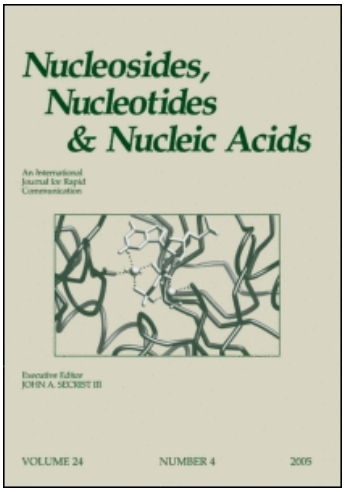

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Suppression of HIV-1 Replication by a Combination of Endonucleolytic Ribozymes (RNase P and tRNase ZL)

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To cite this Article Ikeda, Masahiro , Habu, Yuichiro , Miyano-Kurosaki, Naoko and Takaku, Hiroshi(2006) 'Suppression of HIV-1 Replication by a Combination of Endonucleolytic Ribozymes (RNase P and tRNase ZL)', Nucleosides, Nucleotides and Nucleic Acids, 25: 4, 427 — 437
To link to this Article: DOI: 10.1080/01457630600684120
URL: http://dx.doi.org/10.1080/01457630600684120

Nucleosides, Nucleotides, and Nucleic Acids, 25:427–437, 2006

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/01457630600684120

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SUPPRESSION OF HIV-1 REPLICATION BY A COMBINATION OF ENDONUCLEOLYTIC RIBOZYMES (RNase P AND tRNase ZL)

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□ *We examined the combinatorial action of RNase P and tRNase ZL-mediated specific inhibition of HIV-1 in cultured cells. We designed two short extra guide sequences (sEGS) that specifically recognize the tat and vif regions of HIV-1 mRNA and mediate the subsequent cleavage of hybridized mRNA by the RNase P and tRNase ZL components. We constructed an RNase P and tRNase ZL-associated vif and tat sEGS expression vector, which used the RNA-polymerase III dependent U6 promoter, as an expression cassette for EGS. Together, the RNase P and tRNase ZL-associated sEGS molecules allow more efficient suppression of HIV-1 mRNA production when separately applied. The possibilities offered by the vector to encode sEGS will provide a powerful tool for gene therapy.*

Keywords RNase P; tRNase ZL; External guide sequence; HIV-1; Gene therapy

INTRODUCTION

Small inhibitory RNAs (siRNAs) are small RNAs of 21-nucleotide length that trigger the destruction of target mRNA with which they share complementarity.[1] RNAi has emerged as a powerful tool to probe the function of genes of known sequence *in vitro* and *in vivo*. Advances in vector design

Received 1 December 2005; accepted 23 January 2006.

This work was supported in part by a Grant-in-Aid for High Technology Research, from the Ministry of Education, Science, and Culture, Japan, and also by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare, Japan (H17-AIDS-002). Y. H. was a Research Fellow of the HTR until June 2005, and has been a Research Fellow of the Japanese Foundation for AIDS Prevention since July 2005. Both authors Ikeda and Habu contributed equally to this work.

This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.

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permit the effective expression of siRNA in human cells by transfer of short hairpin RNA (shRNA) expression cassettes.[2] Recent investigations have described the ability of RNAi to decrease the replication of human immunodeficiency virus type 1 (HIV-1) in lymphocytic cells using siRNA targeting viral (e.g., tat, gag, rev, nef)[3–7] and host (e.g., CCR5, CXCR4)[8,9] proteins. RNAi can be used as a form of genetic therapy for HIV-1 and associated infections.

When profound inhibition of virus replication is obtained by means of RNAi technology, one has to consider the possibility of viral escape. This potential problem is particularly true for viruses that exhibit significant genetic variation due to an error-prone replication machinery. Thus, this problem may be more severe for RNA viruses and retroviruses than DNA viruses. Indeed, RNAi-resistant poliovirus and HIV-1 variants have already been reported.[10–12]

