densities between the disrupted membrane fragments and the live cell surface. Special areas, termed lipid-rafts, on the cell membrane contain pathogen-recognition receptors in high densities and facilitate the efficiency of pathogen entry [20,41,42]. Thus, virus–receptor complexes may be efficiently formed at the microenvironments of the cell surface in concentrations considerably higher than those in our reaction mixtures of virions and membrane fragments within test tubes. Alternatively, virion-membrane fusion may occur rapidly, but uncoating or disassembly process of the core may be slow and may be the rate-limiting step. This hypothesis requires involvement of putative uncoating regulation factor(s) that facilitates initiation of disassembly and rapid completion of core trafficking into the cytoplasm. Our cell-free reaction system seems to be usefully adapted to address the possibility and may rescue the slow p24 release kinetics through reconstitution of the biochemical reactions with subcellular fractions from target cells.

The fusion process between HIV-1 and target cells takes place at the cell surface, and HIV-1 enters by a specific receptor/coreceptor-mediated pathway. However, another non-specific entry pathway of HIV-1 has recently been reported [19–23]. These papers described that HIV-1 was internalized into cells via endocytosis and independently of the specific entry receptors. In addition, a recent model of endocytic routes used by viruses implicates the involvement of a number of cytosolic factors and/or molecules [23]. As several endocytic routes may be involved in the non-specific HIV-1 entry, biochemical studies of the entry mechanisms require relevant assays discriminating between the receptor/coreceptor-mediated pathway and endocytsis. In this study, membrane fractions were separated from the cytoplasm, and therefore, it appears not to carry over such cytosolic factors. Indeed, no increasing amounts of p24 were released from NL432 and JRFL virions upon reaction to $CD4^-$ HeLa cell membrane fraction. In addition, VSV/NL432env(-) virus that is allowed to infect through the endocytic pathway failed to release p24. Therefore, the p24 increase in the S_{100} fraction resulted not from non-specific endocytic entry response but more likely from specific receptor-mediated fusion/entry response of HIV-1, suggesting that the cell-free reaction discriminates the two entry pathways of HIV-1 infection. Our cell-free system may provide a useful tool for biochemically analyzing the specific entry mechanisms of HIV-1.

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Regulation of Human Immunodeficiency Virus Type 1 Env-Mediated Membrane Fusion by Viral Protease Activity

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We and others have presented evidence for a direct interaction between the matrix (MA) domain of the human immunodeficiency virus type 1 (HIV-1) Gag protein and the cytoplasmic tail of the transmembrane envelope (Env) glycoprotein gp41. In addition, it has been postulated that the MA domain of Gag undergoes a conformational change following Gag processing, and the cytoplasmic tail of gp41 has been shown to modulate Env-mediated membrane fusion activity. Together, these results raise the possibility that the interaction between the gp41 cytoplasmic tail and MA is regulated by protease (PR)-mediated Gag processing, perhaps affecting Env function. To examine whether Gag processing affects Env-mediated fusion, we compared the ability of wild-type (WT) HIV-1 Env and a mutant lacking the gp41 cytoplasmic tail to induce fusion in the context of an active (PR^+) or inactive (PR^-) viral PR. We observed that PR^- virions bearing WT Env displayed defects in cell-cell fusion. Impaired fusion did not appear to be due to differences in the levels of virion-associated Env, in CD4-dependent binding to target cells, or in the formation of the CD4-induced gp41 six-helix bundle. Interestingly, truncation of the gp41 cytoplasmic tail reversed the fusion defect. These results suggest that interactions between unprocessed Gag and the gp41 cytoplasmic tail suppress fusion.

During or shortly after virus release from the plasma membrane of the infected cell, the human immunodeficiency virus type 1 (HIV-1) protease (PR) cleaves the Gag and Gag-Pol polyprotein precursors to generate the mature Gag and Pol proteins. This PR-mediated processing of Gag and Gag-Pol precursors leads to a striking transformation in virion morphology, a process known as virus maturation. During maturation, noninfectious particles with electron-lucent cores are converted to infectious virions with electron-dense, conical cores (50, 51, 55). It has been postulated that Gag processing induces conformational changes in the matrix (MA) domain of Gag (25, 44, 59). Although it has long been appreciated that immature virions are noninfectious (24, 32, 41), the nature of the infectivity block and the step in virus entry that is affected remain to be determined.

