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Short Communication

Suppression of human immunodeficiency virus type 1 replication by arginine deiminase of *Mycoplasma arginini*

Makoto Kubo,[1,2] Hironori Nishitsuji,[1] Kiyoshi Kurihara,[1] Takaya Hayashi,[1] Takao Masuda[1] and Mari Kannagi[1]

Correspondence
Mari Kannagi
kann.impt@tmd.ac.jp

[1]Department of Immunotherapeutics, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

[2]Japanese Foundation for AIDS Prevention, Tokyo 105-0001, Japan

It was found previously that human immunodeficiency virus type 1 (HIV-1)-irrelevant CD8$^+$ cytotoxic T lymphocytes (CTLs) from uninfected donors suppressed HIV-1 replication in a cell-contact-dependent manner. However, one of these CTL lines (CTL-3) also significantly suppressed HIV-1 replication through its supernatant. Here, the suppressive fraction from CTL-3 supernatant was purified and analysed by mass spectrometry. A protein band specific for the suppressive fraction was identified as arginine deiminase from *Mycoplasma arginini*, which catalyses the hydrolysis of arginine to citrulline. Addition of L-arginine or the use of antibiotics against mycoplasma restored supernatant-mediated but not cell-contact-dependent suppression of HIV-1 replication by CTL-3, clearly indicating that arginine deiminase of *M. arginini* in the supernatants suppressed HIV-1 replication, which is independent of CD8$^+$ T-cell-mediated HIV-1 suppression via cell contact. Arginine deiminase is known to be a chemotherapeutic agent against arginine-requiring tumours and these results suggest that it also has potential application in antiviral therapy.

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CD8$^+$ cells including human immunodeficiency virus type 1 (HIV-1)-specific cytotoxic T lymphocytes (CTLs) play important roles in controlling HIV-1 infection (Borrow *et al.*, 1994; McMichael & Rowland-Jones, 2001). It has been shown that CD8$^+$ cells of asymptomatic carriers produce an unknown CD8$^+$ T-cell antiviral factor that can suppress HIV-1 replication at a transcriptional level without causing cell death (Mackewicz *et al.*, 1995).

We have demonstrated previously that anti-HIV-1 activities of CD8$^+$ cells of asymptomatic carriers exhibit both major histocompatibility complex (MHC) I-restricted and -unrestricted suppression (Kannagi *et al.*, 1990; Ohashi *et al.*, 1999), and that HIV-1-irrelevant CD8$^+$ CTLs derived from uninfected donors also inhibit X4 and R5 HIV-1 replication (Liu *et al.*, 2003). Therefore, we hypothesized that MHC I-unrestricted suppression of HIV-1 replication might be a common property of CD8$^+$ CTLs, regardless of HIV-1 infection in the host. However, in our system, such CD8$^+$ cell-mediated suppression required direct contact between CD8$^+$ cells and infected cells.

Whilst investigating CD8$^+$ cell-mediated HIV-1 suppression, we established four allo-specific CD8$^+$ CTL lines, CTL-1 (Liu *et al.*, 2003), CTL-2, CTL-3 and CTL-4, from four uninfected healthy donors by stimulating peripheral blood mononuclear cells with mitomycin C (MMC)-treated Raji (Pulvertaft, 1964) cells in a long-term culture in the presence of recombinant human interleukin-2 (rhIL-2) as described previously (Liu *et al.*, 2003). These CTLs were not cytotoxic to autologous CD4$^+$ cells, but significantly suppressed HIV-1 replication in HIV-1-infected autologous CD4$^+$ cells when directly co-cultured.

Among these CTL lines, we found that culture supernatants of CTL-3 suppressed HIV-1 replication in addition to cell-mediated suppression. In the present study, we purified and identified the suppressive factor in the supernatant of CTL-3 by serial high-performance liquid chromatography (HPLC) and mass spectrometry.

Fig. 1 shows representative data of HIV-1 suppression by the established allo-specific CD8$^+$ CTL lines. CTLs from both the CTL-2 and CTL-3 lines markedly suppressed HIV-1 replication when directly co-cultured with HIV-1-infected autologous CD4$^+$ cells, whereas culture supernatants from CTL-3 but not CTL-2 suppressed HIV-1 replication (Fig. 1a). Culture supernatants of CTL-3 suppressed replication of both X4 HIV-1 strain NL4-3 (Adachi *et al.*, 1986) and R5 HIV-1 strain JR-CSF (Koyanagi *et al.*, 1987) (Fig. 1b). CTL-2 and CTL-3 culture supernatants did not alter the viability of CD4$^+$ T cells during 4 days of culture.

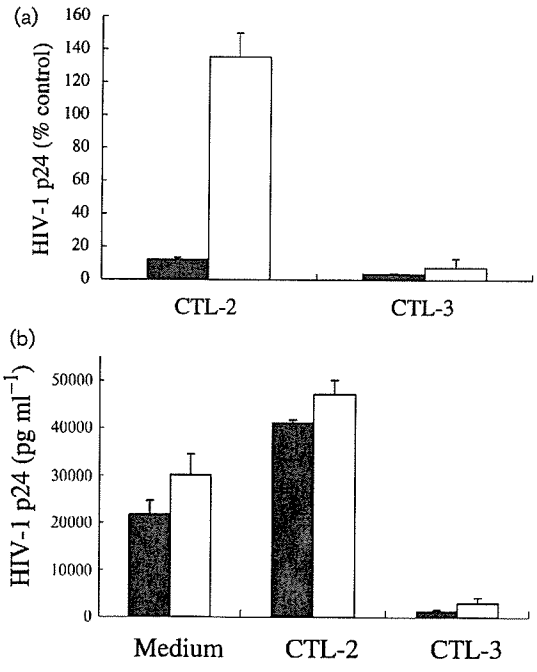
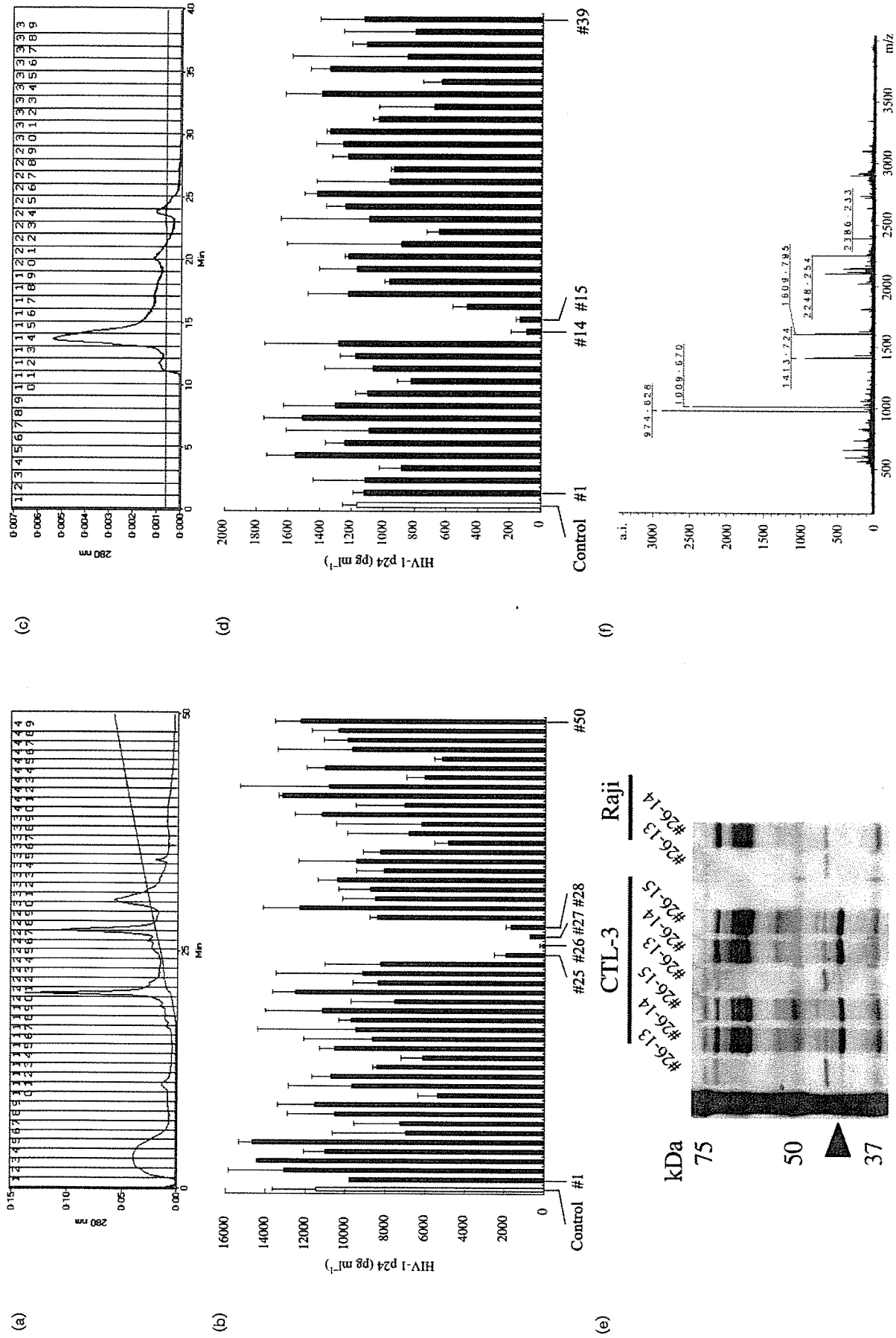


Fig. 1. Cell- and soluble factor-mediated suppression of HIV-1 replication by CD8$^+$ CTLs. (a) Cells (3×10^5 cells per well) (filled bar) or culture supernatants (50% vol.) (open bar) of two allo-specific CD8$^+$ CTL lines (CTL-2 and -3) established from two uninfected donors were co-cultured with phytohaemagglutinin-stimulated autologous CD4$^+$ cells (10^5 cells per well) infected with HIV-1 NL4-3 for 2 h *in vitro*. After culturing for 4 days, the amount of HIV-1 p24 in the supernatants was measured by ELISA (Cellular Products). The results indicate the amounts of HIV-1 p24 (%) compared with infected CD4$^+$ cells alone. The CTL culture supernatants used were prepared as fetal bovine serum (FBS) free and supplemented with 10% FBS for use. To prepare FBS-free supernatants, CTLs were washed 24 h after stimulation with MMC-treated Raji cells and the supernatants were harvested following another 48 h of culture at a concentration of 5×10^6 cells ml^{-1} in FBS-free RPMI 1640 in the presence of 50 U rhIL-2 ml^{-1}. The supernatants were passed through 0·22 μm filters before use. (b) Similarly prepared culture supernatants of CTL-2 and -3 were added at 50% concentrations into acutely HIV-1 NL4-3 (filled bar)- or JR-CSF (open bar)-infected CD4$^+$ cell cultures for 4 days and the amount of HIV-1 p24 in the supernatants was measured by ELISA. The results indicate the mean ± SD of duplicate wells. Similar results were obtained when CTL supernatants prepared in the presence of FBS were used. The viability of CD4$^+$ T cells after 4 days of culture with medium or with CTL-2 and CTL-3 culture supernatants was 95·37 ± 0·13, 97·84 ± 0·15 and 98·64 ± 0·08%, respectively, as determined by flow cytometry following staining with fluorescein isothiocyanate–annexin V and 7-aminoactinomycin D.

