Simon and coworkers determined that every amino acid position dispersed throughout the linear sequence of Vif is important for Vif function, because all the amino acid positions analyzed in their scanning mutation studies of the Vif protein either decreased or increased infectivity.[27] Therefore, we hypothesized that combining targets that together either enhanced or decreased HIV-1 infectivity, in mass block in-frame shift mutants in HIV-1 vectors, could result in novel target sites that might be useful for HIV gene therapy applications. Gene therapy has recently emerged as a promising therapeutic tool for the treatment of genetic diseases, cancers, and chronic infectious conditions, such as AIDS.[28–32] These include the intracellular expression of decoy RNAs, ribozymes, single-chain antibodies, trans-dominant proteins, and antisense RNAs.[33–45] Antisense RNAs targeted to various HIV-1 major structural genes, accessory genes, and receptors, successfully inhibited viral replication in the target cells.[46,47]

In the present study, we initially constructed HIV-1 Vif mutants as the basis for determining the effective HIV-1 Vif target domain(s) that would directly attenuate the Vif-dependent infectivity in the cells. HIV-1 *vif* antisense RNA expression vectors of various sizes were subsequently constructed within the established effective target domains of the HIV-1 *vif* gene. Thus, we used antisense RNA to indirectly block at the transcriptional level, targets within the same region as in the mutant constructs that mediated the downregulation of HIV-1 Vif-dependent infectivity in the cells. The potential anti-HIV-1 activity of these HIV-1 *vif* antisense RNAs was evaluated with the view of establishing highly effective therapeutic target(s) in the HIV-1 *vif* gene, which could be further developed for HIV gene therapy applications for the control and management of HIV-AIDS.

RESULTS

Vector Design Strategy and PCR-Mediated Site-directed Mutagenesis

The target sites used in this study for the construction of both HIV-1 *vif* deletion mutants and the HIV-1 *vif* antisense RNA expression vectors are schematically represented in Figures 1B and 1C, and were based on the HIV-1pNLE genome (Figure 1A). The nucleotide deletions extended from the 5′ end to the middle of *vif* (5′-Δ*vif*; 5271-5560), and from the middle to the 3′ end of *vif* (3′-Δ*vif*; 5561-5849) and were each approximately 288 bp. The ORF-Δ*vif* mutant deletion was approximately 589 bp, extending from the start codon to the stop codon (Figure 1B). The HIV-1 *vif* antisense RNA expression vectors, hereafter referred to as the M-5-AS (5417-5560), M-M-AS (5488-5632), M-3′-AS (5561-5705), 3′-M-AS (5633-5778), and 3′-3′-AS (5706-5849) vectors, were designated as short *vif* antisense RNA expression vectors and were approximately 145 bp each (Figure 1C).

