Potent and Specific Inhibition of Retrovirus Production by Coexpression of Multiple siRNAs Directed Against Different Regions of Viral Genomes

Kaiming Ye*,† and Sha Jin‡

Biomedical Engineering Program, College of Engineering, University of Arkansas, Fayetteville, Arkansas 72701, and Department of Molecular Genetics & Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

siRNA-mediated RNA degradation has been demonstrated to act as an antiviral system in many species. Here we describe inhibition of retrovirus production by multiple siRNAs designed to target various regions of the viral genomes. Using murine leukemia virus (MuLV) as a model, we demonstrate that the virus production can be inhibited by 77% in siLTR2 (a siRNA targeting the U3 region of MuLV) expression vector transfected cells. Coexpression of siLTR2 with siPsi2 (a siRNA targeting the 3′ Psi (packaging signal sequence) results in 93% suppression of the virus production, suggesting that an increased inhibition of the virus production can be achieved by coexpression of multiple siRNAs to target different regions of the viral RNA simultaneously. Our results also indicate that not all sequences of the viral RNA are equally accessible to siRNA. We show that U3 region of MuLV is more accessible to siRNA, whereas the packaging signal sequence, especially the region adjacent to 5′LTR, is less accessible to siRNA, partly as a result of the binding of Gag precursors. Furthermore, we demonstrate that coexpression of siLTR2 with siPsi2 in virus producer cells leads to 88% knockdown of viral titer, showing the benefit of coexpression of multiple siRNAs for potent suppression of virus production in the setting of an established infection. Moreover, we demonstrate that infection of MuLV in cells that stably coexpress siLTR2 with siPsi2 diminishes by 77%. Taken together, we establish that siRNA-mediated gene silencing can suppress multiple steps of the retrovirus life cycle, offering a potential for both treating virus-associated diseases and preventing viral infection.

Introduction

Until recently, vaccines and drugs, which target to specific viral enzymes or other viral proteins, were the major two ways to treat virus-associated diseases. With the discovery of RNA interference (RNAi) (*20*), we now have another approach to combat viral infection by using either synthetic siRNA duplexes (*22, 25, 35*) or plasmid-derived hairpin siRNA (*23, 33*) to attack viral genomes and the mRNA molecules that they encode.

siRNAs are 21- to 23-base pair double-stranded RNA molecules with a characteristic 2-nucleotide overhanging 3′ ends (*18, 21*). They act as intermediates in the RNAi pathway, triggering the specific degradation of homologous RNAs only within the region of identity with the siRNA (*34, 41, 48*). siRNA-mediated RNA degradation has been demonstrated to function as an antiviral system in plants, where it represents a potent form of sequence-specific immunity (*24*). siRNA-mediated RNA degradation has also been discovered in vertebrate cells (*19, 21, 36*). These findings raised a possibility to use siRNA to activate an intracellular immune defense mechanism against viruses (*3, 30*). Compared to vaccines and drugs, siRNA has no significant side effects because of its high sequence-specific RNA degradation mechanism. In vivo, siRNAs are associated with a number of proteins, forming siRNA-protein complexes. These complexes recognize their mRNA targets by matching RNA sequences and subsequently program degradation of RNAs. If there is even one nucleotide that differs between a siRNA and its target, the suppression effect will be greatly diminished (*12, 37*). The gene silencing function of a siRNA is, therefore, highly sequence-specific. This feature greatly improves the safety of using siRNA for in vivo administration, although caution needs to be taken to avoid suppression of other mRNAs' expression with which they share some identical sequence. Besides, siRNA could serve as antiviral reagents, providing long-term treatment to inhibit virus production.

A number of hairpin siRNA expression vectors have been developed and demonstrated to be useful for suppression of replication of viruses including HIV (*23*), hepatitis B virus (HBV) (*33*), hepatitis C virus (*27*), respiratory syncytial virus (RSV) (*45*), and parainfluenza virus (PIV) (*8*). A variety of retroviral and lentiviral siRNA expression vectors have been developed as well, providing a possibility for use of siRNA in gene therapy (*1, 2, 4, 10, 39, 40, 43*). Plasmid-derived siRNA gene silencing has been well demonstrated in primary cells (*4, 42*), adult mice (*32*), transgenic mice (*40*), and embryonic stem cells (*28*). Inducible siRNA expression vectors have also been explored, offering a way to conditionally knockdown target genes by RNAi (*44*).

A number of studies suggest that siRNA can be a good option for the treatment of viral infectious diseases such as AIDS. The ultimate goal of this study is to develop anti-HIV siRNA to fight against AIDS. Nevertheless, HIV is a highly infectious virus and requires a more stringent biosafety facility, which is not always available for most laboratories. MuLV and HIV share most of the features that are common to all of retroviruses, and

* To whom correspondence should be addressed. Ph: 479-575-5315. Fax: 479-575-2846. Email: kye@uark.edu

† University of Arkansas.

‡ University of Pittsburgh.

10.1021/bp050133u CCC: $33.50 © 2006 American Chemical Society and American Institute of Chemical Engineers
Published on Web 09/03/2005

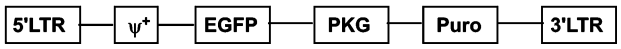


Figure 1. Schematic diagram of retrovirus vector RV_HSCR. LTR, long terminal repeat; ψ^+, packaging signal sequence; EGFP, enhanced green fluorescent protein; PKG, PKG promoter; Puro, puromycin resistance gene.

most important, MuLV can be safely studied in most laboratories. To this end, we selected MuLV as a model to determine if we could effectively inhibit or reduce the virus replication by targeting multiple regions of the viral RNA simultaneously using multiple siRNAs. The experimental results derived from this study could provide some leads to the development of anti-HIV siRNA expression vectors.

Materials and Methods

Vector Construction. Retrovirus vector RV_HSCR as illustrated in Figure 1 was derived from Moloney murine leukemia virus (MoMuLV). EGFP (enhanced green fluorescence protein) was subcloned immediately downstream of the packaging signal sequence, serving as a reporter for the transcription of the viral genome from the 5′ LTR.