In order to purify the suppressive factor(s), first we separated the CTL-3 culture supernatant on an anion-exchange UNO-Q1 column (Bio-Rad) with a linear gradient from 0 to 1·0 M NaCl in 10 mM potassium phosphate buffer (pH 7·0) using HPLC (Fig. 2a). The HIV-1-suppressive

Fig. 2. Purification and identification of an HIV-1-suppressive factor in CTL-3 culture supernatants by HPLC. (a) FBS-free culture supernatants of CTL-3 prepared as described in Fig. 1 legend were concentrated with Amicon Ultra-4 (Millipore), applied to an anion-exchange column (UNO-Q1; Bio-Rad) and eluted with a linear gradient (0–1·0 M NaCl) in 10 mM potassium phosphate buffer at a flow rate of 1 ml min^{-1} using an HPLC system (BioCAD; Applied Biosystems). Protein peaks, conductivity and fraction numbers are indicated. (b) Each fraction was dialysed against PBS, cleared through a 0·22 μm filter, supplemented with 10% FBS and added to an HIV-1 NL4-3-infected CD4$^+$ cell culture at a concentration of 50%. The same volume of PBS with 10% FBS was used as a control (open bar). After 4 days of incubation, the amount of HIV-1 p24 in the culture supernatants was measured by ELISA. The results indicate the mean ± SD of duplicate wells. (c) Fraction #26 separated by anion-exchange HPLC was further subjected to gel filtration on a TSK gel 2000 column (Tosoh) using HPLC at a flow rate of 0·5 ml min^{-1} in PBS. Protein peaks, conductivity and fraction numbers are indicated. (d) Each gel-filtrated HPLC fraction was evaluated for HIV-1-suppressive activity as described in (b). (e) Fractions #26-13, -14 and -15, separated as described in (c) from two independently prepared samples, were subjected to 7·5% SDS-PAGE at a constant current of 24 mA per gel for 5 h. The proteins were visualized by silver staining (ProteoSilver Plus Silver Stain kit; Sigma-Aldrich). Similarly purified fractions from culture supernatants containing MMC-treated Raji cells alone served as controls. The arrow at 45 kDa indicates the bands specific for the fractions with HIV-1-suppressive activity. (f) The protein band at 45 kDa was excised from the SDS-polyacrylamide gel, digested with lysyl endopeptidase and subjected to MALDI-TOF-MS using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics) as described previously (Yamagata *et al.*, 2002). The MS spectrum of this protein as shown was analysed by MASCOT software (Matrix Science) with the NCBInr database and identified as arginine deiminase from *M. arginini*.


(a)
280 nm
Min
(b)
HIV-1 p24 (pg ml^{-1})
Control
#1
#25
#26
#27
#28
#50
(c)
280 nm
Min
(d)
HIV-1 p24 (pg ml^{-1})
Control
#1
#14
#15
#39
(e)
CTL-3
Raji
#26-13
#26-14
#26-15
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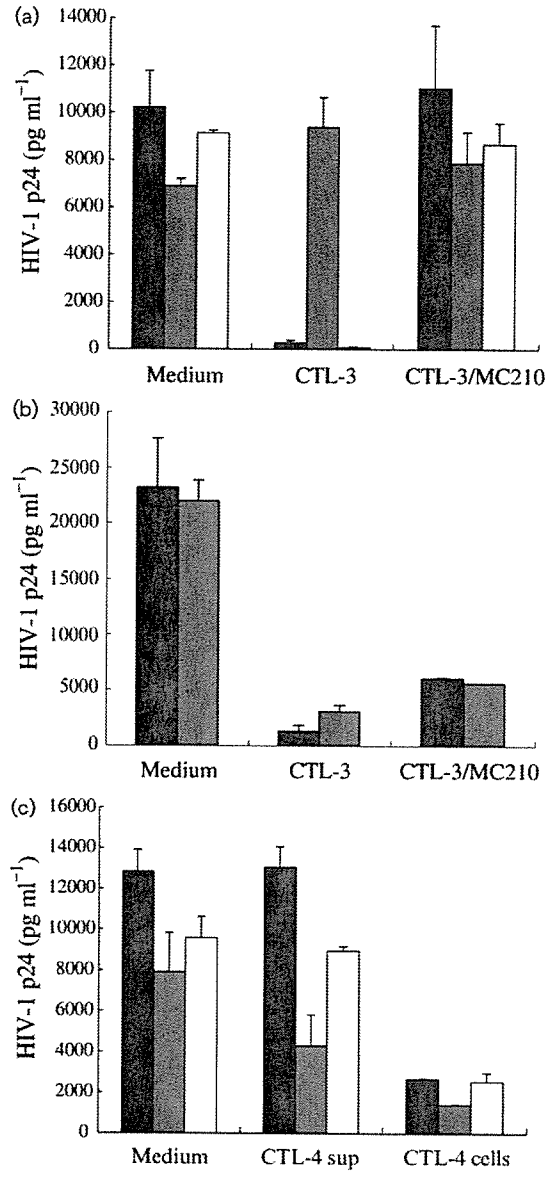

Fig. 3. L-Arginine and MC210 restore the supernatant- but not cell-mediated suppression of HIV-1 replication by CTL-3. (a) Culture supernatants from CTL-3 cells and CTL-3 cells treated with an antibiotic against mycoplasma (MC210; Dainippon Pharmaceutical) for 2 weeks (CTL-3/MC210) were added to an acutely HIV-1 NL4-3-infected CD4$^+$ cell culture in the absence (filled bar) or presence of 10 mM L-arginine (shaded bar) or L-glycine (open bar). HIV-1 p24 concentration in the supernatants was measured by ELISA 4 days after infection. (b) CTL-3 or CTL-3/MC210 cells were directly co-cultured with phytohaemagglutinin-stimulated autologous CD4$^+$ cells (10^5 cells per well) acutely infected with HIV-1 NL4-3 at a CTL : CD4$^+$ cell ratio of 3 in the absence (filled bar) or presence (shaded bar) of 10 mM L-arginine for 4 days. The amount of HIV-1 p24 in the supernatants was measured by ELISA. (c) Culture supernatants or cells from another mycoplasma-free CTL line (CTL-4) were co-cultured with HIV-1 NL4-3-infected autologous CD4$^+$ cells in the absence (filled bar) or presence of 10 mM L-arginine (shaded bar) or L-glycine (open bar) for 4 days and HIV-1 p24 concentration in the supernatants was measured. The results indicate the mean ± SD of duplicate wells.

activity of each fraction (1 ml) was evaluated following direct co-culture with CD4$^+$ cells infected with HIV-1 NL4-3. As shown in Fig. 2(b), fractions #25 to #28 of anion-exchange HPLC markedly suppressed the replication of HIV-1. The suppressive activity peaked at fraction #26 in more than three independent experiments.

Fraction #26 was subsequently separated by gel filtration on a TSK gel 2000 column (Tosoh) using HPLC (Fig. 2c). The resulting fractions, #14 and #15 (designated #26-14 and #26-15, respectively, hereafter) markedly suppressed HIV-1 replication (Fig. 2d).

Next, HIV-1-suppressive fractions #26-14 and -15, serially purified by anion-exchange and gel-filtrated HPLC from CTL-3 supernatants, were resolved by 7·5 % SDS-PAGE. As a control, similarly purified fractions from culture supernatants of MMC-treated Raji cells were used. As shown in Fig. 2(e), silver staining showed protein bands at 45 kDa that were observed only in #26-14 and -15, and not in the control fractions. The 45 kDa protein bands were cut from a separately prepared negatively stained gel (Wako) and subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Fig. 2(f) demonstrates the MS spectrum of peptides extracted from the 45 kDa band after digestion with lysyl endopeptidase, obtained using an Ultraflex TOF/TOF mass spectrometer (Kristensen *et al.*, 2000; Yamagata *et al.*, 2002). Several peaks were analysed further by MS/MS (data not shown). Computer analysis of the MS and MS/MS spectra using MASCOT software (Matrix Science) with the NCBInr database identified the protein as arginine deiminase from *Mycoplasma arginini*. The results strongly indicated that the CTL-3 cells had been contaminated by *M. arginini*, although growth of the CTL-3 cells was not affected.

Finally, we examined whether the suppressive effects of CTL-3 supernatants on HIV-1 replication were attributed to arginine deiminase. Arginine deiminase is a mycoplasma enzyme that catalyses the imine hydrolysis of arginine to citrulline and ammonia. When L-arginine (10 mM) was added to an HIV-1-infected CD4$^+$ cell culture together with CTL-3 culture supernatant, the suppression of HIV-1 replication was almost completely restored (Fig. 3a), clearly indicating that the suppressive effects were mediated mostly by arginine deiminase. L-Glysine (10 mM) as a control showed no effect. In addition, treatment of the CTL-3 culture with antibiotic MC210 against mycoplasma for approximately 2 weeks abolished the HIV-1-suppressive activity of the culture supernatants (Fig. 3a).

In contrast, CTL-3 and MC210-treated CTL-3 cells continued to show significant levels of suppressive effects on HIV-1 replication when directly co-cultured with HIV-1-infected autologous CD4$^+$ cells in the presence of L-arginine (Fig. 3b), indicating that cell-mediated suppression of HIV-1 replication was not attributed to arginine deiminase. The presence of cell-mediated but not supernatant-mediated

suppressive effects on HIV-1 replication was also confirmed by using another mycoplasma-free $CD8^+$ CTL line (CTL-4) (Fig. 3c).

Our results clearly indicate that arginine deiminase from *M. arginini* suppresses HIV-1 replication in $CD4^+$ cells *in vitro*. Although arginine is a non-essential amino acid for humans and mice, some cancers have an elevated requirement for arginine. Arginine deiminase inhibits the growth of arginine-requiring tumours such as human melanomas and hepatocellular carcinoma *in vitro* and *in vivo*, suggesting its potential use as a chemotherapeutic reagent (Curley *et al.*, 2003; Ensor *et al.*, 2002).

Mycoplasma contamination of HIV-1-infected culture causes various effects *in vitro*, such as enhancement of the cytopathic effects associated with HIV-1 replication (Lo *et al.*, 1991), inhibition of CD4 expression and gp120 binding (O'Toole & Lowdell, 1990) and apparent reduction of reverse transcriptase activity, probably due to nuclease activity (el-Farrash *et al.*, 1994; Shang *et al.*, 1995; Vasudevachari *et al.*, 1990). *Mycoplasma penetrans* isolated from HIV-1-infected individuals potentially activates T lymphocytes and HIV-1 replication, suggesting its contribution to disease progression (Sasaki *et al.*, 1995). This variety of effects might partly be due to the variety of *Mycoplasma* species.

In the present study, we identified arginine deiminase as a suppressive factor of HIV-1 replication. Although we found it in a $CD8^+$ CTL line possessing HIV-1-suppressive activity, only supernatant-mediated and not cell-contact-dependent suppression of HIV-1 was attributed to arginine deiminase. The precise mechanisms of arginine deiminase-mediated inhibition of the HIV-1 replication cycle remain to be clarified.