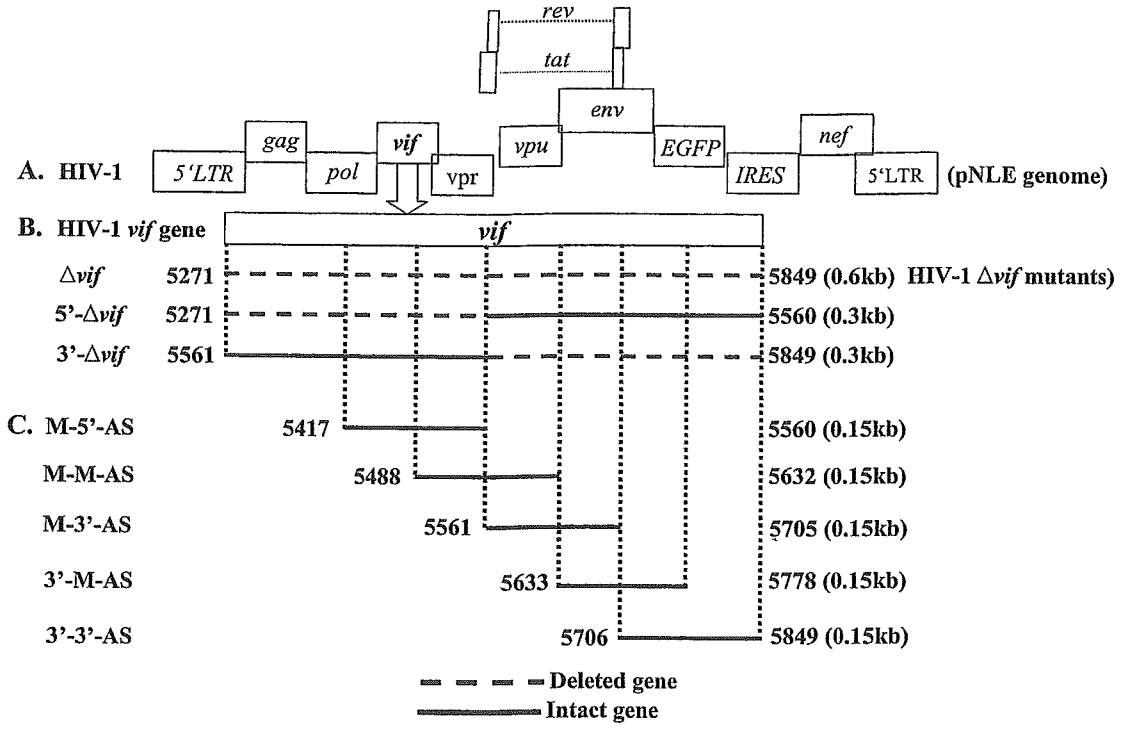

FIGURE 1 Scheme for the construction of both HIV-1 *vif* mutants and the HIV-1 *vif* antisense RNA expression vectors. (A) Schematic representation of the HIV-1pNLE genome, showing the open reading frames, and the 5′and 3′ long terminal repeats. (B) The HIV-1 mutants were constructed by PCR-mediated site-directed mutagenesis. The final amplified fragments were cloned back into the AgeI and EcoRI sites in HIV-1pNLE. The horizontal broken lines represent the deleted portion of the *vif* gene. (C) The selected *vif* targets were amplified by PCR, with EcoRV and XhoI cloning sites added to the fragments, which were then cloned into the EcoRV and XhoI sites in the pcDNA3.1 vector in both the antisense and sense orientations.

H9 Cell-derived Virions Exhibited a Defective Phenotype in MT-4 Cells

To predetermine the target sites for the construction of the HIV-1 *vif* antisense RNA expression vectors, HIV-1 mutant vectors carrying the various extensive deletions in the *vif* gene were generated (Figure 1B), and the intracellular HIV-1 Gag p24 antigen production level for each vector was measured by CLEIA,[48] using the cell-free culture supernatants of the transfected COS, H9, and HeLa-CD4$^+$ cells. The generated virions were normalized at 100 pg of HIV-1 *gag* p24 antigen equivalents, and were then titrated for their relative infectivity in MT-4 cells and H9 cells. The results indicated significant differences in the replication competencies of the H9 and HeLa-CD4$^+$ derived virions on MT-4 cells (Figure 2). The virions generated from H9 cells exhibited significantly lower infectivity in the MT-4 cells compared to those generated from the HeLa-CD4$^+$ cells. The infectivity of the ORF *vif* mutant was twofold lower than that of the 3′*vif* deleted mutant and both were low-titer viruses, while there was no significant difference in the titers of the wt (positive control) and