We used the human U6 (hU6) promoter to drive the efficient transcription of hairpin siRNAs (*39*) in cells. All of the hairpin siRNAs adopted in this study featured a TTCAAGAGA loop situated between sense and antisense targeting sequences and a TTTTTT RNA poly III termination signal at the 3′ end. A single G was placed at position 1 of all of the sense targeting sequences, as required for enhancing the efficient transcription initiation from the hU6 promoter (*38*). siLTR1 contains the sense targeting sequence of cagagagacagcagaatat corresponding to the 118−136 nt positions of the 5′LTR region of RV_HSCR, whereas siLTR2 targets the sequence of ctaaccaatcagttcgctt to the 286−304 nt positions of 5′LTR of RV_HSCR. siPsi1 contains the sequence of tgtttgcgcctgcgtctgt to the 127−145 nt of the packaging signal sequence of RV_HSCR. siPsi2 has the sequence of ccagtcggtagatgtcaag corresponding to the 541−561 nt positions of the packaging signal sequence of RV_HSCR. Two complementary DNA oligos for forming hairpin structure of siLTR1 are 5′ GGATCCCGCAGAGAGACAGCAGAATATTTCAAGAGAATATTCTGCTGTCTCTCTGTTTTTTGGAAA 3′ (forward) and 5′ AAGCTTTTCCAAAAAACAGAGAGACAGCAGAATATTCTCTTGAAATATTCTGCTGTCTCTCTGCGG 3′ (reverse). Oligos for constituting siLTR2 are 5′ GGATCCCGCTAACCAATCAGTTCGCTTTTCAAGAGAAAGCGAACTGATTGGTTAGTTTTTTGGAAA 3′ (forward) and AAGCTTTTCCAAAAAACTAACCAATCAGTTCGCTTTCTCTTGAAAAGVGAACTGATTGGTTAGCGG 3′ (reverse). Oligos for forming siPsi1 are GGATCCCGTGTTTGCGCCTGCGTCTGTTTCAAGAGAACAGACGCAGGCGCAAACATTTTTTGGAAA 3′ (forward) and 5′ AAGCTTTTCCAAAAAATGTTTGCGCCTGCGTCTGTTCTCTTGAAACAGACGCAGGCGCAAACACGG 3′ (reverse). Oligos for constituting siPsi2 are GGATCCCGCCAGTCGGTAGATGTCAAGTTCAAGAGACTTGACATCTACCGACTGGTTTTTTGGAAA 3′ (forward) and 5′ AAGCTTTTCCAAAAAACCAGTCGGTAGATGTCAAGTCTCTTGAACTTGACATCTACCGACTGGCGG 3′ (reverse). Underlined nucleotides were added to produce two cohesive sites of *BamH* I and *Hind* III at the 5′ and 3′ ends of the annealed DNA fragments. To construct the hairpin siRNA expression cassette, two complementary DNA oligos (see above) were chemically synthesized, annealed, gel purified, and inserted between *BamH* I and *Hind* III sites immediately downstream of the hU6 promoter in the p*Silencer* 2.1-U6 hygro (Ambion, Austin, TX). All constructs were verified with DNA sequencing analysis. Endotoxin-free DNA vectors were prepared from *E. coli* DH5α using an Endofree plasmid Maxi kit from Qiagen (Valencia, CA) as previously described (*46*).

Cells. GP293 is a packaging cell line producing MuLV *gag-pol* as described previously (*46*, *47*). The human embryonic kidney cells 293T (ATCC CRL-11269, Manassas, VA) and GP293 were propagated in the DMEM (Fisher Scientifics, Pittsburgh, PA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ATCC) and 2 mM L-glutamine at 37 °C in a 5% CO_2 incubator. Phoenix_a (ATCC) is a packaging cell line expressing amphotropic *env* and MuLV *gag-pol*, and Phoenix_e (ATCC) is a packaging cell line expressing ecotropic *env* and MuLV *gag-pol* as reported elsewhere (*47*). Both cell lines were maintained in the DMEM supplemented with 10% FBS and 2 mM L-glutamine. NIH 3T3 (ATCC CRL-1658) cells were grown in the DMEM complemented with 10% bovine calf serum (BCS) and 4 mM L-glutamine.

Transient and Stable Transfection. Except where otherwise noted, we performed all of the transfections in six-well plates in which $\sim 6 \times 10^5$ cells/well were seeded in 2 mL of the DMEM supplemented with 10% heat-inactivated FBS 1 day prior to the transfection. siRNA expression vectors were transiently transfected into the cells with the PolyFect transfection reagent according to the instruction provided by the manufacture (Qiagen, Valencia, CA). For stable transfection, we plated 2.5×10^6 of cells in a 60-mm dish 1 day prior to the transfection and exchanged the medium 12 h post-transfection. We added 90 μg/mL of hygromycin to the medium 36 h post-transfection for the selection. Colonies formed after cultivation of the cells in the selection medium for 2 weeks were selected for establishing siRNA-expressing cell lines. To obtain a virus producer cell line, we transfected the virus vector RV_HSCR into the Phoenix_e cells as described above. The supernatants were collected 48 h post-transfection and used to infect Phoenix_a cells in the presence of 4 μg/mL of Polybrene. The cells expressing high levels of EGFP were sorted by FACS (fluorescence activated cell sorting) and expanded to establish a virus-producer cell line, RV_HSCR-c.

Virus Production and Infection. Retroviruses were produced by transfecting the virus vector RV_HSCR into the Phoenix_a as described above. The virus-containing supernatants were collected 48 h post-transfection, filtrated through a 0.45 μm cellulose membrane filter (Sigma, St. Louis, MO), and stored at −70 °C until use. To infect NIH 3T3 cells, we plated 1×10^5 cells per well in a six-well plate 1 day prior to the infection. The medium was removed from each well just before the infection, and 0.5 mL of virus cocktails (10-fold serially diluted) was added to each well to infect the cells in the presence of 8 μg/mL of Polybrene (Sigma). The mixtures were incubated for 2 h at 37 °C in a 5% CO_2 incubator, an additional 1.5 mL of fresh medium was added, and the cultures were incubated for a further 18 h, followed by the exchange of the media with fresh media. The cells were trypsinized and applied to flow cytometric analysis 48 h post-infection. The virus titer was determined as follows: titer (cfu/mL) = $(N \cdot P \cdot R)/v$, where v is the volume of the virus cocktails used for the infection (mL), N is the number of the cells in each well, P is the percentage of the EGFP positive cells, and R is the dilution rate. The cell numbers were determined with a hemacytometer (Sigma). The virus titers were also determined by a traditional titration approach as described elsewhere (*46*).

To infect 293T cells, we seeded 4×10^5 cells per well in six-well plates 1 day prior to infection. Cells were infected at a multiplicity of infection (MOI) of 1.0 in the presence of 4 μg/mL of Polybrene for 2 h at 37 °C in a 5% CO_2 incubator. The medium was then exchanged with fresh medium. We detected the expression of the EGFP in the virus-transduced cells with flow cytometry, and all of the experiments were conducted in triplicates.

