HIV-1 RT-dependent DNAzyme expression inhibits HIV-1 replication without the emergence of escape viruses

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ABSTRACT

DNAzymes are easier to prepare and less sensitive to chemical and enzymatic degradation than ribozymes; however, a DNA enzyme expression system has not yet been developed. In this study, we exploited the mechanism of HIV-1 reverse transcription (RT) in a DNA enzyme expression system. We constructed HIV-1 RT-dependent lentiviral DNAzyme expression vectors including the HIV-1 primer binding site, the DNA enzyme, and either a native tRNA (Lys-3), tR^{M}DtR^L, or one of two truncated tRNAs (Lys-3), tR^{M}DΔARMtR^L or tR^{M}D3′-endtR^L. Lentiviral vector-mediated DNAzyme expression showed high levels of inhibition of HIV-1 replication in SupT1 cells. We also demonstrated the usefulness of this approach in a long-term assay, in which we found that the DNAzymes prevented escape from inhibition of HIV. These results suggest that HIV-1 RT-dependent lentiviral vector-derived DNAzymes prevent the emergence of escape mutations.

INTRODUCTION

RNAi has emerged as a powerful tool for probing the function of genes of a known sequence both *in vitro* and *in vivo*. Recent studies describe the ability of RNAi to decrease the replication of human immunodeficiency virus type 1 (HIV-1) in lymphocytes using siRNAs targeting viral proteins (for example, *tat*, *gag*, *rev, env* and *nef*) (1–9) as well as host proteins (for example, CCR5 and CD4) (10–12). Thus, this technique has the potential to be used as a form of gene therapy for HIV-1 and associated infections. More recently, several groups have reported that the antiviral activity of short hairpin RNA (shRNAs) targeting HIV-1 is abolished owing to the emergence of viral quasi-species harboring a point mutation in the shRNA target region (13–16). This finding is particularly relevant for viruses that exhibit significant genetic variation due to error-prone replication machinery, and the risk is likely to be more severe for RNA viruses and retroviruses than for DNA viruses.

Ribozyme technologies are major tools used to inactivate genes in gene therapy (17–19). One model, termed the deoxyribozyme (Dz) model, is especially useful because it can bind and cleave any single-stranded RNA at purine/pyrimidine junctions (20–22). The DNAzyme is similar to hammerhead ribozymes in terms of its secondary structure, which contains two binding arms and a catalytic loop that captures the indispensable catalytic metal ions (23–25). Previously, we described a new system designed for single-stranded DNA (ssDNA) expression using HIV-1 reverse transcriptase (26). The expressed DNAzymes were shown to possess *in vitro* site-specific cleavage activity.

Here, we describe the inhibition of HIV-1 replication by an HIV-1 reverse transcription (RT)-dependent lentiviral vector-transduced DNAzyme. In addition, we describe the construction of a lentiviral vector encoding the DNAzyme, the HIV-1 primer binding site, a native transfer RNA (tRNA)$^{Lys-3}$, and the flanking arms complementary to the HIV-1 V3-loop of the *env* messenger RNA (mRNA) (10,25–28) downstream of the Pol III promoters, tRNA$_i^{Met}$ (29) or U6 (30). The HIV-1 RT-dependent lentiviral vector-transduced DNAzyme inhibited HIV-1 replication and prevented the emergence of resistant viruses in long-term assays.

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MATERIALS AND METHODS

Construction of lentiviral vectors

Plasmids pVAX-Dz-tRNA^{Lys-3}-ter, pVAX-Dz-ΔARMtRNA^{Lys-3}-ter and pVAX-Dz-3′-endtRNA^{Lys-3}-ter were constructed as described earlier (26) and digested with *Kpn* I and *Eco*R I. DNA was then extracted with phenol/chloroform, precipitated with ethanol, and ligated into the *Kpn* I and *Eco*R I sites of pSV2neo (L6) (31) with the tRNA$_i^{Met}$ promoter (29). DNAzyme expression vectors (pL6-tRNA$_i^{Met}$-Dz-tRNA^{Lys-3}-ter, pL6-tRNA$_i^{Met}$-Dz-ΔARMtRNA^{Lys-3}-ter and pL6-tRNA$_i^{Met}$-Dz-3′-endtRNA^{Lys-3}-ter) were digested with *Eco*R I and then cloned into the same site in the lentiviral transfer vector (CS-CDF-CG-PRE).

The shRNA sequences were chemically synthesized as two complementary DNA oligonucleotides: 5′-GACAAG CACATTCTAACATTTCAAGAGAATGTTAGAATG TGCTTGTCTTTTTGGGCCC-3′ and 5′-TCGAGGGCC CAAAAAGACAAGCACATTCTAACATTCTCTTGA AATGTTAGAATGTGCTTGTCGTAC-3′. These oligonucleotides were annealed and ligated into pU6-ter (*Kpn* I and *Xho* I cloning sites). pU6-shRNA-ter, pU6-Dz-tRNA^{Lys3}-ter, pU6-Dz-3′-endtRNA^{Lys3}-ter and the controls, pU6-ter and pU6-Dz-I-3′-endtRNA^{Lys3}-ter (DNAzyme with an inverted catalytic core sequence) (32,33), were each digested with *Eco*R I and *Nhe* I and then cloned into the same sites in the CS-CDF-CG-PRE vector.