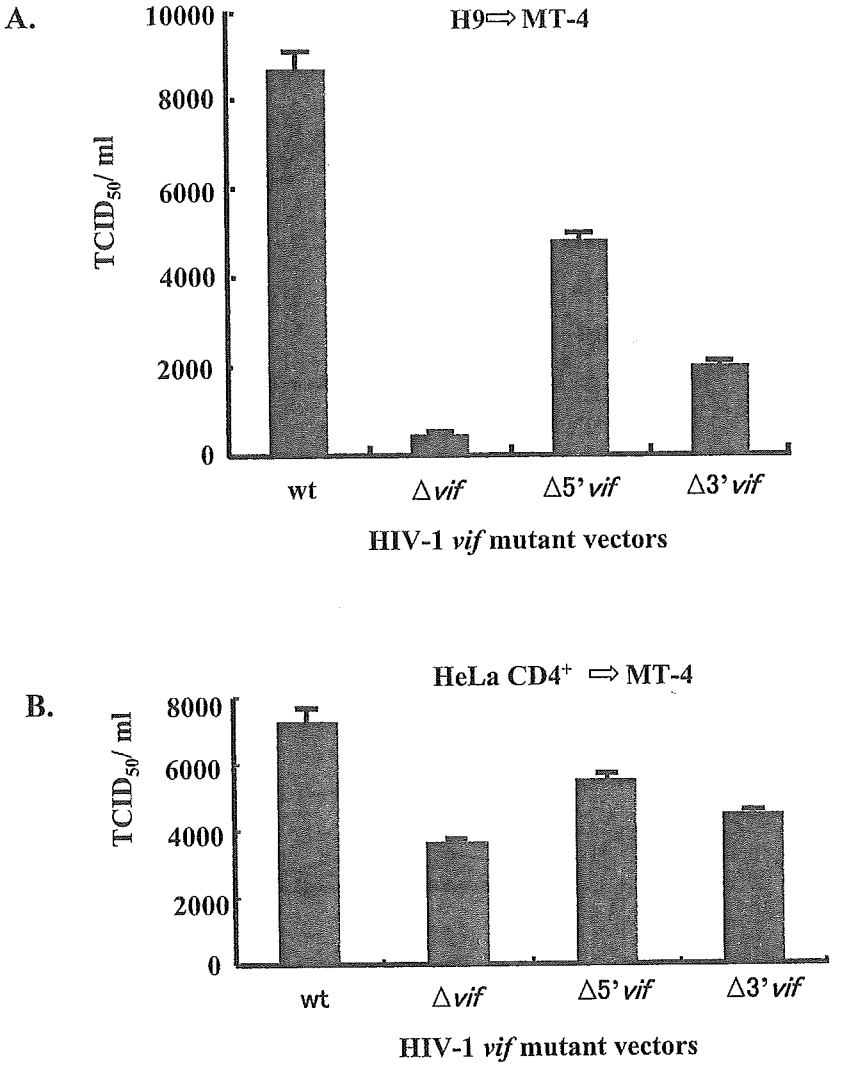

FIGURE 2 Infectivity of wt and mutant viruses. Mutant viruses generated from the co-transfected HeLa-CD4$^+$ and H9 cells were normalized at 100 pg of HIV-1 Gag p24 antigen, and titrated in fivefold serial dilution steps in MT-4 cells. Cell-free culture supernatants were harvested at 6 d posttransfection, and the HIV-1 Gag p24 antigen was measured by ELISA and expressed as $TCID_{50}$/mL. Average and standard deviation of three independent experiments are shown.

5′ *vif* deleted mutants (2A). Generally, there was no significant difference in the titers of the virions generated from the HeLa-CD4$^+$ cells (2B). Results presented as the mean ±SD of three independent experiments.

Growth Kinetics of HIV-1 *vif* Mutants

Transfecting HeLa-CD4$^+$ cells (1×10^6) generated input viruses to further study the growth characteristics of these mutants. H9 and MT-4 cells were infected with HeLa-CD4$^+$-derived viruses at an equal concentration of HIV-1 p24 (100 pg) and cultured for 10 days. All the mutants exhibited a similar growth pattern in MT-4 cells in relation to the wt as they all peaked