This problem can be overcome with the catalytic RNA subunit RNase P which can in principle be targeted to cleave any target RNA using the external guide sequences (EGS).[13] 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.[14,15] RNA-based EGSs have been expressed endogenously as transgenes in both bacteria and mammalian cells,[14,16] and have been effective in inhibiting gene expression by HIV-1.[17,18] 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.[19] The long-form enzyme (tRNase ZL) is unique in that it can cleave any RNA at any site when directed by a small-guide RNA (sgRNA) in vitro.[20–22] Recently, we demonstrated the efficacy of this method in specifically targeting RNA in HIV-1 infected T-cells by introducing sgRNAs encoded by expression retroviral vector. Mo-MLV-based sgRNA-SL4 targeting the HIV-1 gag gene could suppress sgRNA-dependent HIV-1 expression in human T cells.[23]

In this article, we demonstrate the combinatorial action of RNase P and tRNase ZL-mediated specific inhibition of HIV-1 in cultured cells. We designed two truncated short extra guide sequences (sEGS) specifically recognize the tat and vif regions of HIV-1 mRNA and mediate subsequent cleavage of hybridized mRNA by the RNase P and tRNase ZL components. Combination of RNase P and tRNase ZL-associated sEGS molecules allows more efficient suppression of HIV-1 mRNA than separate application.

RESULTS AND DISCUSSION

Design and Construction of the U6–EGS Driven Expression System

In a previous paper, we demonstrated the inhibition of HIV-1 products using sEGS and RNase P to cleave an HIV-RNA target (substrate) in cultured

cells.[18] The short EGS, 12 nucleotides long, was designed to hybridize as an sEGS to a region of the viral RNA with the expected cleavage site located 5′ to the double stranded region (Figure 1B). The greatest inhibitory effect on HIV-1 replication was detected with the sEGS (sEGS-tat) vector as the target of the HIV-1 tat gene (Figures 1A and B). Furthermore, 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.[23] The greatest inhibitory effect on HIV-1 expression was achieved using sgRNA targeting the HIV-1 gag gene.

In this article, we selected the vif (5521-5533) and tat (5921-5940) as the target sites and tested the RNase P and tRNase ZL-associated sEGS (RNase P-tat-sEGS and tRNase-ZL-vif-sEGS) in one molecule for anti-HIV activity (Figures 1B–D). The complete T loop of RNase P-*tat*-sEGS was replaced by single-stranded sequence UUCA, whereas the T-stem loop of tRNase ZL-vi *f*-sEGS included the T-stem loop (5′-CCAGGUUCGACUCCUGG-3′) of wild-type tRNA^{Arg}.

Alternatively, small RNA molecules might also be expressed in the cell following the cloning of siRNA templates into RNA polymerase III (pol III) transcription units, which are based on the sequences of the natural transcription units of the small nuclear RNA U6.[24,25] We cloned the vif-sEGS, tat-sEGS, and vif-tat-sEGS lined with linker (5′-CGCUCGAGU-3′) between either the vif-sEGS-5′ end and tat-sEGS-3′ end genes into Kpn I and BamH I sites downstream of the human U6 snRNA promoter[24] of mammalian expression vector (pSV2neo-U6) (Figure 2). These vectors have been previously described with a high cleavage affinity.[18,23] Furthermore, the control vector designs were constructed to express pSV2neo-U6-mutant-EGS expression vectors (U6-mvif-sEGS, U6-mtat-sEGS, and U6-mvif-mtat-sEGS) (Figure 2).

Expression of the sEGS in Target Cells

To characterize the effect of transge expression, we monitored green-fluorescent protein (GFP) expression in the COS cells transfected with three different plasmids (U6-vif-sEGS, U6-tat-sEGS, and U6-vif-tat-sEGS). GFP expression in these transfected COS cells were observed for 3 days (Figure 3A). To ensure that the therapeutic gene expression by a plasmid vector, pU6-vif-tat-sEGS in target cells, we carried out Northern blot analysis on total RNAs extracted from transduced COS cells. As shown in Figure 3B, a combination of U6-vif-tat-sEGS was expressed at readily detectable levels.