Cell culture

SupT1 and 293T cells were grown in RPMI 1640 medium or Dulbecco's modified Eagle's medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

RT–PCR analysis (RNA expression)

Total RNA from vector-transduced cells was extracted using a GenElute mammalian total RNA kit (Sigma-Aldrich). After isolation, RNA samples were treated with DNase I (Takara Shuzo, Kyoto, Japan) according to the manufacturer's specifications. Reverse transcription polymerase chain reaction (RT–PCR) was then performed using an RNA PCR high-plus kit (Toyobo, Osaka, Japan) with the following primers: forward primers, 5′-AGCAGAGTGGCGCAGCGGAAG-3′ for the tRNA$_i^{Met}$ promoter F1, 5′-GTACCCAAGCACTCG TT-3′ for the U6 promoter F4 and 5′-GTACCCAAGCAC TTCCGATC-3′ for the U6-D-I-3′-endtRNA^L F5; and reverse primers, 5′-CACTCGTTACAAGGCTAGCTACAAC-3′ for the tRNA$_i^{Met}$ promoter R1 and 5′-TGGCGCCGAA CAGGGACTT-3′ for the U6 promoter R3. RT–PCR products were amplified using the following thermal cycling program: 60°C for 30 min, 94°C for 2 min, and then 25 cycles at 94°C for 1 min and 50°C for 90 s, followed by 51°C for 7 min. As an internal control, the mRNA of the human control gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Accession No. NM_002046.3) was amplified simultaneously using G3PDH-F (nucleotides 628–647) and G3PDH-R (nucleotides 1060–1079) primers.

Southern hybridization

SupT1 cells stably expressing the DNAzyme were infected with HIV-1$_{NL4-3}$. After 8 h, cytoplasmic extracts were obtained using a nuclear extraction kit (Marligen Bioscience, Ijamsville, MD, USA). Cytoplasmic extracts were then digested with RNase (Promega, Madison, WI, USA) according to the manufacturer's specifications and separated by 18% polyacrylamide-8 M urea sequencing gel electrophoresis. Next, the DNA was transferred to Zeta-Probe nylon membranes (Bio-Rad, Hercules, CA, USA). The filters were prehybridized for 1 h at 42°C in ECL gold hybridization buffer (GE Healthcare, Chalfont St Giles, UK) and then incubated for 16 h in hybridization buffer containing DNAzyme- or DNAzyme inverse-specific probes labeled with 5′-biotin (5′-GTACCCAAGCACTCGTTGTAGCTAGCCTTGT AAC-3′ or 5′-GTACCCAAGCACTTCCGATCGATGT TGCTGTAAC-3′). The blots were immersed in a chemiluminescent nucleic acid detection module kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and exposed to Kodak XAR-5 film.

RT–PCR analysis (mRNA cleavage)

Total RNA from vector-transduced cells was extracted using a GenElute mammalian total RNA kit (Sigma-Aldrich). RT–PCR was then performed using an RNA PCR high-plus kit (Toyobo) with *env* upstream (NL4-3 7070–7099), *env* neutral (NL4-3 7241–7271) and *env* downstream (NL4-3 7570–7600) forward primers F2 (5′-ACAGCTGAACACATCTGTAGAAATTAATT G-3′) and F3 (5′-AAACAGATAGCTAGCAAATTAAG AGAACAA-3′) and with the reverse primer R2 (5′-GTTG TTATTACCACCATCTCTTGTTAATAG-3′). RT–PCR products were amplified using the following thermal cycling program: 60°C for 30 min, 94°C for 2 min and then 25 cycles of 94°C for 1 min and 53°C for 90 s, followed by 51°C for 7 min. As an internal control, the mRNA of the human control gene G3PDH was amplified simultaneously with G3PDH-F (nucleotides 628–647) and downstream G3PDH-R (nucleotides 1060–1079) primers.

Lentiviral vector preparation

A vector construct (15 μg) was co-transfected into 293T cells with helper constructs encoding gag/pol (pMDLg/p.RRE; 15 μg), the rev-expressing construct pRSV-rev (5 μg), and the VSV-G-expressing construct pMD.G (5 μg), using the calcium phosphate precipitation method. Supernatants were harvested 48 h posttransfection, filtered through a 0.45-μm filter disc, and concentrated 100-fold by centrifugation at 6000*g* overnight. The resultant viral pellet was resuspended in serum-free and antibiotic-free RPMI medium and stored at −80°C until use. To determine the viral titer, SupT1 cells were transduced with the prepared viral stock, and the number of EGFP-positive cells was determined using

flow cytometric analysis (BD Biosciences, Pharmingen, San Diego, CA, USA) after 72 h of culture.

Flow cytometry

Transduced SupT1 cells were washed twice in phosphate-buffered saline (PBS) and then fixed in PBS containing 1% formaldehyde. Direct fluorescence of EGFP was analyzed using a FACSCalibur system (BD Biosciences). Data acquisition and analysis were performed with CellQuest software (BD Biosciences). Gates for EGFP detection were established using mock-transduced cells as a background.

Fluorescence microscopy

We investigated the efficiency of EGFP expression as an index for SupT1 cells expressing the transgenes. For the intracellular fluorescence studies, SupT1 cells were fixed with 3.7% formaldehyde on alternating days. Fluorescent cells were examined under a fluorescence microscope (Biozero BZ-8000; KEYENCE, Osaka, Japan) at an excitation wavelength of 488 nm using a 10× objective lens. Images were acquired at a resolution of 512 × 512.

HIV-1 challenge and culture assay

After transduction with the lentiviral vectors, EGFP-positive SupT1 cells were sorted using a FACSVantage system (Becton Dickinson, Franklin Lakes, NJ, USA) and infected with HIV-1$_{NL4-3}$ or mutant virus HIV-1$_{NL4-3-env-mut-a}$ at a multiplicity of infection of 0.1. After the harvested culture was centrifuged, the cell-free medium was used for an HIV-1 p24 chemiluminescent enzyme immunoassay (Fujirebio, Tokyo, Japan) (34).