The HIV-1 Env glycoproteins are synthesized as a 160-kDa precursor protein, gp160, which is cleaved by cellular proteases during trafficking to the plasma membrane to generate the mature surface glycoprotein gp120 and the transmembrane glycoprotein gp41. The gp120/gp41 Env glycoprotein complex is incorporated into virus particles during the assembly process. On the mature HIV-1 virion, gp120 and gp41 act in concert to catalyze the fusion of viral and target cell membranes, resulting in the delivery of the viral core into the cytoplasm. Env-mediated fusion takes place in a series of steps: binding of gp120 to the HIV-1 receptor CD4, interaction between gp120 and a coreceptor (typically CXCR4 or CCR5), formation of a gp41 ectodomain six-helix bundle (6HB), hemifusion, and fusion pore formation (2, 16, 21).

A number of studies have provided evidence for a functional interaction between the long cytoplasmic tail (CT) of gp41 and the MA domain of Gag. (i) Deletions (57) and point mutations (22) in MA block Env incorporation into virus particles. (ii) Truncation of the gp41 CT reverses the Env incorporation defect imposed by MA mutations (20, 22, 37). (iii) An Env incorporation defect resulting from a small deletion near the middle of the gp41 CT is reversed by a specific point mutation in MA (38). In addition, we and others found that viral cores prepared from PR^- virions contained high levels of $Pr55^{Gag}$ and gp41 (58; T. Murakami and E. O. Freed, unpublished data). Interestingly, this detergent-resistant interaction between gp41 and $Pr55^{Gag}$ is dependent on the gp41 CT, again suggesting that the CT is required for the Gag-gp41 interaction. Furthermore, several lines of evidence suggest a connection between the CT of retroviral Env glycoproteins and membrane fusion. For example, in the case of certain retroviruses (e.g., murine leukemia virus and Mason-Pfizer monkey virus), the Env CT is cleaved by the viral PR after virus release, and this cleavage event activates Env fusogenicity (5, 42, 43). In addition, truncation of the CT of the maedi-visna virus (58), simian immunodeficiency virus (SIV) (45, 49, 60), HIV-1 (13, 15, 20, 54), and human T-cell leukemia virus type 1 (31) transmembrane glycoproteins increases fusion activity. Together, these observations raise the possibility that interactions between HIV-1 Gag and the gp41 CT may affect Env-mediated fusion and that fusion could thus be influenced by the state of Gag processing.

In this study, we investigated whether Gag processing and virion maturation affect HIV-1 Env-mediated membrane fu-

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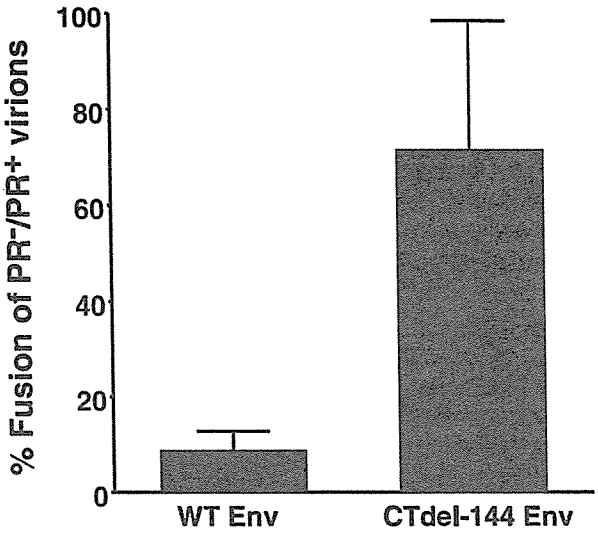


FIG. 1. PR⁻ virions display a fusion defect in a cell-cell fusion-from-without assay. *env*-defective HIV-1 NL4-3 (NL4-3/KFS) (19, 22) and its PR⁻ counterpart were cotransfected into 293T cells with pUC19, with vectors expressing the WT NL4-3 Env (pIIINL4env) (39) or the CT truncation mutant CTdel-144 (pNL4envCTdel-144) (39). Virus-containing supernatants were harvested 2 days posttransfection, and virions were concentrated (10 to 20×) by centrifugation (20,000 × *g* for 2 h). The concentrated pseudovirions were added to Jurkat cells. After a 20-h cultivation, the number of syncytia (whose diameters were more than four times those of unfused Jurkat cells) was scored. The number of syncytia was expressed as a ratio (%) of those obtained with PR⁻ versus PR⁺ virions. The average number of syncytia induced by PR⁺ virions bearing WT and CTdel-144 Env was 171 and 116, respectively. Data are means of the results of four independent experiments. Error bars indicate standard deviations.

