(b) For the 5′-Δ*vif* (5271-5560), we used *forward primer-1*: 5′ LF-1 (5′-CAG CTT CAC TCT TAA GTT CCT CTA AAA GCT AAT CCC TGA TGA TCT TTG CTT TTC TTC TTG GCA-3′), and *reverse primer-3*: 5′ LR-2 (5′-AAT TAG TTG GTC TGC TAG GTC AGG GTC TAC TAA TCC CTG ATC TTT GCT TTT CTT CTT GGC A-3′) and *forward primer-2*: 3′ RF-2 (5′-CAA GAA GAA AAG CAA AGA TCA TCA GGG ATT AGT AGA CCC TGA CCT AGC AGA CCA ACT AAT T-3′), *reverse primer-2*: 3′ RR (5′-GGC TGA CTT CCT GGA TGC TTC CAG GGC TCT-3′). (c) For the 3′-Δ*vif* (5561-5849), we used *forward primer-1*: 5′ LF (5′-GGA TTA AAG TAA GGC AAT TAT GTA AAC TTC-3′), *reverse primer-1*: 5′ LR-3 (5′-CAG CTT CAC TCT TAA GTT CCT CTA AAA GCT TGT GTG CTA TAT CTC TTT TTC CTC CAT TCT-3′), and *forward primer-2*: 3′ RF-3 (5′-AGA ATG GAG GAA AAA GAG ATA TAG CAC AAG CTT TTA GAG GAA CTT AAG AGT GAA GCT GTT A-3′), and *reverse primer-2*: 3′ RR (5′-GGC TGA CTT CCT GGA TGC TTC CAG GGC TCT-3′). The above PCR amplifications were designed to generate fragments with extended ends in the first round of the PCR fusion reaction. Briefly, the resulting pair of fragments with overlapping extensions were then used for further amplification by PCR as primers for the extension of the 3′ ends, subsequently "A-tailed," cloned into the pGEM-T Easy vector (Promega, Japan) using T4-DNA ligase, and designated as pGEM-TΔ*vif*. The AgeI and EcoRI recognition sites in the cloned fragments in pGEM-TΔ*vif* allowed vector recircularization by treating the vector with AgeI and EcoRI. Finally, the purified fragments were cloned into the AgeI and EcoRI sites in HIV-1pNLE to generate the (HIV-1pNLE-Δ*vif*) mutants.

Construction of the HIV-1 *vif* Antisense RNA Expression Vectors

The HIV-1 *vif* antisense and sense RNA vectors were also constructed by amplifying various targets of the *vif* gene from HIV-1pNLE (Figure 1C) by PCR using KOD plus polymerase with the forward and reverse primers containing the EcoRV and XhoI recognition sites, respectively. The short *vif* antisense RNA fragments were generated with the following sets of primers: (a) for the M-5′ *vif* antisense RNA (M-5′-AS), (5417-5560), the *forward primer* was V-4FecoV (5′-GAT ATC CAA AAA TAA GTT CAG AAG TAC ACA TCC C-3′) and the *reverse primer* was V-4Rxho (5′-CTC GAG TGT GTG CTA TAT CTC TTT TTC CTC-3′), (b) for the Mid-Mid *vif* antisense RNA (M-M-AS), (5488-5632), the *forward primer* was V-4MFecoV (5′-GAT ATC ATA CAG GAG AAA GAG ACT GGC AT-3′), and the *reverse primer* was V-4MRXho: (5′-CTC GAG CTT ATA GCA GAT TCT GAA AAA CAA TCA AAA TA-3′). (c) For the Mid-3′ *vif* antisense RNA (M-3′-AS), (5561-5705), the *forward primer* was the same as V-MFecoV and the *reverse primer* was the same as V-4MRXho, (d) while the *forward primer* for the 3′-Mid *vif* antisense

RNA (3′-M-AS), (5633-5778) was V-3′ MFecoV (5′-GAT ATC AAT ACC ATA TTA GGA CGT ATA GTT AGT CC-3′) and the *reverse primer* was V-3MRXho: (5′-CTC GAG TCA GTT TCC TAA CAC TAG GCA AAG GTG GCT-3′). (e) Finally, the set of primers for the 3′-3′ *vif* antisense RNA (3′-3′-AS), (5706-5849) were as follows: the *forward primer* was V-3FecoV: (5′-GAT ATC CAG TAC TTG GCA CTA GCA GCA TTA-3′) and the *reverse primer* was V-Rxho (5′-CTC GAG CTA GTG TCC ATT CAT TGT ATG GCT-3′). The PCRs were performed according to the manufacturers' protocol, and the integrity of the resulting *vif* fragments was confirmed by automated sequencing. These amplified fragments were then cloned into the EcoRV and XhoI sites in the pcDNA3.1 (+/−) vector in both the antisense and sense orientations, to generate the *vif* antisense RNA and the control sense expression vectors.

Transfections and HIV-1 Gag p24 Assay

The HIV-1 Gag p24 antigen production level was used to determine the degree of attenuation in the HIV-1 vif-dependent infectivity mediated by the HIV-1 *vif* mutants and the expressed HIV-1 *vif* antisense RNA transcripts in the transfected cells. In some of the experiments, the infectious molecular clone HIV-1pNLE and the HIV-1 *vif* mutants were separately transfected, while in other experiments, the HIV-1 *vif* antisense RNA vectors were either separately transfected or co-transfected with HIV-1pNLE into COS (3×10^5), HeLa-CD4$^+$ (2×10^5), or H9 at 5×10^5 cells per 60-mm culture dish. The FuGENE^{TM} 6 transfection reagent (Roche Diagnostics, Japan) and Lipofectamine 2000 (Life Technologies, Japan) were used according to the manufacturers' protocols. Briefly, 24 h before transfection, the adherent cells were seeded as described above. COS and HeLa-CD4$^+$ cells were either transfected with 3.0 μg of mutant DNA or antisense vectors or co-transfected with 2 μg antisense DNA and 2 μg HIV-1pNLE DNA using 3 μL of FuGENE 6 reagent. H9 cells were transfected with 3 μL Lipofectamine 2000 transfection reagent, optimized with 50 μL serum-free Opti-MEM. After 72 h of culture, the supernatants were harvested and cleared by centrifugation, and HIV-1 p24 antigen production was measured using an enzyme-linked immunosorbent assay system (CLEIA). The remaining cells were washed and fixed in 1% formaldehyde in phosphate-buffered saline. The co-transfected cells were subsequently monitored for down regulation of the expressed reporter gene (EGFP) using fluorescence microscopy.

Replication Competency of Mutants in H9 and MT-4 Cells

To evaluate the replication competencies of the HIV-1 *vif* mutants, stock virions from multiple samples generated from mutant-transfected

HeLa-CD4$^+$ and H9 cells were further normalized at 100 pg of HIV-1Gag p24 antigen equivalents each, and assayed for replication competency using the terminal dilution micro-assay in susceptible MT-4 cells. Endpoint titration was performed in flat-bottomed micro-titer wells using four parallel series of five-fold dilutions. After 5 to 7 d of incubation, cell-free supernatants were harvested and the presence of the major viral core p24 protein was examined using an HIV-1 p24 CLEIA. The $TCID_{50}$ was calculated by the method of Reed and Muench.[53]

HIV-1 *vif* Mutations Affected Splicing in Transfected Cells

Total RNA was extracted from HeLa-CD4$^+$ cells after 48 h posttransfection with mutant vectors using Trizol (Invitrogen Co.). The RNA was resuspended in 50 μL of RNase-free water, and then contaminating DNA that could interfere with the subsequent amplification experiments was digested with RNase free DNase by treating the sample (10 μL) with 0.2 μg of the enzyme in 10 mM Tris-acetate (ph 7.5), 10 mM magnesium acetate, and 50 mM potassium acetate for 30 min at 37°C, followed by phenol-chloroform and ethanol precipitation. The integrity of the RNA sample was verified by standard agarose gel electrophoresis. Total RNA (2 μg) was concurrently amplified with the human housekeeping gene (G3PDH) using the RT-PCR high plus kit (Toyobo) under the following conditions: One cycle of 60°C for 30 min and 94°C for 2 min, 40 cycles of 94°C for 1 min and at 50°C for 2 min, and 1 cycle of 50°C for 1 min, using the following specific primers within the splicing donor and splicing acceptor sites located in the pNL4-3 DNA; *forward* HIV-SPF (551-573) 5′-AAG TAG TGT GTG CCC GTC TGT TG-3′ and *reverse* HIV-SPR (5823-5849) 5′-CTA GGA TCT ACT GGC TCC ATT TCT TGC-3′. The resultant products were fractionated on 8% native polyacrylamide gels, stained in ethidium bromide, and visualized under an ultraviolet (UV) illuminator.

