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Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins

Daniel Boden, Oliver Pusch, Rebecca Silbermann, Fred Lee, Lynne Tucker and Bharat Ramratnam*

Laboratory of Retrovirology, Division of Infectious Diseases, Department of Medicine, Brown Medical School, 4th Floor, 55 Claverick Street, Providence, RI 02903, USA

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ABSTRACT

Post-transcriptional inhibition of HIV-1 replication can be achieved by RNA interference (RNAi). The cellular expression of short interfering RNA (siRNA) or short hairpin RNA (shRNA) homologous to regions of the HIV-1 genome decreases viral replication by the selective degradation of targeted RNA. Here, we demonstrate that another class of noncoding regulatory RNA, termed microRNA (miRNA), can be used to deliver antiviral RNAi. By incorporating sequences encoding siRNA targeting the HIV-1 transactivator protein *tat* into a human miR-30 premicroRNA (pre-miRNA) backbone, we were able to express *tat* siRNA in cells. The *tat* siRNA delivered as pre-miRNA precursor was 80% more effective in reducing HIV-1 p24 antigen production than *tat* siRNA expressed as conventional shRNA. Our results confirm the utility of expressing HIV-1 specific siRNA through a miR-30 precursor stem–loop structure and suggest that this strategy can be used to increase the antiviral potency of RNAi.

INTRODUCTION

Post-transcriptional regulation of gene expression can be mediated by small noncoding RNAs such as short interfering RNA (siRNA) and microRNA (miRNA). siRNA are short RNA duplexes that direct the degradation of homologous mRNA through the RNA interference (RNAi) pathway (1,2). miRNA, in contrast, are single-stranded RNAs that bind to partially complementary (50–85%) 3′ untranslated regions of mRNA leading to translational repression without target degradation (3–6). Both siRNA and miRNA are ~22 nt in length and are produced from longer double-stranded precursor RNA by the multidomain ribonuclease III enzyme Dicer (7–9). The ability of siRNA to specifically degrade selected mRNA species has rendered RNAi an indispensable tool and has allowed investigators to study the biological effects of gene disruption *in vitro* and *in vivo*. Furthermore, the ability to stably express siRNA/short hairpin RNA (shRNA) in human cells using viral vectors raises the possibility of using RNAi as a form of gene therapy to selectively inhibit the expression of disease specific genes such as oncogenes (10).

Several recent reports have demonstrated that the cellular expression of siRNA or shRNA targeting HIV-1 genes such as *gag*, *nef*, *tat* and *rev* inhibits viral replication (11–17). These studies have involved the introduction of synthetic siRNA or the genetic transfer of DNA expression cassettes capable of producing siRNA/shRNA in human cells. We hypothesized that another potential approach for delivering siRNA and inhibiting HIV-1 could involve the use of the structure of pre-microRNAs (pre-miRNAs). miRNAs have been identified in a wide range of organisms including *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens*. Though >100 different miRNAs have been identified, little is known regarding their function. An exception has been described in the experimental model system *C.elegans* where two miRNAs (*lin-4* and *let-7*) are temporally expressed and repress the translation of mRNA encoding proteins involved in the developmental timing pathway of the worm (18,19).

A unique structural feature of miRNAs is their initial transcription as a long primary transcript (pri-miRNA) that is then processed by the nuclear enzyme Drosha into ~70 nt precursor stem–loop hairpin RNAs (pre-miRNA) (20). Upon nuclear export, pre-miRNA is further processed by Dicer to yield the mature miRNA. One example of a miRNA is the miR-30 species that has been identified in the human HeLa cell line (5). Zeng *et al.* (21) have recently demonstrated that the miR-30 miRNA backbone can be altered to include sequences encoding siRNA and have used this approach to effectively degrade mRNA of endogenous human genes such as the polypyrimidine tract binding protein. McManus *et al.* have demonstrated that siRNA sequences incorporated into the stem of mir-26a function as effectors of RNAi with the specific degradation of homologous mRNA (4). In both reports, the nucleotide sequence introduced into the stem was 100% complementary to the targeted gene, a feature thought to be critical for commencing the RNAi cascade (22).

