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# Effective Suppression of Human Immunodeficiency Virus Type 1 through a Combination of Short- or Long-Hairpin RNAs Targeting Essential Sequences for Retroviral Integration

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**Small interfering RNA (siRNA) could provide a new therapeutic approach to treating human immunodeficiency virus type 1 (HIV-1) infection. For long-term suppression of HIV-1, emergence of siRNA escape variants must be controlled. Here, we constructed lentiviral vectors encoding short-hairpin RNAs (shRNA) corresponding to conserved target sequences within the integrase (*int*) and the attachment site (*att*) genes, both of which are essential for HIV-1 integration. Compared to shRNA targeting of the HIV-1 transcription factor *tat* (shTat), shRNA against *int* (shIN) or the U3 region of *att* (shU3) showed a more potent inhibitory effect on HIV-1 replication in human CD4$^+$ T cells. Infection with a high dose of HIV-1 resulted in the emergence of escape mutants during long-term culture. Of note, limited genetic variation was observed in the viruses resistant to shIN. A combination of shINs against wild-type and escape mutant sequences had a negative effect on their antiviral activities, indicating a potentially detrimental effect when administering multiple shRNA targeting the same region to combat HIV-1 variants. The combination of shIN and shU3 *att* exhibited the strongest anti-HIV-1 activity, as seen by complete abrogation of viral DNA synthesis and viral integration. In addition, a modified long-hairpin RNA spanning the 50 nucleotides in the shIN target region effectively suppressed wild-type and shIN-resistant mutant HIV-1. These results suggest that targeting of incoming viral RNA before proviral DNA formation occurs through the use of nonoverlapping multiple siRNAs is a potent approach to achieving sustained, efficient suppression of highly mutable viruses, such as HIV-1.**

Gene targeting in mammalian cells through the use of short-hairpin RNAs (shRNAs) has been advanced by the development of vector systems for efficient delivery and stable expression of shRNA sequences (4, 7, 22, 30). Upon delivery into cells, shRNAs are converted into short double-stranded RNAs, termed small interfering RNAs (siRNAs), that mediate a sequence-specific RNA degradation process termed RNA interference (RNAi) (12, 14, 42). Antiviral therapy based on siRNA has been proposed as a new method for intracellular immunization against human immunodeficiency virus type 1 (HIV-1) (16, 31, 32) and hepatitis C virus (HCV) (34). When viral genes are targeted, viruses can escape from RNAi-mediated inhibition due to their high mutation rate (6, 11, 39). An alternative approach that shows promise is the use of siRNAs targeting cellular genes essential for virus replication. In the case of HIV-1, siRNAs against the cell surface CD4 receptor (31) or CXCR4 and CCR5 coreceptors for HIV-1 entry conferred viral resistance (2, 3, 33). However, CD4 and CXCR4 are essential for T-cell development and proper immunologic function. In addition, although CCR5 might be nonessential for normal function (23), not all HIV-1 strains require CCR5. Downregulation of an essential cellular coreceptor could potentially result in the emergence of HIV-1 variants that use another coreceptor(s) for viral entry into the cell.

To achieve long-term control of viral replication by siRNA and prevent the emergence of escape variants, it is important to target highly conserved and/or essential HIV-1 sequences. For example, many sites in the *cis*-regulatory regions, as well as the protein-coding regions, of HIV-1 have been examined as potential targets for siRNA. These regions include the primer-binding site, the polypurine tract, the long terminal repeat, and the *gag*, *pol*, *env*, *tat*, *rev*, *vif*, and *nef* genes (6, 10, 11, 16, 18, 21, 30, 39). The degree to which siRNAs inhibited HIV-1 replication and the underlying mechanisms varied considerably, depending on the target sequence (10, 11). For example, RNAi-resistant HIV-1 variants can emerge not only through mutations in the siRNA target sequence but also through mutations that alter the local RNA structure (39). These results emphasize the need for empirical studies to determine effective siRNA target sites within the HIV-1 genome.

In the present study, we selected several sequences for lentivirus-mediated shRNA expression based on a preliminary screening of HIV-1 RNAi target sites using synthetic siRNA duplexes. These sequences mapped within the integrase (IN) gene (*int*) and the attachment site (*att*), which are essential for HIV-1 integration. We evaluated the anti-HIV-1 activity of these expressed shRNAs using a highly susceptible CD4$^+$ T-cell line. Genetic analysis of HIV-1 escape mutants that emerged after treatment with combinations of shRNAs revealed that two or more shRNAs targeting different essential sequences had the strongest impact on

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antiviral activity. The results also suggest that shRNAs or long-hairpin RNA (lhRNA) that targets incoming viral RNA before proviral DNA formation is more efficient at mediating RNAi antiviral therapy.

## MATERIALS AND METHODS

**Construction of plasmids.** A series of small-hairpin-RNA-expression vectors were constructed using pGEM-H1 and pCS-H1 vectors described previously (30). Sense (S) and antisense (AS) sequences for shRNA were as follows: shTat-S, 5′-GAT CCC CTG CTT GTA CCA ATT GCT ATT CAA GAG ATA GCA ATT GGT ACA AGC AGT TTT TGG AAA G-3′; shTat-AS, 5′-TCG ACT TTC CAA AAA CTG CTT GTA CCA ATT GCT ATC TCT TGA ATA GCA ATT GGT ACA AGC AGG G-3′; shIN-S, 5′-GAT CCC GGA GAG CAA TGG CTA GTG ATT CAA GAG ATC ACT AGC CAT TGC TCT CCT TTT TGG AAA G-3′; shIN-AS, 5′-TCG ACT TTC CAA AAA GGA GAG CAA TGG CTA GTG ATC TCT TGA ATC ACT AGC CAT TGC TCT CCG G-3′; shU3-S, 5′-GAT CCC GAC TGG AAG GGC TAA TTC ATT CAA GAG ATG AAT TAG CCC TTC CAG TCT TTT TGG AAA G-3′; shU3-AS, 5′-TCG ACT TTC CAA AAA GAC TGG AAG GGC TAA TTC ATC TCT TGA ATG AAT TAG CCC TTC CAG TCG G-3′; shIN-G4288A-S, 5′-GAT CCC GGA AAG CAA TGG CTA GTG ATT CAA GAG ATC ACT AGC CAT TGC TTT CCT TTT TGG AAA G-3′; shIN-G4288A-AS, 5′-TCG ACT TTC CAA AAA GGA AAG CAA TGG CTA GTG ATC TCT TGA ATC ACT AGC CAT TGC TTT CCG G-3′; shIN-A4293T-S, 5′-GAT CCC GGA GAG CAT TGG CTA GTG ATT CAA GAG ATC ACT AGC CAA TGC TCT CCT TTT TGG AAA G-3′; and shIN-A4293T-AS, 5′-TCG ACT TTC CAA AAA GGA GAG CAT TGG CTA GTG ATC TCT TGA ATC ACT AGC CAA TGC TCT CCG G-3′. To generate pCS-H1-shTat, pCS-H1-shIN, pCS-H1-shING4288A, pCS-H1-shIN-A4293T, and pCS-H1-shU3, pGEM-H1-shTat, pGEM-H1-shIN, pGEM-H1-shING4288A, pGEM-H1-shIN-A4293T, and pGEM-H1-shU3 were digested with EcoRI and SalI. Each fragment was then inserted into the 7.9-kb EcoRI-XhoI fragment of pCS-CDF-PRE.