Suppression of HIV-1 Replication by the EGS

To test the ability of the sEGS-expressed by the mammalian expression vectors (pSV2-neo-U6-sEGS) to inhibit HIV-1 replication in a transient assay,

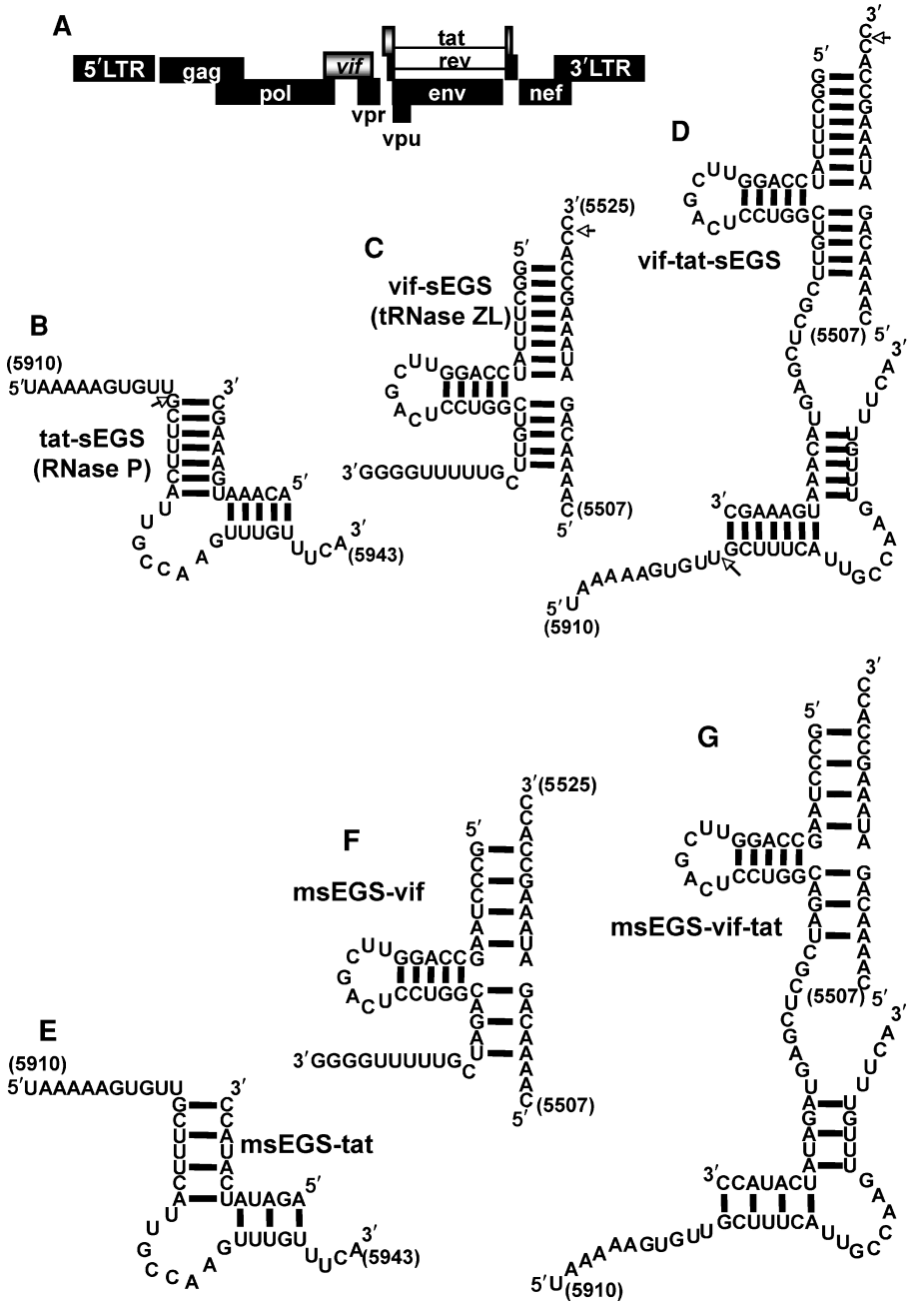

FIGURE 1 Locations of the target sites and secondary structures in the HIV-1 gene of sgRNAs. (A) Locations of the target sites in the HIV-1 gene of sEGS. (B-G) Plausible secondary structures of complexes of the two target sites within the HIV-1 genome (the vif and tat within the HIV-1 gene) with the modified 5′-half-tRNA^{Arg} (tat-sEGS) containing 9 and 5 nt sequences and the T-stem and loop as well as the acceptor stem to the complementary to the target HIV-1 vif site and the tat gene. The arrow indicates the RNase P and tRNase ZL cleavage point.