No reports, to our knowledge, have directly compared the efficiency of HIV-1 gene silencing that results from conventional shRNAs compared with siRNAs incorporated into a miRNA backbone. To determine whether any differences exist in these two approaches, we replaced a part of the miR-30

*To whom correspondence should be addressed. Tel: +1 401 444 5219; Fax: +1 401 444 2939; Email: bramratnam@lifespa.org

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

pre-miRNA stem sequence with a siRNA duplex sequence targeting the HIV-1 transactivator protein *tat*. Two miR-*tat* RNA expression constructs were designed that differed only with respect to the sequence of the miR-30 terminal loop (Fig. 1). The miR-*tat* RNA expression vectors were tested along with a previously described conventional *tat* shRNA expression vector in mammalian cell culture (15). We found that *tat* siRNA was more effectively processed from a pre-miRNA precursor as opposed to a shRNA transcript. In viral challenge experiments, we demonstrate that miR-*tat* RNA is 80% more potent in inhibiting HIV-1 replication than *tat* siRNA expressed as a conventional shRNA precursor.

MATERIALS AND METHODS

Construction of *tat* shRNA and *tat* pre-miRNA expression cassettes

The 21 nt long HIV-1 *tat* sequence expressed as shRNA or pre-miRNA corresponds to nucleotide positions 5993–6013 of HIV-$1_{NL4.3}$. To generate the *tat*-shRNA expression plasmid, two oligonucleotides, SHT-S, 5′-CTA GAG ACG AAG AGC TCA TCA GAA CAT TCA AGA GAT GTT CTG ATG AGC TCT TCG TCT TTT TC-3′, and SHT-AS, 5′-TCG AGA AAA AGA CGA AGA GCT CAT CAG AAC ATC TCT TGA ATG TTC TGA TGA GCT CTT CGT CT-3′ were annealed and introduced into XbaI/XhoI sites of plasmid pAAV-U6-MCS, as described previously (17).

The two miR-*tat* constructs (miA-*tat* and miB-*tat*) were generated by PCR extension of two partly complementary oligonucleotides. To create expression plasmid miA-*tat*, we used the oligonucleotide pair MIA-S, 5′-GTG AAC TCT AGA GCG AGA CGA AGA GCT CAT CAG AAC AGT GAA GCC ACA GAT GTG TTC TGA-3′ and MIA-AS, 5′-ATT GGA CTC GAG GCA GGA CGA AGA GCT CAT CAG AAC ACA TCT GTG GCT TCA CTG TTC TGA-3′; for miB-*tat* MIB-S, 5′-GTG AAC TCT AGA GCG AGA CGA AGA GCT CAT CAG AAC ACT GTG AAG CCA CAG ATG GGT GTT-3′ and MIB-AS, 5′-ATT GGA CTC GAG GCA CGA CGA AGA GCT CAT CAG AAC ACC CAT CTG TGG CTT CAC AGT GTT-3′. Oligonucleotides were annealed and primer extended with the following thermal profile, 95°C for 5 min, 50°C for 30 s, 68°C for 20 min, using Pfx-Taq polymerase (Invitrogen, Carlsbad, CA). Products were gel purified, restriction enzyme digested with XbaI/XhoI and introduced into plasmid pAAV-U6-MCS. A control vector, pAAV-U6-luc-Neo, was generated that synthesizes shRNA targeting the luciferase gene, as described previously (16).

Cell culture and transfection

293 cells were trypsinized and plated at 4×10^5 cells per well in six-well plates 24 h prior to transfection in DMEM containing 10% fetal bovine serum. One microgram of sh-*tat* or miA/B-*tat* DNA was co-transfected with 1 μg of HIV-$1_{NL4.3}$ using 6 μl of Lipofectamine 2000 (Gibco-Invitrogen). Two days later, cell-free supernatant was collected and viral production was quantified by measuring HIV-1 p24 antigen levels in cellular supernatant by ELISA (Beckman-Coulter, Fullerton, CA) according to the manufacturer's instructions.