To introduce the point mutation, T5901C, into the *tat* target sequence of HIV-1 (infectious molecular clone NL-EGFP), total DNA was isolated from MT-4/shTat cells infected by the shTat-resistant HIV-1 variant. The *tat* region of the mutant was amplified by PCR using primers Tat-F (5′-GCA GGA GTG GAA GCC ATA ATA AG-3′) and Tat-R (5′-CAT TAT CAT TCT CCC GCT ACT AC-3′), followed by TA cloning of the PCR product into pT7Blue vector (Merck-Novagen). A 0.28-kb EcoRI-HindIII fragment from the pT7Blue was inserted into pcDNA-NL-RN (pcDNA-TatT5901), which contained a 1.5-kb EcoRI-NheI fragment from NL-EGFP cloned into the EcoRI-NheI sites of pcDNA3.1 (+) (Invitrogen). Finally, the 1.5-kb EcoRI-NheI fragment from pcDNA-TatT5901 was cloned into the EcoRI-NheI site of pNL-EGFP. pNL-EGFP vectors encoding point mutations within the shIN target sequence (G4288A and A4293T) were generated by using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol, with mutagenic primers and pNL-EGFP as a template (20). Mutagenic primers were as follows: G4288A, 5′-TCA CAG TAA TTG GAA AGC AAT GGC TAG TG-3′ and 5′-CAC TAG CCA TTG CTT TCC AAT TAC TGT GA-3′; and A4293T, 5′-TCA CAG TAA TTG GAG AGC ATT GGC TAG TG-3′ and 5′-CAC TAG CCA ATG CTC TCC AAT TAC TGT GA-3′. To generate pCS-hU6-shIN50#1, pCS-hU6-shIN50#2, piGENE-hU6-shIN50#1, and piGENE-hU6-shIN50#2 were constructed by inserting the annealing product of shIN50#1 (5′-CAC CGA TGG AGT AGG TAA GGT CCA AGG AGA GCA TGA GGA ATG TCA TAG TAG TTG TTC AAG AGA CAA TTA CTG TGA TAT TTC TCA TGT TCT TCT TGG GCC TTA TCT ATT CCA TCT TTT TT-3′ and 5′-GCA TAA AAA AGA TGG AAT AGA TAA GGC CCA AGA AGA ACA TGA GAA ATA TCA CAG TAA TTG TCT CTT GAA CAA CTA CTA TGA CAT TCC TCA TGC TCT CCT TGG ACC TTA CCT ACT CCA TC-3′) or shIN50#2 (5′-CAC CCA AGA GGA ACG TGA GAG ATA TTA CAG TAG TTG GAG AGT AGT GGC TGG TGA TTC AAG AGA TCA CTA GCC ATT GCT CTC CAA TTA CTG TGA TAT TTC TCA TGT TCT TCT TGT TTT TT-3′ and 5′-GCA TAA AAA ACA AGA AGA ACA TGA GAA ATA TCA CAG TAA TTG GAG AGC AAT GGC TAG TGA TCT CTT GAA TCA CCA GCC ACT ACT CTC CAA CTA CTG TAA TAT CTC TCA CGT TCC TCT TG-3′) into the BspMI site of the piGENE hU6 vector. The EcoRI-PvuII fragment from piGENE-hU6-shIN50-1 or piGENE-hU6-shIN50-2 was inserted into the EcoRI-EcoRV site of pcDNA3.1 (−). The resultant plasmids were digested with EcoRI and XhoI, and the 0.6-kb fragment was ligated into the EcoRI-XhoI site of pCS-CDF-CG-PRE.

**Cells.** 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μU/ml penicillin, and 100 μg/ml streptomycin. MT-4 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μU/ml penicillin, and 100 μg/ml streptomycin. Human peripheral blood lymphocytes were derived from HIV-1-seronegative, healthy donors. Briefly, peripheral blood mononuclear cells were separated over a Ficoll-Hypaque gradient (Ficoll-Paque Plus; Amersham Pharmacia Biotech Inc., Tokyo, Japan) by centrifugation. Peripheral blood mononuclear cells were allowed to adhere to 150-mm plastic tissue culture dishes (Iwaki, Tokyo, Japan) by incubation in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) containing 5% human AB serum (Sigma or Nippon Bio-SupplyCenter, Tokyo, Japan) for 2 h. Nonadherent cells (peripheral blood lymphocytes) were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 units of recombinant interleukin-2 (Shionogi, Osaka, Japan)/ml.

**Virus preparation.** 293T cells ($4 \times 10^6$) plated in 100-mm dishes were cotransfected with the appropriate lentiviral-shRNA expression vector (17 μg), vesicular stomatitis virus G expression vector pMD.G (5 μg), *rev* expression vector pRSV-Rev (5 μg), and *gag-pol* expression vector pMDLg/pRRE (12 μg) using the calcium phosphate precipitation method. After 4 h, cells were washed three times with phosphate-buffered saline, 5 ml of new medium was added, and cells were incubated for 48 h. Culture supernatants were harvested and filtered through 0.45-μm-pore-size filters. Lentivirus was concentrated ~40-fold by low centrifugation at 6,000 × *g* for 16 h and resuspended in 2 ml of RPMI 1640 medium. In all experiments, cells were transduced with equal amounts of the shRNA lentivirus at a multiplicity of infection of 10. Replication-competent HIV-1 carrying green fluorescent protein (GFP) was generated by transfection of 293T cells with pNL-EGFP (1 μg) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Level of HIV-1 p24 antigen was determined using an enzyme immunoassay (RETRO-TEK; ZeptoMetrix Corp., Buffalo, N.Y.).

**Analysis of provirus sequence.** Viral DNA was isolated from NL-EGFP-infected MT-4 cells. Viral DNA spanning the shRNA target sequence of interest was amplified by PCR using the following primer pairs: shIN target region primers, 5′-CAC CAT GGG ATT TTT AGA TGG AAT AGA TAA GGC CC-3′ and 5′-ATC CTC ATC CTG TCT ACT TGC-3′; shTat target region primers, 5′-GCA GGA GTG GAA GCC ATA ATA AG-3′ and 5′-CAT TAT CAT TCT CCC GCT ACT AC-3′; and shU3 target region primers, 5′-CGG AAT TCT ACC TTA TC TGG CT-3′ and 5′-TCG CCA CAT ACC TAG AAG AAT AAG AC-3′. These PCR products were inserted into the pGEM-T Easy vector (Promega) by TA cloning, followed by DNA sequence analysis using the ABI310 sequencer (Perkin-Elmer Applied Biosystems).