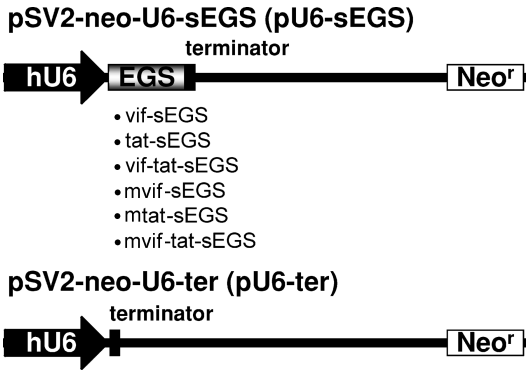

FIGURE 2 Schematic representation of sEGS-expression vectors. The pSVneo-U6-sEGS vectors, pU6-vif-sEGS, pU6-tat-sEGS, pU6-vif-ta-sEGS, pU6-mvif-sEGS, pU6-mtat-sEGS, and pU6-mvif-tat-sEGS. Control vector, pSVneo-U6-ter, lacked sEGS in pSVneo-U6-sEGS.

a viral plasmid (pNL4-3-luc)[26] and the pSV2-neo-U6-sEGSs (U6-vif-sEGS, U6-tat-sEGS, and U6-vif-tat-sEGS) or pSV2-neo-U6-ter (U6-ter) with the U6 promoter and terminator as the control plasmid, were cotransfected into COS cells by the transfection reagent, FuGENE6. The virus production in the culture supernatant was monitored by the HIV-1 p24 antigen (gag gene

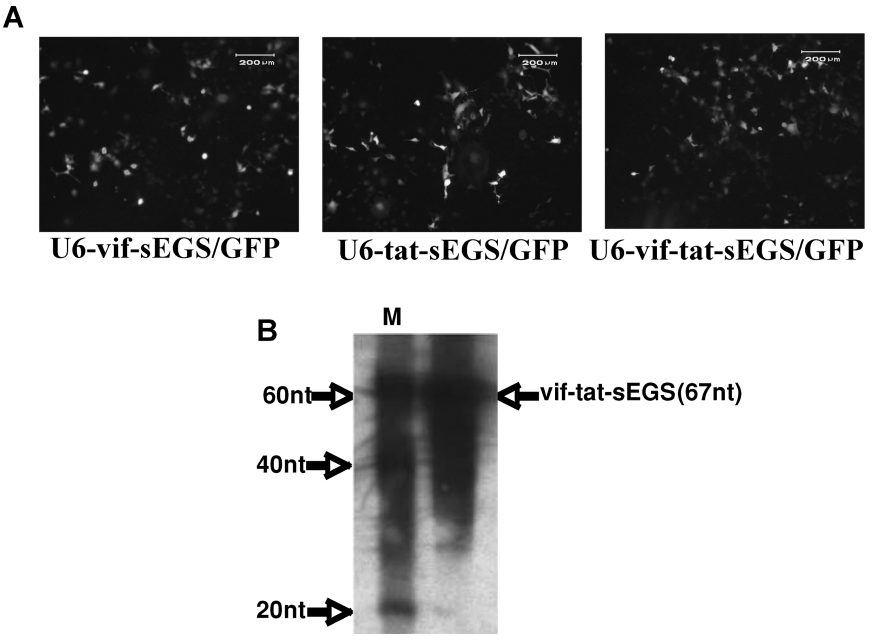

FIGURE 3 Expression of transgenes. (A) To monitor the continuous expression of the transgenes in the transduced cells throughout the culture period, the cells were transferred onto microscope slides and examined for GFP expression during the sampling of the culture cells and supernatants. (B) The presence of RNA was analyzed by northern blotting. Plasmids encoding vif-tat-sEGS were introduced into COS cells. After 72 h, the cells were collected, total RNA was isolated and fractionated on a 15% polyacrylamide gel.