Genotypic sequence analysis of the vif siRNA target region of HIV-1$_{NL4-3}$

Viral RNA from HIV-1$_{NL4-3}$-challenged CS-env-shRNA transduced cultures was analyzed for siRNA-mediated mutations in the env-shRNA target region at 43 days postinfection, as described earlier (35,36). Viral RNA was isolated from the cell-free culture supernatant using a QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Viral RNA (5 µl) was used in an RT–PCR reaction containing Powerscript reverse transcriptase (Clontech, Mountain View, CA, USA), 1 µM each of the deoxynucleotide triphosphates, 1 × first-strand buffer (Clontech), 200 ng random hexamers (Promega) and 10 U RNasin (Promega). Reverse transcription was performed at 42°C for 1 h, followed by heat inactivation of the reverse transcriptase at 70°C for 15 min. cDNA (2 µl) was added to a 48-µl PCR mixture containing 1 × Qiagen Taq PCR buffer, 1.5 mM MgCl$_2$, 20 pmol each of the sense primer *env* F (5′-ATG GAA AAC AGA TGG CAG GTG AT-3′) and the antisense primer *env* R (5′-CTA GTG TCC ATT CAT TGT ATG GCT-3′), 1 mM deoxynucleotide triphosphates and 2.5 U Taq polymerase (Qiagen). PCR was performed in a gradient PCR thermal cycler (Astec, Fukuoka, Japan)

using the following thermal program: 95°C for 1 min and then 35 cycles at 95°C for 15 s, 58°C for 30 s and 72°C for 30 s, followed by 72°C for 5 min. The PCR product was fractionated, analyzed in a 1% SeaKem gel, and purified using a QIAEX II gel extraction kit (Qiagen). Nucleotide sequencing was performed using dye-labeled terminator chemistry.

Generation of viruses

After 43 days, the harvested supernatant containing the *env* mutation virus, HIV-1$_{NL4-3-env-mut-a}$, was titered, stored at −80°C, and later used as the HIV-1$_{NL4-3-env-mut}$ virus.
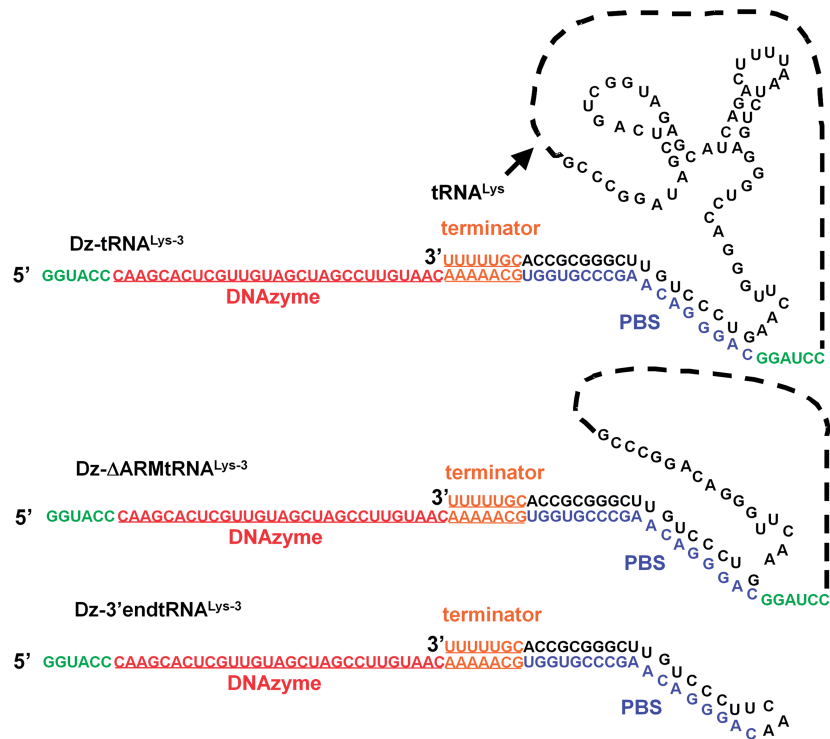
RESULTS

Construction of lentiviral vectors

The design and *in vitro* strategies used to generate the DNAzyme are shown in Figure 1. First, we show the predicted secondary structure of the HIV-1 RT-dependent DNAzyme expression vector (Figure 1A). As shown in Figure 1A, the native tRNA^{Lys-3} or one of two truncated tRNAs-(ΔARMtRNA^{Lys-3} and 3′-endtRNA^{Lys-3})-PBS complexes served as the primer for HIV-1 RT. Next, we constructed the ssDNA lentiviral expression vectors tR^M-D-tR^L, tR^M-D-ΔARMtR^L, tR^M-D-3′-endtR^L, U6-D-tR^L and U6-D-3′-endtR^L containing the DNAzyme, the HIV-1 primer binding site (PBS), and either a native tRNA^{Lys-3} or one of two truncated tRNAs [tR^M-D-ΔARMtR^L, which lacks the D-stem loop, the anticodon-stem loop and the variable loop or tR^M-D-3′-endtRNA^{Lys-3}, which lacks the D-stem-loop, the anticodon-stem loop, the variable loop and the 50-end strand from the full-length tRNA (Lys-3)] that are under the control of the tRNA$_i^{Met}$ or U6 promoter (Figure 1B). We also constructed the control lentiviral vectors tR^M, U6, U6-D-I-tR^L (DNAzyme with the inverted catalytic core sequence) (32,33) and U6-env-shRNA (NL4-3 *env* 7193–7213) (8) for comparison with the DNAzyme. Sequences encoding an active fragment of the DNA enzyme that contained the 10–23 catalytic motif (24,37), the HIV-1 primer binding site and three different tRNA lengths (Lys-3) were inserted between the *Kpn* I and *Eco*R I restriction sites of the RNA transcription vector, pVAX1 (Figure 1B). The DNA enzyme sequence was placed between two oligonucleotide arms that were complementary and able to specifically target the HIV-1 *env* mRNA (7196–7210, according to GenBank accession number AF324493; env-Dz of Figure 1C).

