. Images of the gel were scanned with a Twain Scan Duo 600 (Mustek) and Color it version 3.0. The signal intensities of the competitor and

FIG. 1. (A) Schematic representation of the retroviral vectors expressing anti-HIV-1 ribozyme (Rz) and SL II sequences of the HIV-1 RRE. The fusion RNA is driven by an internal human tRNA^{val} promoter. (B and C) Inhibition of the expression of p24 antigen after challenge with HIV-1_{SF2} at an MOI of 0.01. Δ , MOY-1 cells (expressing anti-Rev ribozyme); $\circ$, MSLOY-1 cells (expressing anti-Rev ribozyme linked to SL II); $\square$, parental Molt-4/8 cells; $\blacktriangle$, MMJT (expressing anti-U5 ribozyme); $\blacksquare$, MdMJT (expressing disabled anti-U5 ribozyme); $\bullet$, MSLMJT (expressing anti-U5 ribozyme linked to SL II). Culture supernatants were used for the measurement of HIV-1 p24 antigen. The experiments whose results are presented in panels B and C were repeated, with similar results.

test PCR products were analyzed by using NIH image version 1.54 and a Macintosh computer.

RESULTS

RRE decoy effect of SL II-ribozyme fusion RNA. To specifically examine the RRE decoy effect of an SL II-hairpin ribozyme fusion RNA, we utilized SF2 as a challenge virus for cells expressing anti-U5 and anti-Rev ribozymes fused to SL II (Fig. 1A). We have reported previously that the SF2 virus is refractory to the anti-Rev (OY-1) ribozyme because of a single nucleotide substitution of G $\rightarrow$ U at the cleavage site (29), while the U5 target sequence is conserved in this virus. Expression of the anti-Rev ribozyme in MOY-1 cells and MSLOY-1 cells was observed by RT-PCR, as previously described (30) (data not shown). As shown in Fig. 1B, only marginal protection against SF2 infection was shown in MOY-1 cells compared with that in Molt-4/8 cells, consistent with our previous data. However, the expression of the p24 antigen of HIV-1 was significantly suppressed in MSLOY-1 cells. We conclude that the protection in MSLOY-1 cells was due to an RRE decoy effect of the fusion

FIG. 2. Ribozyme expression levels in stable cell lines. Total cellular RNAs from MMJT (A), MdMJT (B), MSLMJT (C), and MSLdMJT (D) cultured for 25 weeks after transfection were subjected to RT-PCR amplification in the presence of different amounts of competitor RNA. Ten microliters of each PCR product was loaded onto a 5% low-melting-point agarose gel and stained with ethidium bromide. Video images of the gel were inverted with Adobe Photoshop version 3.0. The numbers of copies of competitor RNA added to each PCR were 10^8 (lanes 1), 10^7 (lanes 2), 10^6 (lanes 3), 10^5 (lanes 4), and 0 (lanes 5). This experiment was repeated, with similar results.

molecule. In contrast, HIV-1_{SF2} expression was inhibited in both MMJT and MSLMJT cells expressing either the anti-U5 ribozyme or the SL II-anti-U5 ribozyme fusion molecule (Fig. 1C).

Quantitation of anti-U5 ribozyme expression in stable cell lines. The expression of the ribozyme or disabled ribozyme in MJT, dMJT, MSLMJT, and MSLdMJT cells was examined by RT-PCR, as described previously, using the Rib 4 and Rib 2 primer pair and oligonucleotide probes to selectively detect the functional or disabled ribozyme. Amplified products were specifically detected only when PCR was carried out after RT (data not shown). By using a 5' primer corresponding to the SL II sequence, the expression of SL II-ribozyme fusion RNA was detected at 25 weeks after transfection in both cell lines (data not shown). Then the expression levels of ribozyme in stable cell lines were examined by QC-RT-PCR using the Rib 4 and Rib 2 primer pair. Figure 2 shows the inverted gel images after ethidium bromide staining. The number of competitor RNA molecules resulting in equal signal intensities for the amplified products of competitor and test RNAs was calculated from the regression line by the least-squares method. The ribozyme expression level was thus estimated to be 5.3×10^7 to 6.2×10^7 copies per 0.5 μ g of total cellular RNA in the four cell lines examined (Fig. 2). Since the amount of total cellular RNA is generally assessed at 1 μ g of RNA per 10^5 cells, we estimated that each cell expressed approximately 1,000 to 1,200 copies of ribozyme-containing RNA. These constitutive levels of ribozyme or fusion RNA expression had no apparent deleterious effect on Molt-4/8 cells, as all transfected cell lines and parental Molt-4/8 cells were indistinguishable with respect to cell growth rate and viability over a period of 6 months, with the passage of cells every 4 days (data not shown).