**Quantitative PCR analysis.** Total DNA was extracted from cells 1 or 8 days postinfection by using the urea lysis method. Briefly, cells were lysed with 0.3 ml of urea lysis buffer (7 M urea, 2% sodium dodecyl sulfate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0], 0.35 M NaCl). Total DNA was purified from the cell lysates by phenol-chloroform extraction followed by ethanol precipitation. Analysis of HIV-1 DNA was performed by quantitative PCR with the HIV-1-specific primers vif-F (5′-GAG ATA TAG CAC ACA AGT AGA CC-3′) and vif-R (5′-GCT AGT GCC AAG TAC TGT GAG AT-3′) using *Taq* DNA polymerase (Invitrogen). The thermal cycle consisted of 1 min at 94°C, followed by 30 cycles of 94°C for 1 min (denaturation), 65°C for 2 min (annealing), and 72°C for 2 min (extension). PCR products were separated on 2% agarose gels and stained with SYBR green.

## RESULTS

**Inhibition of HIV-1 replication by lentiviral-shRNA targeting of *tat*, integrase, and U3 *att* sequences.** Upon HIV-1 infection, the viral enzyme integrase catalyzes the integration of viral DNA into the host cell chromosome, an obligatory step for HIV-1 gene expression. In preliminary experiments using synthetic siRNA duplexes targeting essential motifs within HIV-1 IN (17, 27, 35), we identified several candidate sequences for shRNA-mediated targeting of HIV-1 (data not shown). Earlier studies showed that introduction of a single-amino-acid substitution within the HHCC motif of HIV-1 IN completely abolished virus infectivity (27, 29), indicating that sequence variation in this region is not tolerated by the virus. Indeed, the selected sequences are highly conserved among

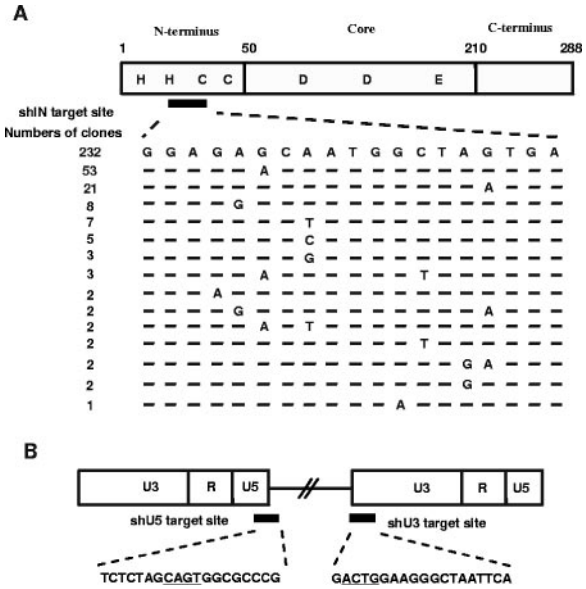


FIG. 1. shRNA target sequences of HIV-1 *int* and the U3 and U5 *att* sites. (A) Schematic representation of HIV-1 integrase (top squares) and shIN target region (bold bar). The conserved HHCC residues in the zinc-binding motif and enzyme active-site residues (DDE) located in the N terminus of the core domain of HIV-1 IN are indicated. The sequence of the shIN target region corresponding to the HIV-1 NL43 clone (1) and used in the present study is shown on the first line. Sequences of this region in 345 different isolates were aligned according to the HIV-1 sequence database published by Los Alamos National Laboratory (http://hiv-web.lanl.gov). Nucleotide differences in comparison with NL43 and numbers of clones that carried them are indicated. (B) Target sequences of shU3 and shU5 in the HIV-1 NL43 clone are shown. Location of each target region in a whole HIV-1 genome is indicated by the bold bar. Conserved sequences in the U3 *att* and U5 *att* regions among all HIV-1 strains are underlined.

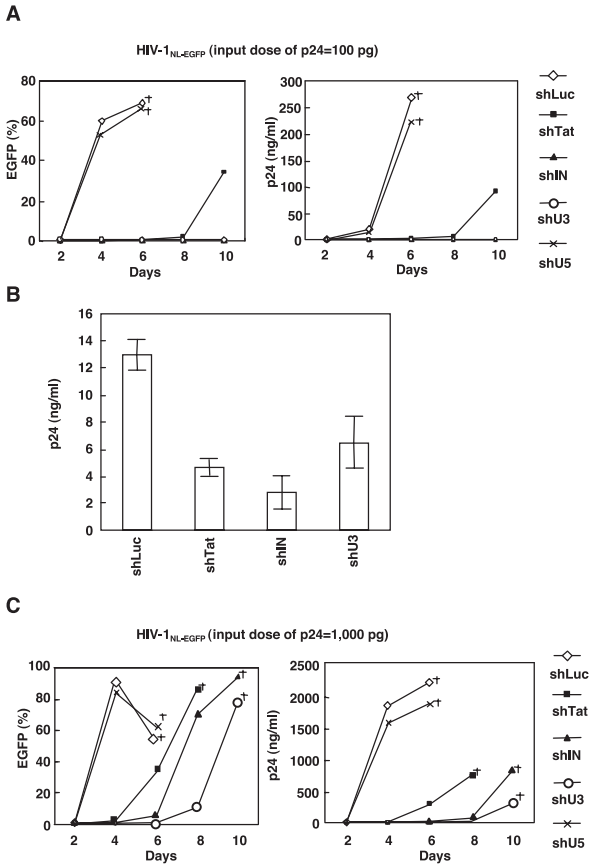


FIG. 2. HIV-1 replication in shRNA-transduced MT-4 or primary $CD4^+$ T cells. (A) MT-4 cells were transduced by the indicated shRNA using a lentivirus vector system (shLuc, shTat, shIN, shU3, and shU5). Seven days later, transduced MT-4 cells were infected by $HIV\text{-}1_{NL\text{-}EGFP}$ at an input dose of 100 pg of p24 (HIV-1 core antigen) per $10^6$ cells. Culture supernatants were collected periodically after infection as indicated. HIV-1 replication was monitored by measuring percent EGFP-positive cells by fluorescence-activated cell sorting (left) or level of HIV-1 p24 by enzyme-linked immunosorbent assay (right). The cross symbol indicates cell death associated with HIV-1 replication. Representative results of three independent experiments are shown. (B) Inhibition of HIV-1 replication by each shRNA in primary $CD4^+$ T cells. $CD4^+$ T cells ($1 \times 10^6$) were transduced with the indicated lentiviral vectors by low centrifugation at 2,000 rpm for 1 h in the presence of 10 μg/ml polybrene. Transduced cells were infected with $HIV\text{-}1_{NL43}$ for 3 h, and p24 antigen levels in culture supernatants 4 days postinfection were measured. Values represent the means and standard deviations for three independent experiments. (C) MT-4 cells transduced with the indicated shRNAs (shLuc, shTat, shIN, shU3, or shU5) were infected by $HIV\text{-}1_{NL\text{-}EGFP}$ at a high input dose (1,000 pg of p24 per $10^6$ cells), and HIV-1 replication was monitored as described for panel A. (C) MT-4 cells transduced with each shRNA (shLuc, shTat, shIN, shU3, or shU5) were infected by $HIV\text{-}1_{NL\text{-}EGFP}$ with a high input dose (1,000 pg of p24 per $10^6$ cells), and HIV-1 replication was monitored as described for panel A.