RT-PCR. mRNA was extracted from 1.5×10^5 cells using a mRNA capture kit (Pierce, Rockford, IL). Sense primer EGFP1: 5′ GTGAGCAAGGGCGAGGAGCT 3′ and anti-sense primer EGFP1R: 5′ TTATCTGATACCGGTGGATC 3′ were used to detect EGFP mRNA in RT-PCR. The housekeeping gene β-actin served as a control for quantitative analysis of the expression level of the EGFP mRNA. The primers used for probing β-actin mRNA were 5′ ATGGATGATGATATCGC-CGC 3′ (sense primer) and 5′ GAGTCCATCACGATGCCAGT 3′ (anti-sense primer). A Kodak 1 D image analysis system (Kodak, Tokyo, Japan) was utilized to semiquantify the expression level of the EGFP mRNA.

Flow Cytometry. Cells were detached from six-well plates with trypsin-EDTA (0.25% trypsin and 0.1% EDTA) (Mediatech, Herndon, VA) and washed once with cold PBS buffer containing 1% FBS. Next, 1×10^5 cells were applied to FACScan (Becton Dickinson, San Carlos, CA) to analyze the expression of the EGFP. The data analysis was performed with CELLQUEST Pro (Becton Dickinson, San Carlos, CA) software.

Fluorescence Microscopy. Cells were examined under an inverted fluorescence microscope (Olympus IX-70). A narrow band of EGFP filter set (exciter D480/20; emittor D520/20; Chroma, Brattleboro, VT) was used to detect the expression of the EGFP in the cells.

Results

Suppression of MuLV Production by Hairpin siRNAs against the Viral RNAs. Retroviral replication is a very complicated process, involving viral entry, reverse transcription of the viral genome, integration of the provirus into the chromosomal DNA of the host cells, transcription of the viral genome and the viral proteins, assembling of the virion proteins and the progeny RNA, budding of the progeny virus, and subsequent maturation of the progeny virus into infectious virus. Failure of any of these steps will lead to defectiveness in replication. In this experiment, we investigated the feasibility of decomposing the MuLV RNA by siRNAs. We have designed a variety of hairpin siRNAs to target different regions of the viral RNA as described below.

siLTR1 was designed to target the 5′ U3 region, the sequences containing promoter and enhancer elements, whereas siLTR2 matched the 3′ U3 region. Both siRNAs were transcribed from the hU6 promoter and formed short hairpin structures after the transcription. siRNA expression vectors were cotransfected into Phoenix_a cells with the virus vectors, RV_HSCR. The viruses were collected at 48 h post-transfection, and the infectivity of the viruses was determined by titering the viruses with NIH 3T3 cells. We collected the viruses 48 h post-transfection, as we found that the titer of the viruses reached maximum approximately 48 h post-transfection as shown in Figure 2. We used the siRNA−, whose sequence has no significant homology to mouse, human, and rat gene sequences including MuLV genomes, as a negative control for the experiments. The expression level of the EGFP in cotransfected cells was determined using an inverted fluorescence microscope 48 h post-transfection. We observed a significant decrease in the expression of the EGFP in the siLTR1- and siLTR2-transfected cells, as shown in Figure 3b and c, as compared with the expression level of the EGFP in the negative control siRNA-transfected cells (Figure 3a). Flow cytometric analysis revealed that the expression of the EGFP dropped by 70% in the siLTR1-transfected cells and 74% in the siLTR2-transfected cells (Figure 3d−g). The results demonstrated that the production of the retrovirus could be significantly inhibited by siRNAs directed against the U3 regions of the MuLV. Moreover, siLTR2 was capable of suppressing the virus production even more effectively, suggesting that the selection of the siRNA targeting sequence affects the activity of siRNA remarkably.

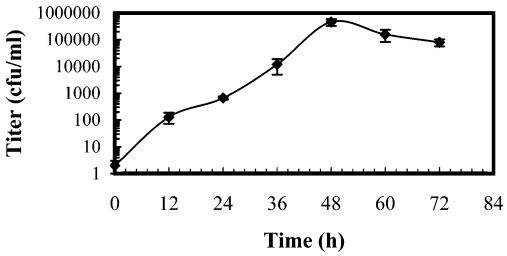


Figure 2. Time course of the virus production. The experiments were carried out in triplicate, and error bars indicate the standard deviations.

Potent Suppression of MuLV Production by Coexpression of Multiple Hairpin siRNAs Targeting Different Regions of the Viral RNA Simultaneously. Having established the inhibitory system of the virus production using siRNA, we next sought to assess the effect of the siRNA target region on the activity of siRNA. We examined a panel of siRNA or the combination of siRNAs as presented in Figure 4. In this experiment, the virus vector RV_HSCR was cotransfected into Phoenix_a cells with siRNA expression vectors. The mean fluorescence intensity was measured 48 h post-transfection as described in Material and Methods. siPsi1 was designed to match the 3′ Psi (packaging signal sequence) of MuLV, whereas siPsi2 targeted the 5′ Psi. As detected by flow cytometric analysis, the expression level of the EGFP reduced 8% in the siPsi1-transfected cells and 24% in the siPsi2-transfected cells. However, siLTR1 and siLTR2 inhibited virus production up to 72% by average. These experiments suggest poor accessibility of the siRNAs to the packaging signal region.

One of the issues relating to the suppression of retrovirus production by RNAi is the mutation of target sequences through retrovirus' error-prone reverse transcriptase, leading to escape of the virus from siRNAs (*9*, *17*). To counteract this weakness, coexpression of multiple siRNAs, which target to either conserved sequences or different regions of viral RNA, could reduce the emergence of single siRNA-resistant virus and achieve a comparable suppression of virus production. To investigate the inhibition of MuLV replication by coexpression of multiple siRNAs, we cotransfected RV_HSCR into Phoenix_a cells with a combination of two siRNAs expression vectors and determined the fluorescence intensity of the EGFP 48 h post-transfection. As illustrated in Figure 4, the expression of the EGFP declined 56% in the siPsi1 and siPsi2 cotransfected cells and 83% in the siPsi1 and siLTR2 cotransfected cells. The expression of the EGFP dropped further by 90% in the siPsi2 and siLTR2 cotransfected cells. The results suggested that a significant inhibition of virus production could be reached by coexpressing multiple hairpin siRNAs to target different regions of the viral RNA simultaneously.