Protection against cell-cell transmission of HIV-1_{HXB2} in fusion RNA-expressing cells. The relative antiviral potencies of the ribozyme and SL II-U5 ribozyme vectors were compared in a system with cell-associated virus as the challenge. Jurkat cells chronically infected with HIV-1_{HXB2} were cocultured with sta-

FIG. 3. Inhibition of p24 expression in cell-cell transmission of HIV-1. MLNL6 (■), MMJT (△), MdMJT (▲), MSLMJT (○), and MSLdMJT (●) cells (10^5) were suspended in 1 ml of 10% fetal calf serum-supplemented RPMI 1640 medium with 100 (1,000:1; ratio of uninfected cells to infected cells) (A) or 1,000 (100:1) (B) Jurkat cells chronically infected with HXB2. Four days after infection, cells were split to adjust the cell concentration to 2×10^5 cells per ml; they were further split 1:5 every 3 days thereafter. Culture supernatants were used for the measurement of p24 antigen levels. This experiment was conducted in duplicate, yielding very similar values, and mean values were used for plots.

ble ribozyme-expressing cell lines at different ratios for infection (1,000:1 and 100:1; ratios of uninfected cells to infected cells). Low levels of p24 expression were detected in all cultures early, probably from infected Jurkat cells directly. HIV transmission and expression appeared to be biphasic in cocultures (Fig. 3). The expression of p24 in MdMJT and MLNL6 cells first increased in the first 2 to 3 weeks and then rose sharply at day 25 (Fig. 3A) at 1,000:1 infection or at day 19 at 100:1 infection (Fig. 3B). The level of virus expression in the first phase was about 10-fold higher in the culture with the higher input of infected cells. We speculate that the initial rise was predominantly due to cell-cell transmission, which was relatively much more efficient, while the later phase involves predominantly cell-free virus transmission. The emergence of the second rise in virus expression in MMJT and MSLdMJT cells was delayed to days 31 and 25, respectively, at the low input and high input of infected cells. Thus, a single antiviral gene (ribozyme or SL II decoy) had a detectable, though modest, inhibitory effect on viral transmission and replication. In contrast, the p24 level remained low in MSLMJT cells at 1,000:1 infection throughout 40 days of culture (Fig. 3A). Even at 100:1 infection, the p24 level increase in MSLMJT cells was

FIG. 4. Reduction of the proviral DNA burden during a first-round infection. After the infection of MMJT, MdMJT, MSLMJT, and MSLdMJT cells with HIV-1_{HXB2} for 7 h, cell lysates were prepared from infected cells as the template DNA for QC-PCR. PCR was carried out by using a ³²P-end-labeled SK29 and SK30 primer pair derived from the HIV-1 LTR in the presence of different concentrations of competitor DNA. The expected sizes of the amplified products were 105 and 123 bp for test and competitor DNAs, respectively. (A) After PCR, 3 μ l of each PCR product was loaded on an 8% polyacrylamide gel, electrophoresed for 16 h, and autoradiographed. (B) Images of the gel were scanned with a Twain Scan Duo 600 (Mustek) and analyzed by using NIH image version 1.54 and a Macintosh computer. Ratio (C/S), ratio of the signal intensities of the competitor DNA and test DNA products. A repeated experiment yielded very similar results.

delayed for an additional 3 days (to day 28) compared with that in MMJT or MSLdMJT cells. These results indicated that the SL II-ribozyme combination was more effective than either the ribozyme or SL II decoy alone in the inhibition of HIV-1. The lesser antiviral effect at the high infected-cell dosage is probably due to the relatively higher effective MOI of virus challenge.

Comparison of the ribozyme activities in MMJT and MSLMJT cells in a first-round infection. To examine the ribozyme activity of this fusion RNA, we measured the reduction in proviral DNA synthesis in the first round of replication after virus challenge. The RRE decoy effect should not be relevant in this early part of the replication cycle. The proviral DNA levels in stable cell lines were determined by QC-PCR 7 h after challenge with HIV-1_{HXB2}. Proviral DNA was amplified in the presence of different concentrations of competitor DNA with ³²P-end labeled SK29 and SK30 as the primer pair. The autoradiograph after QC-PCR and the results after the analysis of gel images are shown in Fig. 4A and B, respectively. The number of added competitor DNA molecules resulting in equal signal intensities for the amplified products of test and competitor DNAs was estimated from the regression line by the least-squares method and should correspond to the proviral DNA copy number in 2×10^5 cells. As expected, no difference in the proviral DNA copy number was observed between MdMJT and MSLdMJT cells, suggesting the lack of an RRE decoy effect on preintegration events. The DNA copy number for MSLMJT cells was reduced to 1/7 of that for MSLdMJT cells, whereas the DNA copy number for MMJT was reduced to 1/3. A similar QC-PCR using a primer pair for β -globin DNA confirmed that an equal number of cells was used to generate cell lysates for quantitative analyses (data not shown). This experiment was repeated, with similar results. Consequently, the results demonstrated that the SL II-ribozyme fusion RNA did indeed function as a ribozyme, and the reproducible difference observed between MSLMJT and MJT cells suggested that the linkage of the SL II sequence further improved ribozyme activity. The reduction in the DNA