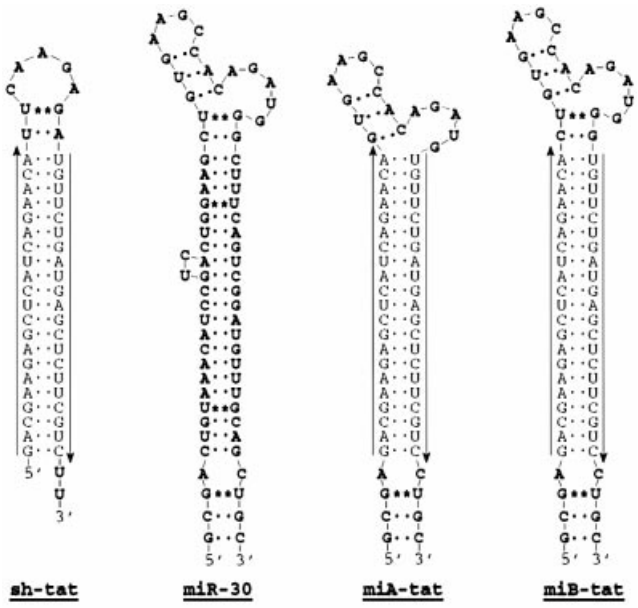

Figure 1. Schematic representation of predicted short hairpin *tat* and pre-micro mir-30/*tat* RNA sequences. Underlined arrows indicate sense and antisense *tat* sequences embedded in a shRNA or a miR-30 precursor RNA backbone. Bold letters in miA-*tat* and miB-*tat* represent stem–loop sequences derived from pre-miR-30. G:U wobbles are indicated with a star.

Western blot

Supernatant was harvested from 293 cells transfected with HIV-$1_{NL4.3}$ and passed through a 0.45 μm filter prior to western blot analyses. Protein extracts were prepared by washing cells with phosphate-buffered saline and lysing in TNT lysis buffer (20 mM Tris/200 mM NaCl/1% Triton X-100) containing protease inhibitors. After 20 min on ice, lysates were spun at 15 000 *g* for 15 min to remove insoluble material. Protein concentrations were determined by the Bradford procedure. Proteins were resolved on 12% SDS–polyacrylamide gels and transferred onto Hybond PVDF membranes (Amersham, Piscataway, NJ). For sequential probing, blots were stripped in Restore Western Blot Stripping Buffer (Pierce, Rockford, IL). A monoclonal antibody to HIV-1 p24 (183 clone H12-C) was obtained through the NIH AIDS Reagent and Reference program and used at a 1/100 dilution. The monoclonal antibody to α-tubulin (clone DM7 1A) (Sigma, St Louis, MO) was used at a 1/500 dilution. Immunodetection was performed using the peroxidase-based ECL Plus detection system (Amersham) according to the manufacturer's instructions.

Real-time RT–PCR for intracellular HIV-1 RNA

Total cellular RNA was isolated from 293 cells with Trizol reagent (Invitrogen) and treated with RQ1 DNase I (Promega, Madison, WI) according to the manufacturer's protocol. One microgram of DNase I-treated RNA was added to a reverse transcription (RT) reaction containing Powerscript Reverse Transcriptase (Clontech, Palo Alto, CA), 1 mM each of dNTP, 1× First-Strand buffer (Clontech), 200 ng of random hexamers (Promega), 10 U RNasin (Promega). RT was performed at 42°C for 1 h followed by heat-inactivation of the RT enzyme at 70°C for 15 min. PCR was performed with

1 µl of cDNA in a final volume of 30 µl and contained 1× Titanium Taq PCR buffer (Clontech), 20 pmol of sense primer *Tat*-A, 5′-ATG GAG CCA GTA GAT CCT A-3′, and antisense primer *Tat*-B, 5′-TGC TTT GAT AGA GAA ACT TGA TG-3′, 1 mM dNTPs, SYBR Green I (1:75 000), 10 nM fluorescein, and 1× Titanium Taq polymerase (Clontech). To normalize the samples for absolute RNA amount, a GAPDH-PCR was performed with primers G1, 5′-GAT TTC TCC CCC TTC TGC TGA TG-3′ and G2, 5′-CCT TGG CTG GGG GTG CTA A-3′. An external standard curve was created using spectrophotometrically determined copy number standards of plasmid pNL4.3. Real-time PCR was carried out in an iCycler (Bio-Rad, Hercules, CA) using the following thermal cycling profile: 95°C for 1 min, followed by 40 cycles of amplification (95°C for 15 s, 63°C for 30 s, 68°C for 30 s). All samples were run in triplicate.

