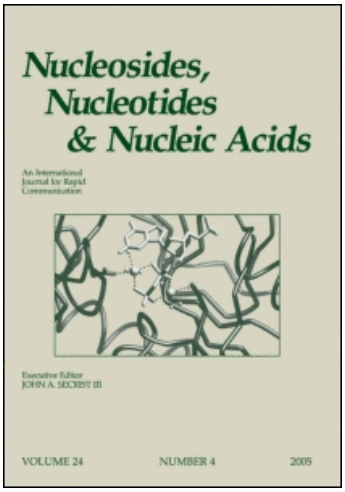

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SILENCING OF HIV-1 GENE EXPRESSION BY siRNAs IN TRANSDUCED CELLS

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□ *The RNA interference (RNAi) phenomenon is a recently observed process in which the introduction of a double-stranded RNA (dsRNA) into cells causes the specific degradation of an mRNA containing the same sequence. To study dsRNA-mediated gene interference targeted to the* env *gene (NL4-3: 7490-7508) in HIV-1 infected cells, we constructed tandem-type and hairpin-type siRNA expression vectors, which were under the control of two U6 promoters. We also constructed lentiviral-based siRNA expression vectors for further assessment of their antiviral activity in transduced cells. At both the transient plasmid and lentiviral-mediated RNA expression levels, the siRNA encoding the* env *fragment exhibited sequence-specific suppression of target gene expression and strongly inhibited (≥ 90%) HIV-1 infection in the cells, as compared to the antisense RNA expression vector. Targeting the HIV-1* env *gene with siRNAs encoding the* env *gene fragment (7490–7508) might be an effective strategy for gene therapy applications in HIV-1/AIDS treatment and management.*

Keywords RNAi; shRNA; siRNA; HIV-1 *env*; Lentiviral vector

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INTRODUCTION

RNA interference (RNAi) is a phenomenon in which double-stranded RNA (dsRNA) affects silencing of the expression of genes that are highly homologous to either of the RNA strands in the duplex. RNAi is initiated by the enzyme Dicer, a member of the RNase III family of double-stranded RNA-specific endonucleases, which promotes the processive cleavage of a long dsRNA into 21- to 23-nt long duplexes, each with a 2-nt, 3′ overhanging end.[1] These products, termed short interfering siRNAs, are then incorporated into an inactive protein complex. ATP-dependent unwinding of the double-stranded siRNA generates an active RNA-induced silencing complex (RISC), which then uses the antisense siRNA sequence to identify homologous mRNA through complementary base pairing. Degradation of the target mRNA is thought to proceed from the center of the region spanned by the guide siRNA.

We previously reported that four designed siRNA oligonucleotides containing HIV-1 *env* gene fragments (E7145, E7361, E7457, and E7490) mediated dsRNA gene interference in HIV-1 infected cells. The E7490 siRNA (NL4-3: 7490–7508) displayed ≥90% inhibitory efficacy in HIV-1 transfected cells.[2,3]

In this study, we analyzed the siRNA-mediated silencing of the *env* gene with the E7490 siRNA to establish its potential targets in the gene for HIV-1 gene therapy. For safe delivery of this gene fragment (E7490), which was chosen based on its efficient HIV-1 inhibitory potential identified in our previous study, we constructed a lentiviral-based siRNA (E7490) expression vector for further assessment of its antiviral activity in transduced MT-4 cells. Our results suggested that siRNA-mediated RNAi targeted to the HIV-1 *env* gene can be used in gene therapy for HIV/AIDS.

RESULTS AND DISCUSSION

To evaluate the gene interference mediated by siRNA (E7490) expression in HIV-1 infected cells, we constructed an siRNA (E7490) expression vector for the assessment of its antiviral activity. In order to express the siRNA targeted to HIV-1 (NL4-3: 7490–7508), we constructed a tandem-type siRNA expression vector, which was driven under the control of two U6 promoters, and an alternate hairpin-type siRNA expression vector, which was controlled by one U6 promoter (Figure 1B). For the control vector, a **G** was mutated to **C** in the sequence of the siRNA (Figure 1A). The sequence and orientation of the constructed vector inserts were confirmed by nucleotide sequence analysis.