Growth Kinetics of HIV-1 Virions in H9 and MT-4 Cells

To analyze the growth characteristics of the mutant virions generated from HeLa-CD4$^+$ cells, the growth kinetics was compared in infected H9 and MT-4 cells. Virions harvested from cell-free supernatant of transiently transfected HeLa-CD4$^+$ cells (1×10^6), were further normalized at 100 pg HIV-1 Gag p24 and infected to H9 (2×10^5), and MT-4 (3×10^5) cells in 6-well plates. Briefly, the cells were seeded 24 h prior to infection (100 pg of p24 antigen) and incubated for 6 h at 37°C with intermittent agitation. Cells were then washed three times to remove residual virus. Culture supernatant was sampled on days 2, 4, 6, 8, and 10, and the quantitative levels of HIV-1 Gag p24 measured by CLEIA.

RNA Purification and RT-PCR

Total cellular RNA was isolated from transfected and co-transfected COS cells with the GenElute Mammalian Total RNA Kit (Sigma-Aldrich Co., St. Louis, Missouri), according to the manufacturer's instructions. The isolated RNA samples were pretreated with DNase I (Promega, Madison, Wisconsin) and then subjected to one-step RT-PCR assays (RT-PCR high-plus-kit; Toyobo, Japan) with specific HIV-1 *vif* mRNA primers, *forward primer*: vmRNA-F, (5′-CAA GAA GAA AAG CAA AGA TCA TCA G-3′) and *reverse primer*: vmRNA-R (5′-CTA GTG TCC ATT CAT TGT ATG GCT-3′), according to the manufacturer's instructions. Briefly, the RNA samples were normalized at 1 μg per reaction and concomitantly amplified with G3PDH as a control. To analyze the extent of the RNA expression in the cells, the products from the RT-PCR amplified RNAs were electrophoresed through a non-denaturing 1.8% agarose gel in TAE buffer.

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LENTIVIRAL-MEDIATED DELIVERY OF COMBINED HIV-1 DECOY TAR AND Vif siRNA AS A SINGLE RNA MOLECULE THAT CLEAVES TO INHIBIT HIV-1 IN TRANSDUCED CELLS

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▫ *RNA interference (RNA_i) silences gene expression via short interfering 21–23 mer double-stranded RNA (siRNA) segments that guide cognate mRNA degradation in a sequence-specific manner. On the other hand, HIV-1 decoy TAR RNA are known to competitively interact with the HIV-1 Tat protein, to downregulate the enhanced gene expression from the long terminal repeat (LTR) promoters. Here we report that a novel expression construct, encoding both HIV-1 decoy TAR and Vif siRNA, as a single RNA substrate, was expressed under the control of the human U6 promoter, and later the TAR and siRNA were cleaved into their respective separate RNA by the endogenous RNase III-like enzyme. Each of the cleaved HIV-1 anti-genes then synergistically contributed toward enhancing the inhibition efficacy (>80%) of HIV-1 replication in transduced Jurkat cells. These results suggest that targeting HIV-1 mRNA with simultaneously expressed intracellular decoy TAR and Vif-siRNA could lead to an effective gene therapy strategy for the control and management of HIV-AIDS.*

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INTRODUCTION

The role of double-stranded RNA (dsRNA) as a potent silencer of homologous genes in the nematode *Caenorhabditis elegans* was unveiled through the pioneering work of Fire et al.[1] in 1998. This technique, termed as RNA interference (RNAi), has proven to be a powerful tool and has been used to disrupt the function of genes in both plants and animals. Recently, the use of RNAi has been extended to differentiated cultured mammalian cells.[2] Importantly, siRNA expressed from DNA templates are able to silence gene expression as effective as exogenously introduced synthetic siRNA. RNAi and other gene therapy strategies have been effectively used to inhibit the replication of several different pathogenic viruses including HIV-1, by targeting the Gag-Pol, Env, Vif, and the small regulatory proteins, such as Tat and Rev, in culture.[3] In this study, we have combined the RNAi and HIV-1 decoy TAR RNA mechanisms via a single RNA molecule, delivered for intracellular expression by a novel lentiviral-based vector construct (CS-Vif siRNA-TAR) under the control of the human U6 promoter, which was later cleaved in the cells by the endogenous RNase III-like enzyme. The dual HIV-1 anti-genes efficiently inhibited HIV-1 replication in a dose-dependent manner. They further mediated a substantial down-regulation of the HIV-1 viral Vif mRNA and the reporter gene (EGFP) expression in transduced Jurkat cells. Our results have provide clear evidence that targeting the HIV-1 genes with simultaneously expressed intracellular dual HIV-1 anti-genes, such as Vif siRNA and decoy TAR RNA, could be a promising gene therapy approach for HIV-1.

RESULTS AND DISCUSSION

To evaluate the enhancement of siRNA in the sequence-specific inhibition of HIV-1 replication, we constructed the U6 vectors (Figure 1A) by linking the HIV-1 Vif siRNA with the decoy TAR, and assessed the mRNA expression in HeLa CD4$^+$

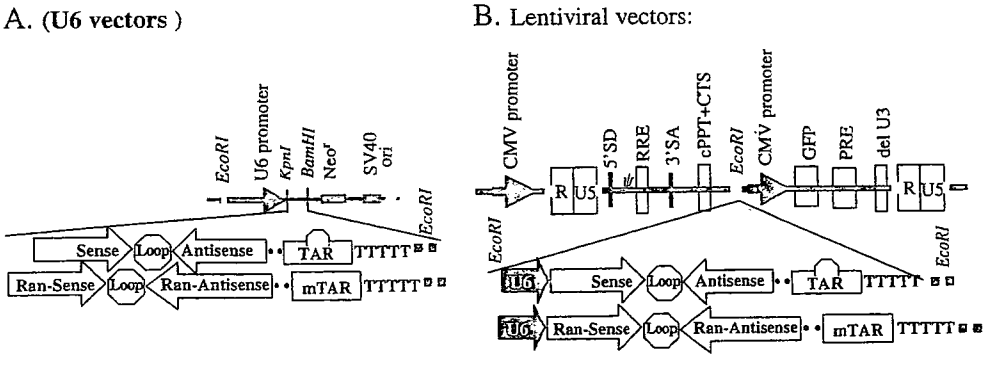

FIGURE 1 Construction of the U6 plasmid and lentiviral vectors. A. Sense and antisense strands of the synthetic DNA oligonucleotides were annealed at 95°C for 5 min, and cloned into the KpnI and BamHI cloning sites in the U6 vector. B. The EcoRI sites upstream from the U6 promoter and downstream from the terminating sequence of the generated U6 vectors were digested, and the fragments were cloned into the CS-CDF-CG-PRE vector to generate the lentiviral vectors.

cells (3×10^5) transfected with the Lipofectamine 2000 reagent. Northern blot analysis of total RNA extracted with Trizol showed an in vivo cleavage activity of the RNA molecule 72 h post-transfection in the cells. Further in-vitro cleavage assays using recombinant human dicer proved that the Vif siRNA-decoy TAR RNA substrate was mostly cleaved into its separate components, as the Vif siRNA and decoy TAR RNA, respectively (data not shown).