DNA expression in human T cells stably expressing the DNAzyme

SupT1 cells were infected with ssDNA lentiviral expression vectors, and the corresponding template RNAs were expressed from the tRNA$_i^{Met}$ promoter (Figure 2A). Total cellular RNA was isolated from SupT1 cells stably expressing the DNAzyme and analyzed by RT–PCR. The template RNA for the DNAzyme, the HIV-1 primer
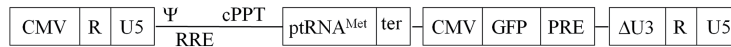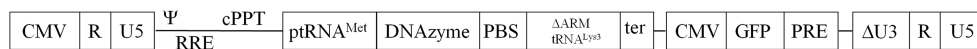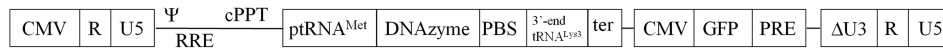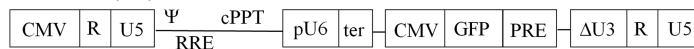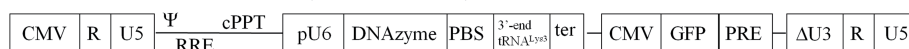
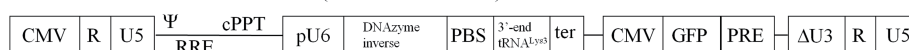**Figure 1.** DNAzymes, lentiviral vectors and Dz structures. (**A**) The predicted secondary structure of the ssDNA-expressing lentivirus vector. Sequence of the template RNA containing the DNA enzyme, HIV-1 primer binding site (PBS), and the native tRNA^{Lys-3}, ΔARMtRNA^{Lys-3} (lacking the D-stem loop, the anticodon-stem loop and the variable loop) and 3′-endtRNA^{Lys-3} (lacking the D-stem loop, the anticodon-stem loop and the variable loop, and the 50 end strand from the full-length tRNA^{Lys-3}). (**B**) Lentiviral vector (CS-CDF-CG-PRE) containing the packaging signal (ψ) comprising the 5′-untranslated region (UTR) and 5′-sequences of the Rev-responsive element, the central polypurine tract and the woodchuck hepatitis virus posttranscriptional regulatory element. The 3′-long-terminal repeat contains a large deletion in the U3 region (ΔU3). CMV, human cytomegalovirus immediate early promoter; EGFP, enhanced green fluorescent protein; ptRNA^{Met}, transfer RNA-methionine promoter (tR^M); U6, U6 promoter; ter, terminator; D, DNAzyme; PBS, primer binding site; shRNA, short hairpin RNA; D-I, DNAzyme with inverted catalytic core sequence. (**C**) Structure of an active or inactive catalytic (I) motif containing DNAzymes targeting the AU dinucleotides present in the HIV-1 mRNA including the V3-loop of the *env* mRNA region. Cleavage occurs at the position indicated by the arrow.
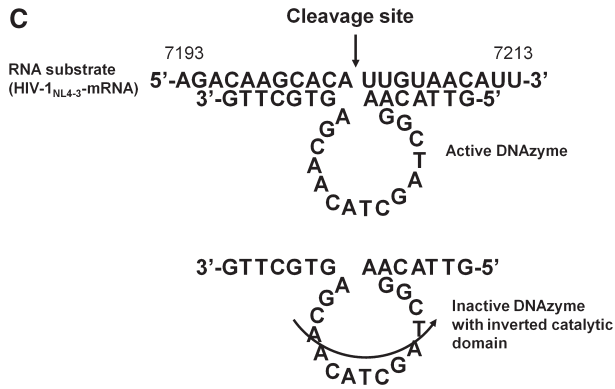
C

Figure 1. Continued.

binding site and a native tRNA^{Lys-3} driven by the tRNA$_i^{Met}$ promoter were expressed in SupT1 cells stably expressing the DNAzyme (Figure 2A, lanes 2–4). However, the direct expression of RNA from the ssDNA lentiviral expression vector resulted in the expression of sense sequences to HIV-*env* mRNA target regions and consequently did not inhibit HIV-1 replication. The control lentiviral vector, CS-tRNA$_i^{Met}$-ter (tR^M), did not express the corresponding RNA (Figure 2A, lane 1).

Next, to confirm ssDNA expression in SupT1 cells stably expressing the DNAzyme, we infected them with HIV-1$_{NL4-3}$. Cytoplasmic extracts obtained using digitonin lysis buffer were digested with RNase A, and ssDNA expression was demonstrated by Southern blot
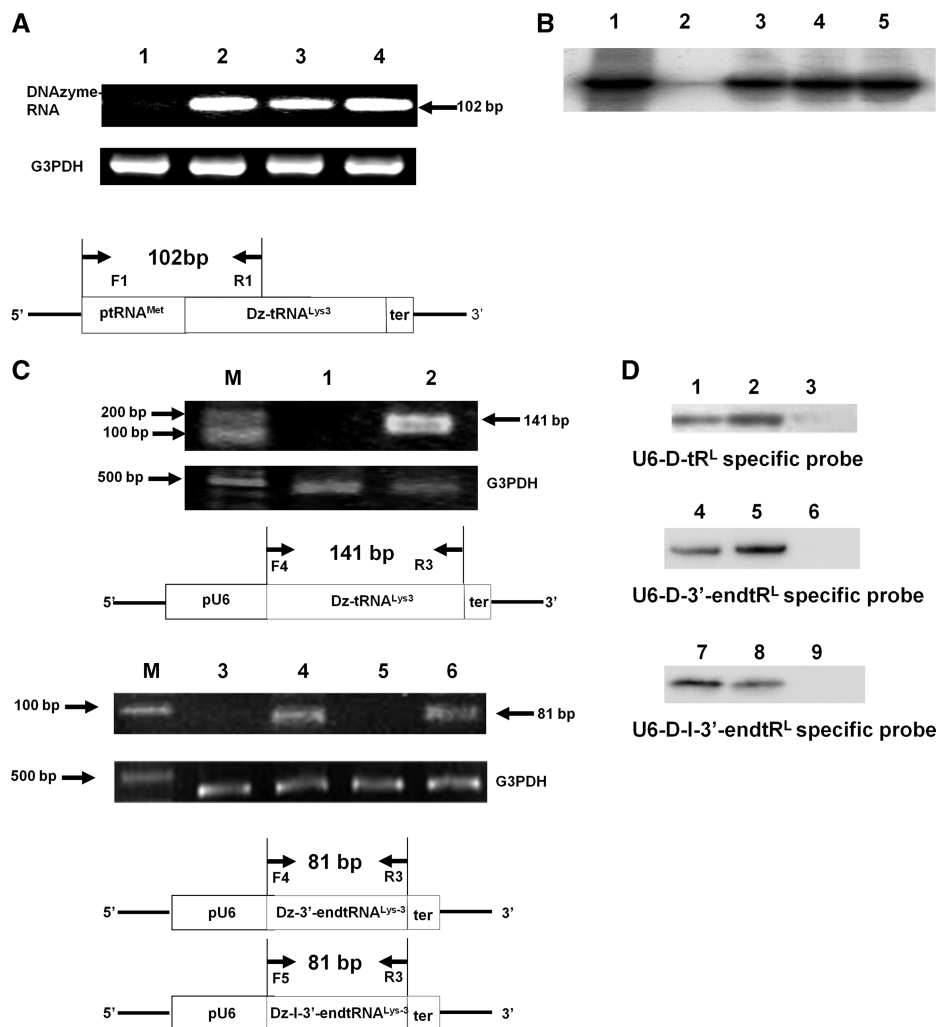