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Identification of a Novel Human Immunodeficiency Virus Type 1 Integrase Interactor, Gemin2, That Facilitates Efficient Viral cDNA Synthesis In Vivo

Seiji Hamamoto,[1,2]† Hironori Nishitsuji,[1]† Teruo Amagasa,[2] Mari Kannagi,[1] and Takao Masuda[1]*

Department of Immunotherapeutics, Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan,[1] and Department of Maxillofacial Surgery, Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan[2]

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Retroviral integrase (IN) catalyzes the integration of viral cDNA into a host chromosome. Additional roles have been suggested for IN, including uncoating, reverse transcription, and nuclear import of the human immunodeficiency virus type 1 (HIV-1) genome. However, the underlying mechanism is largely unknown. Here, using a yeast two-hybrid system, we identified a survival motor neuron (SMN)-interacting protein 1 (Gemin2) that binds to HIV-1 IN. Reduction of Gemin2 with small interfering RNA duplexes (siGemin2) dramatically reduced HIV-1 infection in human primary monocyte-derived macrophages and also reduced viral cDNA synthesis. In contrast, siGemin2 did not affect HIV-1 expression from the integrated proviral DNA. Although Gemin2 was undetectable in cell-free viral particles, coimmunoprecipitation experiments using FLAG-tagged Gemin2 strongly suggested that Gemin2 interacts with the incoming viral genome through IN. Further experiments reducing SMN or other SMN-interacting proteins suggested that Gemin2 might act on HIV-1 either alone or with unknown proteins to facilitate efficient viral cDNA synthesis soon after infection. Thus, we provide the evidence for a novel host protein that binds to HIV-1 IN and facilitates viral cDNA synthesis and subsequent steps that precede integration in vivo.

When a cell is infected with a retrovirus, the viral genome is subjected to several processes that include uncoating, reverse transcription of the viral genomic RNA into a cDNA copy by use of reverse transcriptase (RT), transport of this cDNA into the nucleus, and integration of the cDNA into the host chromosome. These early events are mediated through the interactions of several viral proteins and host factors with the viral genome, often referred as the reverse transcription complex or preintegration complex (4, 8, 16). The integration of a viral cDNA copy into a host cell chromosome is accomplished by integrase (IN) (24).

Mutational analyses of human immunodeficiency virus type 1 (HIV-1) IN have suggested putative roles for IN at steps prior to integration, such as uncoating (25, 29, 32), reverse transcription (11, 29, 37, 39), and nuclear import of viral cDNA (5, 20, 37). However, the mechanisms for these pleiotropic effects of IN mutations are largely unknown. Several cellular proteins, including integrase interactor 1 (23, 41) and human lens epithelium-derived growth factor-transcription coactivator p75 (27, 28), have been reported to interact directly with HIV-1 IN for chromosomal targeting of HIV-1 IN. Meanwhile, there has been increasing evidence of physical interactions between IN and RT during reverse transcription of HIV-1 (12, 19, 42), murine leukemia virus (MLV) (13), and *Saccharomyces cerevisiae* retrovirus-like element Ty3 (33). The results of an endogenous RT assay using purified HIV-1 virus particles also suggested that a cellular cofactor(s) might be required to complete reverse transcription in vivo (29).

In this study, we identified a novel host protein that binds to HIV-1 integrase and plays a critical role in HIV-1 infection in vivo. Survival motor neuron (SMN)-interacting protein 1 (Gemin2) (26) is a member of the SMN complex that mediates the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) (3, 15, 21, 26, 30). Our results suggest that Gemin2 interacts with IN in the incoming virus genome complex and is essential for HIV-1 infection and viral cDNA synthesis and subsequent steps that proceed to integration.

MATERIALS AND METHODS

Plasmids. DNA fragments of the full-length HIV-1 IN were amplified by PCR from the HIV-1 pNL4-3lucΔenv vector by use of the oligonucleotide sense primer GBT9IN-1R (5′-CCGGAATTCTTTTTAGATGGAATA-3′) and the oligonucleotide antisense primer GBT9INenBH (5′-ACGGATCCTTAATCCT CATCCTG-3′). In the pNL4-3lucΔenv vector, the *env* gene has been deleted and the *nef* gene has been replaced with the firefly luciferase (Luc) gene (29). The amplified PCR products were digested with the restriction enzymes EcoRI and BamHI and ligated into the pGBT9 vector (BD Biosciences, San Jose, CA) (pGBT-IN). The pGBT9 vector constructs with truncated forms of IN (pGBT-ΔN-IN, pGBT-ΔN/ΔC-IN, and pGBT-INΔC) were similarly prepared using the following primer pairs: for pGBT-ΔN-IN, the sense primer GBT9IN50R (5′-CCGGAA TTCCATGGACAAGTAGAC-3′) and the antisense primer GBT9INenBH (corresponding to IN amino acid positions 51 to 288); for pGBT-ΔN/ΔC-IN, the sense primer GBT9IN50R and the antisense primer GBT9IN210BH (5′-ACGG ATCCAGTTTGTATGTCTGT-3′) (corresponding to IN amino acid positions 51 to 210); and for pGBT-IN-ΔC, the sense primer GBT9IN-1R and the antisense primer GBT9IN210BH (corresponding to IN amino acid positions 1 to 210). The pGAD-GH vector containing a HeLa cDNA library pretransformed into yeast strain Y187 was purchased from BD Biosciences. For preparation of a lentiviral vector expressing FLAG-tagged Gemin2, an EcoRI-XbaI fragment

* Corresponding author. Mailing address: Department of Immunotherapeutics, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81-3-5803-5799. Fax: 81-3-5803-0235. E-mail: tmasu.impt@tmd.ac.jp.
† S.H. and H.N. contributed equally to this work.

from pTRE-FLAG-Gemin2 (34) (kindly provided by G. Dreyfuss, University of Pennsylvania) was ligated into the pCSII-CMV-MCS vector (31) (kindly provided by H. Miyoshi, RIKEN Tsukuba Institute) or the pEF6/V5-HisA expression vector (Invitrogen). For construction of an small interfering RNA (siRNA)-resistant Gemin2 expression vector, silent point mutations were introduced into the target sequences of siGemin2#372 by use of mutagenic oligonucleotides (5′-CCTCCCTTGCTTAGCATCGTAAGCAGAATGAATC-3′).

Yeast mating and cDNA isolation. The pGBT-IN plasmid was transformed into yeast strain AH109, and yeast mating was performed according to the manufacturer's instructions (BD Biosciences). Positive transformants were verified for beta-galactosidase activity as described in the instructions.

Cells. HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; MP Biomedicals Inc., Irvine, CA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 2 μg/ml sodium hydrogen carbonate (Wako, Osaka, Japan), 0.88 μg/ml tissue culture powdered DMEM amino acid and vitamin medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. Human peripheral blood mononuclear cells were isolated from HIV-1-seronegative healthy individuals by use of Ficoll-Paque Plus (Amersham Pharmacia Biotech Inc., Tokyo, Japan) density centrifugation. Human monocyte-derived macrophages (MDMs) were subsequently isolated from the peripheral blood mononuclear cells and cultured with RPMI 1640 (GIBCO, Invitrogen) supplemented with 5% human AB serum (Nippon Bio-supply Center, Tokyo, Japan) as described previously (37).

Construction of siRNAs. An siRNA duplex (small interfering green fluorescent protein [siGFP]) targeting the sequence 5′-AAGGUGCUCUGAAGUGA GGCU-3′ in the open reading frame of human Gemin2 (siGemin2) and a control double-stranded RNA targeting the 5′-CGGCAAGCUGACCCUGAA GUUC-3′ sequence in siGFP were purchased from QIAGEN K. K. (Tokyo, Japan). The targeting sequences of Gemin2 for the chemically modified synthetic siRNA duplexes (Stealth RNAi) purchased from Invitrogen were as follows: for siGemin2#372, 5′-CCU UGC UUA GUA UUG UUA GCA GAA U-3′; for siGemin2#373, 5′-GGA UAG CAA AGA UGA UGA GAG GGU U-3′; for siGemin2#374, 5′-UGA CCA ACG UGA UUU AGC UGA UGA G-3′; for siGemin2#375,5′-CAA GAA GGU GCU CUG AAG UGA GGC U-3′; for siGemin2#mm375, 5′-CAA GGA CGU UCU AAG GUG GAG AGC U-3′; for small interfering SMN#271 (siSMN#271), 5′-UAC UGG CUA UUA UAU GGG UUU CAG A-3′; for siSMN#272, 5′-CCA AAA GAA GAA UAC UGC AGC UUC C-3′; for siGemin3#430, 5′-CCA GUG AUC CAA GUC UCA UAG GUU U-3′; for siGemin3#431, 5′-GCU GCC GCU UCU CAU UCA UAU UAU U-3′; for siGemin3#432, 5′-GCU GUU GGA UCU CCU GGC AGA AUU A-3′; for siGemin4#354, 5′-GAA CUG CCU GAU GAG UCC CGU GAA A-3′; for siGemin4#355, 5′-AGG GAU UCC AGU GGC UGC UCU UCU U-3′; for siGemin4#356, 5′-UCU CGG AGA GGA UGC UGU CUC UCU U-3′; for siGemin6#950, 5′-CCC UUA GAA UGG CAA GAU UAC AUU U-3′; for siGemin6#951, 5′-GCA AAG CAU ACA GCC CAG AGG AUC U-3′; and for siGemin6#952, 5′-UCU GUC GCG UGU UCA GGA UCU UAU U-3′.

Transfection of siRNA. Cells were transfected with 40 nM of siRNA (siGemin2 or siGFP) by use of Oligofectamine or Lipofectamine 2000 (Invitrogen, CA). After 4 h of incubation, the cells transfected with the siRNA were added to 250 μl DMEM supplemented with 30% (vol/vol) heat-inactivated FBS. After 12 to 18 h of incubation, the transfected cells were washed and replaced with DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate. After 24 h of incubation, the siRNA duplex was transfected again to achieve efficient depletion of the target protein.

Virus preparation and infection. Pseudotyped viruses were generated as described previously (29). Briefly, 293T cells were transfected with the pNL43lucΔenv vector together with an ampho-MLV (pJD-1) (29) or vesicular stomatitis virus-G expression vector (pHCMVG) (40) by use of Lipofectamine (Invitrogen). The culture supernatants of the transfected cells (5 ml) were harvested 48 h posttransfection, filtered through 0.45-mm-pore-size filters, and used as virus preparations. The virus preparation was treated with DNase I (Worthington, Lakewood, NJ) (20 μg/ml) in the presence of 10 mM $MgCl_2$ at 37°C for 40 min to avoid plasmid DNA contamination. An aliquot of the virus preparation was incubated at 65°C for 30 min and used as a heat-inactivated control. To monitor the amount of virus in each preparation, HIV-1 p24 antigen levels were determined using an enzyme-linked immunosorbent assay. To monitor viral gene expression from each plasmid vector, luciferase activity in the transfected cells was also measured. At 48 h posttransfection, the 293T cells were lysed with 1 ml of 1× cell culture lysis reagent (Promega, Madison, WI), and 10 μl of each cell lysate was subjected to the luciferase assay. After incubation for 6 h, the viruses were removed and the cells were washed and incubated with fresh culture medium at 37°C in a 5% CO_2 incubator.