We then examined the dose-dependent anti-HIV-1 efficacy of the RNA substrate by co-transfecting various amounts (0.1, 1.0, and 3.0 μg) of U6 vector DNA and 0.2 μg of HIV-1 plasmid DNA into HeLa $CD4^+$ cells, and measuring the HIV-1 gag p24 antigen production level from the cell-free culture supernatant by a chemiluminescence enzyme-linked immunosorbent assay system (CLEIA) after 72 h, as an index for inhibition. The highest inhibition was observed at a concentration of 3 μg U6 vector DNA, while at the 1 μg U6 vector DNA concentration, there was still an appreciable level of inhibition. The decoy TAR U6 vector alone at 3 μg DNA did not mediate as much inhibition as that of the Vif siRNA U6 vector alone at 3 μg DNA. Our results therefore suggest that the TAR component of the U6 Vif siRNA-decoy TAR RNA molecule only complemented the inhibition efficacy in the co-transfected cells. The observed inhibitions correlated with the down-regulation of the HIV-1 viral mRNA (data not shown).

We further elucidated the inhibitory capacity of the RNA substrate, by constructing the lentiviral versions of the U6 plasmid Vif siRNA-TAR and Vif Ran-mTAR vectors (Figure 1B). 293T cells were transfected by the calcium phosphate precipitation method, and the viral titers of transduced MT-4 cells (3×10^5) were calculated using the expressed EGFP from FACS analysis. Jurkat cells (5×10^5) were then transduced with 10 MOI of the lentivirus expressing the Vif siRNA-TAR, including its random siRNA-mutant TAR version in addition to mock transduced Jurkat cells as control,[4] and were finally challenged with 0.02 MOI of $HIV\text{-}1_{NL4\text{-}3}$.

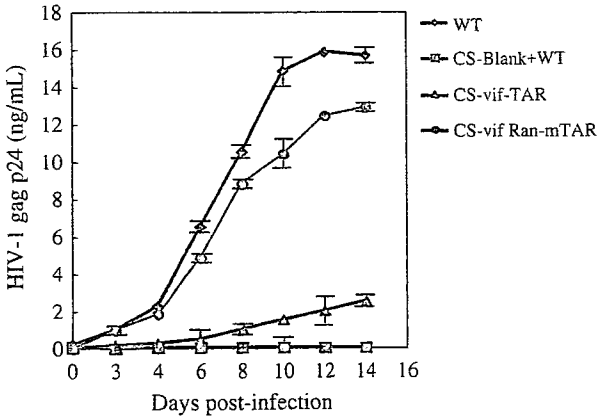

FIGURE 2 Evaluation of the inhibition efficacy of the trans-genes. Transiently transduced Jurkat cells expressing the Vif-TAR, Ran-Vif-mTAR and empty lenti-vectors were challenged with 0.02 MOI of $HIV\text{-}1_{NLE}$ and cultured over a period of 14 days. Cell-free supernatants were sampled over the period and analyzed for HIV-1 *gag p24* antigen production by CLEIA. Data represent the mean values in the supernatants ± SD of three independent experiments.

Cell-free culture supernatants were sampled over a period of 14 days and evaluated for HIV-1 gag p24 antigen production, to determine the sustainable inhibition efficacy of the lentivirus-delivered dual HIV-1 anti-genes. The results indicated the sustained inhibition of HIV-1 replication by the lentivirus-mediated Vif siRNA-TAR RNA molecule, compared to the lentivirus-mediated random siRNA-mutated TAR version (Vif Ran siRNA-mTAR), the positive controls (lentivirus-mediated CS-empty vector + HIV-1_{NL4-3}. (wt)), and the mock infected, empty vector transduced Jurkat cells (negative control) over the same period of 14 d (Figure 2). In conclusion, our findings suggest that targeting the HIV-1 genes with the intracellularly expressed HIV-1Vif siRNA and decoy TAR RNA, as a single RNA substrate, enhances both delivery efficiency to the target cells and the inhibition efficacy on HIV-1 replication. This strategy will be a promising tool for HIV-1 gene therapy.

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Inhibition of HIV-1 gene expression by retroviral vector-mediated small-guide RNAs that direct specific RNA cleavage by tRNase ZL

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ABSTRACT

The tRNA 3′-processing endoribonuclease (tRNase Z or 3′ tRNase; EC 3.1.26.11) is an essential enzyme that removes the 3′ trailer from pre-tRNA. The long form (tRNase ZL) can cleave a target RNA *in vitro* at the site directed by an appropriate small-guide RNA (sgRNA). Here, we investigated whether this sgRNA/tRNase ZL strategy could be applied to gene therapy for AIDS. We tested the ability of four sgRNA-expression plasmids to inhibit HIV-1 gene expression in COS cells, using a transient-expression assay. The three sgRNAs guide inhibition of HIV-1 gene expression in cultured COS cells. Analysis of the HIV-1 mRNA levels suggested that sgRNA directed the tRNase ZL to mediate the degradation of target RNA. The observation that sgRNA was localized primarily in nuclei suggests that tRNase ZL cleaves the HIV-1 mRNA when complexed with sgRNA in this location. We also examined the ability of two retroviral vectors expressing sgRNA to suppress HIV-1 expression in HIV-1-infected Jurkat T cells. sgRNA-SL4 suppressed HIV-1 expression almost completely in infected cells for up to 18 days. These results suggest that the sgRNA/tRNase ZL approach is effective in downregulating HIV-1 gene expression.

INTRODUCTION

RNA-based gene-interference strategies for the treatment of HIV-1 infection have often used technology based on antisense oligonucleotides, ribozymes or double-stranded interference RNA (RNAi) (1–11). A more recent approach uses external guide sequences (EGSs) to induce cleavage of a target mRNA by endogenous RNase P. This strategy is unique in that cleavage of a specific target mRNA occurs after hybridization of the EGS to form a structure resembling a tRNA substrate (12,13). RNA-based EGSs have been expressed endogenously as transgenes in both bacteria and mammalian cells (12,14) and have been effective in inhibiting gene expression by HIV-1 (15,16). We previously designed a short RNase P-associated EGS to target HIV-1-U5 and evaluated its ability to inhibit HIV-1 replication (17). Mammalian cells contain the essential enzyme, tRNA 3′-processing endoribonuclease (tRNase Z or 3′-tRNase; EC 3.1.26.11), which removes 3′ trailers from pre-tRNAs (18). The human genome contains two tRNase Z genes, which encode a 362 amino acid short form (tRNase ZS) and an 829 amino acid long form (tRNase ZL) (19,20). Although the C-terminal half of tRNase ZL has a high level of similarity to the whole of tRNase ZS, they each require different reaction conditions for optimal activity (20). Interestingly, the human tRNase ZL gene was first identified as a candidate prostate cancer-susceptibility gene (21). Two types of mutation in the human tRNase ZL gene, an insertion/frameshift and a missense change, segregate with prostate cancer in two different pedigrees. Furthermore, two additional common missense mutations seem to be associated with prostate cancer. However, a causal association between the missense mutations and prostate cancer has not been proven, because these amino acid substitutions do not alter the enzymatic activities of tRNase ZL (22). The long-form enzyme is unique in that it can cleave any RNA at any site when directed by a small-guide RNA (sgRNA) *in vitro*

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(23–27). tRNase ZL functions in the same way as the 4 nt-recognizing RNA cutter RNase 65, by forming a complex with a 3′-truncated tRNA (23). Partial HIV-1 RNA targets can be cleaved site specifically by the enzyme, once the targets form pre-tRNA-like structures with the aid of appropriate 5′-half-tRNAs (24). RNA heptamers that form acceptor-stem-like duplexes with their targets through base pairing can also direct the specific cleavage of target RNAs by tRNase ZL *in vitro* with the same efficiency as the original 5′-half-tRNAs (25,26). However, in this case, the target sites are restricted to regions immediately downstream of stable hairpin structures resembling the T stem/loop. Together with such flexibility in substrate recognition, the ubiquity and constitutive expression of tRNase ZL suggests that this enzyme can be utilized for specific cleavage of cellular RNAs by introducing the appropriate sgRNAs into living cells (27).

Recently, we demonstrated the efficacy of this method in specifically targeting RNA in living cells by introducing sgRNAs encoded either by expression plasmids or by 2′-*O*-methyl RNAs (28). The expression of the exogenous reporter genes for *Escherichia coli* chloramphenicol acetyltransferase and firefly luciferase were downregulated for at least 48 h by appropriately designed sgRNAs in cultured human and dog cells. A 2′-*O*-methyl heptamer designed to target the endogenous Bcl-2 mRNA was also successful in Sarcoma 180 cells.