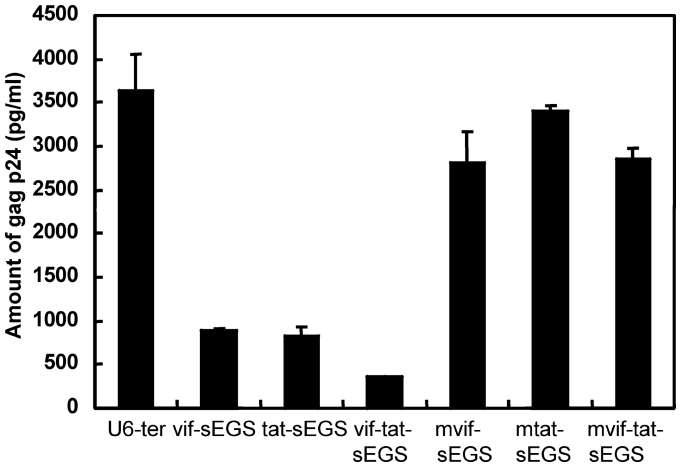

FIGURE 4 Inhibition of production of HIV-1 gag p24 antigen in COS cells transduced with pU6-sEGS. COS cells were transduced with pU6-sEGS and pNL4-3-luc. The pU6-vif-tat-sEGS showed significant suppression of the HIV-1p24 antigen expression in COS cells. Data are representative of three independent experiments.

product) assay.[27] Low levels of p24 product for both U6-vif-sEGS and U6-tat-sEGS used in the challenge assays were detected, and no differences between the EGS efficiencies were observed (Figure 4). In contract, combination of RNase P and tRNase ZL associated sEGSs molecules significantly reduced the level of HIV-1 p24 antigen as compared with that of either U6-vif-sEGS and U6-tat-sEGS (Figure 4). On the other hand, the control sEGSs (U6-mvif-sEGS, U6-mtat-sEGS, and U6-mvif-tat-sEGS) had no inhibitory effect on HIV-1 p24 antigen. These results suggest that the combination of two different antiviral RNAs provides more than separate application.

Effect of a Combination of RNase P and tRNase ZL-Associated sEGS

We also examined the HIV-1 mRNA levels to identify the contribution of HIV-1 mRNA cleavage to the sEGS-mediated anti-HIV-1 effect. The RT-PCR reactions were used to establish the level of uncleaved HIV-1 mRNA (product I and II).[28] The uncleaved HIV-1 mRNA was amplified by the vif-F1and tat-F1 primers, and the vif-R and tat-R primers (Figure 5A). The level of product I and II were expected to decrease after cleavage of the HIV-1 mRNA. The results, which are shown in Figure 5B, indicate that the vif-sEGS and tat-dependent expression system reduced the amount of full-length HIV-1 mRNA (product I and II), whereas COS cells transfected with pNL4-3-luc/mutant-sEGS did not exhibit a significantly altered level of intact HIV-1 mRNA (Figure 5B). The reduction in functional full-length HIV-1 mRNA was consistent with the cleavage effects of vif-sEGS and tat-sEGS at the