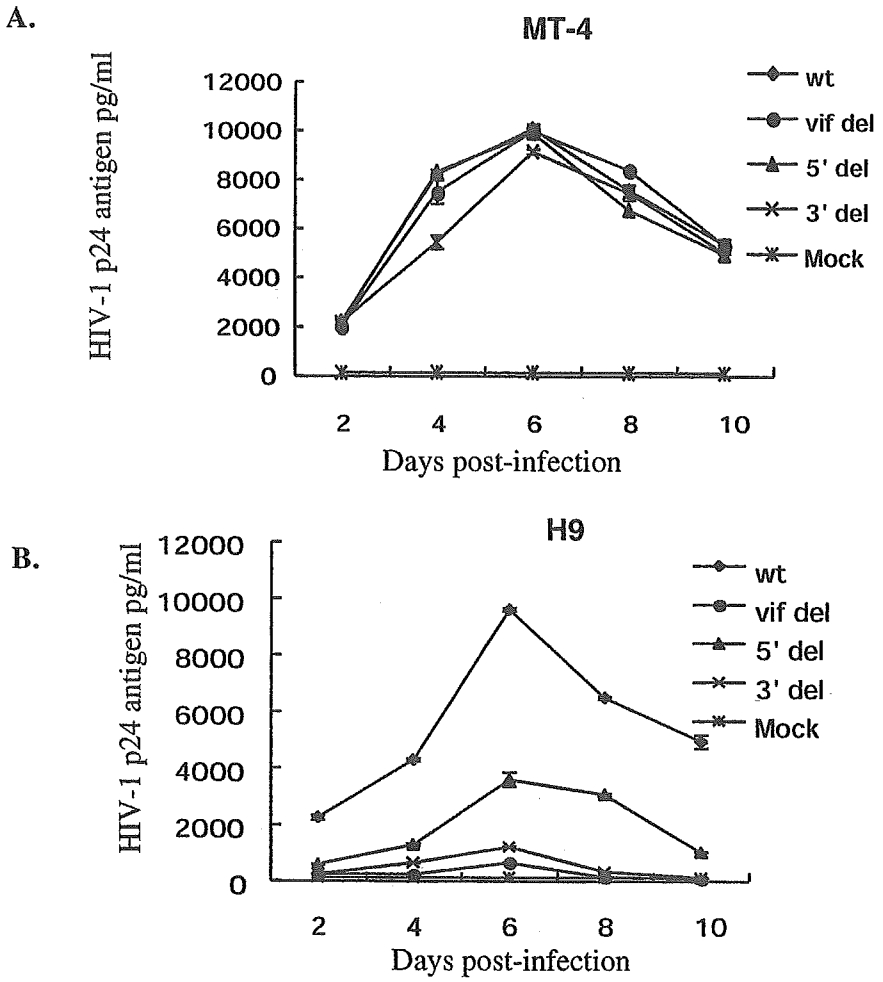

FIGURE 3 Growth kinetics of wt and mutant viruses in H9 and MT-4 cells. Viruses were generated in HeLa-CD4$^+$ cells (1×10^6), normalized at 100 pg p24 antigen and used to infect H9 and MT-4 cells, with the residual virus removed by washing. Subsequently, HIV-1 p24 antigen production was monitored at the indicated time intervals. Average and standard deviation of three independent experiments are shown. (The error bars are shown, but are very small.)

on day 6 (Figure 3A). Significantly, these same virions grew poorly in the H9 cells as compared to the wt. Nevertheless, the 5′ end *vif* mutant grew slightly more than the ORF and the 3′*vif*-deletion mutants, thus indicating that these mutants are defective in the H9 cells (Figure 3B). Results are shown as the mean ±SD of three independent experiments.

Mutation Affected HIV-1 Splicing in Transiently Transfected HeLa-CD4$^+$ Cells

To further determine the extent of the mutant defects, HIV-1 splicing was observed in HeLa-CD4$^+$ cells transiently transfected with the vectors. Total RNA was extracted 48 h posttransfection and RT-PCR amplified with