In the present study, we investigated whether the sgRNA/tRNase ZL strategy could also be an effective approach to gene therapy for AIDS. Several sgRNAs targeted against HIV-1 mRNA, and expressed by plasmid and retroviral vectors, were tested for their ability to repress its expression. Our findings confirmed that they were effective in both COS and Jurkat cells.

MATERIALS AND METHODS

Plasmid construction and retroviral vector production

Expression cassettes for the sgRNA were constructed under the control of the human methionine tRNA promoter. The expressed sgRNA was targeted against either the packaging signal or the *gag* portion of the HIV-1_{NL4-3} strain (Figure 1B). Enhanced green fluorescent protein (EGFP) is a red-shifted variant of wild-type GFP (29,30), which has been optimized for brighter fluorescence and higher expression in mammalian cells (excitation maximum = 488 nm; emission maximum = 507 nm). A DNA fragment containing EGFP was excised from the plasmid pLEGFP (Promega, Madison, WI) by digestion with BamHI–NaeI. The EGFP fragment was ligated to the EcoRI and XhoI sites of pSV2neo/sgR to generate pSV2neo/sgRG. The Moloney strain of the murine leukaemia virus (MoMLV)-based sgRNA pLsgRGSN (Figure 1B) was constructed by inserting the EcoRI and XhoI fragments from plasmid pSV2neo/sgRG, along with sgRNAs and EGFP genes, into the EcoRI and XhoI sites of the retroviral vector pLXSN (31).

Vesicular stomatitis virus glycoprotein-pseudotyped retrovirus vector supernatants were generated by transient transfection of 293T cells, as described previously (32), using 20 μg of vector plasmid, 10 μg of pMLVΔψΔenv (33) and 10 μg of pVSVG envelope plasmid (32,34). Thereafter, supernatants were collected every 12 h for 3 days, filtered through a 0.45 μm pore-size filter (Nalgene, Rochester, NY) and concentrated 100- to 1000-fold by ultracentrifugation (35,36). Pellets were resuspended in serum-free DMEM and stored at −80°C until they were used.

Transduction of target cells

A total of 3×10^5 Jurkat cells per well were plated out in 6-well plates 1 day before transduction. After 24 h, virus supernatant was added together with polybrene (final concentration = 5–8 μg/ml) and the cells were incubated at 25°C overnight. The medium was then replaced with fresh medium containing G418 (500 μg/ml; GIBCO-BRL, Rockville, MD). After 10 days, cell pools that were resistant to G418 were established.

Cells and transfection

COS and Jurkat cells were grown in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Transfection was carried out using the FuGENE™6 reagent (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's protocol.

Luciferase assay

Luciferase activity was measured with the PicaGene kit (Toyo-inki, Tokyo, Japan) according to the manufacturer's protocol. The envelope-defective HIV-1 NL_{4-3}-based retroviral vector containing a luciferase-expression marker (pNL_{4-3}-luc) (32) was generated as follows. The *nef* gene sequences of the HIV-1 NL_{4-3} genome were substituted with the firefly luciferase gene, and the envelope gene sequences located between two Bgl II restriction endonuclease sites were deleted (Figure 1B). The transfection reagent, FuGENE™6, was used to transfect COS cells with the plasmids expressing sgRNA and pNL_{4-3}-luc. The COS cells were lysed using 200 μl of PicaGene cell lysis buffers (Toyo-inki) for 15 min and detached from the plate by scraping. Cellular debris were then removed by centrifugation. The luminescent signal was quantitated by adding 10 μl clarified lysate to 100 μl luminous substrate, and the level of fluorescence was recorded immediately using a luminometer (Lumat LB 9507; Berthold, Bad Wildbad, Germany).

The amount of firefly luciferase activity was normalized with reference to the protein concentration in the lysate. The protein was quantitated using the BCA Protein Assay Reagent kit (Pierce, IL), which is based on bicinchoninic acid.

Localization of sgRNA

The cytoplasmic fraction was prepared from collected cells after washing twice with phosphate-buffered saline (PBS). The cells were resuspended in digitonin lysis buffer (50 mM HEPES-KOH, pH 7.5, 50 mM potassium acetate, 8 mM $MgCl_2$, 2 mM EGTA and 50 μg/ml digitonin) and incubated on ice for 10 min. The lysate was centrifuged at 1000 *g* for 5 min and the resultant supernatant was collected and used as the cytoplasmic fraction. The pellets were resuspended in NP-40 lysis buffer (20 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM NaCl, 1 mM EDTA and 0.5% NP-40) and incubated on ice for 10 min. The resultant lysate was used as the nuclear fraction. Cytoplasmic and nuclear RNA were extracted and purified

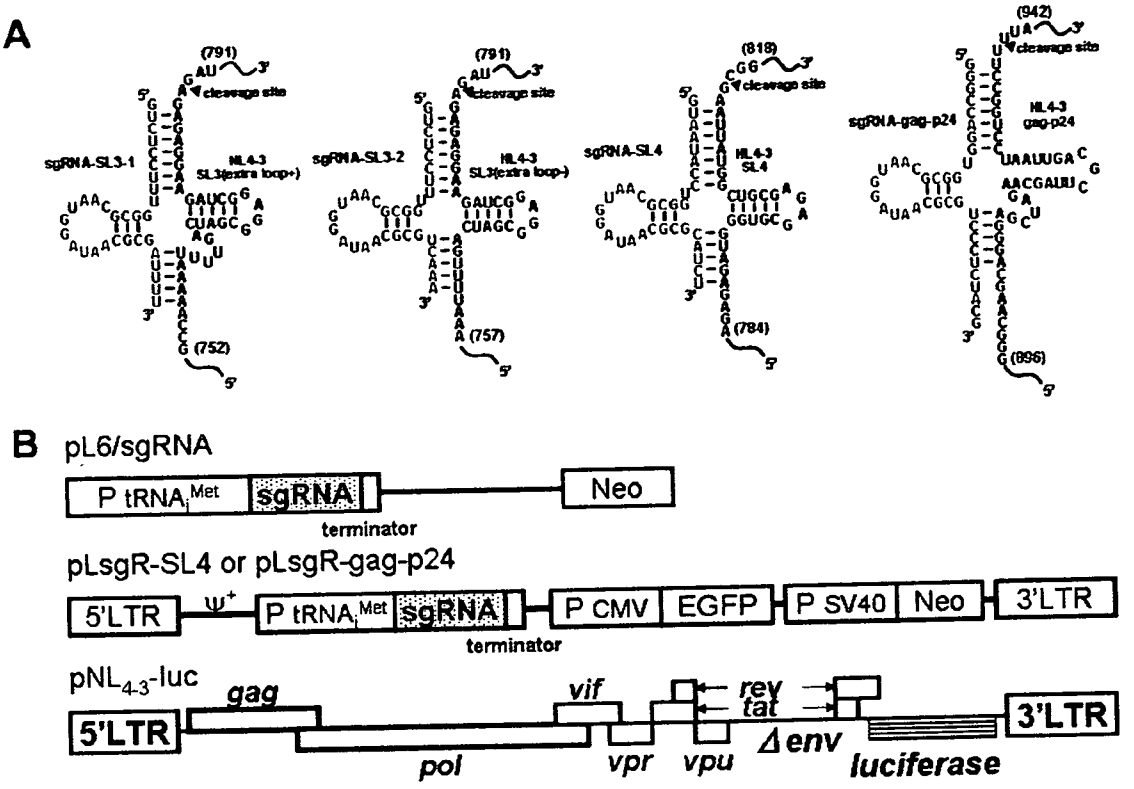

Figure 1. (A) Plausible secondary structures of complexes of the three target sites within the HIV-1 genome [the ψ site (SL3-stem loop), the SL4-stem-loop site and gag-24 within the HIV-1 gene] with the modified 5′-half-tRNA^{Arg} (sgRNA-SL3-1, 2, SL-4 and gag-p24) containing 7 and 5 nt sequences complementary to the target HIV-1 ψ site and the *gag* gene. The arrow indicates the tRNase Z cleavage point. (B) Schematic diagrams of the sgRNA-expression plasmids, the retroviral vector and the HIV-1 luciferase reporter-vector constructs. Methods for the construction of the sgRNA-expression plasmids (pL6-sgRNA-SL3-1, pL6-sgRNA-SL3-2, pL6-sgRNA-SL4 and pL6-sgRNA-gag-p24) and the retroviral vectors (pLsgRGSN-SL4 and pLsgRGSN-gag-p24) are detailed in Materials and Methods. The envelope-defective HIV-1 NL$_{4-3}$-based retroviral vector contained a luciferase-expression marker (pNL$_{4-3}$-luc). This HIV-1-based vector was generated by substituting the *nef* gene sequences of the HIV-1$_{NL4-3}$ genome with the firefly luciferase gene, and deleting the envelope gene sequences located between two Bgl II restriction endonuclease sites (32).