Northern blot analysis of *tat* siRNA expression

Forty-eight hours after transfection, RNA samples were prepared from 293 cells using Trizol reagent. To determine the level of *tat* shRNA expression, 40 µg of total RNA was separated by electrophoresis on a 15% polyacrylamide–7 M urea gel and electroblotted for 4 h at 400 mA on a Zeta-Probe GT membrane (Bio-Rad). RNA was immobilized by UV crosslinking and baking for 1 h at 80°C. Hybridization was carried out at 42°C using Ultrahyb-Oligo Hybridization Buffer (Ambion). HIV-1 specific siRNA was probed with a ^{32}P-labeled *tat* antisense oligonucleotide (5′-TGT TCT GAT GAG CTC TTC GTC-3′). Membranes were washed twice in 2× SSC, 0.1% SDS and 0.2× SSC, 0.1% SDS at 37°C.

RESULTS

Our experiments describe an alternative strategy to achieve intracellular synthesis of siRNA. By incorporating *tat* siRNA sequences into the stem of the human *miR-30* miRNA, we were able to efficiently produce active HIV-1 specific siRNA in human cells under the control of the human U6 pol III promoter. We first followed the structural motif described by Zeng and Cullen (23) who used a loop structure (miA-*tat*) that differed from the endogenously produced *miR-30* by 4 nt. We also created a loop structure (miB-*tat*) identical to that of endogenously produced *miR-30* (Fig. 1). In co-transfection experiments, we observed that HIV-1 production was most effectively suppressed by miB-*tat*. Quantification of HIV-1 p24 antigen by immunoassay in culture supernatant revealed that miA-*tat* and miB-*tat* reduced viral activity by 25- and 45-fold, respectively (Fig. 2A and B). Most importantly, both miRNA constructs were up to 82% more effective in inhibiting HIV-1 replication than conventional *tat* shRNA. These results were confirmed by western blot analysis of HIV-1 p24 antigen isolated from culture supernatant and cell lysate. The production of p24 antigen was reduced in both compartments and mirrored the p24 immunoassay results. miB-*tat* RNA showed the most effective reduction of p24 antigen in both compartments followed by miA-tat RNA (Fig. 3).

It has been demonstrated that a duplex RNA incorporated in a miRNA sequence context operates as siRNA and triggers destruction of a perfectly complementary target mRNA (4,23). To verify that the antiviral effect was indeed siRNA mediated and not due to miRNA related translational repression of protein synthesis, we further evaluated the nature of the observed reduction of viral particle production. We assessed HIV-1 RNA target degradation by quantitative real-time PCR. Once again, we observed the same pattern of antiviral activity with miB-*tat* being the most effective, followed by miA-*tat* in degrading HIV-1 RNA (Fig. 4). These results demonstrate that

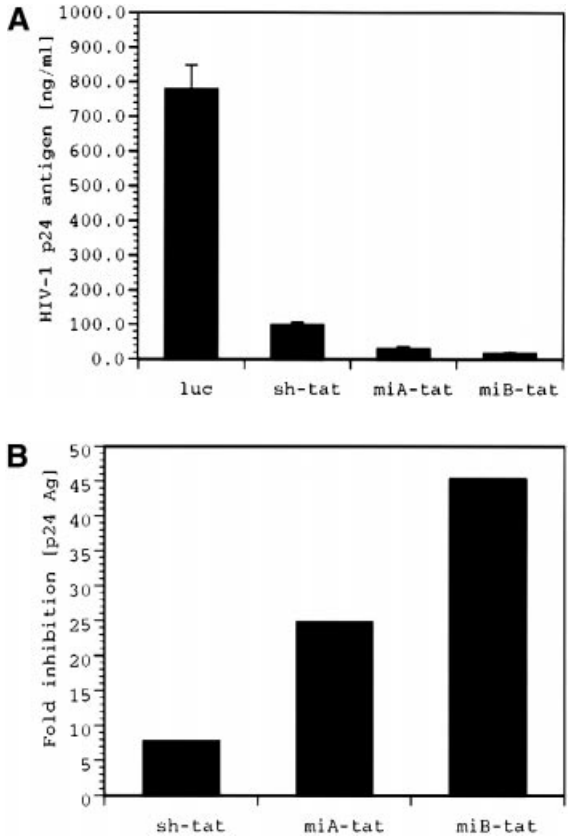

Figure 2. HIV-1 p24 antigen production in culture supernatant of 293 cells transfected with HIV-$1_{NL4.3}$ and the indicated *tat* siRNA expression plasmids determined by p24 immunoassay 48 h post-transfection; luc indicates a shRNA expression plasmid targeting luciferase. (**A**) Absolute p24 antigen in nanograms per milliliter of culture supernatant. (**B**) Antiviral potency of the various *tat* siRNA expression plasmids relative to control plasmid luc shown as fold inhibition. Values represent averages of three independent experiments, with the range indicated.