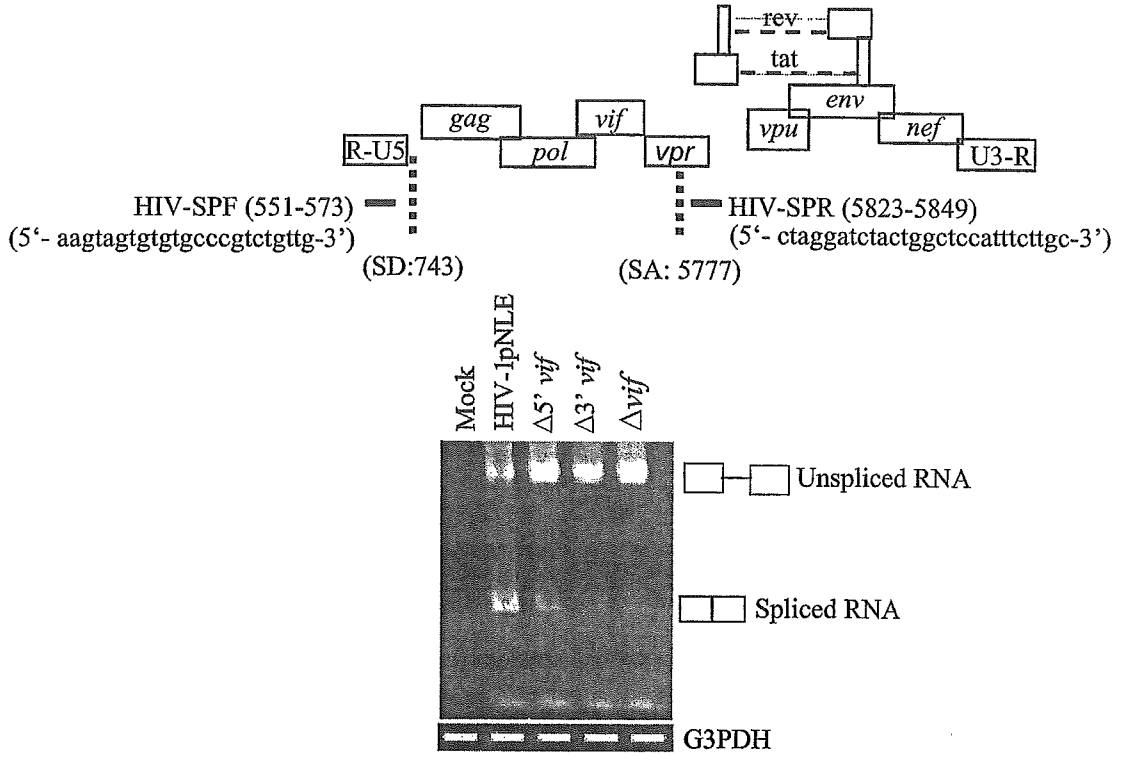

FIGURE 4 HIV-1 mutant splicing in transfected-HeLa-CD4$^+$ cells. (A) HIV-1 genome and RT-PCR primers were positioned around the splicing donor site (SD) and the splicing acceptor (SA) region as forward and reverse primers, respectively. (B) Total and nuclear RNA extracted from transiently transfected HeLa-CD4$^+$ cells with mutants after 48 h was subjected to RT-PCR and fractionated on 8% polyacrylamide gels stained with ethidium bromide. Lane 1: mock-transfected HeLa-CD4$^+$ cells; Lane 2: HIV-1pNLE-transfected HeLa-CD4$^+$ cells; Lane 3: 5′ *vif*-deletion mutant-transfected HeLa-CD4$^+$ cells; Lane 4: 3′ *vif*-deletion mutant transfected HeLa-CD4$^+$ cells; and Lane 5: ORF *vif* deletion mutant-transfected HeLa-CD4$^+$ cells.

the specific forward primer that was positioned in the splicing donor region, while the reverse primer was positioned in the splicing acceptor region of the *vpr* gene (Figure 4A). The resultant products were analyzed on polyacrylamide gels and the wt had more spliced pre-mRNA than unspliced pre-mRNA. In contrast, the mutants had more unspliced pre-mRNA than spliced pre-mRNA. Therefore, the deletions affected HIV-1 splicing in the HeLa-CD4$^+$ cells (Figure 4B).

Intracellular Expression Level of Antisense RNA in the Cells

Because the antisense mechanism is partly dependent on the expressed antisense RNA in the cells, we determined the level of mRNA expression for all the antisense RNA constructs in transiently transfected HeLa-CD4$^+$ and COS cells. The following primer pair was used to amplify total RNA from co-transfected cells; 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