from their respective fractions using ISOGEN reagent (Wako, Osaka, Japan). RNA samples were treated with DNase I (Takara Shuzo, Shiga, Japan) according to the manufacturer's instructions. RT–PCR assays were performed using an RT–PCR High-Plus kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The nucleotide sequences of the sgRNA-SL4 primers were 5′-TACTGTGAGACCGTGTGC-TT-3′ (F-primer) and 5′-TACTGTGAGACCGTGTGCTT-3′ (R-primer). The nucleotide sequences of the U6 primers were 5′-CAGACATGATAAGATACATTGATGAGTT-TG-3′ (F-primer) and 5′-CGGGATCCCGGCAATAGCAT-CACAAATTTC-3′ (R-primer).

Cleavage activities of sgRNA in COS cells

COS cells were grown to ~80% confluence (3×10^5 cells) and transfected with 3 μg each of the sgRNA vector and the pNL$_{4-3}$-luc plasmid. The cells were incubated for 2 days before total cellular RNA was isolated. RNA samples were treated with DNase I according to the manufacturer's specifications, and RT-PCR assays were carried out as described above. The levels of cleaved and uncleaved HIV-1 mRNAs were quantified by RT–PCR with an endogenous internal standard, GAPDH. A sample of 1 μg of total RNA was used as the template with the SL3, SL4, p24 and GAPDH primers (20 pmol each). Reverse transcription in a final reaction volume of 50 μl was carried out at 60°C for 30 min. The cDNA products were then amplified by PCR using amplification conditions comprising 40 cycles of 94°C for 60 s and 60°C for 90 s. The SL3-F1 (5′-TTGCTGAAGCGCGCACGGCA-3′) and p24-F1 (5′-TAAGGCCAGGGGGAAAGAAACAA-TATAAAC-3′) primer pair, and the SL3-R and SL4-R (5′-GTTCTTCTGATCCTGTCTGAAGGGATGGTT-3′) and p24-R (5′-GCCCCTGGAGGTTCTGCACTATAGGGTA-AT-3′) primer pair, only generated a cDNA product from the uncleaved HIV-1 mRNA (RT–PCR products I and III, SL3 = 310 bp; RT–PCR product V, gag-p24 = 360 bp). The SL3-F2 (5′-TAAATGGGAAAAAATTCGGT-3′), SL4-F2 (5′-ATTCGGTTAAGGCCAGGGGG-3′) and p24-F2 (5′-AGACAAATACTGGGACAGCTACAACCATCC-3′) primer pairs were used to generate cDNA products corresponding to the cleaved and uncleaved sgRNA sequences, respectively, thereby identifying both sets of products. The SL3, SL4 and p24 yielded the RT–PCR products II (SL3 = 182 bp), IV (SL4 = 168 bp) and VI (p24 = 250 bp), whereas the GAPDH-F and R-primers amplified a fragment of the GAPDH gene (0.45 kb) as an internal control.

In vitro RNA synthesis

The partial HIV-1 RNA targets (T-SL3-1 and T-gag-p24) and the sgRNAs (sgRNA-SL3-1 and sgRNA-gag-p24) were synthesized from the corresponding synthetic DNA templates with an additional unencoded 'G' at the 5′ end corresponding to the −1 in a tRNA to enhance its transcriptional efficiency, using T7 RNA polymerase (Takara Shuzo). The sequences of the target RNAs and sgRNAs were as follows: 5′-GCCAAA-AAUUUUGACUAGCGGAGGCUAGAAGGAGAGAGAU-GGGUGC-3′ (T-SL3-1); 5′-GCAAGCAGGGAGCUAGAA-CGAUUCGCAGUUAAUCCUGGCCUUUUAGAGACA-3′ (T-gag-p24); 5′-GUCUCCUUUGGCGCAAUGGAUAACG-CGAUUUU-3′ (sgRNA-SL3-1); and 5′-GGGCCAGGU-GGCGCAAUGGAUAACGCGUCCCUCUACG-3′ (sgRNA-gag-p24). The transcription reactions were carried out under the conditions recommended by the manufacturer (Takara Shuzo), and after synthesis the RNAs were purified by denaturing gel electrophoresis. The RNA transcripts for T-SL3-1 and T-gag-p24 were subsequently labelled with fluorescein (F) according to the manufacturer's protocol (Amersham Pharmacia Biotech, NJ). Briefly, after the removal of the 5′-phosphates of the transcribed RNAs using bacterial alkaline phosphatase (Takara Shuzo), the RNAs were phosphorylated with T4 polynucleotide kinase (Takara Shuzo) and ATPγS. Then, a single fluorescein moiety was appended to the 5′-phosphorothioate site. The resulting fluorescein-labelled RNAs were gel-purified before being used in the assays.

In vitro RNA-cleavage assays

The *in vitro* RNA-cleavage assays for the fluorescein-labelled target RNA, T-SL3-1 or T-gag-p24 (0.1 pmol), were performed with pig liver tRNase ZL (20 ng) in the presence of unlabelled sgRNA-SL3-1 or sgRNA-gag-p24 (5 or 10 pmol) in a mixture (6 µl) containing 10 mM Tris–HCl (pH 7.5), 1.5 mM DTT and 10 mM $MgCl_2$ at 50°C for 10 min. After resolution of the reaction products by electrophoresis through a 10% polyacrylamide/8 M urea sequencing gel, the gel was analysed using a Typhoon 9210 (Amersham Pharmacia Biotech).

HIV-1-challenge assay

G418-resitant cell pools were incubated for 4 h with HIV-1_{NL4-3} at a multiplicity of infection of 0.01. After two washes with PBS, the cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) heat-inactivated FBS. The supernatant was collected on days 1, 3, 6, 9, 12, 15 and 18 after viral infection, and the culture medium was assayed for HIV-1 gag-p24 antigen using CLEIA (Lumipulse, Fujirebio, Tokyo, Japan) according to the manufacturer's protocol (37).

RESULTS AND DISCUSSION

sgRNAs can inhibit HIV-1 gene expression

We demonstrated in a previous study that antisense phosphorothioate oligonucleotides (S-ODNs) complementary to the *gag* mRNA (SL4-stem loop), containing the HIV-1 *gag* AUG initiation codon, have potent anti-HIV activity in infected cultured cells. This activity was strong compared with oligonucleotides targeted to the splice acceptor of the *tat* gene and the AUG initiation codon of the *rev* gene (38). We have also shown *in vitro* that mammalian tRNase ZL with the aid of a 5′-half-tRNA-like sgRNA can cleave a partial HIV-1 mRNA substrate containing the p24 site of the HIV-1 *gag* gene (24). Therefore, it would be reasonable to select the gag AUG site (designated SL4) and the gag-p24 site to examine the efficacy of the sgRNA method in cultured cells. The HIV-1 packaging signal (ψ) that efficiently targets genomic RNA into nascent virions (39,40) and the ψ site (designated SL3) was chosen as an additional target site for tRNase ZL.