Analysis of HIV-1 cDNA synthesis. Total cells were harvested from each well periodically after infection with pseudotyped viruses. After washes with phosphate-buffered saline (PBS), total DNA was extracted by the urea-lysis method (29). Quantitative analyses of the amplified products and the rate of viral cDNA synthesis were performed using real-time quantitative PCR (LightCycler; Roche Diagnostics, Mannheim, Germany) as described previously (20).

Antibodies. The anti-Gemin2 monoclonal antibody (MAb), the anti-Ran MAb, and the anti-SMN MAb were purchased from BD Bioscience. The anti-Gemin3 MAb was purchased from ImmuQuest (ImmuQuest Ltd., Barwick TS175AL, United Kingdom). The anti-Gemin4 and anti-Gemin6 MAbs were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-HIV-1 IN MAb was purchased from Microbix Biosystems Inc. (Toronto, Canada). Anti-HIV-1 p24 MAb was purchased from Chemicon International (Temecula, CA).

GST pull-down assay. DNA fragments encoding the N-terminal (amino acid positions 1 to 55), central (amino acid positions 50 to 212), and C-terminal (amino acid positions 213 to 288) HIV-1 IN regions or full-length (amino acid positions 1 to 288) HIV-1 IN were amplified by PCR using pNL43lucΔenv as a template. The amplified products were ligated to BamHI-EcoRI-digested pGEX-2T vector (Amersham Pharmacia Biosciences Inc., Uppsala, Sweden). Recombinant glutathione-*S*-transferase (GST)–IN was prepared as described previously (20). HeLa cell lysate (100 to 200 μg) was incubated with each GST-IN protein (200 nM) immobilized on glutathione-Sepharose beads in binding buffer (1.0% Triton X-100-1 mM phenylmethylsulfonyl fluoride in 1× PBS) for 15 min at 4°C. The beads were then washed five times with wash buffer (0.3% Triton X-100 in 1× PBS) and eluted with elution buffer. An aliquot of the pulled-down fraction was subjected to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and Western blotting analysis.

Immunoprecipitation experiments. Total cell extracts were prepared as described previously (34). Briefly, cell pellets were suspended in RSB-100 (10 mM Tris-HCl [pH 7.4], 2.5 mM $MgCl_2$, 100 mM $NaCl_2$) containing 0.1% Nonidet P-40 and protease inhibitors followed by centrifugation at 10,000 × g for 15 min. Extracts were incubated with an anti-FLAG antibody (Sigma) for 1 h and subjected to immunoprecipitation using the Catch and Release system according to the manufacturer's instructions (Upstate, Lake Placid, NY).

RESULTS

Identification of a cellular factor that binds to HIV-1 IN. To identify host proteins that bind to HIV-1 IN, we used the yeast two-hybrid system and the yeast-mating method (BD Biosciences, San Jose, CA). A plasmid carrying the entire HIV-1 IN fused with the GAL4 DNA binding domain-coding region (pGBT-IN) was used as a bait vector. Five positive-testing clones were obtained from ~2×10^7 prey plasmids containing a human HeLa cDNA library expressed as GAL4 activation domain fusion proteins. DNA sequence analysis of three of these positive-testing clones resulted in identification of a single cDNA clone encoding an amino acid fragment corresponding to residues 137 to 238 of SMN-interacting protein 1 (Gemin2; formerly SIP1) (26). We therefore termed residues 137 to 238 of Gemin2 IBDG2 (for "IN binding region of Gemin2"). Various bait vectors carrying the full-length or truncated forms of HIV-1 IN were cotransformed with the GAL4 activation domain vector carrying IBDG2 (pGAD-IBDG2) into yeast strain AH109 or HF7c. HIV-1 IN comprises three distinct functional domains (10, 38). Deletion of the COOH-terminal domain of IN (pGBT-DC-IN) significantly reduced the binding of IN to IBDG2, whereas deletion of the NH_2-terminal domain of IN (pGBT-DN-IN) had little effect on the binding activity (Fig. 1A). Deletion of both the NH_2-terminal and the COOH-terminal domains (pGBT-DN/DC-IN) resulted in low-level but significant binding to IBDG2. These results suggest that the COOH-terminal domain of the IN is the minimum domain responsible

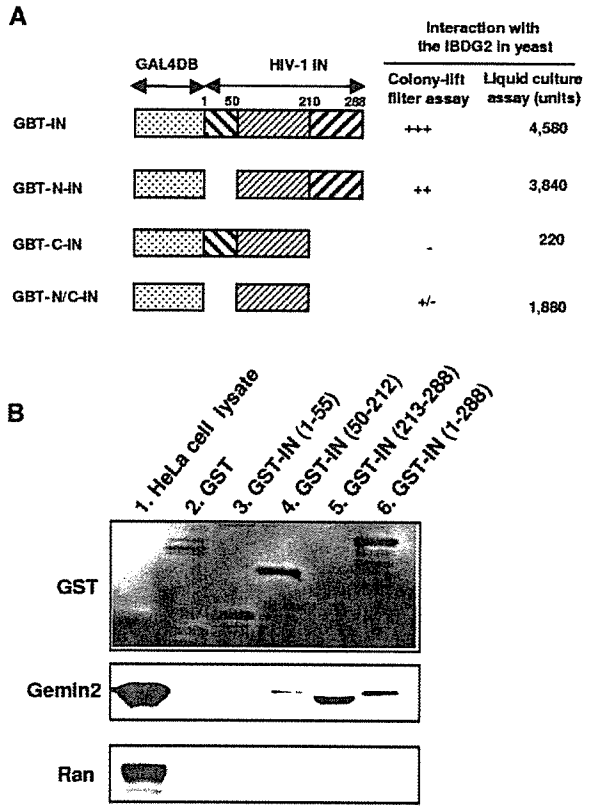

FIG. 1. Interaction of IN with Gemin2. (A) Yeast AH109 or HF7c cells were cotransformed with the pGAD-IBDG2 vector carrying the IBDG2 domain of Gemin2 together with various pGBT9 plasmids carrying full-length (pGBT-IN) or truncated (pGBT-DN-IN, pGBT-DC-IN, and pGBT-DN/DC-IN) forms of HIV-1 IN. Dashed boxes in the diagram indicate the region of HIV-1 IN retained in each pGBT-IN vector. The amino acid positions of HIV-1 IN are numbered according to the NL43 sequence. Interaction of coexpressed proteins was determined with a beta-galactosidase colony-lift filter assay (+++, dark-blue colony; +, medium-blue colony; +/−, light-blue colony; −, white colony) or a liquid-culture assay for estimating beta-galactosidase activity (expressed in units). The value for bait vector only (pGBT-IN) was used as the background value for the liquid-culture assay. (B) Untreated (lane 1) or pull-down (lanes 2 to 6) fractions of HeLa cell lysates on glutathione-Sepharose beads bound to GST-IN protein containing the N-terminal (1-55), central (50-212), C-terminal (213-288), or full-length (1-288) HIV-1 IN were subjected to Western blotting analysis using anti-GST, anti-Gemin2, and noninteracting (control) anti-Ran antibodies.

for the binding to the IBDG2 and that the central domain of IN partly contributed to the binding. We next examined the specific interaction of HIV-1 IN with endogenous Gemin2 in human cells. Recombinant GST fused with the entire IN protein or with the NH_2-terminal, central core, or COOH-terminal domain of HIV-1 IN was used for the pull-down experiment (Fig. 1B). Neither the NH_2-terminal domain of IN (GST-IN1-55) nor the control GST showed any specific binding activity to the endogenous Gemin2; however, the COOH-terminal domain of IN (GST-IN213-288) and full-length IN (GST-IN1-288) each bound to Gemin2. The central core domain of IN (GST-IN55-212) also bound to Gemin2 but with much weaker affinity than the COOH-terminal domain (GST-IN213-288). Thus, we confirmed that HIV-1 IN interacts specifically with full-length Gemin2 endogenously expressed in human cells.

Interaction of Gemin2 with IN of HIV-1 preintegration complex. Gemin2 interacts tightly with the SMN protein to form a macromolecular complex termed the SMN complex (26, 35). To address the interaction of Gemin2 and HIV-1 IN during the viral infection cycle, we first measured the amounts of Gemin2 and other constituents of the SMN complex (SMN and Gemin3) (6, 26) in purified, cell-free virus particles. None of these proteins were detected in the virus particles (Fig. 2A), suggesting that Gemin2 was not incorporated into HIV-1 virus particles. We next used coimmunoprecipitation to address the interaction of Gemin2 and IN during acute infection of HIV-1. Since the antibodies for Gemin2 or HIV-1 IN were available only for immunoblotting but not for immunoprecipitation, we used a lentivirus-vector gene delivery system to transduce FLAG-tagged Gemin2 (34) into HeLa cells (Fig. 2B). The HeLa cells expressing FLAG-tagged Gemin2 (Flag-Gemin2/HeLa) or control HeLa cells transduced with empty vector were infected with HIV-1 pseudotype virus. At 2 or 6 h postinfection, IN efficiently coimmunoprecipitated with FLAG-Gemin2 in the Flag-Gemin2/HeLa cell extract (Fig. 2C), and a significant amount of HIV-1 cDNA synthesized de novo was detected by PCR at each time point. The HIV-1 cDNA in the similarly prepared immunoprecipitate (IP) fraction from the control HeLa cells was below the detectable level following infection (Fig. 2D, upper panel). Quantitative PCR analysis of the HIV-1 cDNA in the IP fraction of the Flag-Gemin2/HeLa cells at 2 h and 6 h postinfection showed that 1,200 and 2,500 copies were present, respectively (Fig. 2D, lower panel), corresponding to 10% to 20% of the total cDNA in the input fraction at each time point. These results suggest that Gemin2 might interact with an incoming HIV-1 preintegration complex through IN after the entry and uncoating of viral genome.

Functional role of Gemin2 during the HIV-1 cycle. We next addressed the functional role of Gemin2 during the HIV-1 infection cycle by using the siRNA technique (36) to specifically deplete Gemin2 from cells. We directed the 21-nucleotide siRNA duplexes (9) against a coding region of the Gemin2 gene (siGemin2). The level of Gemin2 was monitored periodically after siRNA transfection. A reduction in the Gemin2 level was evident 1 day after the siRNA transfection and persisted for at least 4 days in culture (data not shown). At 48 h post-siRNA transfection, the siGemin2 specifically reduced the level of Gemin2 to from 20% to 40% of the level seen with the mock-treated or negative-control siGFP-treated HeLa cells (Fig. 3A). Slight reductions in levels of the SMN complex constituents, SMN and Gemin3 (6), were also noticed following the siGemin2 treatment.