Figure 2. Detection of DNAzyme RNAs and ssDNAs in SupT1 cells stably expressing DNAzyme. (**A**) RT–PCR amplification products of RNAs containing the DNAzyme, HIV-1 PBS and a native tRNA^{Lys-3} driven by the tRNA$_i^{Met}$ promoter. Lane 1, control; lane 2, tR^M-D-tR^L; lane 3, tR^M-D-ΔARMtR^L and lane 4, tR^M-D-3′-endtR^L. (**B**) Detection of ssDNA expression by Southern blot analysis. Lane 1, synthetic ssDNA (102 bp); lane 2, tR^M-D-tR^L-without HIV-1$_{NL4-3}$; lane 3, tR^M-D-tR^L; lane 4, tR^M-D-ΔARMtR^L and lane 5, tR^M-D-3′-endtR^L. (**C**) RT–PCR amplification of RNAs containing the DNAzyme, HIV-1 PBS and a native tRNA^{Lys-3} driven by the U6 promoter. Lane M, DNA marker; lane 1, U6; lane 2, U6-D-R^L; lane 3, U6; lane 4, U6-D-3′-endtR^L; lane 5, U6; lane 6, U6-D-I-3′-endtR^L. (**D**) Detection of ssDNA expression by Southern blot analysis. Lane 1, synthetic ssDNA (34 bp); lane 2, U6-D-tR^L; lane 3, U6-D-tR^L-without HIV-1$_{NL4-3}$; lane 4, synthetic ssDNA (34 bp); lane 5, U6-D-3′-endtR^L; lane 6, U6-D-3′-endtR^L without HIV-1$_{NL4-3}$; lane 7, synthetic ssDNA (34 bp); lane 8, U6-D-I-3′-endtR^L and lane 9, U6-D-I-3′-endtR^L without HIV-1$_{NL4-3}$.

analysis (Figure 2B). The authentic sample, ssDNA containing the tRNA$_i^{Met}$ promoter, and DNAzyme sequences were synthesized using RT (Figure 2B, lane 1). All ssDNAzymes (tR^M-D-tR^L, tR^M-D-ΔARMtR^L and tR^M-D-3′-endtR^L) were found to be expressed at readily detectable levels (Figure 2B, lanes 3–5). We also examined ssDNA expression in HIV-1-uninfected SupT1 cells stably expressing tR^M-D-tR^L. Although we observed the corresponding template RNA (Figure 2A), the corresponding ssDNAzyme (tR^M-D-tR^L) was not expressed (Figure 2B, lane 2). These data suggest that ssDNA expression is achieved by HIV reverse transcriptase through the template RNA after HIV-1 infection of SupT1 cells stably expressing the DNAzyme.

To compare the U6 and tRNA$_i^{Met}$ promoters, we constructed the ssDNA lentiviral expression vectors U6-D-tR^L and U6-D-3′-endtR^L, which were under the control of the U6 promoter (Figure 1B). The template RNA containing the DNAzymes (U6-D-tR^L and U6-D-3′-endtR^L) was expressed by the lentiviral vectors (U6-D-tR^L and U6-D-3′-endtR^L) in infected SupT1 cells (Figure 2C, lanes 2 and 4). After stable DNAzyme-expressing SupT1 cells (U6-D-tR^L) were challenged with HIV-1$_{NL4-3}$, cytoplasmic extracts were digested with RNase A, and ssDNA expression was demonstrated by Southern blot analysis. The authentic sample and ssDNA containing the DNAzyme sequences (34 bases) were synthesized by RT (Figure 2D, lanes 1 and 4), and ssDNAzymes (Dz) were found to be expressed at readily detectable levels (Figure 2D, lanes 2 and 5). We also examined ssDNA expression in HIV-1-uninfected SupT1 cells stably expressing U6-D-tR^L and U6-D-3′-endtR^L. The corresponding template RNAs (Figure 2C, lanes 2 and 4), but not the corresponding ssDNAzymes (Figure 2D, lanes 3 and 6), were expressed. Furthermore, the control lentiviral vector U6-D-I-3′-endtR^L (DNAzyme with the inverted catalytic core sequences) also expressed the corresponding template RNA (Figure 2C, lane 6) and ssDNAzyme (Figure 2D, lane 8). ssDNA from the corresponding U6-D-tR^L, U6-D-3′-endtR^L and U6-D-I-3′-endtR^L produced the expected 34-bp band (Figure 2D, lanes 2, 5 and 8).