HIV-1 strains (Fig. 1A). Therefore, we chose this site for lentiviral-shRNA-mediated gene targeting. We also chose the conserved regions within the U3 *att* and U5 *att* sites as the shRNA targets (Fig. 1B). The attachment sites at both viral DNA ends (U3 *att* and U5 *att*) are *cis*-acting regions required for retroviral integration by IN, and point mutations or deletion of HIV-1 U3 or U5 *att* sites resulted in severe impairment of integration in vivo (25, 27). The lentiviral-based shRNA expression system we used in these studies was previously shown to efficiently inhibit HIV-1 replication in 293T and MT-4 cells and primary macrophages (30).

First, we examined the effect of expressing shRNAs targeting IN (shIN), U3 *att* (shU3), or U5 *att* (shU5) on HIV-1 replication in a highly susceptible human $CD4^+$ T-cell line, MT-4 (15). The MT-4 cell system has been used successfully by us and others to isolate HIV-1 mutants acquired with resistance to the neutralization antibody (26) or anti-HIV-1 drugs (13). As a positive control for shRNA-mediated inhibition of HIV-1, we constructed a lentivirus vector expressing shRNAs for the HIV-1 transactivator protein gene *tat* (shTat) that corresponded to the region described by Boden et al. (6). Following transduction with lentiviral shRNAs, MT-4 cells were infected at various doses with an HIV-1 clone carrying enhanced GFP (pNL-EGFP) (20). Expression of shIN, shU3, and shTat profoundly inhibited HIV-1 replication compared with the control shRNA, shLuc, at an input dose of 100 pg of HIV-1 p24 (*gag* gene product), or the equivalent of a 50% tissue culture infective dose of about 200 (Fig. 2). The inhibitory effect of shU5 expression was very weak, possibly due to a high G+C content in its 3′ region (36). At 10 days postinfection, viral replication was detected in the shTat-transformed MT-4 cells,

followed by cell death. In contrast, HIV-1 replication was undetectable in MT-4 cells transduced by shIN or shU3 up to 1 month postinfection, indicating complete inhibition of HIV-1 by shIN or shU3 during this time frame. Thus, in MT-4 cells, shIN and shU3 conferred stronger resistance against HIV-1 than shTat. The antiviral effect of each shRNA was also observed in human primary $CD4^+$ T cells (Fig. 2B), where shIN exhibited the strongest antiviral activity. Prolonged antiviral activity by shIN or shU3 was abolished by increasing the level of input HIV-1 to 1,000 (Fig. 2C) or 10,000 pg of p24 (not shown). Under conditions of increased infectious dose, HIV-1 replication was observed 10 days postinfection in MT-4 cells transduced with shIN or shU3.

**Genetic analysis of shRNA-resistant HIV-1.** Although each shRNA could inhibit HIV-1 replication under conditions of low dose of infection, the inhibitory effect was transient when higher input doses were used. This effect was most likely due to acquired mutations within the viral shRNA target sequences. Viruses were harvested from MT-4 cells that had been transduced by each of the shRNAs and used to infect a fresh set of shRNA-transduced MT-4 cells. Viruses harvested from culture supernatants 12 days after infection of shTat-transduced MT-4 cells showed specific resistance against shTat but not against shIN or shU3 (Fig. 3A, left). Viruses harvested from shIN- or shU3-transduced MT-4 cells 10 days after infection with high doses of HIV-1 also showed specific resistance against shIN or shU3 *att*, respectively (Fig. 3B and C, left).

We next examined the genetic profile of shRNA target sites in each shRNA-resistant virus. MT-4 cells were freshly infected with each shRNA-resistant virus, and total DNA was extracted. Viral DNA fragments spanning each shRNA target region were amplified by PCR, followed by TA cloning. Several clones derived from each of the resistant viruses were examined by DNA sequence analysis. Various single-nucleotide substitutions were observed within the shTat target region of shTat-resistant virus DNA (Fig. 3A, right), while the sequences within the shIN and shU3 target regions were unchanged (not shown). Similarly, shIN- or shU3-resistant viruses contained one or two mutations within the corresponding target region. No viruses in which wild-type sequences in each shRNA target region were maintained emerged after long-term culture (16 to 22 days postinfection), indicating a strong selective pressure of these shRNAs toward wild-type virus. Of note, shIN-resistant viruses contained only two types of mutation (G4288A and A4293T), suggesting that mutations in the IN region are more detrimental for virus replication than those in other shRNA targeted regions in *tat* and U3 *att*.

To confirm whether the nucleotide substitutions detected in the above experiments could confer resistance to the corresponding shRNA, we introduced each point mutation into the parental HIV-1 clone (pNL-EGFP) and evaluated its replication ability in shRNA-transduced MT-4 cells. Viruses carrying point mutations within the shTat target site (Tat-T5901C) or the shIN target site (IN-G4288A or IN-A4293T) showed specific resistance against shTat or shIN, respectively (Fig. 4). We also observed that IN-G4288A or IN-A4293T mutants had constantly higher levels of replication in MT-4 cells transduced with shIN than in control MT-4 cells transduced by shLuc (Fig. 3B and C). Although the mechanism underlying the enhanced replication of these escape mutants in the presence of shIN is unknown, enhancement of HIV-1 replication by siRNA has been reported recently by others (10). These results indicate that shRNA-mediated selection pressure can generate HIV-1 escape mutants that can replicate in the presence of each shRNA.