Since the SMN complex regulates the biogenesis of the snRNP complex, we first examined the effects of siGemin2 on gene expression from the HIV-1 provirus and the infectivity of progeny viruses. siGemin2 or a control siGFP duplex (siCont) was cotransfected into 293T cells with an HIV-1 molecular clone, pNL43lucΔenv, and a vector expressing an amphotropic MLV or vesicular stomatitis virus-G envelope for a subsequent single-round infection assay. We measured the levels of p24 (HIV-1 capsid protein) 48 h after transfection. The amount of HIV-1 p24 in the culture supernatants of the siGemin2-transfected 293T cells was comparable to that in control and siCont-

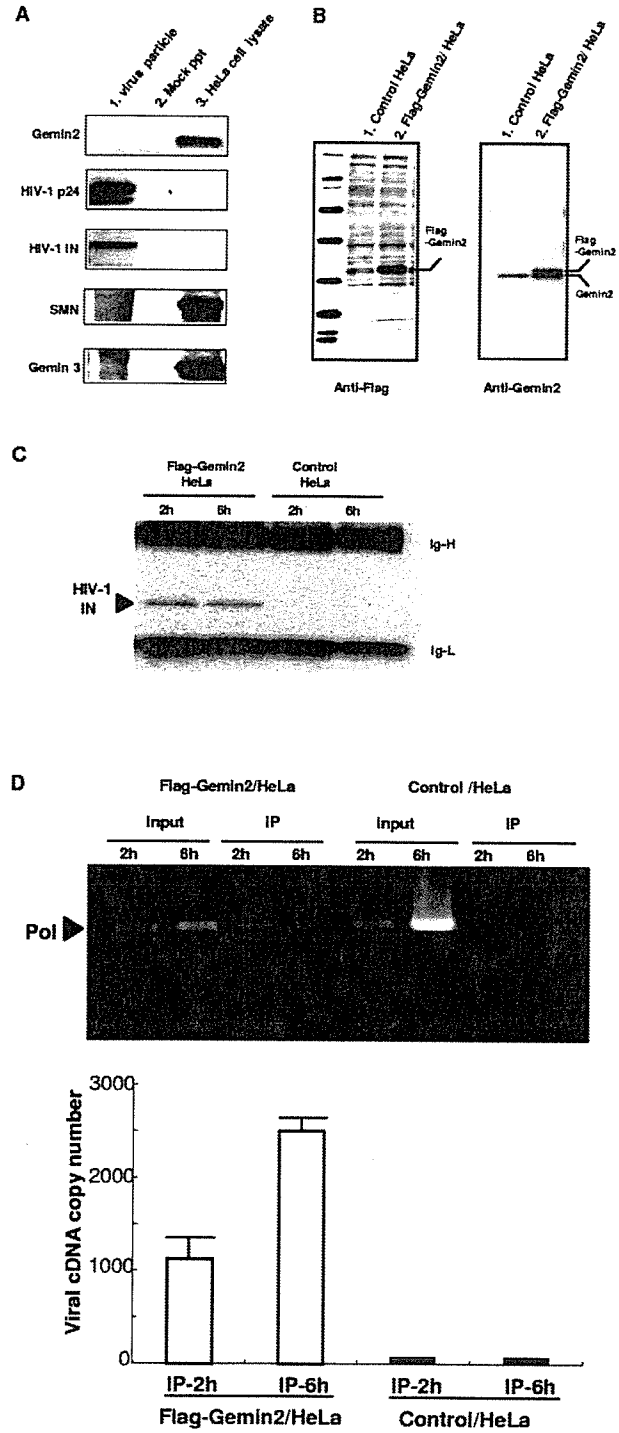

FIG. 2. Gemin2 interacts with the incoming HIV-1 genome complex. (A) Virus particles in the culture supernatant of 293T cells cotransfected with pNL43lucΔenv and pHCMVG were pelleted by ultracentrifugation (1 h at 315,000 × *g*) and subjected to Western blotting analysis using anti-Gemin2, anti-HIV-1 IN, or anti-p24 antibodies. Similarly prepared pellet fractions from mock-transfected cell supernatants (Mock ppt) and a HeLa whole-cell lysate were used as negative and positive controls, respectively. (B) HeLa cells were transduced with an empty vector (Control HeLa) or a FLAG-tagged Gemin2-expressing lentiviral vector (Flag-Gemin2/HeLa) and then subjected to Western blotting using an anti-FLAG antibody (left panel) or anti-Gemin2 antibody (right panel). (C) The transduced HeLa cells were infected with HIV-1 pseudotype virus. At 2 or 6 h postinfection, the cell lysates were immunoprecipitated with an anti-FLAG antibody and then subjected to Western blotting using an HIV-1 IN antibody. IgH, immunoglobulin heavy chain; IgL, immunoglobulin light chain. (D) Nucleic acids extracted from whole-cell lysates (input) or the IP obtained as described for panel C were subjected to PCR analysis of HIV-1 cDNAs. Amplified products (Pol) were separated in a 2% agarose gel and visualized by SYBR green staining (upper). The copy number of viral cDNA (R/gag) in each sample was estimated with real-time quantitative PCR (lower panel). Means ± standard errors (SE) from triplicate assays are shown.

transfected 293T cells (Fig. 3B, left panel). Furthermore, the progeny viruses released from the siGemin2-transfected 293T cell retained their infectivity (Fig. 3B, right panel). Thus, depletion of Gemin2 did not lead to any significant effect on proviral gene expression, virus release, or subsequent viral infectivity.

In contrast, HIV-1 infectivity was significantly reduced when the siGemin2 duplex was introduced into cells before viral infection (Fig. 3C). To exclude nonspecific or off-target effects (22) of the siGemin2 duplex originally tested here, we used four additional chemically modified synthetic siRNA duplexes (Stealth RNAi; Invitrogen) targeting different sequences within Gemin2 (Gemin2#372, Gemin2#373, Gemin2#374, and Gemin2#375) and an siGemin2 duplex carrying several nucleotide substitutions as a mismatch siGemin2 control (mm375). We observed significant reductions of HIV-1 infectivity that correlated well with the amount of specific reduction of Gemin2 with each type of siRNA treatment (Fig. 3C). In addition, we constructed an siRNA-resistant Gemin2 expression vector carrying silent point mutations in the target sequences of siGemin2#372. The siRNA-resistant Gemin2 continued to be expressed in the presence of siGemin2#372, but expression of the endogenous Gemin2 was greatly reduced (Fig. 3D lower). Under this condition, the siRNA-resistant Gemin2 rescued HIV-1 infectivity in siGemin2-treated cells (Fig. 3D upper). These results strongly suggest the functional role of Gemin2 through interaction with incoming HIV-1 genome complexes during the early steps of HIV-1 infection.

Functional role of Gemin2 in HIV-1 infection of primary nondividing cells. Next, we addressed the functional role of Gemin2 in HIV-1 infection of human primary MDMs, a model of major natural targets for HIV-1 infection in vivo. MDMs were isolated from three different healthy donors. Transfection of siGemin2 into MDMs markedly reduced HIV-1 infectivity to from 0.3% to 10% of the levels in the control MDMs (Fig. 4B, left panel). This remarkable effect of siGemin2 observed in MDMs could be partly explained by the fact that primary MDMs constitutively express a lower level of Gemin2 than the 293T and HeLa cell lines, which have been adapted in vitro (Fig. 4A). We confirmed that reduction of Gemin2 also suppressed spreading of replication-competent HIV-1 in MDMs to less than 10% of control level for at least 7 days (data not shown). As with the results obtained using 293T and HeLa cell lines, the siGemin2 duplex did not significantly affect HIV-1 expression when introduced into MDMs 24 h after infection (Fig. 4B, right panel).

Finally, we addressed the effect of depletion of Gemin2 on HIV-1 cDNA synthesis and subsequent integration using real-

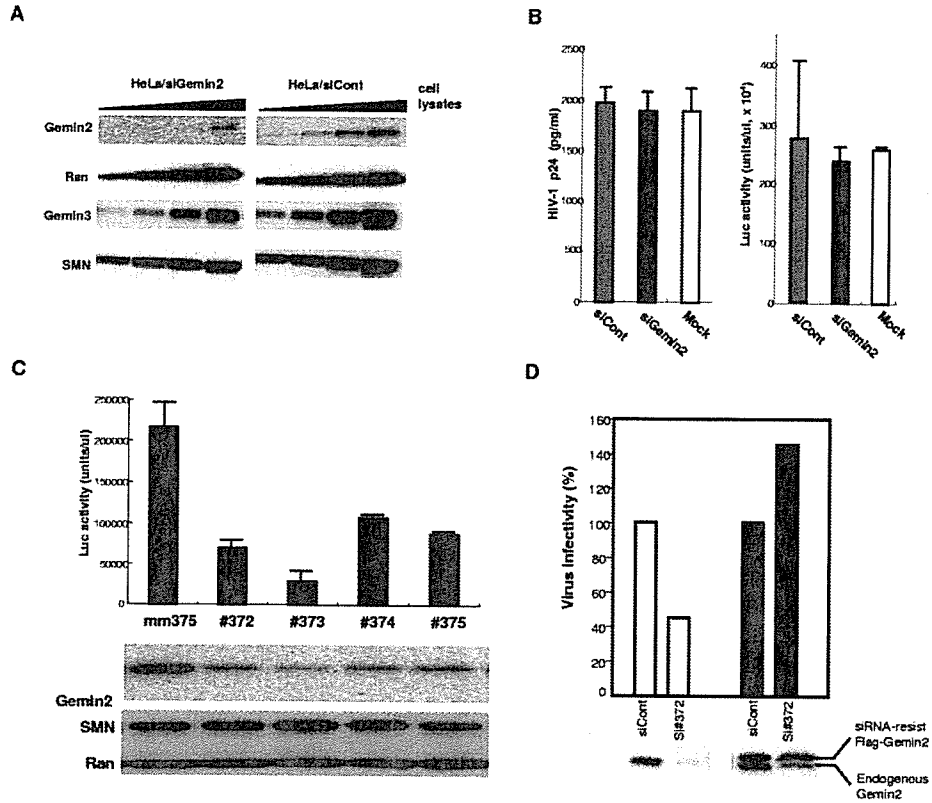

FIG. 3. Involvement of cellular Gemin2 in HIV-1 replication. (A) Total HeLa cell lysates prepared 48 h after transfection of siGemin2 (HeLa/siGemin2) or control siGFP (HeLa/siCont) were serially diluted twofold followed by Western blot analysis with antibodies against Gemin2, Ran, Gemin3, and SMN. (B) 293T cells were transfected with siGemin2 (black bar), siGFP (siCont; gray bar), or no siRNA (Mock; white bar), together with pNL-lucΔenv vector and pJD-1. The level of virus release from these 293T cells was determined by measuring HIV-1 p24 concentrations in the culture supernatant (left panel) 48 h posttransfection. These virus-containing supernatants were then incubated with HeLa cells. The cells were harvested 48 h postinfection and subjected to a luciferase assay (right panel). (C) HeLa cells were transfected with Stealth-siGemin2 (#372, #373, #374, or #375) or the control mismatch siGemin2 (mm375) 48 h before infection with HIV-1 pseudotype virus. The cells were harvested 48 h postinfection and subjected to a luciferase assay (upper panel) and Western blotting for Gemin2, SMN, and Ran (lower panel). (D) 293T cells were transfected with siRNA-resistant Flag-Gemin2 expression vector (black bars) or control empty vector (white bars) together with siGemin2#372 (si#372) or control siRNA (siCont) and then infected with HIV-1 pseudotype virus. The cells were harvested 24 h postinfection and subjected to a luciferase assay (upper) and Western blotting for endogenous Gemin2 and Flag-Gemin2 (lower). Virus infectivity is presented relative to the Luc activity in siCont-transfected cells, which was set to 100%.