Long-term inhibition of HIV-1 gene expression by HIV-1 RT-dependent lentiviral vector-derived DNAzyme

To investigate the long-term inhibition of HIV-1 replication, SupT1 cells were stably transduced with the lentiviral expression vectors and then challenged with HIV-1$_{NL4-3}$. HIV-1 gag p24 antigen levels were measured as an index of viral replication or inhibition by the expressed transgenes at 3 days intervals over a 60 days period. HIV-1 replication was inhibited in SupT1 cells stably expressing the DNAzyme without any viral breakthrough at 60 days postinfection (Figure 3A). By contrast, the control lentiviral vector, tR^M, failed to inhibit viral replication under these experimental conditions. Furthermore, we observed enhanced green fluorescent protein (EGFP) expression at 60 days postinfection in transduced SupT1 cells (Figure 3B). These results suggest that ssDNAzyme expression might initiate from the primer binding site. In

SupT1 cells stably expressing env-shRNA, the siRNA-related escape mutant phenomenon was observed at 33 days postinfection in transduced SupT1 cells, as indicated by the virus breakthrough effect (Figure 3A).

To compare the U6 and tRNA$_i^{Met}$ promoters, we examined the long-term inhibition of HIV-1 replication in the different types of stable DNAzyme-expressing SupT1 cells (U6-D-tR^L, U6-D-3′-endtR^L or tR^M-D-tR^L). HIV-1 replication was completely inhibited in all cell types without any viral breakthrough at 60 days postinfection (Figure 3A). By contrast, the controls, U6 and DNAzyme with an inverted catalytic core sequence (U6-D-I-3′-endtR^L) (32,33), showed no inhibitory effect on HIV-1 replication (Figure 3A). We also observed EGFP expression at 60 days postinfection in transduced U6-D-tR^L, U6-D-3′-endtR^L and U6-D-I-3′-endtR^L SupT1 cells (Figure 3B). Therefore, the efficacy of HIV-1 replication inhibition did not differ between these two promoters. Furthermore, our data demonstrated a DNAzyme-specific inhibitory effect on HIV-1 replication, but not an antisense effect.

The inhibitory effect of the DNAzyme occurs via target RNA degradation

The contribution of HIV-1 mRNA degradation to the DNAzyme-mediated anti-HIV-1 effect was examined by measuring HIV-1 mRNA levels. Two sets of RT–PCR reactions were used to establish the level of HIV-1 mRNA at the target site that was not cleaved by DNAzyme (product 1; 529 bp) and the total amount of HIV-1 mRNA cleaved at the target site (product 2; 351 bp). The uncleaved HIV-1 mRNA was amplified using primers F2 and R2 (Figure 4A) (35). The levels of product 1 were expected to decrease after cleavage of the HIV-1 mRNA, whereas the levels of product 2 reflected the total amount of HIV-1 mRNA, as the 3′-fragment of the cleaved HIV-1 mRNA remained a viable template for RT–PCR amplification. We observed that progeny virus production was decreased in cells expressing DNAzyme or shRNA, whereas the control lentiviral vector, tR^{Met}, did not greatly alter uncleaved HIV-1 mRNA expression after 10 days postinfection (Figure 4A). However, after 43 days postinfection, a band of uncleaved HIV-1 mRNA appeared in the shRNA-dependent expression system (Figure 4A, lane 6) but not in the DNAzyme system (Figure 4A, lane 7). Furthermore, the contribution of HIV-1 mRNA cleavage to the DNAzyme-mediated anti-HIV-1 effect was examined by measuring HIV-1 mRNA levels, which revealed that the DNAzyme degraded the target RNA (Figure 4B, lane 3). These data are consistent with the results of the gag-p24 antigen assays and suggest that the inhibitory effect of the DNAzyme is achieved via degradation of the target RNA by the DNAzyme.

Generation of HIV-1 mutants that escape shRNA-env

RNAi has not been shown to protect cells against HIV-1 in long-term virus replication assays. Here the siRNA-related escape mutant phenomenon was observed at 33 days postinfection in transduced SupT1 cells, as
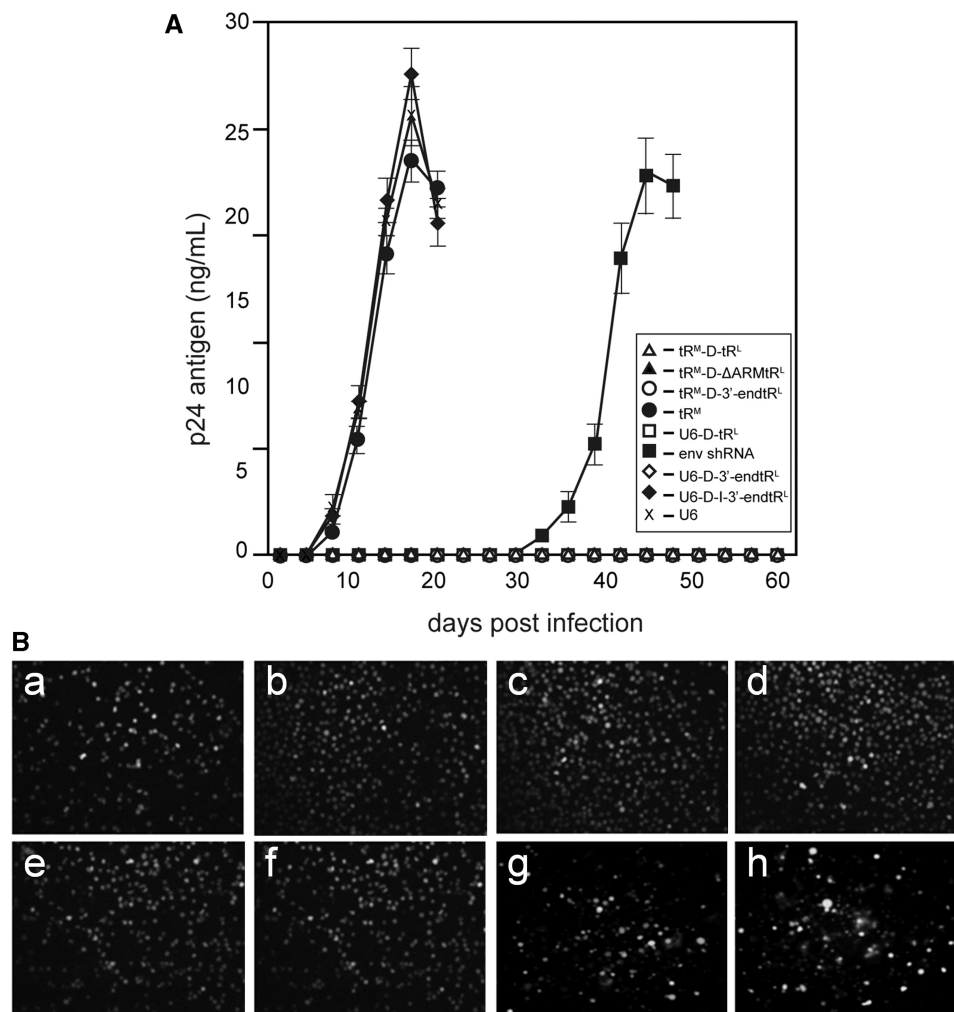
Figure 3. (A) Inhibition of HIV-1 gene expression by tRNA^{met} or U6 promoter-mediated DNAzyme and env-shRNA in human T cells. p24 antigen levels assessed over a 60-days period. Data represent means ± SDs (error bars) from three independent experiments. (B) Long-term EGFP expression in SupT1 cells transduced with the indicated lentiviruses as examined by fluorescence microscopy for EGFP expression at 60 days following transduction. (a) tR^{M}; (b) tR^{M}-D-tR^{L}; (c) tR^{M}-D-ΔARMtR^{L}; (d) tR^{M}-D-3′-endtR^{L}; (e) U6; (f) U6-D-tR^{L}; (g) U6-D-3′-endtR^{L} and (h) U6-D-I-3′-endtR^{L}.