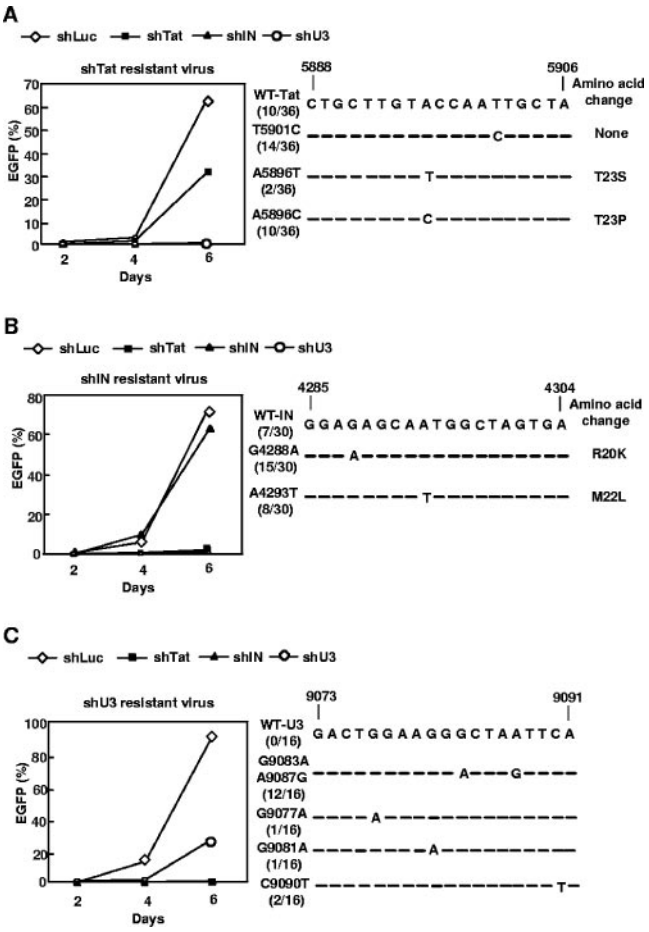


FIG. 3. shRNA-specific resistance of HIV-1 escape mutants in shRNA-transduced MT-4 cells. Culture supernatants of shTat-transduced MT-4 cells infected with a low dose of HIV-1 (100 pg of p24) (A) or shIN (B)- or shU3 (C)-transduced MT-4 cells infected with a high dose of HIV-1 (1,000 pg of p24 per $10^6$ cells) were harvested at 12 days after challenge infection. Culture supernatants containing shRNA-resistant virus (100 pg of p24) were inoculated to newly prepared MT-4 cells transduced by shLuc, shTat, shIN, or shU3, and replication of HIV-1 in these cells was monitored by measuring percent EGFP-positive cells (left). Representative results of three independent experiments are shown. Culture supernatants of shTat-transduced MT-4 cells infected with shTat-resistant virus (A), shIN-transduced MT-4 cells infected with shIN-resistant virus (B), and shU3-transduced MT-4 cells infected with shU3-resistant virus (C) were harvested at 6 days postinfection. Each culture supernatant containing shRNA-resistant viruses (100 pg of p24) was infected with newly prepared MT-4 cells transduced by shTat, shIN, or shU3. Total DNA was extracted from these MT-4 cells at 4 days postinfection. A fragment of viral DNA spanning each shRNA target region was amplified by PCR followed by TA cloning. Then, several clones from each were subjected to DNA sequence analysis. Nucleotide changes in the target sequence for shTat (nucleotides 5888 to 5906 of the *tat* gene), shIN (nucleotides 4285 to 4304 of the *int* gene), and shU3 (nucleotides 9073 to 9091 of the U3 *att* region) are shown on the right, along with the expected amino acid changes. Relative numbers of each clone are indicated in parentheses.

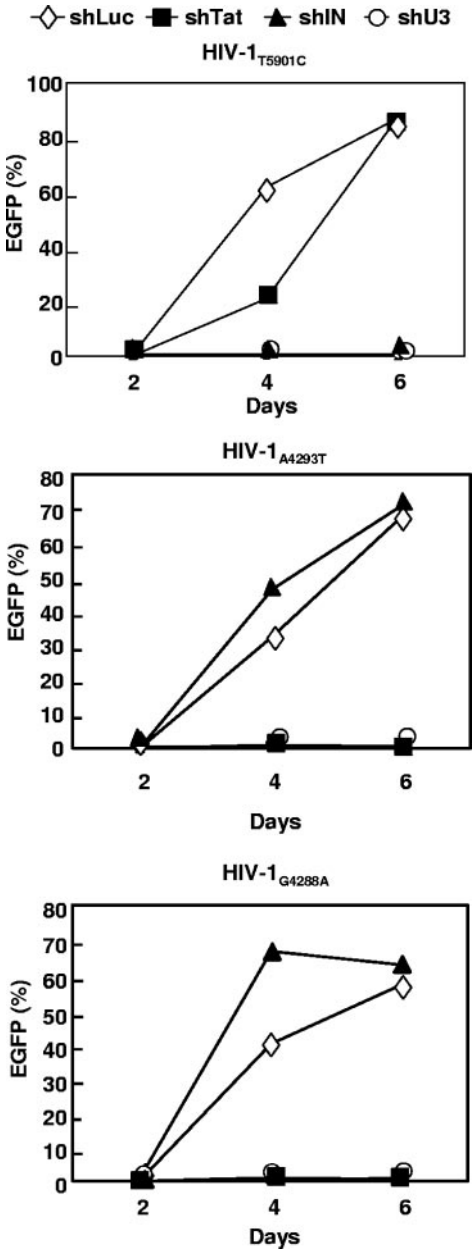


FIG. 4. shRNA-specific resistance of HIV-1 molecular clones carrying point mutations within each shRNA target site. Point mutations within the shTat target site (Tat-T5901C) or shIN target site (IN-G4288A or IN-A4293T) were introduced into the parental HIV-1 clone (pNL-EGFP) through mutagenesis. Each recombinant mutant clone was transfected into 293T cells, and the culture supernatant was harvested and inoculated to MT-4 cells expressing the corresponding shRNA. Replication of each mutant clone was monitored by measuring percent EGFP-positive cells at the indicated days. Representative results of three independent experiments are shown.