time quantitative PCR analysis (20) in MDMs. Over time, the level of late reverse-transcription products (R/gag) was reduced in siGemin2-treated MDM (Fig. 4C) to ~5% of the level in the control siRNA-treated MDM at 48 h after HIV-1 infection (Fig. 4D). Of note, the amount of the early products of reverse transcription (R/U5) in the siGemin2-treated MDM was 30% to 40% of the level in the control siGFP-treated MDM (Fig. 4D). The region amplified for detection of the early reverse-transcription products (R/U5) is duplicated in the complete or nearly complete form of viral cDNA (late reverse transcription; R/gag) during the reverse-transcription step. Therefore, the marked reduction in the late reverse-transcription products by siGemin2 would be in part a consequence of the reduction in the duplication of the R/U5 region. The reduced amounts of the two-long terminal repeat (2-LTR) or integrated forms of cDNA observed in the siGemin2-treated MDMs could also be attributed to the inhibition of viral cDNA synthesis (2-LTR and integration; Fig. 4D). Thus, the dramatic reduction in the levels of late reverse-transcription products in the siGemin2-treated MDMs indicates that the abrogation of the reverse transcription of viral RNA might occur before or during the second template switch needed to complete the double-stranded viral cDNA copy, suggesting a role for Gemin2 during reverse transcription of the HIV-1 genome in vivo. We also confirmed that siGemin2 produced a similar effect on late RT products in HeLa cells. However, the inhibitory effect of siGemin2 on late RT products in HeLa cells was weaker than that in MDMs (data not shown). Therefore, possible roles of Gemin2 at other steps, including nuclear transport and integration of viral cDNA, cannot be ruled out.

Involvement of other constituents of the SMN complex. Under our experimental conditions, reduction of Gemin2 by siGemin2 was accompanied by slight decreases in SMN and Gemin3 levels (Fig. 3A and 4A). We addressed the critical point of whether Gemin2 acts on HIV-1 through the SMN complex or through another, unknown, complex by use of siRNAs targeting the other Gemin2-related proteins constituting the SMN complex. For each constituent of the complex—SMN (26), Gemin3 (6), Gemin4 (7), and Gemin6 (34)—we synthesized two or three chemically modified siRNA duplexes

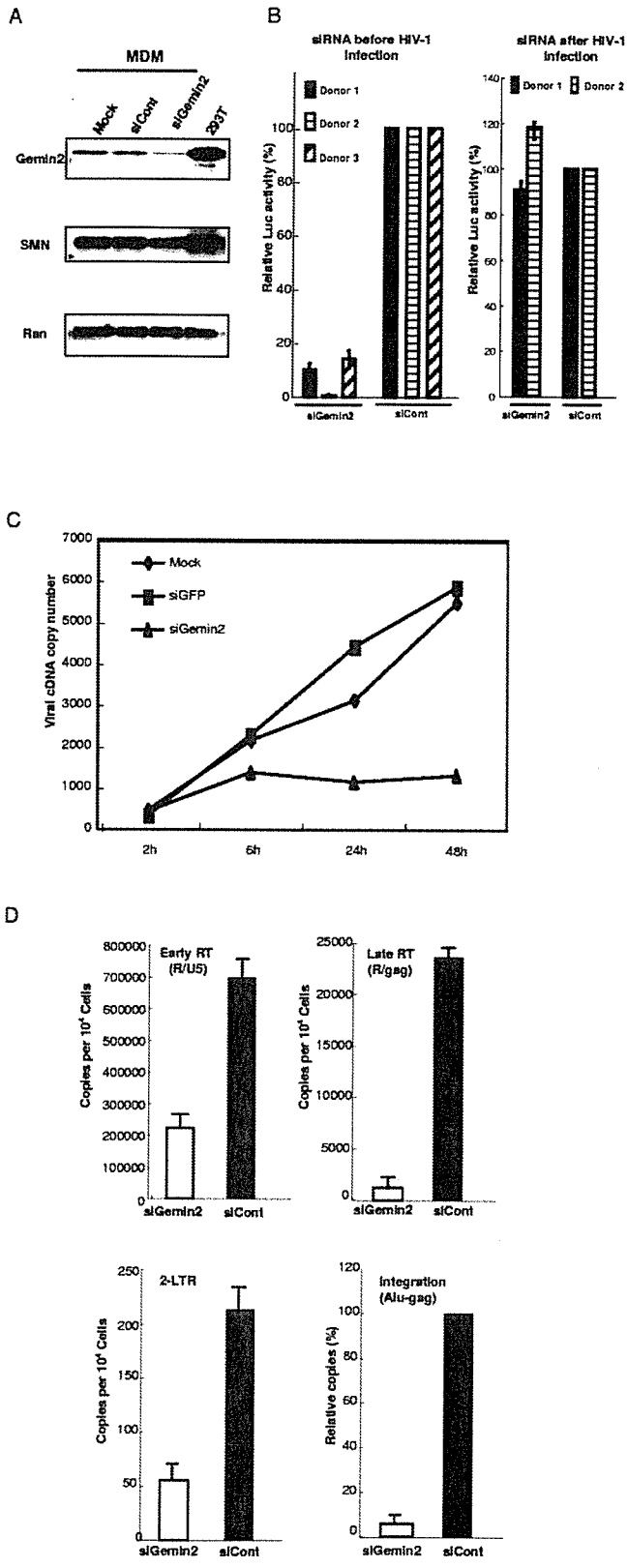

FIG. 4. Effect of siGemin2 on HIV-1 infection and cDNA synthesis in primary MDMs. (A) MDMs were transfected with siGemin2 or control siGFP (siCont) and subjected to Western blot analysis for Gemin2, SMN, and Ran. Mock, mock infected. (B) MDMs were transfected with each siRNA 24 h before (left panel) or after (right panel) infection with HIV-1 pseudotype virus. Cells were harvested and subjected to a luciferase assay 48 h (left panel) or 72 h (right panel) after infection. Relative Luc activity was calculated as a percentage of the control value in siCont-transfected cells. Each bar represents the value determined using MDMs prepared from different donors. Means ± SE from duplicate assays are shown. (C) MDMs were transfected with each siRNA 24 h before HIV-1 infection. Total DNA was extracted from siGemin2- or control siGFP-transfected MDMs at 2, 6, 24, and 48 h postinfection. Each sample was subjected to a quantitative analysis of viral cDNAs using real-time quantitative PCR with the primer pair M667-M661 (R/gag region) to measure the amount of complete or nearly complete viral cDNA (late RT). (D) Total DNA was extracted from MDMs transfected with siGemin2 or siGFP (siCont), infected with HIV-1 pseudotype virus for 48 h, and subjected to a quantitative analysis of viral cDNAs by use of real-time quantitative PCR with primer pair M667-AA55 (R/U5 region) for viral cDNA (Early-RT) or primer pair M667-M661 (R/gag region) for complete or nearly complete viral cDNA (Late-RT). We also monitored the formation of 2-LTR circular DNA by use of a primer pair that amplifies a sequence unique to the 2-LTR DNA junction and monitored the level of the integrated form of each viral cDNA by the Alu-PCR method using HIV-1-specific (M661) and Alu (Integration) primers. Results represent means ± SE.

targeting different sites within the coding sequence. Then we evaluated the effects of each siRNA on HIV-1 infection in HeLa cells (Fig. 5A). In parallel, the specific reduction in protein caused by each siRNA was also determined by examining protein expression profiles of the SMN constituents (Fig. 5B). Reduction of Gemin2 or SMN significantly blocked HIV-1 infection without showing apparent cell toxicity. However, siRNA duplexes against the SMN also significantly reduced Gemin2 levels (siSMN#271 and siSMN#272; Fig. 5B). In repeated experiments, the inhibitory effects of siSMNs on HIV-1 always correlated with the level of indirect reduction of Gemin2. Meanwhile, the reduction of Gemin3, Gemin4, and Gemin6 levels through their respective siRNA duplexes did not significantly affect HIV-1 infectivity, although siGemin3#432 and siGemin6#950 did inhibit HIV-1 infection by causing high cell toxicity. These results suggest that among the SMN constituents, Gemin2 is a critical constituent necessary to support HIV-1 infection. Thus, the effect of Gemin2 on HIV-1 infectivity might be independent of the other constituents of the SMN complex.

DISCUSSION

In this study, we have provided evidence that a novel host protein binds to HIV IN and modulates HIV-1 cDNA synthesis in vivo. We identified residues 137 to 238 of Gemin2 as binding to IN. In both the yeast two-hybrid system and a GST pull-down assay, the COOH-terminal domain of the IN was shown to be the minimum domain responsible for binding to Gemin2 (Fig. 1), although the central domain of the IN partly contributed to the binding. Gemin2 is a constituent of the SMN complex, along with other Gemin family proteins, including the putative DEAD box helicase dp103/Gemin3 (6), Gemin4 (7), Gemin5 (18), Gemin6 (34), and Gemin7 (2). Under our experimental conditions, treatment of cells with siGemin2 reduced the amount of Gemin2 protein and also resulted in slight decreases of SMN and Gemin3 levels (Fig. 3A and 4A).

Although the exact role of Gemin2 in the snRNP complex

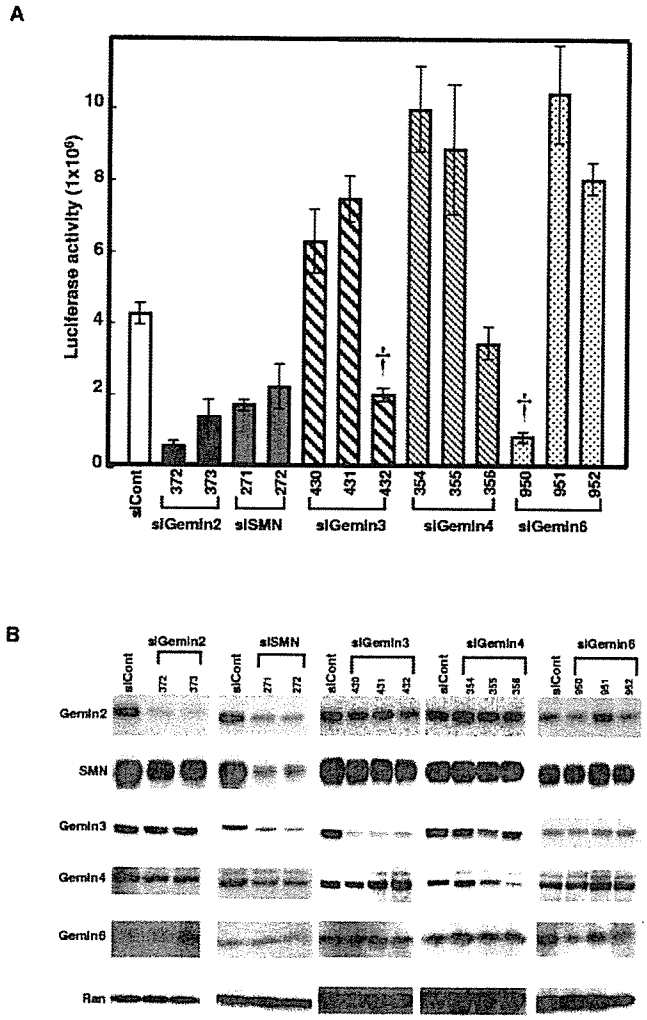

FIG. 5. Involvement of other constituents of the SMN complex. (A) HeLa cells were transfected with the Stealth siRNAs targeting Gemin2, Gemin3, Gemin4, Gemin6, or SMN 48 h before infection with HIV-1 pseudotype virus. The mismatch siGemin2 (mm375) was used as a negative control siRNA (siCont). The cells were harvested 48 h postinfection and subjected to a luciferase assay. Each bar represents the means ± SE. †, less than 20% of cells were viable 48 h after siRNA transfection. (B) Aliquots of the same cells harvested for the luciferase assay as described for panel A were subjected to Western blot analysis for Gemin2, SMN, Gemin3, Gemin4, Gemin6, and Ran.