Effect of the Viral Protein Binding Site on the Activity of siRNA. As retroviruses are assembled in cytoplasm, their

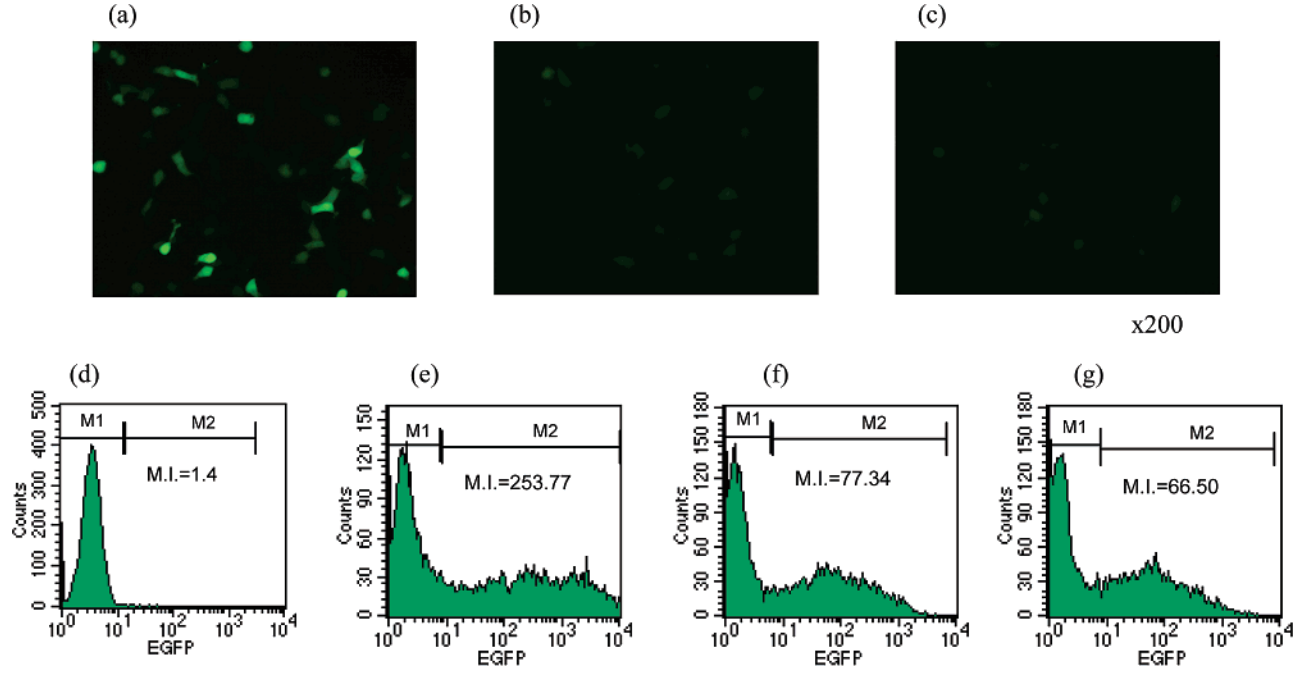


Figure 3. Suppression of MuLV replication by siRNA targeting the viral LTR. Viral vector pRV_HSCR was cotransfected into Phoenix_a cells with siRNA expression vectors: (a and e) siRNA−; (b and f) siLTR1; (c and g) siLTR2. (d) untransfected Phoenix_a cells. The expression of EGFP from the 5′LTR was determined by flow cytometry and fluorescence microscopy 48 h post-cotransfection. M1, EGFP negative region; M2, EGFP positive region; M.I., mean fluorescence intensity.

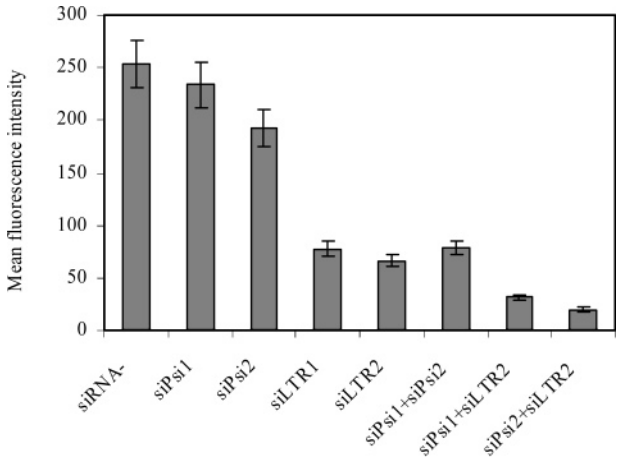


Figure 4. The activity of siRNAs in Phoenix_a cells. Viral vector pRV_HSCR was cotransfected into Phoenix_a cells with the indicated siRNA expression vectors. The expression of the EGFP from the 5′ LTR was measured 48 h post-transfectionas. The results represent the average of three independent experiments, with the standard deviations indicated as error bars.

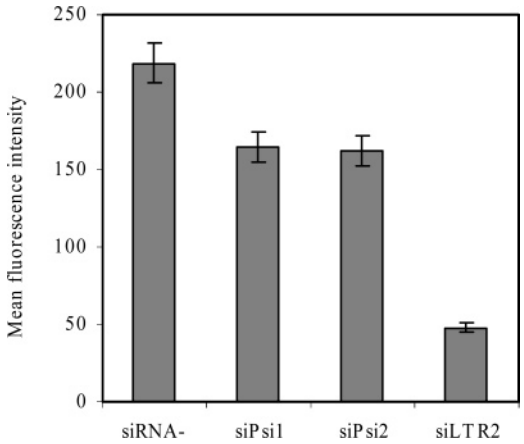


Figure 5. The activity of siRNAs in 293T cells. Viral vector pRV_HSCR was cotransfected to 293T cells with the indicated siRNA expression vectors. The expression of EGFP from the 5′ LTR was measured 48 h post-transfection. Data represent the averages of at least two independent experiments with treatments performed in triplicate wells with standard deviations indicated.

genomes are selected from a cytosolic pool that contains a substantial excess of cellular RNAs (*5*). This specificity is mediated predominantly by interactions between the nucleocapsid (NC) domains of the assembling Gag polyproteins and the packaging signal sequence (*6, 7, 15, 16, 29*). These interactions could affect the recognition or the accessibility of siRNA to these sequences and thus diminish the activity of siRNAs to suppress retrovirus production. To verify this hypothesis, we cotransfected 293T cells with RV_HSCR and siRNA expression vectors. Since the sequences encoding the *gag-pol* genes were deleted from the viral genome of RV_HSCR, no Gag precursors were synthesized in the transfected cells and thus no binding of Gag precursors to the packaging signal sequence would occur. As shown in Figure 5, the expression level of the EGFP from the 5′ LTR dropped by 25% in the siPsi1-transfected cells and 26% in the siPsi2-transfected cells. The suppression of the EGFP expression by these two siRNAs was almost at the same level, suggesting that the binding of the Gag precursors to the packaging signal region impaired RNAi by sheltering the sequence from being accessed to siRNAs. By contrast, the absence of Gag precursors did not affect the activity of siLTR2 in 293T cells. The expression level of the EGFP in the siLTR2-transfected 293T cells declined 78%, which was almost at the same level as we observed in the siLTR2 and RV_HSCR cotransfected Phoenix_a cells. These experiments suggest that proteins bound to the viral RNA might affect the accessibility of the sequence to siRNAs.