level in MMJT cells was 10 to 20 times less than the result given in our previous paper (30). This may be due to the fact that a higher MOI (0.2 instead of 0.1) was used for infection in the present study or to differences in the assay procedures (QC-PCR versus semiquantitative PCR).

DISCUSSION

Recent studies of the dynamics of HIV replication in patients under antiviral therapy have reaffirmed the central role of the virus in disease progression and provided a strong rationale for the development of effective, long-term antiviral therapy (7, 12, 28). One interesting parameter from these studies is the extremely short life span of an HIV-1-infected CD4⁺ lymphocyte (half-life, 1 to 2 days), in contrast to other studies giving an estimated life span of months to years for uninfected lymphocytes (2, 3). These observations are particularly relevant for antiviral gene therapy since an intracellular immunized cell resistant to viral infection will be strongly selected for in vivo. However, because of the high rate of viral replication, the possibility of escape mutants is still a major issue with gene therapy.

In previous studies, we have demonstrated the efficacies of several anti-HIV-1 hairpin ribozymes in inhibiting virus replication in human T-cell lines (29, 30, 32, 34). With an anti-U5 ribozyme which targets a highly conserved region of the HIV-1 genome, we further showed that intracellular immunization of primary lymphocytes or hematopoietic progenitor cells could lead to resistance to both lymphotropic and macrophage tropic HIV-1 strains (15, 31). To further increase the antiviral potency of the ribozyme vector, as well as reduce the chance of viral resistance, we explored the possibility of adding other antiviral genes to ribozyme constructs.

Rev, an early gene product of HIV, controls the expression of the HIV-1 structural genes through binding to an RRE present in unspliced or partially spliced viral transcripts and facilitates the nuclear export and utilization of such transcripts in the cytoplasm (9, 10, 14, 19, 20). We reasoned that linkage

of the RRE sequence to a ribozyme would be a good strategy. Not only would this molecule be bifunctional (ribozyme and decoy effect), but ribozyme activity should also be facilitated by linking to RRE for several reasons. The RRE would further stabilize the ribozyme molecule; the binding of Rev to the RRE-ribozyme fusion molecule would allow it to traffic the same nuclear-cytoplasmic pathway as HIV mRNA does, thereby increasing the opportunity of interaction between enzyme and substrate; and the binding of Rev to the ribozyme-substrate complex is expected to increase ribozyme turnover, resulting in increased catalytic activity. The HIV-1 RRE is 234 nucleotides in length and is predicted to form a central stem and five stem-loop structures (19). First, we inserted the entire RRE sequence into the ribozyme expression cassette. Although a stable cell line expressing such fusion RNA demonstrated strong virus inhibition, the expression of this fusion RNA was turned off at week 15 after transfection (data not shown). Since RRE RNA is known to bind to one or more mammalian cellular proteins (27; 28a), such binding may induce cellular toxicity and provide a negative selection for cells expressing this fusion RNA. However, a minimal sequence consisting of the SL II of RRE binds to Rev (6, 8, 11, 16, 35), but not to known cellular factors. Hence, the SL II sequence was subsequently introduced into ribozyme transcription cassettes. Indeed, SL II-anti-U5 ribozyme fusion RNA was shown to be persistently expressed in stable cell lines over 25 weeks. It was also found to be more effective in virus inhibition than was the ribozyme alone or SL II linked with a disabled ribozyme (Fig. 3). The decoy effect of this fusion RNA was demonstrated by HIV-1_{SF2} infection of a stable cell line, MSLOY-1, expressing the SL II sequence linked to an anti-Rev ribozyme. Since SF2 is refractory to inhibition by the anti-Rev ribozyme because of a substitution at the G residue at the site of cleavage (Fig. 1B) (29), the observed inhibitory effect of this fusion RNA is likely to be due to the SL II sequence acting as a decoy. We also demonstrated the ribozyme activity of this fusion RNA by showing a reduction in the proviral DNA burden in a first-round infection (Fig. 4). Additionally, this fusion RNA appeared to exert a twofold-greater reduction in viral DNA synthesis than that of the ribozyme alone. Bertrand and Rossi (1) reported that adding the nucleocapsid protein of HIV-1 or heterogeneous ribonucleoprotein A1 to the cleavage reaction of hammerhead ribozymes increased the binding, specificity, and turnover of ribozymes in vitro without inhibiting cleavage, depending on the length of the ribozyme-substrate duplexed region.