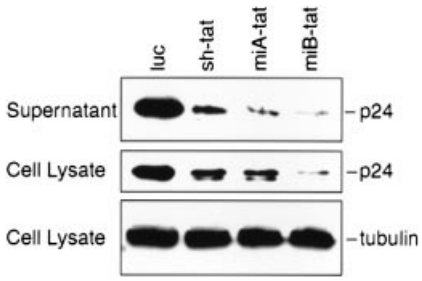

Figure 3. Reduction of HIV-1 p24 antigen in cell-free supernatant and cell lysate determined by western blot analysis. 293 cells were co-transfected with HIV-$1_{NL4.3}$ and the indicated *tat* siRNA expression vectors. The control plasmid luc expresses a luciferase hairpin siRNA. Tubulin served as a loading control.

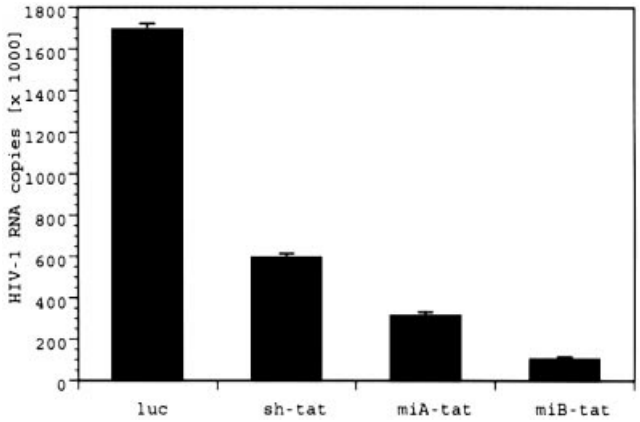

Figure 4. Reduction of total intracellular HIV-1 RNA determined by real-time PCR. Variation of RNA input was normalized by quantifying GAPDH expression by real-time PCR. To convert threshold cycles to copy number, an external standard curve was created with known copy numbers of HIV-$1_{NL4.3}$. Values represent averages of three independent experiments, with the range indicated.

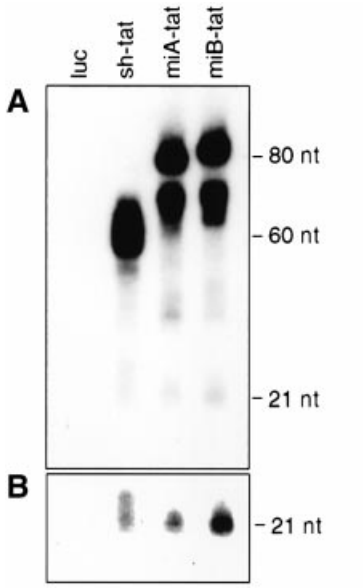

Figure 5. Northern blot analysis of *tat* shRNA, pre-miRNA and siRNA expression from 293 cells co-transfected with HIV-$1_{NL4.3}$ and the indicated DNA constructs. Total cellular RNA was extracted from 293 cells 48 h after transfection and separated on a 15% polyacrylamide–7 M urea gel. Hybridization was performed using a ^{32}P-labeled DNA oligonucleotide probe complementary to the tat sense strand. (**A**) Northern blot was exposed for 1 h. (**B**) Detection of processed *tat* siRNA effector molecules after 10 h of exposure.

siRNA incorporated in a pre-miRNA backbone enters the RNAi pathway and exerts its gene silencing effect by target degradation.

To examine whether the increased HIV-1 RNA degradation obtained with miB-*tat* was due to enhanced processing of precursor RNA to siRNA, we performed northern blot experiments to quantify the production of siRNA effector molecules. Most efficient processing of siRNA from *tat* short hairpin or pre-miRNAs was obtained with miB-*tat* RNA. Expression of sh-*tat* RNA resulted in the lowest level of siRNA production (Fig. 5A and B).