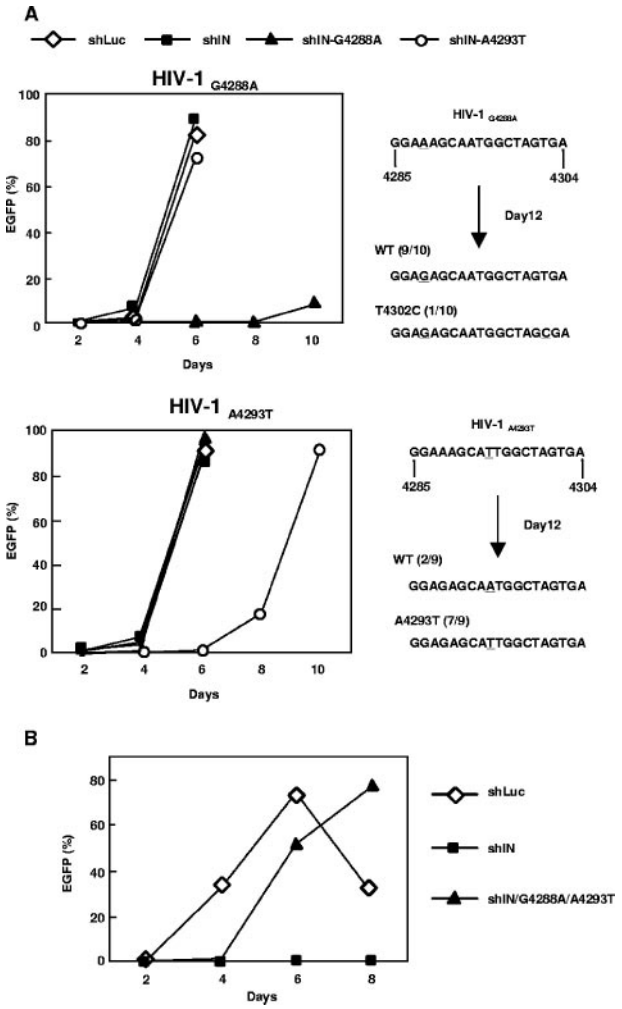


FIG. 5. Reversion of escape mutants in the presence of modified shRNAs targeting mutant sequences. (A) MT-4 cells were transduced with shIN or its modified shIN, which targeted escape mutant sequences (shIN-G4288A or shIN-A4293T). As a negative control, shLuc was introduced into MT-4 cells as well. The transduced cells were infected by HIV-$1_{NL\text{-}G4288A}$ or HIV-$1_{NL\text{-}A4293T}$ at a dose of 1,000 pg of p24 antigen per $10^6$ cells. Ten or 12 days after challenge infection, total DNA was isolated, viral DNA spanning the shIN target region was amplified by PCR and subjected to TA cloning, and sequences were analyzed. WT, wild type. (B) Effect of sequential transduction of shRNAs targeting *int* from wild-type and escape mutant viruses. MT-4 cells were transduced with shIN, shIN-G4288A, and shIN-T4293A sequentially (shIN/G4288A/T4293A). In parallel, MT-4 cells were transduced with shIN or shLuc alone. Transduced MT-4 cells were infected with HIV-$1_{NL\text{-}EGFP}$ at a dose of 100 pg of p24 per $10^6$ cells. Virus replication was monitored by measuring percent EGFP-positive cells at the indicated days. Representative results of three independent experiments are shown.

**Combination of shINs against wild-type and escape mutants.** Two different single-nucleotide substitutions were identified in shIN escape mutants (G4288A and A4293T). We examined HIV-1 replication in MT-4 cells expressing shRNAs targeting wild-type IN and both of the variant sequences (G4288A and A4293T). We constructed shRNA expression vectors encoding the IN escape mutant sequences, shIN-G4288A and shIN-A4293T, and confirmed their specific abilities to suppress the replication of the corresponding viral mutants, IN-G4288A and IN-A4293T, respectively (Fig. 5A). However, significant viral replication was detected 8 to 10 days postinfection, with IN-G4288A or IN-A4293T in MT-4 cells expressing the corresponding mutant shRNA (Fig. 5A). Sequence analysis of clones isolated from shIN-G4288A-trans-

duced MT-4 cells had revealed that 9 out of 10 had wild-type IN sequences, suggesting that these viruses had reverted to the wild type. In nine clones isolated from shIN-A4293T-transduced MT-4 cells, two had IN sequences that had reverted to the wild type, and seven clones retained the original mutation (Fig. 5A, lower panel). Differences in the efficiencies of reversion of the two mutants may reflect different selection pressures conferred by shIN-G4288A or shIN-A4293T.

Reversion to wild-type sequences was detected only when each escape mutant was treated with its corresponding mutant-specific shRNA. We next examined the effect of combining shRNAs targeting the wild-type and shIN escape mutant viruses on the emergence of mutant and/or wild-type virus. MT-4 cells were sequentially transduced with shIN, shIN-G4288A, and shIN-A4293T and then infected with wild-type HIV-1. Contradictory to our expectations, the combination of two different shRNAs weakened HIV-1 suppression by shRNAs (Fig. 5B). We detected significant HIV-1 replication 6 days postinfection, under the same conditions that resulted in complete suppression by shIN alone. Sequence analysis revealed that only wild-type HIV-1 had persisted, and escape mutant viruses were not detected (data not shown). These experiments suggest that several shRNAs targeting the same region might have a detrimental effect on their suppression capabilities, perhaps due to competition between the same target RNAs, with less effective shRNAs carrying a mismatch point mutation.

**The combination of shRNA targeting different sites of HIV-1 for efficient suppression of HIV-1.** We next evaluated the antiviral effect of combining shRNAs that target different sites within the HIV-1 genome. MT-4 cells were simultaneously transduced with three different combinations of shRNAs: shIN/shU3, shTat/shU3, and shTat/shIN. The transduced MT-4 cells were infected with a dose of HIV-1 containing 1,000 pg of p24 antigen. These were the conditions under which a single type of shRNA could not control viral replication and escape mutants emerged (Fig. 2). All of the combinations of shRNAs completely inhibited HIV-1 replication, and the inhibitory effect persisted for more than 1 month without emergence of escape mutants (Fig. 6A). These results demonstrated that shRNAs targeting at least two different essential genes might have a positive impact on suppressing viral activity.

Interestingly, we observed that proviral DNA was absent in dual-transformed MT-4 cells after HIV-1 infection. We speculated that the shRNAs might target and degrade incoming viral RNA, preventing subsequent viral cDNA synthesis. We analyzed the levels of viral cDNA synthesized soon after HIV-1 infection of shRNA-transduced MT-4 cells by a quantitative PCR, using primers specific for the type of HIV-1 used for the challenge infection. In control MT-4 cells transduced with shLuc, viral cDNA was detected as early as 6 h postinfection. The levels of viral cDNA increased over time, indicating multiple rounds of viral infection (Fig. 6B). In contrast, at 6 h postinfection, the levels of viral cDNA in MT-4 cells transduced with shIN/shU3, shTat/shU3, or shTat/shIN were significantly reduced to 45%, 49%, or 15%, respectively, of those in control MT-4 cells and then declined to undetectable levels at 24 h and the later time point (8 days) after infection. These results suggested that shRNAs could target incoming viral RNA, thereby preventing subsequent reverse transcription and integration of HIV-1 RNA.