The 5′-half-tRNA-type sgRNAs, designated sgRNA-SL3-1 and sgRNA-SL3-2 (extra loop-), were designed to form

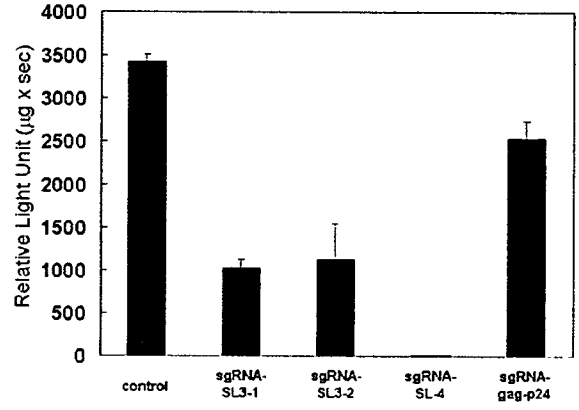

Figure 2. Luciferase activity of the pNL_{4-3}-luciferase (pNL_{4-3}-luc) fusion gene in COS cells. The cells were co-transfected with the target-expressing plasmid pNL_{4-3}-luc and either the pL6 plasmid encoding the sgRNAs or pL6-ter serving as a negative control for inhibition using the FuGENE™6 transfection reagent. The plotted data were averaged from three independent experiments, and the bars represent ±SD.

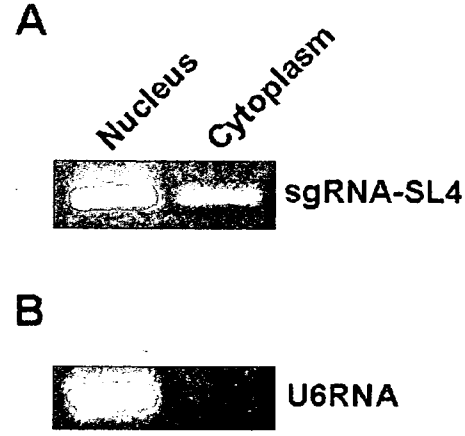

Figure 3. Intracellular localization of the $tRNA_i^{met}$–sgRNA. To analyse the degree of intracellular localization of $tRNA_i^{met}$–sgRNA, nuclear and cytoplasmic fractions were prepared from transformants that expressed sgRNA-SL4 and the total RNA was extracted from each. The transcribed sgRNA-SL4 was detected using RT–PCR analysis with a primer specific for the sgRNA. (**A**) RT–PCR analyses revealed that $tRNA_i^{met}$–sgRNA-SL4 was located almost exclusively in the nucleus, as predicted. (**B**) Nuclear and cytoplasmic fractions were examined with a probe specific for the transcript of the U6 gene (control).

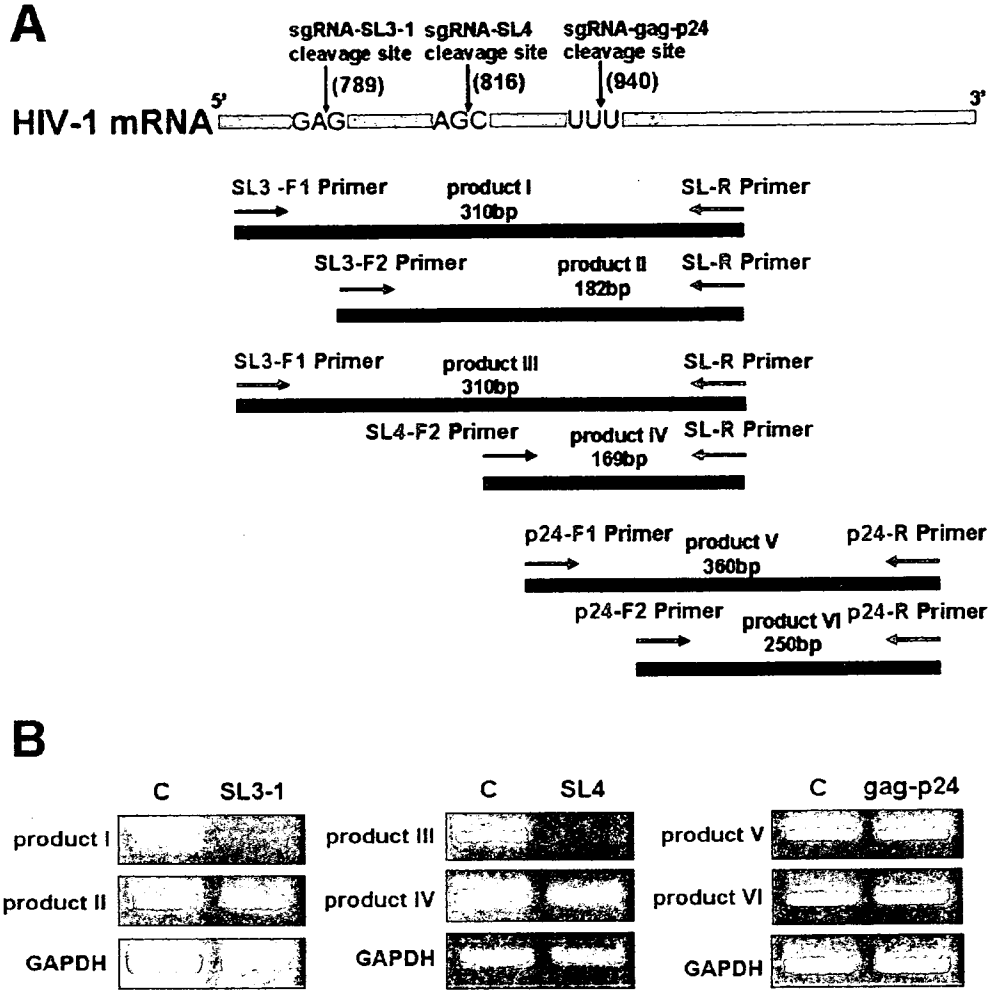

Figure 4. RT–PCR analyses of HIV-1 mRNA expression. RT–PCR analyses of uncleaved (product I), and total cleaved and uncleaved (product II), HIV-1 mRNA were performed using HIV-1 *gag*-specific primers with concurrent amplification of GAPDH mRNA. (A) Schematic representation of HIV-1-specific primer sites with respect to HIV-1 mRNA: F1 primers, SL3 and p24; F2 primers, SL3, SL4 and p24; R-primers, SL3, SL4 and p24. (B) RT–PCR amplification products analysed by 2% agarose gel electrophoresis with ethidium bromide staining.

pre-tRNA-like complexes with SL3. They contained or lacked an extra loop, respectively (Figure 1A). sgRNA-SL4 without an extra loop was intended to make a pre-tRNA-like complex with SL4, and sgRNA-gag-p24 with an extra loop was designed to form a pre-tRNA-like complex with the gag-p24 site (Figure 1A).

The sgRNA-expression plasmids were constructed in the mammalian expression plasmid pL6 (41,42) and (Figure 1B) by inserting synthetic DNA sequences between the human $tRNA_i^{met}$ promoter sequence and the RNA polymerase III termination-signal sequence. RT–PCR analysis was used to examine the expression of the sgRNA by these plasmids in transfected COS cells. A high level of sgRNA was expressed in the transfected cells, driven by a $tRNA_i^{met}$ promoter (data not shown).

A transient-expression assay was used to test the ability of pL6-expressed sgRNA-SL3-1 and 2, sgRNA-SL4 and sgRNA-gag-p24 to inhibit HIV-1 expression. COS cells were co-transfected with the sgRNA plasmids and an HIV-1_{NL4-3}-based vector containing a luciferase-expression maker (pNL_{4-3}-luc) (31), then suppression of HIV-1 was assessed in a single-cycle infectivity assay (Figure 2). HIV-1_{NL4-3}-based luciferase activity was recorded using the control plasmid vector L6-ter with only the $tRNA_i^{met}$ promoter and terminator sequences, rather than the sgRNA-expression plasmid. Both sgRNA-SL3-1 and sgRNA-SL3-2 showed good inhibition of HIV-1 expression in the cultured cells, suggesting that the extra loop in the pre-tRNA-like complex is not important for tRNase ZL recognition (Figure 2). Amazingly, HIV-1 gene expression was almost completely inhibited by sgRNA-SL4, but only moderately suppressed by sgRNA-gag-p24. This might be because endogenous tRNase ZL has difficulty in recognizing the sgRNA-gag-p24/target complex, possibly due to the lack of a stable 'T-stem-loop' structure in the target, as indicated by the *in vitro* cleavage assays described below. These results imply that the sgRNA/tRNase