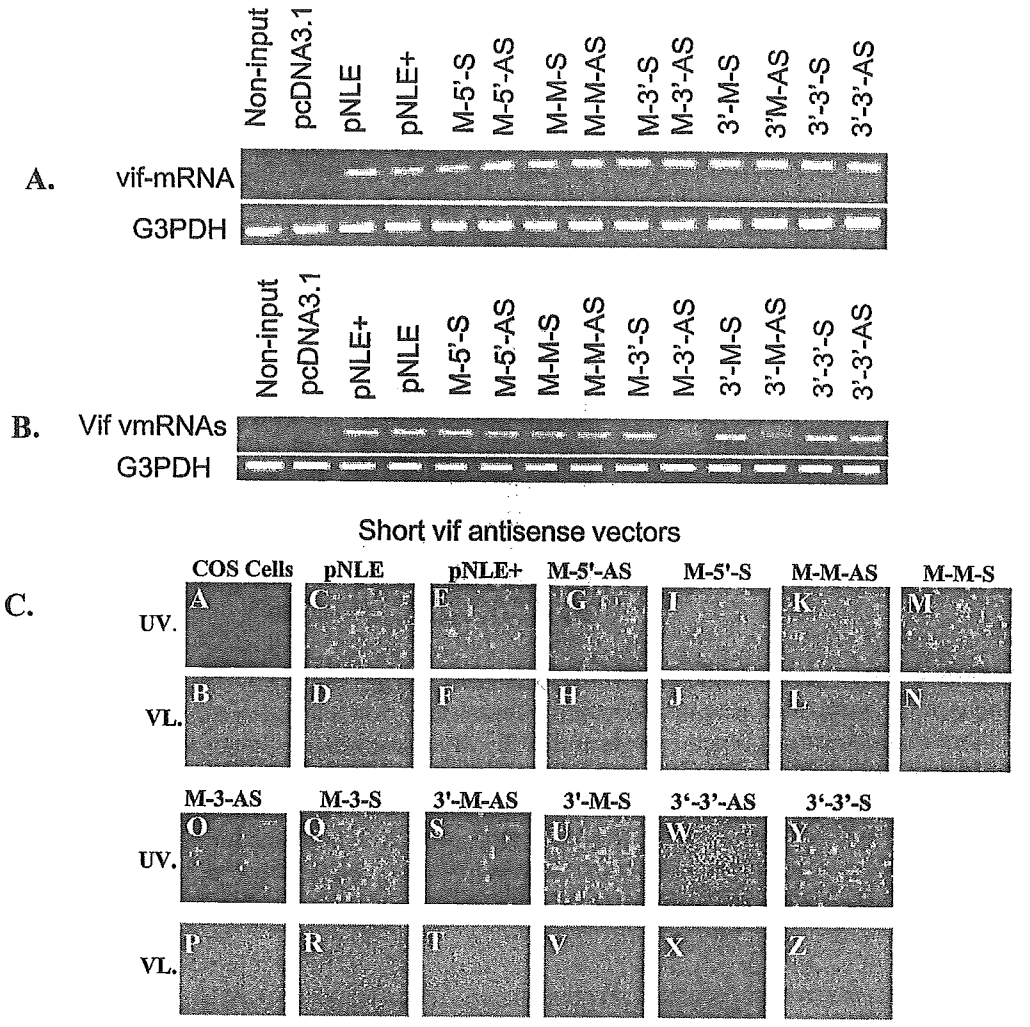

FIGURE 5 RT-PCR analysis of the *vif* antisense and the control RNA expression in COS cells. (A) The expressed short *vif* antisense RNA transcripts from the COS cells was concurrently amplified by RT-PCR with the controls, including the G3PDH RNA, and were resolved on an agarose gel. Lane 1: control G3PDH RNA; lane 2: empty vector (pcDNA3.1); lane 3: pNLE; lane 4: (pNLE + pcDNA 3.1); lane 5: M-5′-S; lane 6: M-5′-AS; lane 7: M-M-S; lane 8: M-M-AS; lane 9: M-3′-S; lane 10: M-3′-AS; lane 11: 3′-M-S; lane 12: 3′-M-AS; lane 13: 3′-3′-S; and lane 14: 3′-3′-AS. Inhibition of the HIV-1 viral *vif* mRNA and reporter gene expression. (B) RNA extracted from COS cells co-transfected with the short *vif* antisense RNA and pNLE was subjected to RT-PCR and separated on an agarose gel. Lane 1: control G3PDH RNA; lane 2: blank vector (pcDNA3.1); lane 3: (pNLE + pcDNA 3.1); lane 4: wt (pNLE); lane 5: M-5′-S; lane 6: M-5′-AS; lane 7: M-M-S; lane 8: M-M-AS; lane 9: M-3′-S; lane 10: M-3′-AS; lane 11: 3′-M-S; lane 12: 3′-M-AS; lane 13: 3′-3′-S; and lane 14: 3′-3′-AS. (C) The expressed antisense RNA transcripts mediated downregulation of the EGFP in COS cells. Notable, the panels representing M-3′-AS (Panel O), and 3′-M-AS (Panel S) showed >98% and >90%, respectively, downmodulation of the gene expression as compared to the HIV-1pNLE (Panel C).

CAT TGT ATG GCT-3′). There were significant levels of expression of both the antisense and sense mRNA in COS cells (Figure 5A), and HeLa-CD4$^+$ cells (data not shown).

RNA Content and HIV-1 *vif* Viral mRNA Reduction

To determine the relative inhibitory efficacies of the short *vif* antisense RNA expression vectors, they were co-transfected with the HIV-1pNLE. The co-transfected vectors were examined for a reduction in the viral *vif* mRNA and the downregulation of the reporter gene expression. Total RNA isolated from the co-transfected HeLa-CD4$^+$ cells, which were concurrently amplified with an internal control RNA (G3PDH) by reverse transcription (RT)-PCR using the specific *vif* viral mRNA detection primers and the following pair of specific primers for the control RNA (G3PDH), the *G3PDH-forward primer* (5′-ACC ACA GTC CAT GCC ATC AC-3′) and the *G3PDH-reverse primer* (5′-TCC ACC ACC CTG TTG CTG TA-3′), demonstrated that the short *vif* antisense RNA expression vectors encoding M-3′-AS and 3′-M-AS equally mediated downregulation of HIV-1pNLE *vif* mRNA expression (Figure 5B) in comparison with the control HIV-1pNLE *vif* mRNA alone (lane 4) and the control HIV-1 plus the empty vector (lane 3). Visualizing the RT-PCR products in ethidium-bromide-stained agarose gels thus provided a quantitative estimate of the degree of the reduction in the expressed HIV-1 *vif* viral mRNA. These reductions in the viral mRNA could be a result of the effective antisense mechanism mediated by the highly expressed *vif* antisense RNA transcripts in the cells (Figure 5A).