Inhibitory Effect of siRNA on the Virus Infectivity. To further examine the inhibitory effect of siRNA on the infectivity of progeny virus, we cotransfected virus vector RV_HSCR and siRNA expression vector, or triple transfected virus vector and the combination of siRNA expression plasmid DNAs into

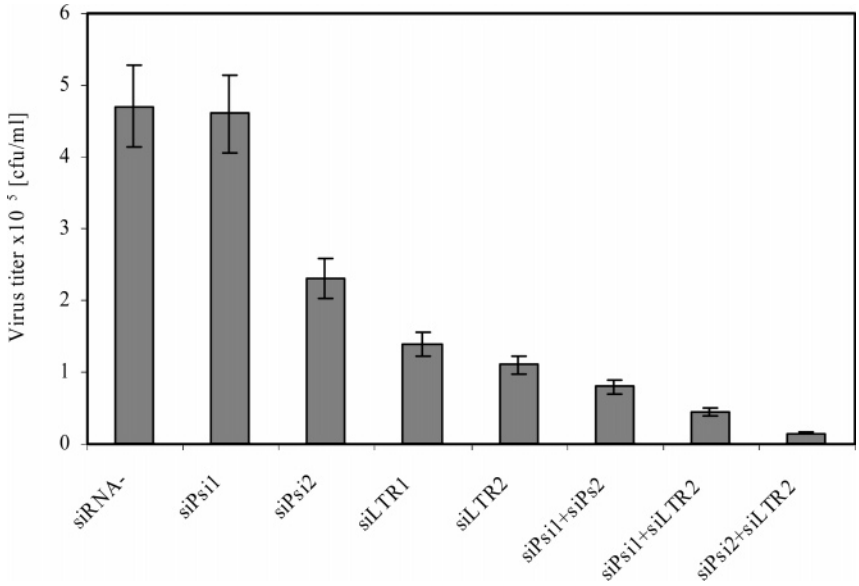


Figure 6. Titer of the viruses produced from siRNAs-transfected cells. The pRV_HSCR vector was cotransfected into Phoenix_a cells with the indicated siRNA expression plasmid. The viral supernatants were collected 48 h post-transfection and titrated with NIH 3T3 cells.

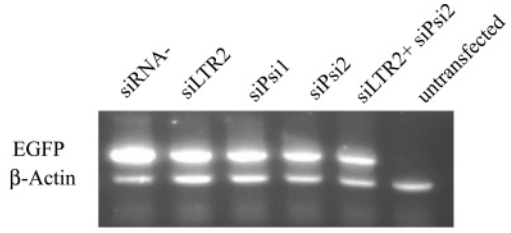
Phonex_a cells. The virus supernatants were collected and clarified 48 h post-transfection and used to infect NIH 3T3 cells to determine viral titer with antibiotic selection as described in Material and Methods. As presented in Figure 6, the titer of the viruses produced from siRNA-transfected cells was $4.7 \pm 0.8 \times 10^5$ cfu/mL. The titer of the viruses produced from the siPsi1-transfected cells was almost the same as that from a negative control experiment. However, the infectivity of MuLV decreased by 51% in the siPsi2-transfected cells, whereas the titers were $1.4 \pm 0.6 \times 10^5$ cfu/mL from the siLTR1-transfected cells and $1.1 \pm 0.3 \times 10^5$ cfu/mL from the siLTR2-transfected cells, resulting in 70% and 77% decrease of viral infectivity, respectively. Importantly, the combination of siRNAs leads to dramatic suppression of virus infectivity. The titer was approximately $0.8 \pm 0.07 \times 10^5$ cfu/mL, in the combination of siPsi1- and siPsi2-transfected cells. Moreover, the titer was $0.44 \pm 0.05 \times 10^5$ cfu/mL in the combination of siPsi1- and siLTR2-transfected cells. The viral titer was only about $1.5 \pm 0.2 \times 10^4$ cfu/mL from the viral supernatant produced from the siLTR2- and siPsi2-transfected cells. This was a 93% decline in the MuLV replication. These experimental results revealed that the expression level of the EGFP correlates to the viral titers. Thus, the expression level of the EGFP can be used as an indicator of virus production and a relative index of viral titer.

Effects of siRNA on Viral mRNA Expression and Virus Production after Viral Integration. RNAi could be adapted to treat a number of virus-associated diseases by reducing or completely blocking virus production in virus-infected cells by long-term expression of siRNAs. To assess this potential clinical benefit of siRNA, we established a virus producer cell line, RV_HSCR-c, which produced MuLV from integrated provirus. We transfected siRNA expression vectors into RV_HSCR-c cells and monitored both the expression level of the EGFP mRNA and the production of the viruses to determine the effect of siRNA on the virus replication after viral integration. EGFP has an estimated half-life of >24 h in vivo (*50*). It appears to be stable when expressed in mammalian cells. Nevertheless, mRNA level reflects the dynamics of transcription of a gene. Hence, we monitored the expression level of the EGFP mRNA rather than the fluorescence intensity of the EGFP in order to determine the suppression of the virus replication by siRNA in RV_HCSR-c cells. A housekeeping gene, β-actin, served as a reference for normalization of the measurement of the EGFP mRNA expression level. As shown in Figure 7, the EGFP mRNA expression level reduced by 56.2% in the siLTR2-transfected RV_HSCR-c cells, indicating the potent suppression of steady-state virus production by siRNA even in the setting of an established infection. The replication of MuLV was inhibited 57% in siLTR2-transfected RV_HSCR-c cells. Expression of siPsi1 and siPsi2 in viral producer cell line resulted in 25.4% and 38.6% inhibition of the expression level of EGFP mRNA, respectively. There was an 82.9% reduction of the expression level of EGFP mRNA in siLTR2 and siPsi2 cotransfected RV_HSCR-c cells. In addition, we observed an 88% decrease of the viral titer when coexpressing siLTR2 and siPsi2 in RV_HSCR-c cells. This remarkable decrease of viral titer suggests the benefit of coexpression of multiple siRNAs for potent knockdown of the virus production after viral integration.