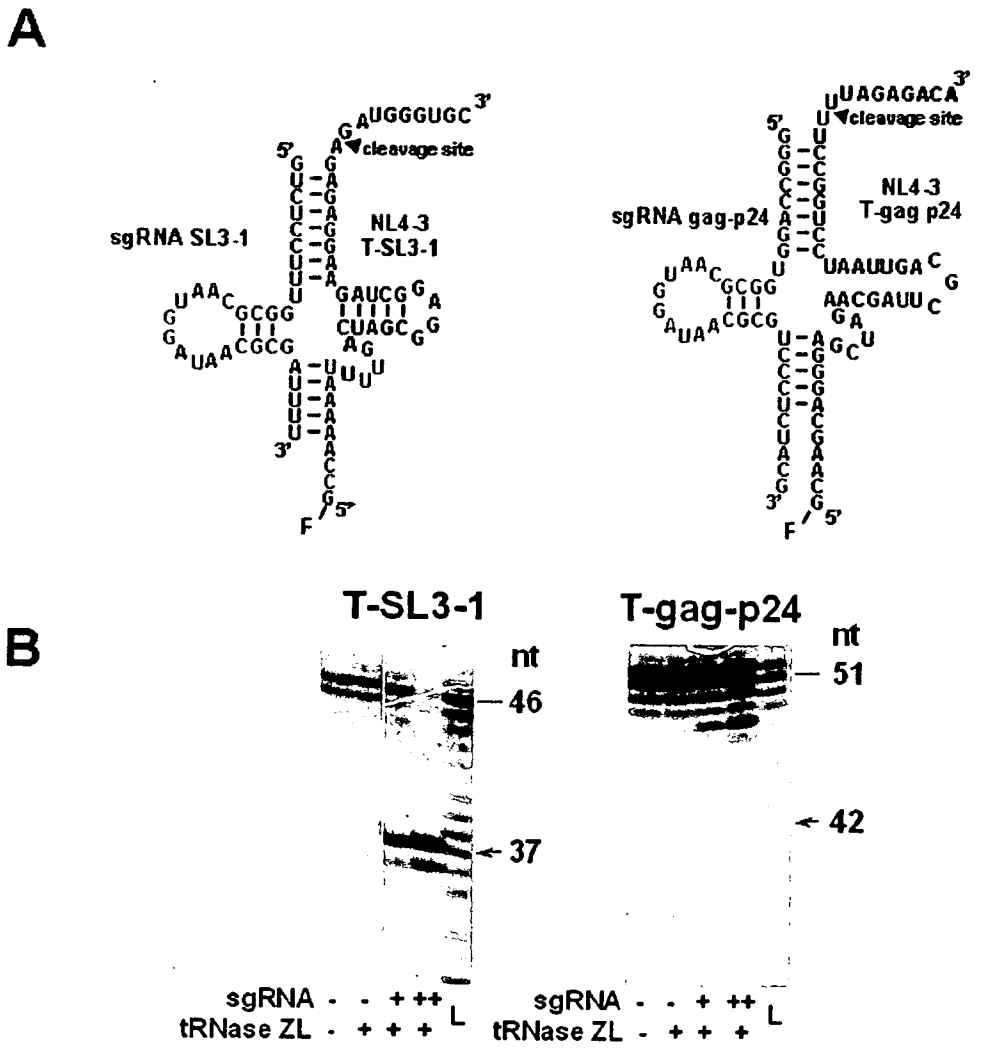

Figure 5. sgRNA guided specific HIV-1 mRNA cleavage by *in vitro* tRNase ZL assays. (**A**) Secondary structures of the substrate-HIV-1 SL3-1 (extra loop) and gag-24 complexes with sgRNA-SL3-1 and sgRNA-gag-p24. (**B**) The assays for the fluorescein (F)-labelled target RNA T-SL3-1 or T-gag-p24 (0.1 pmol) were performed with pig liver tRNase ZL (20 ng) in the presence of the unlabelled sgRNA-SL3-1 or sgRNA-gag-p24 (5 or 10 pmol) at 50°C for 10 min. The cleavage reactions were analysed using a 10% polyacrylamide/8 M urea sequencing gel. The target RNA and the primary 5′-cleavage product are indicated by a bar and arrow, respectively, together with their size in nucleotides. L denotes the alkaline ladder of each fluorescein-labelled target RNA.

ZL strategy is effective in reducing the level of the HIV-1 gene expression, although the efficiency of inhibition differs according to the sgRNAs used.

Intracellular localization of the $tRNA_i^{met}$–sgRNA

Co-localization of sgRNAs, their RNA targets and tRNase ZL is important for efficient downregulation; therefore, we examined the intracellular location of sgRNA-SL4 (41,42). Transformed cells expressing sgRNA-SL4 were separated into nuclear and cytoplasmic fractions, and the total RNA was extracted from each. sgRNA-SL4 was detected using RT–PCR analysis with a primer specific for the sgRNA. This revealed that they were located almost exclusively in the nucleus (80%) (Figure 3).

The inhibitory effect of sgRNA occurs through target RNA degradation by tRNase ZL

The contribution of HIV-1 mRNA cleavage to the sgRNA-mediated anti-HIV-1 effect was examined by measuring HIV-1 mRNA levels (43,44). Two sets of RT–PCR reactions were used to establish the level of HIV-1 mRNA uncleaved at the target site by tRNase ZL [products I (310 bp), III (310 bp) and V (360 bp)], and the total amount of HIV-1 mRNA at the target site [products II (182 bp), IV (169 bp) and VI (250 bp)], i.e. both cleaved and uncleaved. The uncleaved HIV-1 mRNA was amplified by primers SL3-F1 and gag-p24-F1, and the SL3-R, SL4-R and gag-p24-R primers (Figure 4A). The levels of products I, III and V were expected to decrease after cleavage of the HIV-1 mRNA. The levels of products II, IV and VI reflected the total amount of HIV-1 mRNA (cleaved and

uncleaved SL3, SL4 and gag-p24), as the 3′ fragment of cleaved HIV-1 mRNA remained a viable template for amplification in this PCR. The results, which are shown in Figure 4B, indicate that the sgRNA-SL3-1- and SL4-dependent expression system reduced the amount of HIV-1 mRNA [products I (92%) and IV (97%)], whereas transfection with pNL_{4-3}-luc/sgRNA-gag-p24 did not significantly alter uncleaved HIV-1 mRNA expression in COS cells (Figure 4B). These data are consistent with the results of the luciferase assays and suggest that the inhibitory effect of sgRNA is achieved through target RNA degradation by tRNase ZL.

sgRNA-gag-p24 barely directs *in vitro* HIV-1 RNA cleavage by tRNase ZL

sgRNA-gag-p24 was much less effective than the other sgRNAs in suppressing HIV-1 gene expression in cells. We examined the reason for this by assessing its ability to guide HIV-1 RNA cleavage by tRNase ZL *in vitro*. The partial HIV-1 RNAs T-SL3-1 and T-gag-p24 containing SL3 and the gag p24 site, respectively, were synthesized *in vitro* with T7 RNA polymerase and 5′ end-labelled with fluorescein. sgRNA-SL3-1 and sgRNA-gag-p24 were also transcribed by T7 RNA polymerase *in vitro*. The ability of each sgRNA to induce cleavage of the corresponding target RNA by tRNase ZL was tested *in vitro*. The target T-SL3-1 was cleaved efficiently under the direction of sgRNA-SL3-1, whereas cleavage of the target T-gag-p24 by sgRNA-gag-p24 was highly inefficient (Figure 5), this was in agreement with the *in vivo* observations. Cleavage of each target occurred primarily 1 nt downstream of the nucleotide corresponding to the discriminator (Figure 5). These results indicate that sgRNA bound to its target HIV-1 mRNA, and cleavage of the pre-tRNA complexes with tRNase ZL occurred. It was therefore important to determine the localization of both the sgRNA and its target HIV-1 in the cells. The reduction in functional HIV-1 mRNA was consistent with tRNase ZL cleavage occurring at the post-transcriptional level.