Finally, we assessed the thermodynamic properties of all three RNA species. In modeling the secondary structure (MFold version 3.0), we found that the most potent construct (miB-tat) contained the highest local folding potential (lowest ΔG) (sh-*tat* ΔG, –38.5; *miA-tat* ΔG, –41.6; miB-*tat* ΔG, –45.9).

DISCUSSION

The use of small RNAs to decrease HIV-1 replication offers a new approach to antiviral gene therapy. Thus far, HIV-1 specific RNAi has been generated by the cellular introduction of synthetic 21 nt long RNA duplexes or DNA vectors harboring siRNA/shRNA expression cassettes. DNA vectors have followed a stereotypic design with the use of RNA pol III promoters to drive the expression of 19–21 nt long sense and antisense strands either separately or joined by a variably sized loop with the end result of cellular synthesis of siRNA or shRNA, respectively (12,16).

Our results confirm the utility of using the pre-miR-30 backbone as a delivery system for siRNA. The enhanced potency of gene silencing achieved with the miA/B constructs compared with conventional shRNA deserves comment. The effectiveness of RNAi mediated gene silencing is likely multifactorial and reflects the efficiency of siRNA generation, its nucleotide homology with the chosen target, the accessibility of the target RNA, the structural stability of the siRNA delivery molecule (miRNA, shRNA) and its ability to be processed by Dicer to enter the RNAi silencing complex (RISC)/miRNA ribonucleoprotein particle (miRNP). Northern blot analysis revealed that siRNA derived from miB-*tat* RNA was most efficiently processed in the cell. In modeling the secondary structure of the three different RNAs (MFold version 3.0), we note that the most potent construct (miB-tat) contained the highest local folding potential (lowest ΔG) (sh-*tat* ΔG, –38.5; *miA-tat* ΔG, –41.6; miB-*tat* ΔG, –45.9). Thus, the enhanced processing of miB-*tat* RNA to siRNA may be related to the higher structural stability of the stem–loop structure, which folds back more rapidly into its secondary structure that is now available for Dicer binding and cleavage. The higher rate of diced shRNA/miRNA leads to faster formation of the RISC/miRNP with the end result of more potent gene silencing. Presently, structural or sequence motifs important for Dicer recognition of its target RNA are unknown. Site-directed mutagenesis studies of pre-miR-30 demonstrated the requirement of dsRNA structure around the cleavage site of the 5′ and 3′ ends of pre-miR-30 for efficient processing into siRNA (20,23). The sequence and structural requirements of the loop structure of shRNA and miRNA precursors critical for efficient processing have not been fully investigated and remain unclear. McManus *et al.* found that the majority of processing within shRNA stem–loop structures occurs in the loop sequences of the hairpin (4). Thus, it may be that a yet unidentified structural motif in the pre-miRNA loop is better recognized by Dicer, or nucleases other then Dicer, and/or is more efficiently processed, resulting in increased gene silencing. In the future it will be important to determine structural elements of miRNA precursors and shRNA as well as RNase III proteins to better understand the mechanism of pre-miRNA/shRNA processing.

Our results also have a more immediate, practical application. A central challenge in using RNAi is the identification of siRNA/shRNA nucleotide sequences that lead to effective gene silencing. In our experience, nucleotide sequence selection is largely an empiric process initially guided by RNA secondary structure predictions of the target mRNA (e.g. MFold), but ultimately tested by direct experimentation. It is not uncommon for investigators to run through a panel of siRNA/shRNAs before identifying an effective species. We note that the same *tat* siRNA delivered by pre-miRNA is ~80% more effective than when delivered as conventional shRNA. Thus, the use of the pre-miRNA backbone may increase the effectiveness of a moderately effective siRNA species.

Finally, a potentially important attribute of miRNAs is that a significant fraction of them occur naturally in clusters containing multiple miRNAs expressed coordinately from a single precursor RNA and processed into individual miRNAs (3,5). Thus, it may be possible to simultaneously express a series of miRNA/shRNAs targeting different regions of the viral genome, polycistronically transcribed by a single pol III promoter. Such a strategy may yield more potent inhibition of viral replication and may be a basis of effective gene therapy for HIV-1 infection.

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