Sullenger and Cech (26) reported that linkage of the packaging signal sequence to a ribozyme targeting the Moloney leukemia virus genome facilitated virus inhibition, probably because of cotracking of the ribozyme and virus RNA genome. We and others previously showed time-regulated nuclear export of Rev which was correlated with protein expression from RRE-containing mRNAs and proposed that the distribution of Rev reflects its interaction with RRE-containing RNAs and their migration from the nucleolus across a solid phase of nucleus and nuclear membrane to the cytoplasm through a specific export pathway (18, 21). Therefore, SL II-containing ribozyme (the fusion RNA) should traffic through the same cellular compartments as HIV mRNA does through the binding of Rev, thereby increasing the efficiency of the ribozyme catalytic activity. Although our assay of the first-round infection would be unable to address the potential enhancement of ribozyme activity by the RRE through mechanisms of RNA trafficking or Rev binding, as discussed above, SL II could conceivably stabilize the ribozyme molecule by adding a highly folded structure to the 5' end of the molecule (1). It has been

proposed that the 5' half of the ribozyme sequence is less stable than its 3' half, since it has no intrinsic secondary structure. However, since no significant differences were observed in the steady-state levels of ribozyme RNA in MJT and MSLMJT cells (Fig. 2), the reason for the observed improvement of ribozyme activity in MSLMJT cells remains unclear. Nonetheless, the additional mechanisms likely contributed to the greater inhibition of virus replication and expression (Fig. 3). Formal proof of the trafficking hypothesis will require determinations of the cellular localizations of this fusion RNA and Rev-bound complexes by in situ hybridization or in situ RT-PCR coupled with immunofluorescence assays. In any event, by coexpression of a combination of different small therapeutic RNAs (ribozymes and RNA decoys) that function additively or synergistically to target multiple conserved sites of the HIV-1 genome, one may hope to ultimately eliminate the possibility of the emergence of escape virus mutants in long-term in vivo therapy.

ACKNOWLEDGMENTS

We thank D. Kang for technical assistance.

This work was supported in part by the NIH SPIRAT award and NIH grant DK49618 to F.W.S.