indicated by the virus breakthrough effect (Figure 3). Therefore, we investigated the sudden surge of viral replication in cultures expressing *env* shRNA; sequence analyses were performed using different cultures (samples mut-a and -b) at 43 days postinfection. This analysis revealed that the RNAi-resistant viruses contained nucleotide substitutions within the shRNA-*env* target sequence (Figure 5A), whereas the DNAzyme sequences remained unchanged (data not shown). These results suggest that the DNAzyme inhibited HIV-1 replication and prevented the emergence of resistant viruses.

To determine whether inhibition of HIV-1 replication was dependent on the DNAzyme, we infected SupT1 cells stably expressing tR^{M}-D-tR^{L} and U6-D-tR^{L} with the evolved HIV-1$_{NL4-3-env-mut-a}$ carrying mutations corresponding to positions 7201, 7203 and 7206 of the *env* target sequence. Although viral challenge of the DNAzyme-expressing SupT1 cells with HIV-1$_{NL4-3-env-mut-a}$ did not suppress viral replication (Figure 5B), 92% inhibition was observed in

DNAzyme-expressing SupT1 cells with HIV-1$_{NL4-3}$ over a 60 days period (Figure 3A). This lack of suppressive effects on the HIV-1$_{NL4-3-env-mut-a}$ strain might be explained by a single base substitution (AU to CU) at the DNAzyme cleavage site. This finding confirms a DNAzyme-mediated anti-HIV-1 effect and not an antisense effect.

DISCUSSION

Recent reports have documented the emergence of virus escape variants following siRNA treatment in long-term cultures (13–17,37) and have raised doubts about the application of siRNA to HIV-1 gene therapy. RNAi-resistant variants can emerge through mutations in siRNA target regions and also through mutations that alter the local RNA structure (38). Furthermore, Bull *et al.* (39) also reported that antisense RNA directed against the viral gene confers resistance to viral replication. However, multiple shRNA gene therapy
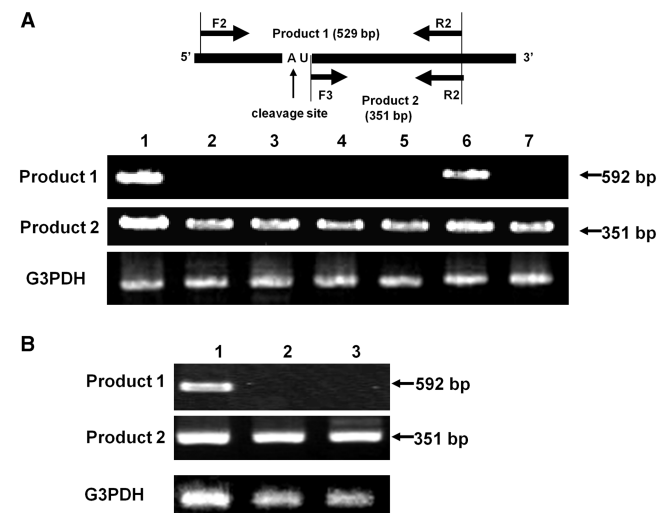
A

B

Figure 4. HIV-1 mRNA expression at the target site after treatment with the DNAzyme. (**A**) RT–PCR analyses of uncleaved (product 1) and total cleaved and uncleaved (product 2) HIV-1 mRNA at 10 days posttransfection in SupT1 cells. Lane 1, tR^M; lane 2, tR^M-D-tR^L; lane 3, tR^M-D-ΔARMtR^L; lane 4, tR^M-D-3′-endtR^L; lane 5, env-shRNA; lane 6, env-shRNA, 43 days and lane 7, tR^M-D-tR^L, 43 days. Schematic representation of HIV-1-specific primer sites with respect to HIV-1 mRNA: forward primers F2 and F3 as well as reverse primer R2. (**B**) RT–PCR analyses of uncleaved (product 1) and total cleaved and uncleaved (product 2) HIV-1 mRNA performed using HIV-1 *env*-specific primers with concurrent amplification of G3PDH mRNA. Lane 1, tR^M; lane 2, tR^M-D-tR^L and lane 3, U6-D-tR^L. Schematic representation of HIV-1-specific primer sites with respect to HIV-1 mRNA: forward primers F3 and F4 as well as reverse primer R3.