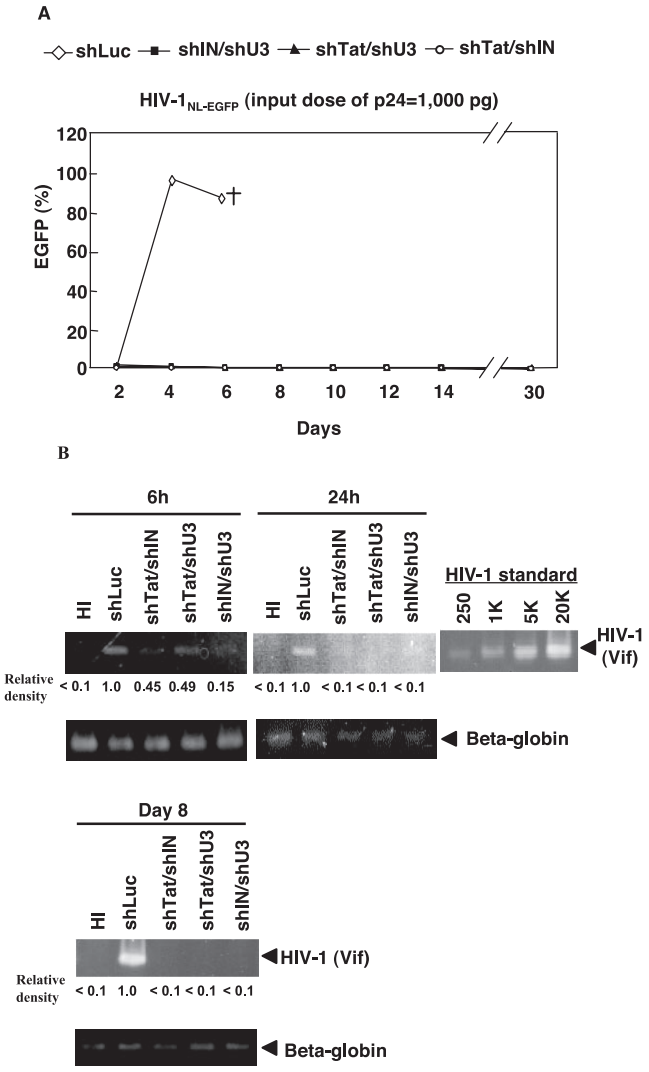


FIG. 6. Efficient antiviral activity with a combination of shRNAs targeting different sites in the HIV-1 genome. (A) MT-4 cells were transduced with combinations of shIN and shU3 (shIN/shU3), shTat and shU3 (shTat/shU3), or shTat and shIN (shTat/shIN). The dual-transduced cells were infected with DNase I-treated HIV-$1_{NL-EGFP}$ at a dose of 1,000 pg of p24 per $10^6$ cells. Virus replication was monitored by measuring percent EGFP-positive cells at the indicated days postinfection. The cross symbol indicates cell death associated with HIV-1 replication. (B) In parallel, total DNA was isolated from MT-4 cells 6 h, 24 h, or 8 days postinfection. Level of viral DNA was determined by quantitative PCR as described previously (27). For PCR, virus incubated at 65°C for 30 min prior to inoculation was used as the heat-inactivated control (HI), and for the HIV-1 DNA standard, a linearized HIV-1 molecular clone (pNL43luc$\Delta$*env*) was amplified. Human β-globin DNA was used as the internal control (17). The gel image was taken by using Image Saver System AE-6905C (ATTO, Tokyo, Japan), and the intensities of the PCR products were quantified by using Adobe Photoshop 7.0 software. The values shown are the intensity of each band relative to that in the control shLuc-transduced MT-4 cells, taken as 1.0.

For successful long-term control of HIV-1 replication by shRNA, targeting the incoming viral RNA before reverse tran-

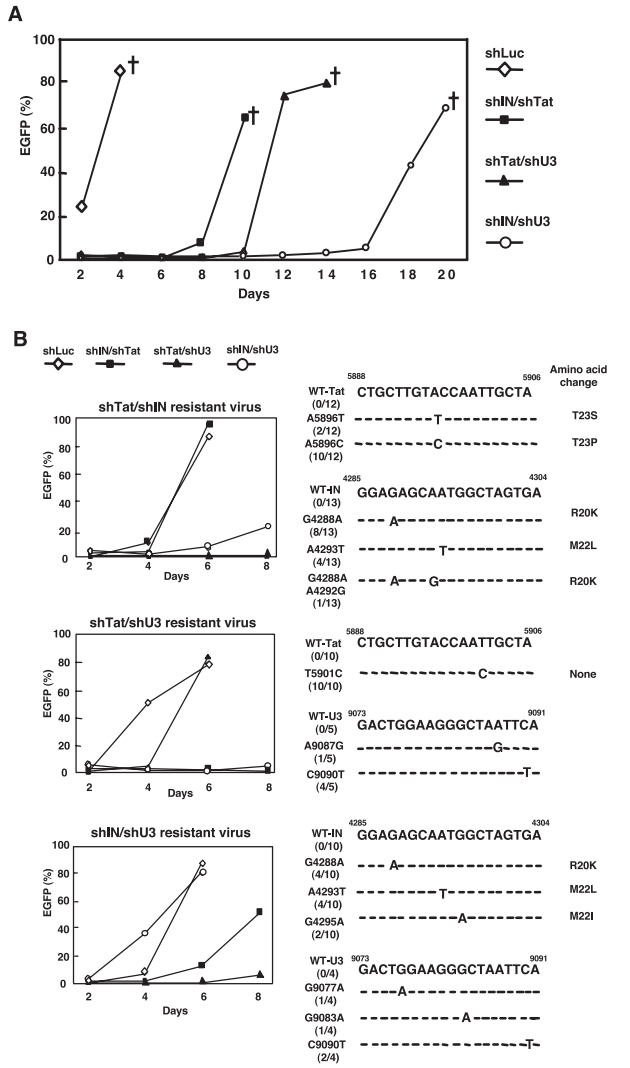


FIG. 7. Emergence of escape mutants from combinations of shRNAs targeting different sites following a high dose of HIV-1 infection. (A) MT-4 cells were transduced with combinations of two shRNAs as described for Fig. 6. The dual-transduced cells were infected with HIV-$1_{NL\text{-}EGFP}$ at 10,000 pg of p24 per $10^6$ cells. Virus replication was monitored by measuring percent EGFP-positive cells at the indicated days postinfection. (B) Viruses were harvested from the culture supernatants of dual-transduced MT-4 cells 12 days postinfection for shTat/shIN, 16 days for shTat/shU3, and 22 days for shIN/shU3. Viruses resistant to each combination of shRNAs were inoculated into MT-4 cells freshly transduced by each combination of the two shRNAs. Virus replication was monitored by measuring percent EGFP-positive cells at the indicated days. The cross symbol indicates cell death associated with HIV-1 replication. Total DNA was harvested from the infected cells when virus replication became evident. Viral DNA spanning each shRNA target region was amplified by PCR and subjected to TA-cloning followed by sequence analysis, as described for Fig. 3. WT, wild type.