Effect of the Expression of Multiple siRNAs on Antiviral Activity. To extend our experimental results and to assess the resistance of siRNAs to viral infection, we next investigated the feasibility of siRNA to inhibit virus production in 293T cells. We established three siRNA-expressing cell lines by stable transfection of siRNAs into 293T cells as described in Material and Methods. siRNA−_e is a cell line that stably expresses siRNA−, and siLTR2_e is a cell line expressing siLTR2. siLTR2-siPsi2_e is a cell line expressing both siLTR2 and siPsi2 simultaneously. We infected these cells with RV_HSCR virus and measured the expression of EGFP 24 h post-infection by flow cytometry (Figure 8). The mean fluorescence intensity of EGFP in cells expressing siRNA− was 150.47 ± 18.22, whereas it dropped to 88.54 ± 10.79 in the cells expressing siLTR2. It decreased further in the siLTR2 and siPsi2 coexpressed cells. The mean fluorescence intensity of the EGFP in the cells expressing both siLTR2 and siPsi2 declined up to 77%. The decrease in the virus production in the siRNA transduced cells indicated the antiviral activity of siRNA.

Discussion

To suppress the replication of a retrovirus one can inhibit the expression of cellular regulatory proteins, viral proteins, or viral genomes with siRNA. However, suppressing the expression



	siRNA-	siLTR2	siPsi1	siPsi2	siLTR2+siPsi2
Normalized EGFP mRNA expression level	8.78	3.85	6.55	5.39	1.50
Virus Titer x10^5 (cfu/ml)	3.5±1.2	1.5±0.6	2.8±1.0	2.0±0.9	0.43±0.04

Figure 7. RT-PCR assay of the suppression of MuLV replication by siRNA after viral integration siRNA expression vectors were transfected into RV_HSCR-c cells. mRNAs were extracted 48 h post-transfection and detected by RT-PCR assay. *β*-Actin served as a housekeeping gene for normalization of the expression level of EGFP mRNA. The viral supernatants were titrated with NIH 3T3 cells.

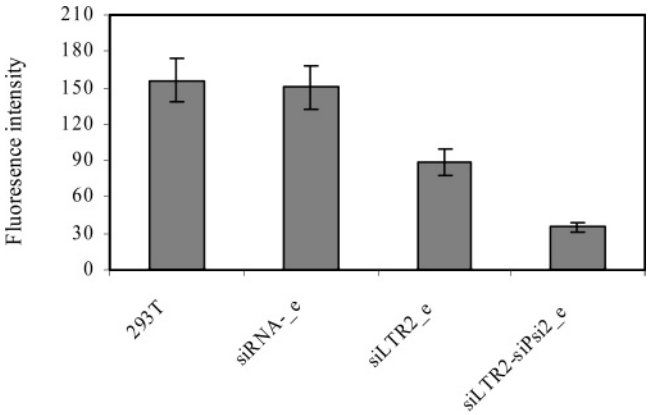


Figure 8. Inhibition of the viral infection in the cells expressing siRNA. 293T and siRNA-expressing cells were infected with RV_HSCR (MOI = 1.0), and the expression of EGFP was determined 24 h post-infection by flow cytometry. Cell lines: 293T, a control cell line; siRNA-_e, a cell line expressing siRNA−; siLTR2_e, a cell line expressing siLTR2; siLTR2+siPsi2_e, a cell line coexpressing siLTR2 and siPsi2.

of cellular regulatory proteins could lead to loss of biological function of the cells. For example, a number of studies demonstrated that HIV infection could be effectively inhibited by suppressing the expression of CD4 (the principle receptor for HIV) and/or CCR5/CXCR4 (the co-receptor for HIV) in T cells and microphages (*31, 35, 39*). CD4 is a cell surface protein involved in immune recognition. Suppressing the expression of CD4 could eventually deplete helper T cells, rendering the human body susceptible to infection by microbes (*14*). The suppression of CCR5/CXCR4 by siRNA has also been studied, with very promising results in terms of inhibiting the HIV infection (*31*). Meanwhile, the silencing of genes encoding viral proteins by RNAi has been extensively investigated. For example, a number of siRNA structures have been designed to block the expression of Tat and/or Rev, leading to potent inhibition of HIV replication (*13*). Gag, as well as Pol, has been chosen as a target for siRNA-mediated RNA degradation (*11, 22*).

In this study, we establish that viral RNA itself can be a good target for RNAi. There are two transcripts including unspliced and spliced transcripts expressed from integrated MuLV provirus. The full-length transcript serves as both the mRNA for the *gag-pol* genes and the genomic RNA of progeny virus. Targeting the full-length transcript with siRNA can reduce not only the expression of *gag-pol* genes but also the number of viral RNAs packaged into virions, leading to a significant suppression of the virus production. However, not all viral RNA sequences are equally accessible to siRNA. By targeting different portions of the sequence in the packaging signal region of MuLV with siPsi1 and siPsi2, respectively, we demonstrated that the packaging signal sequence, especially the region adjacent to 5′LTR, is less accessible to siRNA because of the binding of NC domains of Gag proteins onto this portion of the sequence. We found that the inhibition of the virus production in the siPsi1-transfected 293T cells was almost at the same level as that in the siPsi2-transfected cells, whereas it differed considerably in the siPsi1- and siPsi2-transfected Phoenix_a cells that expressed Gag polyproteins. This suggests that the binding of Gag polyproteins to the packaging signal sequence impairs remarkably the access of the sequence to siRNA, thereby hampering the RNAi. It has been also observed that the viral genomes packaged in early replication complexes are not sufficient substrates for RNAi (*22*). Accordingly, the optimal sequence selected for siRNA targeting should not be the portion of viral RNA containing protein-binding sequences. For example, U3 region is a good target for RNAi. Compared to the packaging signal sequence, no protein binds to the LTR region of MuLV in the cytoplasm. As we observed in this study, both siLTR1 and siLTR2 provide high levels of inhibition of the virus production.