Inhibition of HIV-1 gene expression by retroviral vector-mediated sgRNAs in human T cells

The inhibitory effect of HIV-1 expression by tRNase ZL-mediated sgRNAs was investigated in human T cells by constructing a MoMLV-based sgRNA retroviral vector. Most retroviral vectors used in experimental and clinical gene therapy are derived from the MoMLV (45). Retroviruses integrate into the chromosomal DNA, so their genome is stable in the host cells and is inherited by their progeny. Accordingly, long-term expression of a transduced gene can be achieved through retrovirus-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-titre vectors. It has previously been shown that an amphotropic MoMLV-based retrovirus vector can transduce a human T-cell line (46). We therefore expressed the sgRNA under the control of the promoter of a human $tRNA_i^{met}$ gene via a retroviral vector (Figure 1B). The plasmid pLsgRGSN (Figure 1B) was constructed by inserting the following elements into the EcoRI and XhoI sites of the retroviral vector pLXSN: an EcoRI and XhoI fragment from the plasmid pSV2neo/sgRG, sgRNAs and EGFP genes. We then obtained transduced Jurkat T cells

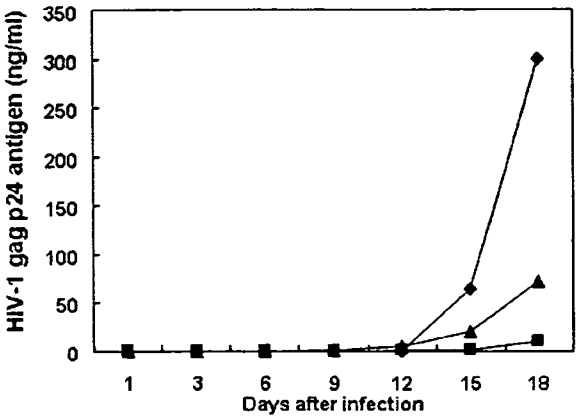

Figure 6. Inhibition of HIV-1 gag-p24 product in Jurkat cells expressing stably retrovirus vector-mediated sgRNA. Cells were cultured for 18 days after infection with $HIV\text{-}1_{NL4\text{-}3}$. Small aliquots of supernatant were prepared from each culture on days 1, 3, 6, 9, 12, 15 and 18. HIV-1 gag p24 antigen was determined using CLEIA (Lumipulse) according to the manufacturer's protocol (37): closed diamonds, pLGSN-Δ*sgR*: closed squares, pLsgRGSN-SL4; and closed triangles, pLsgRGSN-gag-p24.

stably expressing the sgRNAs. These Jurkat T cells were infected with wild-type $HIV\text{-}1_{NL4\text{-}3}$, and HIV-1gag-p24 antigen levels in the cell-free supernatant were measured at weekly intervals over 18 days. By day 18, the HIV-1 gag-p24 product was suppressed almost completely (~97%) in the cell cultures expressing sgRNA-SL4 (Figure 6). In contrast, sgRNA-gag-p24 and sgRNA-SL4 failed to inhibit viral expression under these experimental conditions. The difference between the effects of sgRNA-SL4 and sgRNA-gag-p24 in the HIV-1-challenged assay was due to the lack of base pairing in the hairpin structure resembling the T-stem-loop region.

In conclusion, we demonstrated the inhibition of HIV-1 gene products in cultured cells by inducing HIV-1 mRNA cleavage using a modified 5′-half-$tRNA^{Arg}$ (sgRNA) and mammalian tRNase ZL. The sgRNA/target HIV-1RNA complex formed a pre-tRNA-like structure with 5′-half-tRNA and a stable hairpin (3′-half-tRNA) structure resembling the T-stem-loop region. The $tRNA_i^{met}$–sgRNA transcript was expressed at high levels and localized in the nucleus. The greatest inhibitory effect on HIV-1 expression was achieved using sgRNA-SL4 targeting the HIV-1 *gag* gene. These results suggest that both sgRNA and its target HIV-1 mRNA were located in the nucleus, allowing specific cleavage by tRNase ZL. Furthermore, MoMLV-based sgRNA-SL4 could suppress sgRNA-dependent HIV-1 expression in human T cells. We believe that the use of mammalian tRNase ZL in conjunction with guide sequences represents a promising tool for the inactivation of genes in mammalian cells. Furthermore, the inhibition of HIV-1 using this approach demonstrates its potential as a therapeutic agent for AIDS.

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Solution RNA Structures of the HIV-1 Dimerization Initiation Site in the Kissing-Loop and Extended-Duplex Dimers

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Dimer formation of HIV-1 genomic RNA through its dimerization initiation site (DIS) is crucial to maintaining infectivity. Two types of dimers, the initially generated kissing-loop dimer and the subsequent product of the extended-duplex dimer, are formed in the stem-bulge-stem region with a loop including a self-complementary sequence. NMR chemical shift analysis of a 39mer RNA corresponding to DIS, DIS39, in the kissing-loop and extended-duplex dimers revealed that the three dimensional structures of the stem-bulge-stem region are extremely similar between the two types of dimers. Therefore, we designed two shorter RNA molecules, loop25 and bulge34, corresponding to the loop-stem region and the stem-bulge-stem region of DIS39, respectively. Based upon the chemical shift analysis, the conformation of the loop region of loop25 is identical to that of DIS39 for each of the two types of dimers. The conformation of bulge34 was also found to be the same as that of the corresponding region of DIS39. Thus, we determined the solution structures of loop25 in each of the two types of dimers as well as that of bulge34. Finally, the solution structures of DIS39 in the kissing-loop and extended-duplex dimers were determined by combining the parts of the structures. The solution structures of the two types of dimers were similar to each other in general with a difference found only in the A16 residue. The elucidation of the structures of DIS39 is important to understanding the molecular mechanism of the conformational dynamics of viral RNA molecules.

Key words: DIS, HIV-1, NMR, RNA, structure.

Abbreviations: DIS, dimerization initiation site; HIV-1, human immunodeficiency virus type 1.

Two molecules of viral genomic RNA are packaged in a dimeric state in the virion of human immunodeficiency virus type 1 (HIV-1), and this dimer formation is crucial to maintaining their infectivity (*1–4*). Accumulating evidence from both *in vivo* and *in vitro* experiments has shown that the specific sequence, the dimerization initiation site (DIS) located close to the 5′ terminus of the genomic RNA, is required for spontaneous dimerization of HIV-1 RNA. DIS can form a stem-loop structure with a self-complementary sequence in the loop and a bulge in the stem (*5, 6*). The dimerization of DIS forms the kissing-loop dimer as the first step; then, their intramolecular stems are converted into intermolecular stems, generating the extended-duplex dimer (*7, 8*). This two step dimerization process is called the kissing-loop mechanism. The kissing-loop dimer is converted into the extended-duplex dimer by incubation at 55°C (*9, 10*) or by incubation at physiological temperature with the HIV-1 nucleocapsid protein, NCp7, which includes two basic regions and two zinc-fingers (*11*). A number of experiments have been performed to gain an understanding of the role of the zinc-fingers as well as the basic regions (*12–16*). Our previous results show that, for the two step dimerization from the kissing-loop dimer to the extended-duplex dimer, the two basic regions surrounding the N-terminal zinc finger of NCp7 have RNA chaperone activity by themselves, and the zinc fingers increase the efficiency of the activity (*17, 18*).

A number of three dimensional structural analyses using NMR and X-ray methods have been performed to determine the conformation of each region of DIS, the loop region in the kissing-loop (*19, 20*) or extended-duplex dimers (*21–24*), as well as the bulge-out region (*25–27*). However, our previous studies suggested that the 39mer RNA sequence, DIS39, which covers the entire bulge and loop regions, is necessary and sufficient for the two step dimerization (*28, 29*). Thus, it is still relevant to determine the structures of the kissing-loop and extended-duplex dimers for DIS39 with the same sequence and conditions.

In the present study, we designed two shorter RNA molecules, loop25 and bulge34; loop25 includes the loop-stem region of DIS39, and bulge34 includes the stem-bulge-stem region (Fig. 1), respectively we then determined the solution structures of loop25 in each of the kissing-loop and extended-duplex dimers as well as bulge34. By combining the structure parts, the solution structures of DIS39 in the kissing-loop and extended-duplex dimers were able to be determined.

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