Downregulation of EGFP as Markers for HIV-1 Replication

To determine the suppression level of HIV-1 replication, transfected COS cells were examined under a fluorescence microscope to detect EGFP expression for extrapolations. The results exhibited significantly varied levels of reporter gene expression (Figure 5C). Panels O and S representing M-3′-AS and 3′-M-AS showed >98% and >90% inhibition of HIV-1 replication respectively, as compared to Panel C representing HIV-1pNLE (positive control for HIV replication). The others did not mediate any significant inhibitions.

DISCUSSION

In this study, we screened for highly effective therapeutic targets in the HIV-1 *vif* gene that interfered with HIV-1 Vif-dependent infectivity, due to the critical role Vif has in the infectivity and pathogenicity of HIV-1 in the target cells of the host. For this purpose, to determine the target sites for

the construction of the HIV-1 *vif* antisense RNA expression vectors, we used the HIV-1 *vif* mutant scheme (Figure 1). The Δ*vif* and the 3′Δ*vif* virions generated from H9 cells and titrated on MT-4 cells had low infectivity. The 5′Δ*vif* was not significantly affected compared to the ORF Δ*vif* and the 3′Δ*vif* virions, but was still not as potent as the wt. Contrarily, we did not observe any significant differences among the virions generated from HeLa-CD4$^+$ cells (Figure 2), which is consistent with results from others.[49] We further examined the growth characteristics of the HeLa-CD4$^+$-derived virions by comparing the growth kinetics on H9 and MT-4 cells. There was a direct correlation between the level of infectivity and the growth kinetics of the virions in both cell types. The Δ*vif* and 3′Δ*vif* virions grew poorly on the H9 cells, whereas the growth of the 5′Δ*vif* was slightly better, but not as progressive as the wt (Figure 3B). Comparatively, there were no significant differences observed in the growth kinetics of the HeLa-CD4$^+$-derived virions on MT-4 cells (Figure 3A). We further investigated whether the resultant low infectivity and the poor growth kinetics could eventually affect HIV-1 splicing. Pre-mRNA analysis from transiently-transfected HeLa-CD4$^+$ cells by RT-PCR revealed that the block deletions in the *vif* gene affected splicing. In the wt, there was significantly more spliced mRNA than unspliced. In contrast, the mutants demonstrated a significantly higher level of unspliced mRNA than spliced mRNA (Figure 4), which might be the result of the block deletions affecting elements that are crucial for HIV-1 biologic function in the cells. Based on these data, we further investigated whether HIV-1 *vif* antisense RNAs targeting various sites in the *vif* gene would equally attenuate the Vif-mediated infectivity in the target cells. The expression level and fidelity of the mRNA of the constructs were verified from transiently transfected COS cell and HeLa-CD4$^+$ cells, because the key step in the antisense mechanism is the expression of the antisense mRNA in the cells. There was significant mRNA expression in the cells, which might have led to the specific antisense effect on the HIV-1 *vif* mRNA in the co-transfected cells. As shown in Figure 5B, there was an effective reduction in HIV-1 viral *vif* mRNA by vectors encoding fragments within the overlapping sequences between the middle and 3′ ends of *vif*, referred to as (M-3′-AS) lane 10 and (3′-M-AS) lane 12. This correlatively mediated downregulation of the reporter gene (Figure 5C) and inhibited viral replication. In conclusion, M-3′-AS, spanning nucleic acid positions 5561-5705, which correspond to amino acid residues 96-144, significantly inhibited HIV-1 replication in COS cells. This resulted in marked downregulation of the level of the HIV-1 *vif* mRNA transcripts and reporter gene (EGFP) expression. Although not in all cases does the effect of deletions in a gene correspond to antisense effect if the same target is used (depending on the secondary RNA structure of the target), our results have shown that the amino acid residues stretching

from the middle towards the C-terminal end of Vif, especially 96-144, could be developed as an effective therapeutic target for gene therapy applications because both *vif* deletions and antisense RNA designed to target the same domain abrogated the HIV-1-Vif-dependent infectivity in the target cells.