Local characteristics of the target RNA, including local RNA folding, could be another factor affecting the effectiveness of the activity of siRNAs. siRNA is regarded to be highly sequence-specific; the recognition between siRNA and its target sequence occurs between the siRNA double strand and the target or after a possible proceeding dissociation of the siRNA with its antisense strand. This may explain the difference in the activity of siRNA between siLTR1 and siLTR2. Although a computational program has been developed to predict the accessibility of target RNA to siRNA (*26*), the selection of optimal siRNAs for silencing target genes still heavily relies on the trial-and-error approach.

Another issue affecting the siRNA-mediated gene silencing for suppression of virus production is that viruses often produce mutated progeny molecules, preventing the viruses from being recognized by siRNA (*12*). One approach to overcome this obstacle is to target several viral RNA regions simultaneously with multiple siRNAs. Our results establish that combinations of multiple siRNA can induce potent retroviral gene silencing. By targeting both the LTR and the packaging signal sequences with coexpression of siLTR2 and siPsi2, we show that MuLV replication can be inhibited up to 93%. Nevertheless, the suppression of MuLV replication by expression of siLTR2 was 78%. These results serve as a proof-of-principle that coexpression of multiple siRNA can be used to effectively inhibit retroviral production. It may be reasonably expected that the virus production could be completely blocked by coexpression of a number of siRNAs directed against different regions of viral genomes.

By investigating the suppression of MuLV replication by expression of siRNA in virus producer cells, we demonstrated that siRNA effectively suppresses the virus production after viral integration. This is of particular interest as it raises a possibility to diminish virus production by RNAi in individuals infected by virus. Viral RNAs are transcripted from integrated provirus in the nucleus and transported into the cytoplasm, where they are packaged with Gag polyproteins, forming progeny viruses. Unlike a lentivirus such as HIV-1, the transporting of MuLV RNA is relatively simple and does not require interaction with

any viral proteins. Considering the factor that the siRNA-mediated RNA degradation only affects cytoplasmic mRNAs (*49*), targeting the viral RNA directly in cytoplasm with siRNA can be more potent and specific for inhibition of the virus production.

Moreover, we demonstrate that siRNA can provide protection against viral infection. We found a 77% inhibition of viral infection in the cells coexpressing siLTR2 and siPsi2, which is consistent with the works reported by Capodici et al. (*11*). The inhibition of viral infection could be interpreted by the suppression of early and late stages of reverse transcription by RNAi (*11*), which reduces the copies of provirus in the chromosomal DNA. Likewise, siRNA also down-regulates the virus production after viral integration. Consequently, siRNA can provide a double assurance against viral infection. Taken together, we establish that siRNA can be used to suppress multiple steps of the retrovirus life cycle, raising a great potential for treating virus-associated diseases, as well as for preventing viral infection by RNAi.

However, the development of siRNA-based antiviral therapy can be quite challenging, as only the cells that carry antivirus siRNA expression vectors will have capabilities of inhibiting the virus replications or preventing the cells from virus infection. To ensure that most of the cells in the blood bear antivirus siRNAs, bone marrow transplantation could be a good option, especially for patients who are at a later stage of HIV infection. Antivirus siRNA can be introduced into hemotopoietic stem cells (HSC) and then transplanted into patients. The differentiation of HSC after bone marrow transplantation will endow all of the HSC-derived cells with a capability of inhibiting or preventing virus infection. This approach has been currently tested in a number of laboratories include the authors' lab.

References and Notes

(1) Abbas-Terki, T.; Blanco-Bose, W.; Deglon, N.; Pralong, W.; Aebischer, P. Lentiviral-mediated RNA interference. *Hum. Gene Ther.* **2002**, *13*, 2197−2201.

(2) An, D. S.; Xie, Y.; Mao, S. H.; Morizono, K.; Kung, S. K.; Chen, I. S. Efficient lentiviral vectors for short hairpin RNA delivery into human cells. *Hum. Gene Ther.* **2003**, *14*, 1207−1212.

(3) Andino, R. RNAi puts a lid on virus replication. *Nat. Biotechnol.* **2003**, *21*, 629−630.

(4) Barton, G. M.; Medzhitov, R. Retroviral delivery of small interfering RNA into primary cells. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14943−14945.

(5) Berkowitz, R.; Fisher, J.; Goff, S. P. RNA packaging. *Curr. Top. Microbiol. Immunol.* **1996**, *214*, 177−218.

(6) Berkowitz, R. D.; Goff, S. P. Analysis of binding elements in the human immunodeficiency virus type 1 genomic RNA and nucleocapsid protein. *Virology* **1994**, *202*, 233−246.

(7) Berkowitz, R. D.; Luban, J.; Goff, S. P. Specific binding of human immunodeficiency virus type 1 gag polyprotein and nucleocapsid protein to viral RNAs detected by RNA mobility shift assays. *J. Virol.* **1993**, *67*, 7190−7200.

(8) Bitko, V.; Musiyenko, A.; Shulyayeva, O.; Barik, S. Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* **2005**, *11*, 50−55.

(9) Boden, D.; Pusch, O.; Lee, F.; Tucker, L.; Ramratnam, B. Human immunodeficiency virus type 1 escape from RNA interference. *J. Virol.* **2003**, *77*, 11531−11535.

(10) Brummelkamp, T. R.; Bernards, R.; Agami, R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **2002**, *296*, 550−553.

(11) Capodici, J.; Kariko, K.; Weissman, D. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J. Immunol.* **2002**, *169*, 5196−5201.

(12) Carmichael, G. G. Medicine: silencing viruses with RNA. *Nature* **2002**, *418*, 379−380.

(13) Coburn, G. A.; Cullen, B. R. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J. Virol.* **2002**, *76*, 9225−9231.

(14) Coffin, J. M.; Hugbes, S. H.; Varmus H. E. *Retroviruses*; Cold Spring Harbor Laboratory Press: New York, 1997.

(15) Dannull, J.; Surovoy, A.; Jung, G.; Moelling, K. Specific binding of HIV-1 nucleocapsid protein to PSI RNA in vitro requires N-terminal zinc finger and flanking basic amino acid residues. *Embo J.* **1994**, *13*, 1525−1533.

(16) Darlix, J. L.; Gabus, C.; Nugeyre, M. T.; Clavel, F.; Barre-Sinoussi, F. Cis elements and trans-acting factors involved in the RNA dimerization of the human immunodeficiency virus HIV-1. *J. Mol. Biol.* **1990**, *216*, 689−699.

(17) Das, A. T.; Brummelkamp, T. R.; Westerhout, E. M.; Vink, M.; Madiredjo, M.; Bernards, R.; Berkhout, B. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J. Virol.* **2004**, *78*, 2601−2605.

(18) Elbashir, S. M.; Lendeckel, W.; Tuschl, T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **2001**, *15*, 188−200.