A

B

Figure 5. Generation of HIV-1 variants resistant to RNAi. (**A**) Genotype sequence analysis of escape variants. The postinfection day is indicated. The target nucleotides (AU) are underlined. Substitutions are indicated in bold. (**B**) HIV-1 gag-p24 antigen expression in SupT1 cells stably expressing DNAzymes (tR^M-D-tR^L and U6-D-tR^L) that evolved HIV-1$_{NL4-3-env-mut-a}$ over a 12-days period. Mock: HIV-1$_{NL4-3-env-mut-a}$. Data represent means ± SDs (error bars) from three independent experiments.

strategies are currently being investigated for the treatment of viral diseases such as HIV-1. It is important to use several different shRNAs to prevent the emergence of treatment-resistant strains (40–43).

Here we propose the use of a DNA rather than an RNA molecule, because the DNAzyme has a secondary structure similar to that of hammerhead ribozymes (23,24,44). Previous studies have shown that the DNAzyme can be expressed by an ssDNA expression vector using HIV-1 reverse transcriptase (28). The ssDNA expression vectors used in the present study contained the DNAzyme, the HIV-1 primer binding site and a native $tRNA^{Lys-3}$, as well as flanking arms complementary to the HIV-1 V3-loop of the *env* mRNA. However, their anti-HIV-1 activity was low as a result of low posttransfection efficacy of the plasmid DNA vector in human T cells. Recently, Jakobsen *et al.* (45) also reported the efficient inhibition of HIV-1 expression by DNAzymes.

The DNAzyme-induced inhibition of HIV-1 expression was examined in human T cells by constructing an HIV-1 RT-dependent lentiviral ssDNA vector. Lentiviruses integrate into the chromosomal DNA, and therefore their genomes are stable in host cells and are inherited by host cell progeny (46,47). Accordingly, the long-term expression of a transduced gene can be achieved through lentivirus-mediated gene transfer. Other advantages of this vector include its broad host range and the availability of packaging cell lines for the large-scale production of
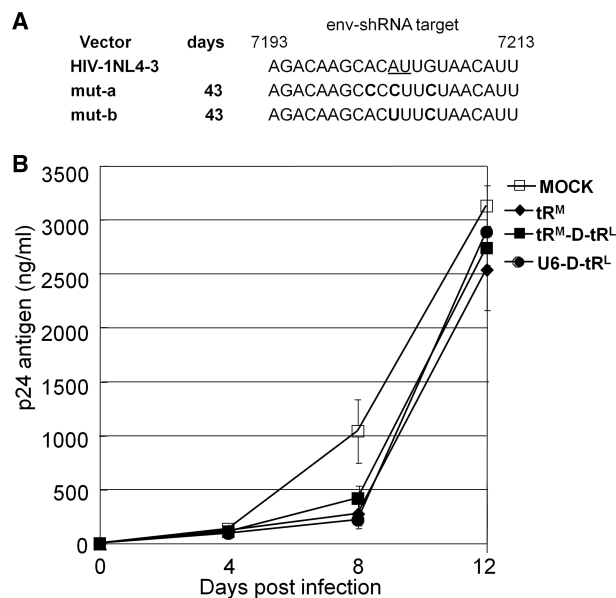
high-titer vectors. The DNAzyme construct was designed to target HIV-1 (NL4-3 *env* 7196–7210), which is more accessible to DNAzyme oligonucleotides. The expression of template RNA containing the DNAzymes driven by the $tRNA_i^{Met}$ or U6 promoters was observed in stable DNAzyme-expressing SupT1 cells [Figure 2A (lanes 2–4) and C (lanes 2, 4 and 6)]. Furthermore, ssDNA expression was achieved from the template RNA by HIV reverse transcriptase [Figure 2B (lanes 3–5) and D (lanes 2, 5 and 8)].

We evaluated the effectiveness of long-term inhibition of HIV-1 replication in HIV-1 RT-dependent lentiviral-mediated DNAzyme-expressing SupT1 cells and found that SupT1 cells stably expressing the DNAzyme showed inhibition of HIV-1 replication for 60 days postinfection (Figure 3A). By contrast, the control lentiviral vectors, tR^M, U6 and U6-D-I-3′-endtR^L (DNAzyme with inverted catalytic core sequence) (32,33), failed to inhibit viral replication under these experimental conditions. These results indicate that DNAzyme-mediated inhibition of HIV-1 replication occurred without an antisense effect. We also found that the primer binding site is required for the expression of ssDNA.

Next, we showed that the effectiveness of the long-term inhibition of HIV-1 replication did not differ under the control of two different promoters: U6 and $tRNA_i^{Met}$ (Figure 3A). The DNAzyme was found to inhibit HIV-1 replication through target RNA degradation (Figure 4A and B), thus preventing the emergence of resistant viruses

(Figure 3A). However, the siRNA-related escape mutant phenomenon was observed in SupT1 cells stably expressing the env-shRNA at 33 days postinfection (Figure 3A). Sequence analyses at 43 days postinfection revealed that the RNAi-resistant viruses contained nucleotide substitutions in the shRNA-*env* target sequence (Figure 5A) (13–17). Furthermore, viral challenge of DNAzyme-expressing (tR^M-D-tR^L and U6-D-tR^L) SupT1 cells with HIV-1$_{NL4-3-env-mut-a}$ did not suppress viral replication (Figure 5B), demonstrating that DNAzyme-mediated specific silencing of HIV genes significantly inhibited HIV-1 replication. The loss of the inhibitory effect in the HIV-1$_{NL4-3-env-mut-a}$ strain occurred via a single base mutation (AU to CU) at the DNAzyme cleavage site. Taken together, our results demonstrate the potential of an anti-HIV-1 DNAzyme for controlling HIV-1 infection (22) and preventing the emergence of resistant viruses in long-term assays. More experiments are needed to verify the role of DNAzyme in the inhibition of HIV-1 replication. The results obtained in the present study for DNAzymes might be useful in the development of an effective gene therapy for HIV-1 infections.

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