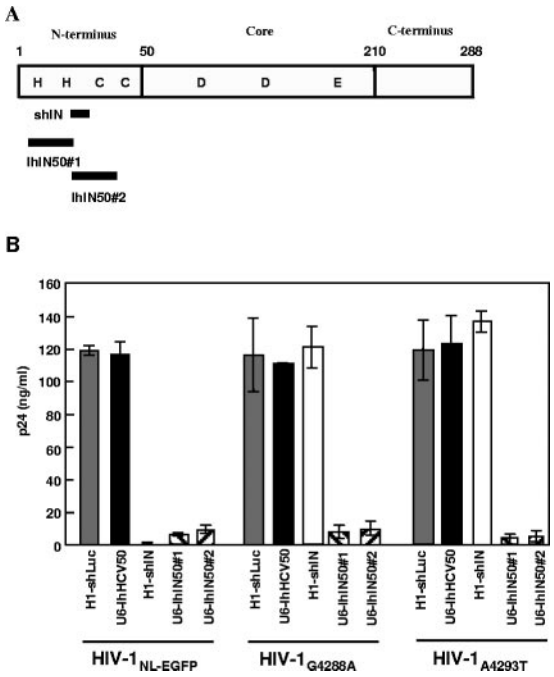


FIG. 8. Inhibitory effect of lhRNA on wild-type or shIN-resistant viral clones. (A) The target sites of lhRNAs against the HIV-1 *int* gene (lhIN). The target sites of two lhINs (lhIN50#1 and lhIN50#2) are indicated by bold bars. lhIN#1 was designed to target the 50 nucleotides upstream of the shIN target sequence. lhIN50#2 targets 50 nucleotides that include the shIN target sequence. (B) Lentiviral vectors expressing each lhRNA under the control of the human H1 promoter or U6 promoter were constructed. As a negative control, lhRNA targeting 50 nucleotides of HCV genome (U6-lhHCV50) was used (38). Transduction of MT-4 cells with each lhRNA was performed as described for Fig. 2. Transduced MT-4 cells were infected by the parental clone (HIV-$1_{NL\text{-}EGFP}$) or the shIN-resistant clone (HIV-$1_{G4288A}$ or HIV-$1_{A4293T}$) at a dose of 100 pg of p24 per $10^6$ cells. Virus replication was monitored by measuring levels of p24 antigen in culture supernatants 4 days postinfection. Values are the means plus standard deviations for three independent experiments.

scription might be a key point of interference. In support of this hypothesis, when the infectious dose of HIV-1 was increased to the level of 10,000 pg of p24 antigen, none of the combinations of shRNAs was able to control HIV-1 replication in long-term cultures (Fig. 7A). When we examined the viruses replicating in the presence of each combination of shRNA, they showed specific resistance against the corresponding shRNAs and corresponding genetic alterations within both shRNA target sites (Fig. 7B). Note, however, that the combination of shIN/shU3 showed the strongest suppressive effects, inhibiting viral replication until 18 days postinfection, with a high dose of HIV-1 in the challenge infection (Fig. 7A).

**Modified long-hairpin RNA can suppress replication of HIV-1 wild-type or shRNA escape mutants in short-term culture.** Recently, it was reported that modified lhRNA, in which multiple point mutations were introduced into the sense strand to prevent activation of the cellular-interferon response (28), could effectively suppress the replication of hepatitis C virus (38). We constructed lentiviral vectors expressing lhRNAs targeting 50 nucleotides that span the shIN target region of the *int* gene (Fig. 8A). When lentiviral lhRNAs were expressed under the control of the human H1 promoter, viral replication was not significantly inhibited, perhaps due to the low expression levels and/or low stability of the transcripts (data not shown). Therefore, we used the human U6 promoter to drive lhRNA expression and evaluated the antiviral activity of the lhRNAs against wild-type or shIN-resistant clones (Fig. 8B). As described above, shIN had antiviral activity against wild-type

HIV-1 but not against the shIN-resistant clones (IN-G4288A or IN-A4293T). In contrast, lhRNAs targeting the *int* gene efficiently blocked replication of both of wild-type virus and the shIN-escape mutants (Fig. 8B). Interestingly, the anti-HIV-1 activity of lhIN50#2 was similar to that of lhIN50#1, which did not contain shIN target sequences, suggesting that viruses could not escape from RNAi caused by the lhRNAs. However, the antiviral effects of lhIN50#1 and lhIN50#2 were transient, and low levels of viral replication were detected 6 days postinfection. Sequence analysis revealed that replicating viruses were genotypically wild type (data not shown). Thus, the antiviral activity of the lhRNAs was not strong enough to induce generation of escape mutants, perhaps due to the low expression levels or poor stability of the expressed lhRNAs. The development of a more efficient expression system for lhRNAs might be necessary to achieve long-term control of HIV-1 replication. Nonetheless, our data suggest that targeting longer sequences of HIV-1 could be beneficial and an alternative approach to suppressing escape mutants.

## DISCUSSION

Expression of siRNAs directed against viral RNA has a potent and sequence-specific antiviral effect. However, viruses can escape from RNAi because of their high mutation rate. One approach to designing an effective siRNA-based therapy against HIV-1 is to target highly conserved regions in the HIV-1 genome. In this study, we showed that HIV-1 replication was efficiently inhibited through the expression of shRNAs that targeted the *int* or U3 *att* region, with no emergence of shRNA escape mutants when low doses of infection were used. However, shRNA escape mutant viruses did emerge with a higher dose of HIV-1 infection. Notably, among the target sequences examined in these studies, the target site for shIN is potentially the least-mutated region of the HIV genome.

Recently, it was shown that accumulation of several point mutations is required for siRNA resistance in an HCV replicon system (40). Several studies have suggested that shRNA-resistant virus can emerge not only by escaping the siRNA-mediated degradation of mRNA but also by micro RNA-mediated translational inhibitory pathways (8, 19, 24, 37, 41). In this paper, we showed that a single point mutation within a target site is sufficient for HIV-1 to escape from shRNA-mediated inhibition. This difference between HIV-1 and HCV might be partly due to suppressor protein function in RNA silencing. HCV has not been shown to encode a suppressor protein for RNA-silencing function, such as HIV-1 Tat (5) or influenza virus NS1 (9). One of the escape mutants in these studies showed enhanced replication in the presence of shRNA (Fig. 3, shIN-resistant virus clone). Similar enhancement by shRNA was also noted by others (10), sounding a cautionary note that if not selected properly, siRNA may enhance, rather than inhibit, virus replication.

The experiments in which several combinations of shRNAs were used revealed important new clues towards understanding siRNA-based therapeutic approaches against HIV-1. Pretreatment of cells simultaneously with shINs targeting wild-type and escape mutant sequences to prevent the emergence of escape mutations resulted in HIV-1 replication of wild-type sequences. Thus, there appears to be a detrimental effect of simultaneously administering shRNAs that target overlapping sequences in an effort to cover variant sequences among different HIV-1 strains. In contrast, multiple shRNAs targeting different essential sequences had a strong impact on antiviral activity.

HIV-1 Tat possesses a suppressor of RNA silencing function to evade elicited RNAi. Importantly, Tat suppresses RNAi mediated by shRNAs but not by synthesized oligonucleotide siRNA duplexes. shRNA requires Dicer-mediated processing to elicit RNAi, whereas presynthesized siRNA does not, suggesting that the role of Tat may be to subvert the cell's Dicer activity and inhibit processing of precursor double-stranded RNAs into siRNAs (5). Therefore, we were interested in testing other siRNAs against the HIV-1 genome in combination with siRNA targeting the *tat* gene. A synergic effect of shTat in combination with either shIN or shU3 was not detected in our studies. Rather, a combination of shIN and shU3 was shown to be most effective against HIV-1. Thus, we demonstrated a positive impact on the antiviral effect of shRNAs by using combinations of siRNAs targeting different regions of the genome. The lhRNAs, which targeted longer sequences, were also effective against viral pools containing divergent sequences or escape mutant sequences. Our lhRNA system, however, needs further modification to increase the expression and/or stability of the precursor transcripts. Taken together, the results of the present study suggest that targeting incoming viral RNA before viral cDNA synthesis through multiple or longer siRNAs is an important key for successful RNAi-mediated antiviral therapy.

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