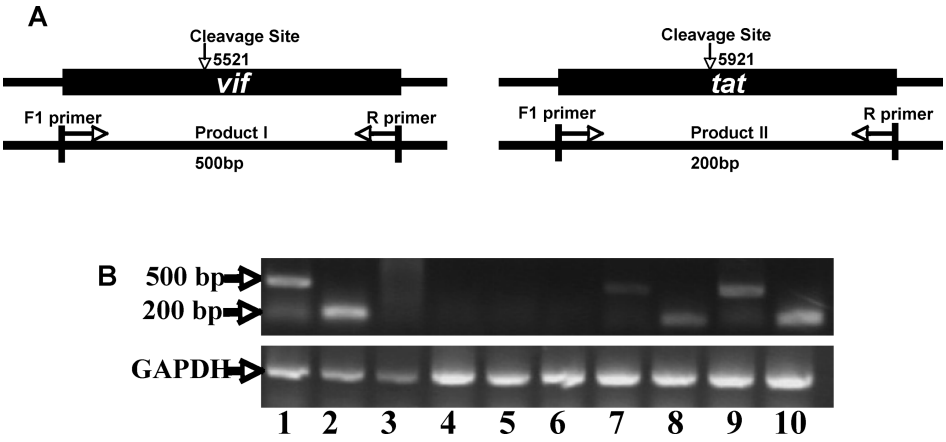

FIGURE 5 RT-PCR analyses of HIV-1 mRNA expression. RT-PCR analyses of uncleaved (product I and II), HIV-1 mRNA were performed using HIV-1 vif and tat-specific primers with concurrent amplification of GAPDH mRNA. (A) Schematic representation of HIV-1-specific primer sites with respect to HIV-1 mRNA: F1 primers, vif and tat; R-primers, vif and tat. (B) RT-PCR amplification products analysed by 2% agarose gel electrophoresis with ethidium bromide staining RT-PCR analysis of HIV-1 mRNA expression in COS cells. RT-PCR analysis of uncleaved HIV-1 mRNA was carried out using HIV-1 specific primers with concomitant amplification of GAPDH mRNA. Lane 1: control vif; lane 2: control tat; lane 3: vif-sEGS; lane 4: tat-sEGS; lane 5: vif-tat-sEGS (vif); lane 6: vif-tat-sEGS (tat); lane 7: mvif-sEGS; lane 8: mtat-sEGS; lane 9: mvif-tat-sEGS (vif); lane 10: mvif-tat-sEGS (tat).

post-transcriptional level. These results indicate that the binding of the sEGS to its target HIV-1 mRNA, and cleavage of pre-tRNA complexes with RNase P and tRNase ZL, might occur in the nucleus. The reduction in functional full-length HIV-1 mRNA was consistent with the RNase P and tRNase ZL cleavage effect at the post-transcriptional level.

This work explores the combination of two inhibitor RNAs (RNase P and tRNase ZL) in one molecule, generating a multifunctional RNA tool, which we have termed an "endonucleolytic ribozymes" (enR). The combination of an RNase P and a tRNase ZL-associated EGSs molecules would be advantageous than separate application. It possible to take advantage of the possibilities offered by the vector to encode sEGS and provides a powerful tool for HIV-1 gene therapy.

EXPERMENTAL PROCEDURES

Cell Cultures

COS and MT-4 cells were grown in complete culture medium consisting of either RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) or D-MEM (Sigma Chemical Co.) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

Construction of U6 Expression Plasmid Vectors

Expression plasmids were constructed using standard techniques. EGS-RNA sequences that were chemically synthesized as two complementary DNA oligonucleotides were mixed in equimolar amounts, heated for 5 min at 95°C, and then gradually cooled down to RT in annealing buffer (10 mM Tris-HCl/100 mM NaCl). The resultant duplex was ethanol precipitated and then ligated into the Kpn I and BamH I cloning sites downstream of the human U6 snRNA promoter of mammalian expression vectors (pSV2neo) (BD Biosciences Clontech, Mountain View, CA) in order to produce the following: the pSV2neo-U6-vif-tat-sEGS vector encoding both HIV-1 vif and tat RNA as dsRNA sense sequences containing KpnI and BamH I cloning sites (5′-GGCTTTATCCAGGTTCGACTCCTGGCTGTT-CGCTCGAGACAAATGAAAGCTTTTTG-3′) and antisense sequences (5′-GATCCCAAAAAGCTTTCATTTCTCTCGAGCGAACAGCCAGGAGTCGAA-CCTGGATAAAGCCGTA-3′); the pSV2neo-U6-vif-sEGS vector encoding HIV-1 vif sense fragment sequences (5′-CGCTTTATCCAGGTTCGACTC-CTGGCTGTTCGTTTTTGGGGTACG-3′) and antisense sequences (5′-GAT-CCGTACCCCAAAAACGAACAGCCAGGAGTCGAACC- TGGATAAAGCCG-TAC-3′); the pSV2neo-U6-tat RNA vector encoding HIV-1 tat sense sequences (5′-ACAAATGAAAGCTTTTTG-3′) and antisense sequences (5′-GATCCAA-AAAGCTTTCATTTGTGTAC-3′); the pSV2neo-U6-mvif-sEGS vector encoding mutated vif sense sequences (5′-CCCCTAAGCCAGGTTCGAC-TCCTGGCAGATCGTTTTTGGGGTACG-3′) and antisense sequences (5′-GATCCGTACCCCAAAAACGATCTGCCAGGAGTCGAACCTGGCTTA-GGCCGTAC-3′); the pSV2neo-U6-mtat-sEGS vector encoding mutated tat sense sequences (5′-AGATATCATACCTTTTTG-3′) and antisense sequences (5′-GATCCAAAAAGGTATGATATCTGTAC-3′); and, finally, the pSV2neo-U6-mvif- tat-sEGS vector encoding mutated vif and tat sense sequences (5′-GCCCTAAGCCAGGTTCGACTCCTGGCAGATCGCTCGAGAGATATC-ATACCTTTTTG-3′) and antisense sequences (5′-GATCCCAAAAAGGTA-TGAATCCTCTCGAGCGATCTGCCAGGAGTCGAACCTGGCTTAGGGCG-TAC-3′); as control expression vectors.