EXPERIMENTAL PROCEDURE

Cell Cultures

COS, HeLa-CD4$^+$, H9, and MT-4 cells were grown in complete culture medium consisting of either RPMI 1640 medium (Sigma Chemical Co., St. Louis, Missouri), or D-MEM (Gibco, Invitrogen Corp., Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL), as previously described.[50] All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

Construction of Plasmid Vectors and Virus

We used the HIV-1pNLE infectious molecular clone,[51] which was based on the previously described HIV-1pNL4-3.[52] Harvesting cell-free virus from the supernatant of transfected HeLa-CD4$^+$ or H9 cells generated the wild-type (wt) HIV-1_{NLE} used in the infection assays. The constructed HIV-1 *vif* antisense RNA expression vectors were based on the eukaryotic vector pcDNA3.1 (+/−) (Invitrogen Co., Carlsbad, California), while the HIV-1 *vif* deletion mutants were based on the HIV-1pNLE infectious molecular clone. The HIV-1 *vif* open reading frame (ORF) and the 5′-*vif* and 3′-*vif* extensive block-in-frame deletion mutants were constructed by polymerase chain reaction (PCR)-mediated site-directed mutagenesis, with primers designed to amplify the AgeI and EcoRI recognition sites on either side of the *vif* gene of the HIV-1pNLE template.

Mutagenic PCR for the Construction of HIV-1 *vif* Mutants

The various fragments of the *vif gene* were amplified by PCR, using KOD plus polymerase (Toyobo, Osaka, Japan) and the following sets of mutagenic primers: (a) For the ORF Δ*vif* mutant (5271-5849), we used *forward primer-1*: 5′ LF (5′-GGAT TAA AGT AAG GCA ATT ATG TAA ACT TC-3′), *reverse primer-1*: 5′ LR-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 *forward primer-2*: 3′ RF-1 (5′-CAA GAA GAA AAG CAA AGA TCA TCA GGG ATT AGC TTT TAG AGG AAC TTA AGA GTG AAG CTG TTA-3′) and *reverse primer-2*: 3′ RR (5′-GGC TGA CTT CCT GGA TGC TTC CAG GGC TCT-3′).

(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 $CD4^+$

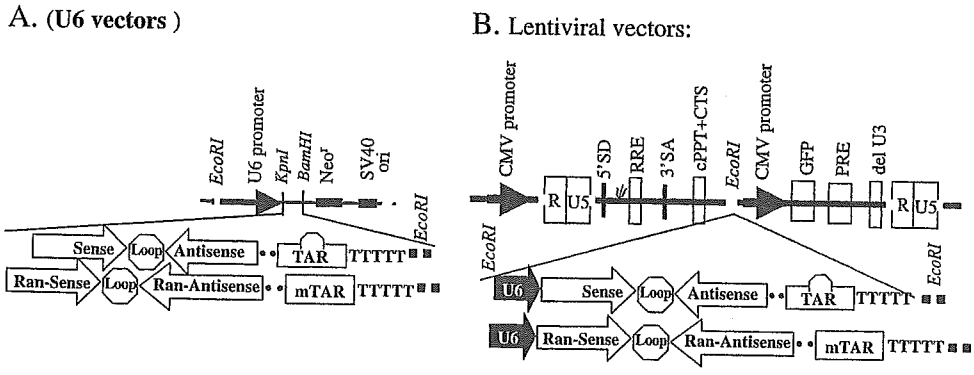

FIGURE 1 Construction of the U6 plasmid and lentiviral vectors. A. Sense and antisense strands of the synthetic DNA oligonucleotides were annealed at 95°C for 5 min, and cloned into the KpnI and BamHI cloning sites in the U6 vector. B. The EcoRI sites upstream from the U6 promoter and downstream from 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 $HIV\text{-}1_{NL4\text{-}3}$.

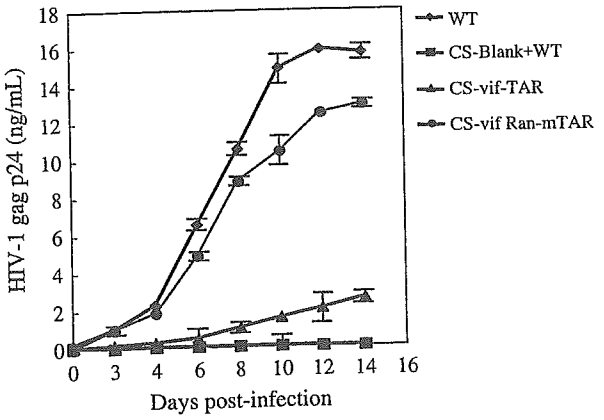

FIGURE 2 Evaluation of the inhibition efficacy of the trans-genes. Transiently transduced Jurkat cells expressing the Vif-TAR, Ran-Vif-mTAR and empty lenti-vectors were challenged with 0.02 MOI of $HIV\text{-}1_{NLE}$ and cultured over a period of 14 days. Cell-free supernatants were sampled over the 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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