remains to be determined, some reports suggest that it has a critical role in the assembly of the snRNP complex in the cytoplasm (3, 21, 30). More recently, the roles of the individual SMN constituents were addressed by using RNA interference (14). Feng et al. showed that a reduction of SMN leads to a decrease in snRNP assembly, the disappearance of bodies called Gems where SMN and Gemin2 are concentrated in the nucleus, and a drastic reduction in the amounts of several Gemins. Moreover, reduction of Gemin2 or Gemin6 levels strongly decreases the activity of the SMN complex. Therefore, we cannot exclude the possibility that a reduction of Gemin2 might also reduce SMN function under our experimental conditions. However, our data obtained using siRNAs targeting SMN, Gemin3, Gemin4, or Gemin6 suggest that Gemin2 is a critical constituent of the SMN complex for support of HIV-1 infection (Fig. 5). It seems likely that Gemin2 acts on the HIV-1 preintegration complex, either alone or with as-yet-unknown proteins other than the SMN constituents, although this point remains to be confirmed. In addition to the full-length Gemin2 (termed Gemin2-alpha), three splicing variants of Gemin2 (Gemin2-beta, -gamma, and -delta) have been identified (1). Gemin2-alpha has found to be ubiquitously expressed at high levels in the various normal tissues. In contrast, Gemin2-beta and -gamma are expressed at very low levels in these normal tissues (1). The Gemin2 residues that we identified as the region of Gemin2 that binds IN in the yeast two-hybrid system (residues 137 to 238, IBDG2) were shared by all splicing variants of Gemin2 except Gemin2-beta, in which residues 174 to 188 are deleted. It will be interesting to identify the contribution of other splicing variants of Gemin2 to HIV-1 infection.

The SMN complex has recently been reported to be generally used by infectious agents for RNP assembly (17). Golembe et al. demonstrated that herpesvirus saimiri uses the SMN complex to assemble Sm cores on its small RNAs (HSURs), just as occurs with host snRNPs. HSURs are the most abundant viral transcripts in latently infected, transformed T cells but are not essential for viral replication. Thus, the biological meaning of their complex formation remains to be determined. However, the authors suggest that infectious agents that engage the SMN complex may burden SMN-dependent pathways, possibly leading to a deleterious reduction in the availability of SMN complexes for essential host functions.

In the case of HIV-1 infection, we showed here that HIV-1 might require Gemin2 for efficient viral cDNA synthesis. Our results suggest that Gemin2, either alone or in concert with unidentified cellular proteins, supports HIV-1 infection, probably by supporting the reassembly of the reverse transcription complex to initiate and complete reverse transcription. Several cases of mutations in HIV-1 IN affecting reverse transcription have been described previously (11, 29, 37, 39, 42). It is, therefore, reasonable that a protein interacting with IN might play a role in reverse transcription and could also be involved in the subsequent nuclear transport and integration of viral cDNA. Since both reverse transcription and integration are essential steps for retrovirus infection, our findings will shed light on the functional role of IN during the reverse transcription of the retroviral genome and will also serve as the basis for a novel therapeutic approach to treat HIV-1 disease.

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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

Hironori Nishitsuji,[1] Michinori Kohara,[2] Mari Kannagi,[1] and Takao Masuda[1]*

Department of Immunotherapeutics, Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan,[1] and Department of Microbiology and Cell Physiology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan[2]

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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

* Corresponding author. Mailing address: Department of Immunotherapeutics, Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Phone: 81-3-5803-5799. Fax: 81-3-5803-0235. E-mail: tmasu.impt@tmd.ac.jp.

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 (4×10^6) 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

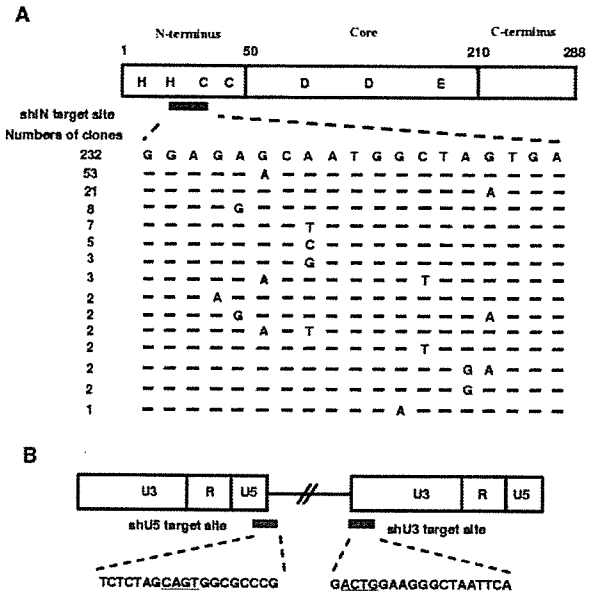

FIG. 1. shRNA target sequences of HIV-1 *int* and the U3 and U5 *att* sites. (A) Schematic representation of HIV-1 integrase (top squares) and shIN target region (bold bar). The conserved HHCC residues in the zinc-binding motif and enzyme active-site residues (DDE) located in the N terminus of the core 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.

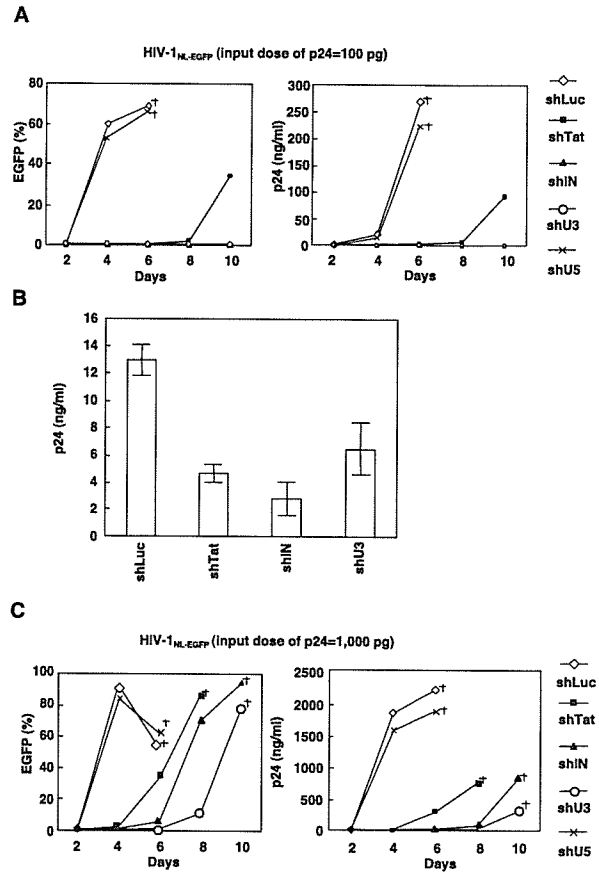

FIG. 2. HIV-1 replication in shRNA-transduced MT-4 or primary $CD4^+$ T cells. (A) MT-4 cells were transduced by the indicated shRNA using a lentivirus vector system (shLuc, shTat, shIN, shU3, and shU5). Seven days later, transduced MT-4 cells were infected by $HIV\text{-}1_{NL\text{-}EGFP}$ at an input 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 (1×10^6) 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.

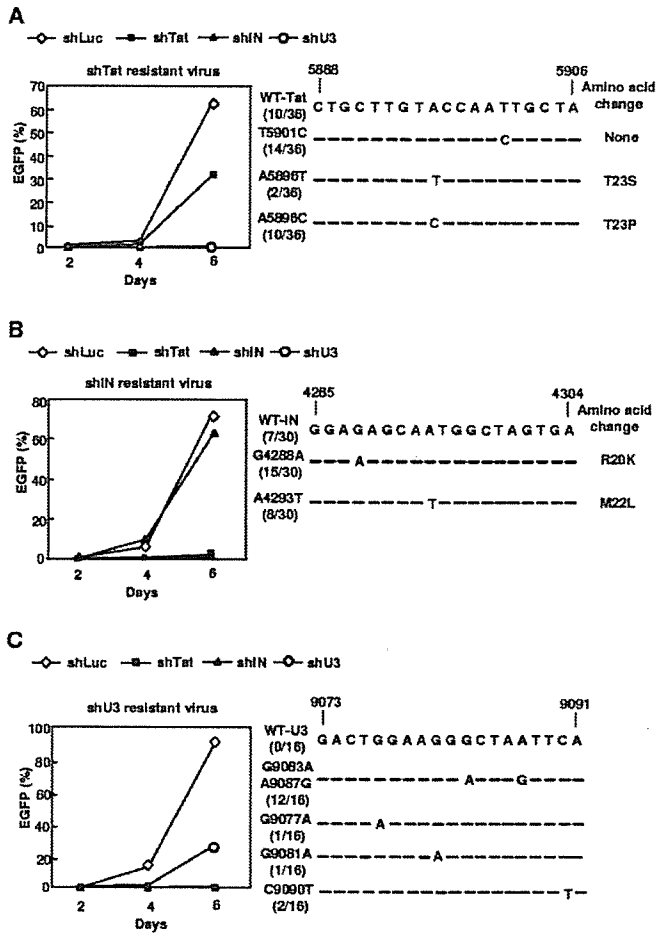

FIG. 3. shRNA-specific resistance of HIV-1 escape mutants in shRNA-transduced MT-4 cells. Culture supernatants of shTat-transduced MT-4 cells infected with a low dose of HIV-1 (100 pg of p24) (A) or shIN (B)- or shU3 (C)-transduced MT-4 cells infected with a high dose of HIV-1 (1,000 pg of p24 per 10^6 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.