In order to evaluate the anti-HIV-1 activity of the constructed vectors, they were cotransfected into COS cells with pNL4-3, using the FuGENE6 (Roche Applied Science, Mannheim, Germany), and cultured for three

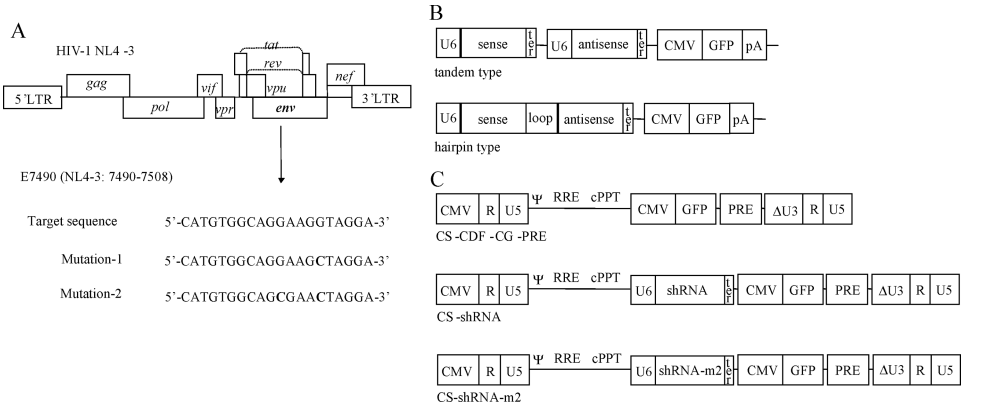

FIGURE 1 Construction of siRNA expression vectors and target sequences for siRNA design. (A) HIV-1 genomic sequence showing the target sequence in the *env* gene used for the siRNA design. (B) The sense and antisense oligos for both the tandem- and hairpin-type siRNAs were annealed at 95°C and gradually cooled down to 4°C. They were then cloned into the Kpn I site, downstream from the U6 promoter and the Xho I site behind the terminator sequence. (C) The generated U6 hairpin vectors were digested with EcoR I and Nhe I and then were cloned into the same site in the CS-CDF-CG-PRE (from Dr. Miyoshi, RIKEN, Japan).

days, and then the GFP expression was monitored by fluorescence microscopy. The GFP expression in these transfected cells was observed. The amount of p24 antigen was measured to check for HIV-1 inhibition by the CLEIA assay (Fujirebio, Tokyo, Japan). Both the tandem- and hairpin-type siRNAs mediated about 90% inhibition, as compared to the empty vector (Figure 2). RT-PCR was performed for the downregulation of viral RNAs. The reduction of HIV-1 RNA was correlation with the p24 antigen products.