(19) Fire, A. RNA-triggered gene silencing. *Trends Genet.* **1999**, *15*, 358−363.

(20) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **1998**, *391*, 806−811.

(21) Hannon, G. J. RNA interference. *Nature* **2002**, *418*, 244−251.

(22) Hu, W. Y.; Myers, C. P.; Kilzer, J. M.; Pfaff, S. L.; Bushman, F. D. Inhibition of retroviral pathogenesis by RNA interference. *Curr. Biol.* **2002**, *12*, 1301−1311.

(23) Jacque, J. M.; Triques, K.; Stevenson, M. Modulation of HIV-1 replication by RNA interference. *Nature* **2002**, *418*, 435−438.

(24) Kasschau, K. D.; Carrington, J. C. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. *Cell* **1998**, *95*, 461−470.

(25) Kitabwalla, M.; Ruprecht, R. M. RNA interference−a new weapon against HIV and beyond. *N. Engl. J. Med.* **2002**, *347*, 1364−1367.

(26) Kretschmer-Kazemi Far, R.; Sczakiel, G. The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides. *Nucleic Acids Res.* **2003**, *31*, 4417−4424.

(27) Kronke, J.; Kittler, R.; Buchholz, F.; Windisch, M. P.; Pietschmann, T.; Bartenschlager, R.; Frese, M. Alternative approaches for efficient inhibition of hepatitis C virus RNA replication by small interfering RNAs. *J. Virol.* **2004**, *78*, 3436−3446.

(28) Kunath, T.; Gish, G.; Lickert, H.; Jones, N.; Pawson, T.; Rossant, J. Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nat. Biotechnol.* **2003**, *21*, 559−561.

(29) Luban, J.; Goff, S. P. Binding of human immunodeficiency virus type 1 (HIV-1) RNA to recombinant HIV-1 gag polyprotein. *J. Virol.* **1991**, *65*, 3203−3212.

(30) Martinez, M. A.; Clotet, B.; Este, J. A. RNA interference of HIV replication. *Trends Immunol.* **2002**, *23*, 559−561.

(31) Martinez, M. A.; Gutierrrez, A.; Armand-Ugon, M.; Blanco, J.; Parera, M.; Gomez, J.; Clotet, B.; Este, J. A. Suppresion of chemokine receptor expression by RNA inteference allows for inhibition of HIV-1 replication. *AIDS* **2002**, *16*, 2385−2390.

(32) McCaffrey, A. P.; Meuse, L.; Pham, T. T.; Conklin, D. S.; Hannon, G. J.; Kay, M. A. RNA interference in adult mice. *Nature* **2002**, *418*, 38−39.

(33) McCaffrey, A. P.; Nakai, H.; Pandey, K.; Huang, Z.; Salazar, F. H.; Xu, H.; Wieland, S. F.; Marion, P. L.; Kay, M. A. Inhibition of hepatitis B virus in mice by RNA interference. *Nat. Biotechnol.* **2003**, *21*, 639−644.

(34) McManus, M. T.; Sharp, P. A. Gene silencing in mammals by small interfering RNAs. *Nat. Rev. Genet.* **2002**, *3*, 737−747.

(35) Novina, C. D.; Murray, M. F.; Dykxhoorn, D. M.; Beresford, P. J.; Riess, J.; Lee, S. K.; Collman, R. G.; Lieberman, J.; Shankar, P.; Sharp, P. A. siRNA-directed inhibition of HIV-1 infection. *Nat. Med.* **2002**, *8*, 681−686.

(36) Paddison, P. J.; Caudy, A. A.; Bernstein, E.; Hannon, G. J.; Conklin, D. S. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* **2002**, *16*, 948−958.

(37) Parrish, S.; Fleenor, J.; Xu, S.; Mello, C.; Fire, A. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Mol. Cell* **2000**, *6*, 1077−1087.
(38) Paule, M. R.; White, R. J. Survey and summary: transciption by RNA polymerase I and III. *Nucletic Acids Res.* **2000**, *28*, 1283−1298.
(39) Qin, X. F.; An, D. S.; Chen, I. S.; Baltimore, D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 183−188.
(40) Rubinson, D. A.; Dillon, C. P.; Kwiatkowski, A. V.; Sievers, C.; Yang, L.; Kopinja, J.; Rooney, D. L.; Ihrig, M. M.; McManus, M. T.; Gertler, F. B.; Scott, M. L.; Van Parijs, L. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* **2003**, *33*, 401−406.
(41) Sharp, P. A. RNA interference− 2001. *Genes Dev.* **2001**, *15*, 485−490.
(42) Stewart, S. A.; Dykxhoorn, D. M.; Palliser, D.; Mizuno, H.; Yu, E. Y.; An, D. S.; Sabatini, D. M.; Chen, I. S.; Hahn, W. C.; Sharp, P. A.; Weinberg, R. A.; Novina, C. D. Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* **2003**, *9*, 493−501.
(43) Tiscornia, G.; Singer, O.; Ikawa, M.; Verma, I. M. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1844−1848.
(44) Wiznerowicz, M.; Trono, D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. *J. Virol.* **2003**, *77*, 8957−8961.
(45) Xu, Z.; Kuang, M.; Okicki, J. R.; Cramer, H.; Chaudhary, N. Potent inhibition of respiratory syncytial virus by combination treatment with 2−5A antisense and ribavirin. *Antiviral Res.* **2004**, *61*, 195−206.
(46) Ye, K.; Dhiman, H. K.; Suhan, J.; Schultz, J. S. Effect of pH on infectivity and morphology of ecotropic moloney murine leukemia virus. *Biotechnol. Prog.* **2003**, *19*, 538−543.
(47) Ye, K.; Jin, S.; Ataai, M. M.; Schultz, J. S.; Ibeh, J. Tagging retrovirus vectors with a metal binding peptide and one-step purification by immobilized metal affinity chromatography. *J. Virol.* **2004**, *78*, 9820−9827.
(48) Zamore, P. D.; Tuschl, T.; Sharp, P. A.; Bartel, D. P. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **2000**, *101*, 25−33.
(49) Zeng, Y.; Cullen, B. R. RNA interference in human cells is restricted to the cytoplasm. *RNA* **2002**, *8*, 855−860.
(50) Zhao, X.; Jiang, X.; Huang, C. C.; Kain, S. R.; Li, X. Generation of a destablilized form of enhanced green fluorescent protein. *Methods Enzymol.* **1999**, *302*, 438−444.

Accepted for publication August 9, 2005.

BP050133U