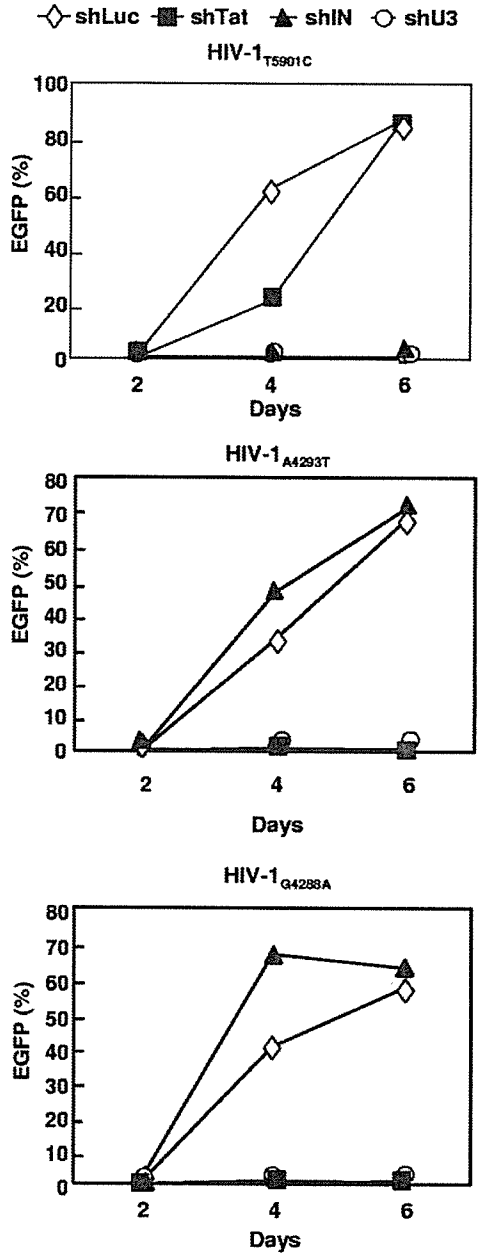

FIG. 4. shRNA-specific resistance of HIV-1 molecular clones carrying point mutations within each shRNA target site. Point mutations within the shTat target site (Tat-T5901C) or shIN target site (IN-G4288A or IN-A4293T) were introduced into the parental HIV-1 clone (pNL-EGFP) through mutagenesis. Each 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.

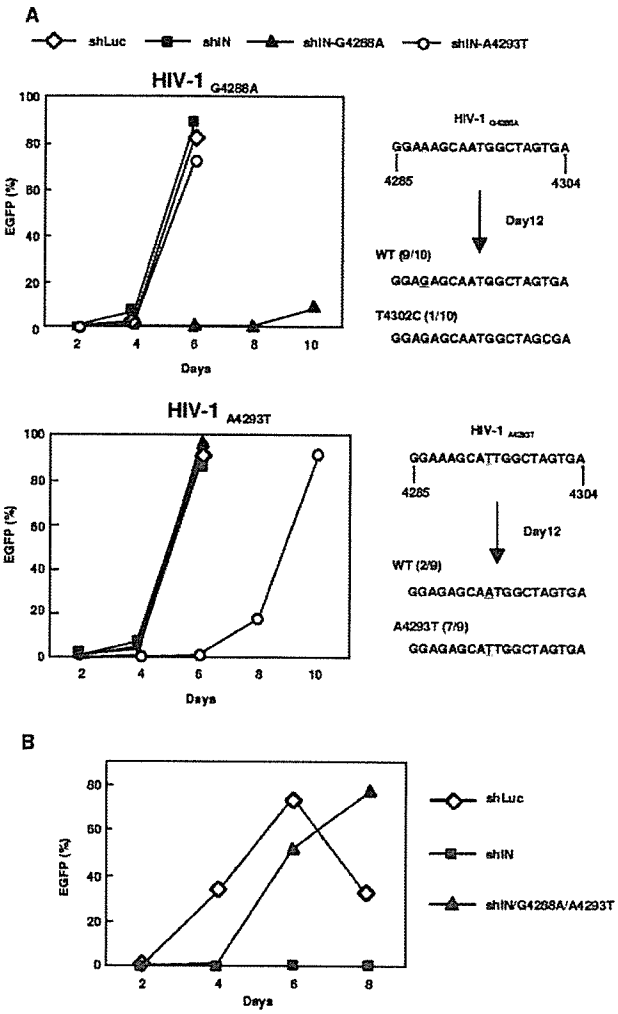

FIG. 5. Reversion of escape mutants in the presence of modified shRNAs targeting mutant sequences. (A) MT-4 cells were transduced with shIN or its modified shIN, which targeted escape mutant sequences (shIN-G4288A or shIN-A4293T). As a negative control, shLuc was introduced into MT-4 cells as well. The 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.

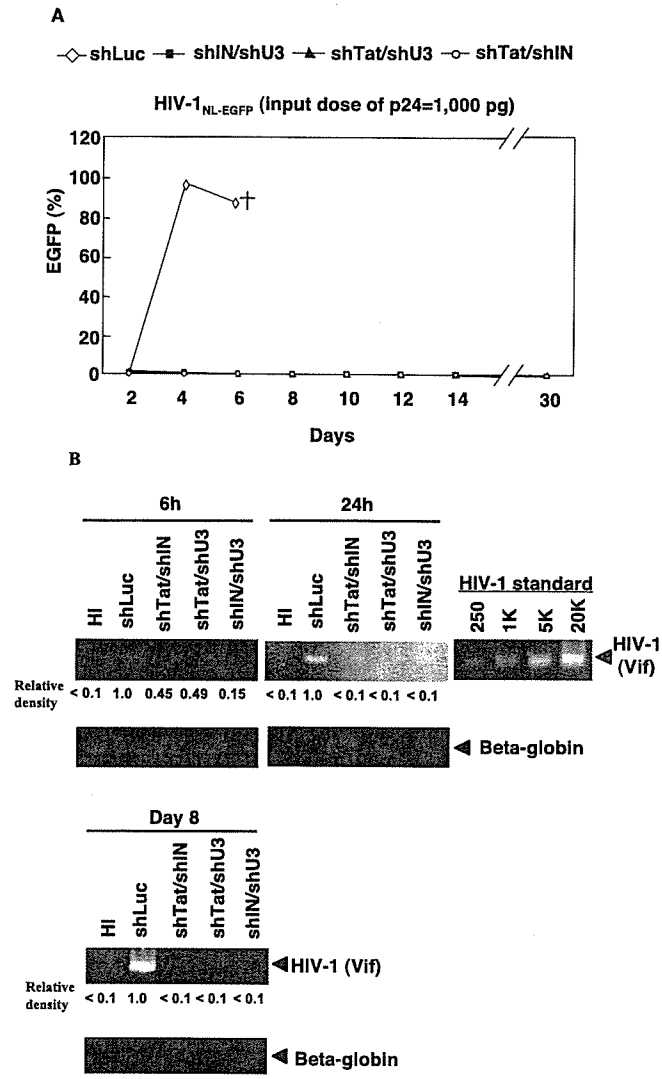

FIG. 6. Efficient antiviral activity with a combination of shRNAs targeting different sites in the HIV-1 genome. (A) MT-4 cells were transduced with combinations of shIN and shU3 (shIN/shU3), shTat and shU3 (shTat/shU3), or shTat and shIN (shTat/shIN). The dual-transduced cells were infected with DNase I-treated HIV-$1_{NL\text{-}EGFP}$ 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 (pNL43lucΔ*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-

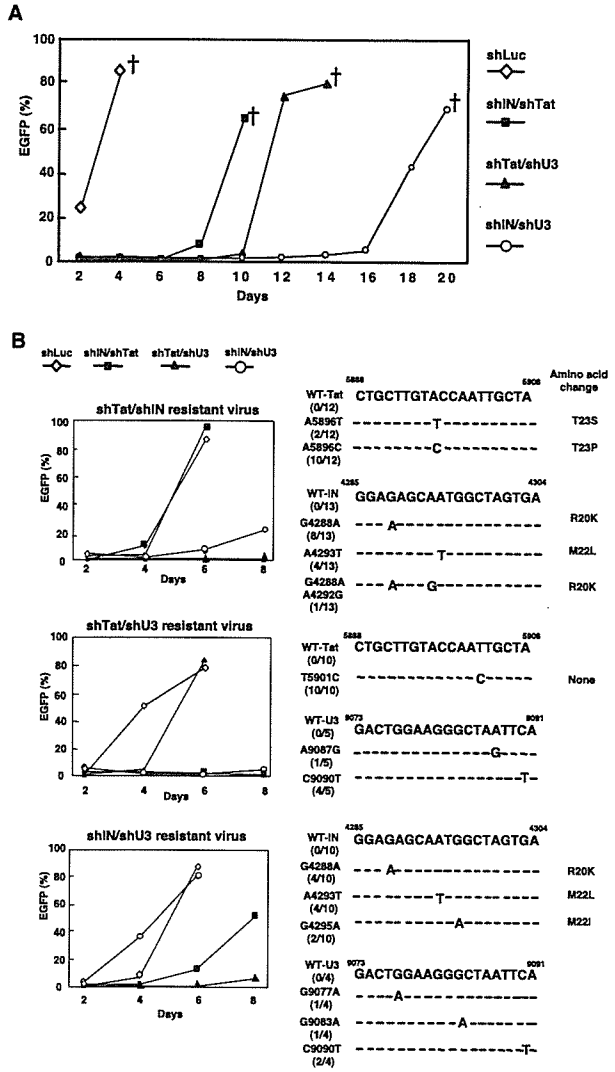

FIG. 7. Emergence of escape mutants from combinations of shRNAs targeting different sites following a high dose of HIV-1 infection. (A) MT-4 cells were transduced with combinations of two shRNAs as described for Fig. 6. The dual-transduced cells were infected with HIV-$1_{NL\text{-}EGFP}$ at 10,000 pg 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.

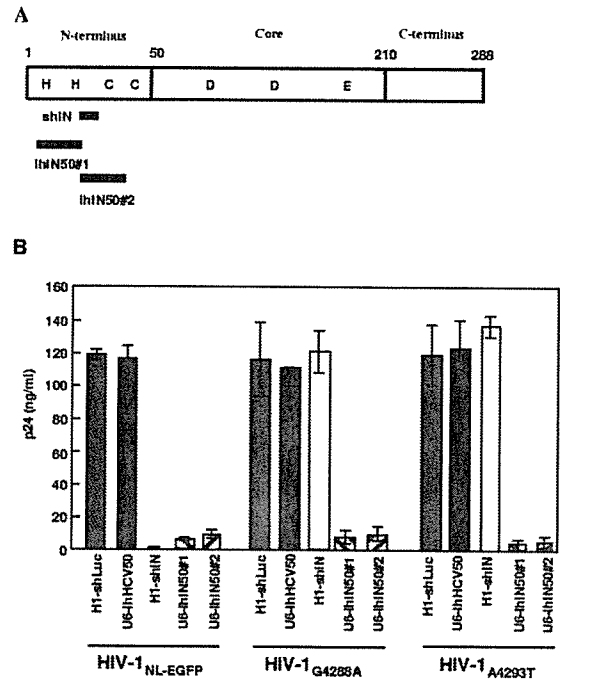

FIG. 8. Inhibitory effect of lhRNA on wild-type or shIN-resistant viral clones. (A) The target sites of lhRNAs against the HIV-1 *int* gene (lhIN). The target sites of two lhINs (lhIN50#1 and lhIN50#2) are indicated by bold bars. lhIN#1 was designed to target the 50 nucleotides upstream of the shIN target 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