Fluorescent Microscopy

COS cells were grown to ~80% confluence (3×10^5 cells) and transfected with 1 μg each of the sEGS-RNA vectors using 3 μl of FuGENE 6 (Roche Applied Science, Indianapolis, IN). The transfected COS cells were trypsinized, washed twice in PBS, and fixed in PBS containing 1% formaldehyde. Direct fluorescence microscopy of GFP was carried out at the mitotic stage of cell division after each passage on day 3; the data were acquired with a DP12 digital microscope camera (Olympus Company, Tokyo, Japan).

Northern Blot Analysis

Total RNA was extracted with Trizol reagent (GibcoBRL Life Technologies, Carlsbad, CA) from vector-transfected cells, and samples (30 μg) were loaded onto a 20% polyacrylamide/8M urea gel. After electrophoresis, the RNA bands were transferred onto a Hybond-N^{TM} nylon membrane (Amersham Co., Buckinghamshire, UK). The membrane was probed with synthetic oligonucleotides that were complementary to the antisense strand of the env shRNA. Hybridization was carried out at 42°C, and was followed by washing with 2 × SSC at 25°C prior to autoradiography.

Antiviral Assay

To each well of a six-well plate were added COS 3×10^5 cells in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal bovoine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Approximately 24 h after plating, when the cells had reached 80% confluence, sEGS-RNA vectors (1 μg) and pNL4-3-luc (0.1 μg) using 3 μl of FuGENE 6 (Roche Applied Science) were generated according to the manufacturer's optimized protocols. The envelope-defective HIV-1NL4-3-based retroviral vector containing a luciferase expression marker (pNL4-3-luc) was generated by substituting nef gene sequences of the HIV-1NL4-3 genome with the firefly luciferase gene and deleting the envelope gene sequences located between two Bgl II restriction endonuclease sites.[26] After 3 days of incubation, the virus replication was monitored in the cell-free culture supernatants with the HIV-1 p24 CLEIA assay (Lumipulse, Fujirebio Inc., Tokyo, Japan).[27]

Cleavage Activities of sEGS in COS Cells

COS cells were grown to ~80% confluence (3×10^5 cells) and transfected with 1 μg each of the sEGS-RNA vectors and the pNL4-3-luc plasmid. The cells were incubated for 3 days before the total cellular RNA was isolated. RNA samples were treated with DNase I according to the manufacturer's specifications. To quantify the level of HIV-1 RNA, amplifications of HIV-1 mRNA and the internal control message GAPDH were incorporated into the reaction mixtures for the RT and PCR steps. A sample of 1 μg of total RNA was used as the template with the vif, tat, and GAPDH primers (20 pmol each). Reverse transcription (final volume: 50 μl) was carried out at 60°C for 30 min. The cDNA products were amplified by PCR (94°C, 60 sec; 60°C, 90 sec; 40 cycles). The vif-F1 (5′-AGGAGAAAGAGACTGGCATTTGGG-3′) and tat-F1 (5′-ATGGAGCCAGTAGATCCTAGATCAGA-3′) primers and the vif-R (5′-CTCCTTCTGTCGAATAACGCCTATTCTG-3′) and tat-R (5′-CTATTCCTTCGGGCCTGTCGGGTC-3′) primers only generated a cDNA product from the uncleaved HIV-1 mRNA (RT-PCR product I, vif = 500 base

pairs, product II, tat = 200 base pairs), whereas the GAPDH-F and R primers generated the GAPDH gene (0.45 kb) as the internal control.

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