REFERENCES

- Bertrand, E. L., and J. J. Rossi. 1994. Facilitation of hammerhead ribozyme catalysis by the nucleocapsid protein of HIV-1 and the heterogeneous nuclear ribonucleoprotein A1. *EMBO J.* **13**:2904-2912.
- Blaese, R. M., K. W. Culver, A. D. Miller, C. S. Carter, T. Fleisher, M. Clerici, G. Shearer, L. Chang, Y. Chiang, P. Tolstoshev, J. J. Greenblatt, S. A. Rosenberg, H. Klein, M. Berger, C. A. Mullen, W. J. Ramsey, L. Muul, R. A. Morgan, and W. F. Anderson. 1995. T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. *Science* **270**:475-480.
- 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.
- Chomczynski, P., and N. Sacchi. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156-159.
- Chowria, B. M., A. Berzal-Herranz, and J. M. Burke. 1991. Novel guanosine requirement for catalysis by the hairpin ribozyme. *Nature (London)* **354**:320-322.
- Cochrane, A., C. Chen, and C. Rosen. 1990. Specific interaction of the human immunodeficiency virus Rev protein with a structured region in the env mRNA. *Proc. Natl. Acad. Sci. USA* **87**:1198-1202.
- Coffin, J. M. 1995. HIV population dynamics in vivo—implications for genetic variation, pathogenesis, and therapy. *Science* **267**:483-489.
- Daefler, S., M. E. Klotman, and F. Wong-Staal. 1990. Trans-activating rev protein of the human immunodeficiency virus 1 interacts directly and specifically with its target RNA. *Proc. Natl. Acad. Sci. USA* **87**:4571-4575.
- Feinberg, M. B., R. F. Jarret, A. Adovini, R. C. Gallo, and F. Wong-Staal. 1986. HTLV-III expression and production involve complex regulation at the levels of splicing and translation of viral RNA. *Cell* **46**:807-817.
- Felber, B. K., M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, and G. N. Pavlakis. 1989. Rev protein of human immunodeficiency virus type 1 affects the stability and transport of the viral mRNA. *Proc. Natl. Acad. Sci. USA* **86**:1495-1499.
- Heaphy, S., C. Dingwall, I. Ernberg, M. J. Gait, S. M. Green, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner. 1990. HIV-1 regulator of virion expression (Rev) protein binds to an RNA stem-loop structure located within the Rev response element region. *Cell* **60**:685-693.
- Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature (London)* **373**:123-126.
- Joseph, S., and J. M. Burke. 1993. Optimization of an anti-HIV hairpin ribozyme by in vitro selection. *J. Biol. Chem.* **268**:24515-24518.
- Kjems, J., A. D. Frankel, and P. A. Sharp. 1991. Specific regulation of mRNA splicing in vitro by a peptide from HIV-1 Rev. *Cell* **67**:169-178.
- 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.
- 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.
17. Lee, T. C., B. A. Sullenger, H. F. Gallardo, G. E. Ungers, and E. Gilboa. 1992. Overexpression of RRE-derived sequences inhibits HIV-1 replication in CEM cells. *New Biol.* **4**:55–74.
 18. Luznik, L., M. E. Marton, G. Kraus, Y. Zang, Y. Xu, M. H. Ellisman, and F. Wong-Staal. 1995. Localization of human immunodeficiency virus Rev in transfected and virus-infected cells. *AIDS Res. Hum. Retroviruses* **11**:795–804.
 19. Malim, M. H., J. Hauber, S.-Y. Le, J. V. Maizel, and B. R. Cullen. 1989. The HIV-1 Rev trans-activator acts through a structured target sequence to activate nuclear export of transplanted viral mRNA. *Nature (London)* **338**:254–257.
 20. Malim, M. H., L. Tiley, D. McCarn, J. Rusche, J. Hauber, and B. Cullen. 1990. HIV-1 structural gene expression requires binding of the Rev trans-activator to its RNA target sequence. *Cell* **60**:675–683.
 21. Meyer, B. E., and M. H. Malim. 1994. The HIV-1 Rev trans-activator shuttles between the nucleus and the cytoplasm. *Genes Dev.* **8**:1538–1547.
 22. Ojwang, J., A. Hampel, D. Looney, F. Wong-Staal, and J. Rappaport. 1992. Inhibition of human immunodeficiency virus type-1 (HIV-1) expression by a hairpin ribozyme. *Proc. Natl. Acad. Sci. USA* **89**:10802–10806.
 23. Ou, C.-Y., S. Kwok, S. W. Mitchell, D. H. Mack, J. W. Sninsky, J. W. Krebs, P. Feorino, D. Warfield, and G. Schochetman. 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. *Science* **239**:295–297.
 24. Rossi, J. J., and N. Sarver. 1990. RNA enzymes (ribozymes) as antiviral therapeutic agents. *Trends Biotechnol.* **8**:179–183.
 25. Sarver, N., E. M. Cantin, P. S. Chang, J. A. Zaia, P. A. Ladne, D. A. Stephens, and J. J. Rossi. 1990. Ribozymes as potential anti-HIV-1 therapeutic agents. *Science* **247**:1222–1225.
 26. Sullenger, B. A., and T. R. Cech. 1993. Tethering ribozymes to a retroviral packaging signal for destruction of viral RNA. *Science* **262**:1566–1569.
 27. Vaishnav, Y. N., M. Vaishnav, and F. Wong-Staal. 1991. Identification and characterization of a nuclear factor that specifically binds to the Rev response element (RRE) of human immunodeficiency virus type 1 (HIV-1). *New Biol.* **3**:142–150.
 28. Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, and et al. 1995. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature (London)* **373**:117–122.
 - 28a. Xu, Y., T. R. Reddy, W. H. Fischer, and F. Wong-Staal. A novel HnRNP specifically interacts with HIV-1 RRE RNA. *J. Biomed. Sci.*, in press.
 29. 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.
 30. 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.
 31. Yu, M., M. C. Leavitt, M. Maruyama, O. Yamada, D. Young, A. 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.
 32. Yu, M., J. 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.
 33. Yu, M., E. Poeschla, and F. Wong-Staal. 1994. Progress towards gene therapy for HIV infection. *Gene Ther.* **1**:13–26.
 34. Yu, M., E. Poeschla, O. Yamada, P. Degrandis, M. C. Leavitt, M. Heusch, J.-K. Yees, F. Wong-Staal, and A. Hampel. 1995. *In vitro* and *in vivo* characterization of a second functional hairpin ribozyme against HIV-1. *Virology* **206**:381–386.
 35. Zapp, M. L., and M. R. Green. 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. *Nature (London)* **342**:714–716.