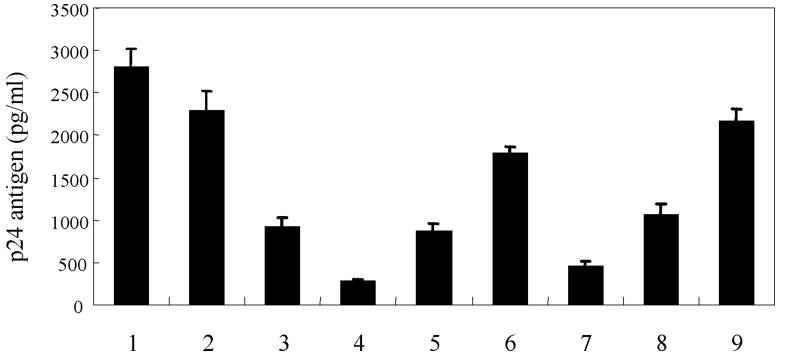

FIGURE 2 Effects of siRNA expression vector targeted against the *env* gene (NL4-3: 7490–7508). COS cells were cotransfected with the siRNA expression vector (1 μg) and pNL4-3 (0.1 μg). At 3 days post cotransfection, p24 antigen production was detected by the HIV-1 p24 CLEIA assay (each bar represents the average of 3 samples/replicate +/− standard deviations). Lane 1: U6-G, empty vector (hairpin type); lane 2: U6-sen, only sense RNA expression; lane 3: U6-ant, only antisense RNA expression; lane 4: U6-sh, hairpin-type siRNA expression; lane 5: U6-sh-m1, hairpin-type siRNA (mutation-1) expression; lane 6: U6-Sh-m2, hairpin- type siRNA (mutation-2) expression; lane 7: U6-SA; tandem- type siRNA expression; lane 8: U6-SA-m1, tandem- type siRNA (mutation-1) expression; lane 9: U6-SA-m2, tandem- type siRNA (mutation-2) expression.

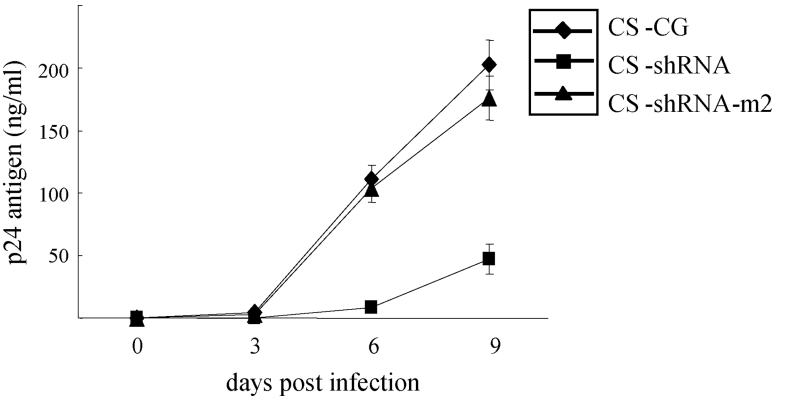

FIGURE 3 Evaluation of the inhibition efficacy of the transgene. The empty (CS-CG), shRNA, and shRNA-m2 lenti-vectors were used to infect MT-4 cells, which were challenged with 0.005 MOI of HIV-1_{NL4-3}. At 3, 6, and 9 days after infection, the amount of p24 was measured by the HIV-1 p24 CLEIA assay (each bar represents the average of 3 samples/replicate ± standard deviations).

Based on these results, siRNAs expressed by lentiviral vectors using the CS-CDF-CG-PRE (CS-CG) site were constructed for the purpose of transducing lymphoid cells and evaluating their anti-HIV-1 activity. The lentiviral vectors were pseudotyped with VSV-G and encoded green fluorescence protein (GFP) as a reporter gene, and the siRNA expression cassettes were inserted into the EcoR I site upstream of the CMV-GFP (Figure 1C).

The lentivirus was packaged in 293T cells by simultaneous transfection of the plasmid vectors. Then the produced virus was titrated for viral infectivity on MT-4 cells, using GFP expression as an indicator. Vector titer ranged from 8.7×10^6 to 1.5×10^7 for CS-CG, CS-shRNA, and CS-shRNA-m2, respectively. MT-4 cells were transduced by the CS-CG, CS-shRNA, and CS-shRNA-m2 at a multiplicity of infection (MOI) of 1. Twenty-four hours after transduction, MT-4 cells (2×10^6 cells/ml in 48-well plate) were challenged with HIV-1_{NL4-3} (MOI of 0.005) and cultured for three, six, and nine days, and the amount of p24 antigen was measured as an index for HIV-1 inhibition by the CLEIA assay. After six days, CS-shRNA showed a 93% inhibition effect, as compared to the empty vector (Figure 3).

Generally, at the plasmid and lentiviral RNA expression levels, the siRNA encoding the *env* fragment (NL4-3: 7490–7508) exhibited sequence-specific suppression of target gene expression and strongly inhibited HIV-1 infection in the cells as compared to the CS-CG and CS-shRNA-m. Hence, targeting the HIV-1 env gene with siRNAs encoding the *env* fragment will be an effective strategy for clinical application against HIV-1/AIDS.

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