sion. We found that inactivating HIV-1 PR suppresses the ability of HIV-1 virions to induce cell-to-cell fusion, a type of fusion known as fusion from without (3, 11, 28). The fusion defect is not the result of differences in gp120/gp41 levels on PR⁺ versus PR⁻ virions and appears to be imposed at a step following CD4 binding and receptor-activated conformational changes in gp41. Interestingly, the fusion defect is reversed by truncating the CT of gp41, suggesting that interactions between unprocessed Gag and the gp41 CT suppress fusion.

PR⁻ virions bearing WT Env display defects in cell-cell fusion assays. To examine whether Gag processing affects HIV-1 Env-mediated fusion, we compared the ability of HIV-1 virions to induce fusion from without (11) in the context of either active (PR⁺) or inactive (PR⁻) viral PR (27). To prevent productive infection and thereby ensure that our assays accurately measure fusion from without, we used pseudovirions produced by cotransfecting an Env-defective HIV-1 NL4-3 molecular clone (NL4-3/KFS) (19, 22) or its PR⁻ counterpart (NL4-3/PR⁻/KFS) with a wild-type (WT) NL4-3 Env expression vector. Concentrated pseudovirions were added to a Jurkat T-cell line, and the extent of fusion was determined by scoring syncytia (whose diameters were more than four times those of unfused Jurkat cells). PR⁺ virions efficiently induced fusion; in contrast, fusion induced by PR⁻ virions was reduced by 90% compared with levels observed with PR⁺ virions (Fig. 1). We postulated that if the fusion defect observed with PR⁻ virions was a consequence of interactions between unprocessed Gag and the gp41 CT, then the suppressive effect on fusion would be reversed by removing the CT. To test this hypothesis, we prepared PR⁺ and PR⁻ pseudovirions bearing an Env mutant (CTdel-144) lacking the CT. This mutant was constructed by introducing two adjacent stop codons in the *env* gene, such that a truncated form of gp41 lacking the C-terminal 144 amino acids was expressed (39). Intriguingly, and consistent with our hypothesis, pseudovirions bearing the CTdel-144 Env induced fusion in both PR⁺ and PR⁻ contexts (Fig. 1). We obtained results essentially identical to those presented in Fig. 1 by using a highly quantitative fusion assay in which Tat-expressing Jurkat (i.e., Jurkat-tat) cells (6) and LuSIV cells containing a luciferase reporter gene under the control of the SIV long terminal repeat (46) were cocultivated in the presence of concentrated pseudovirions (data not shown). These results support the hypothesis that Gag processing activates virus-induced cell-cell fusion mediated by the WT HIV-1 Env.

Differences in the ability of PR⁺ versus PR⁻ virions to induce fusion are not attributable to different levels of virion-associated Env. To explore the possibility that PR⁻ virions are fusion defective due to reduced levels of virion-associated Env, we measured levels of gp41 on PR⁺ and PR⁻ virions by Western blotting. Amounts of virion-associated gp41 were found to be unaffected by whether the virions were PR⁺ or PR⁻; this result was observed for both the WT Env and the CTdel-144 truncation mutant (Fig. 2). We also determined by radioimmunoprecipitation analysis that the state of Gag processing did not affect gp120 levels on virions bearing either WT or CTdel-144 Env (data not shown). Thus, differences in the ability of PR⁺ and PR⁻ virions to induce fusion from without do not result from different levels of virion-associated Env.

The fusion defect displayed by PR⁻ virions is not due to impaired CD4-dependent cell surface binding or major effects on 6HB formation. To investigate which step in virus entry is blocked in the absence of Gag processing, we examined whether Gag processing affects CD4-dependent HIV-1 binding to target T cells. For this purpose, we selected the Molt-4 T-cell line (30), which has been reported to contain low levels of heparan sulfate (40) and therefore displays limited CD4-independent virion binding. Molt-4 cells were incubated (37°C for 30 min) with concentrated HIV-1 pseudovirions in the presence of anti-CD4 monoclonal antibody (MAb) (Beckman 13B8.2) or control immunoglobulin G1 (IgG1). The virus-bound cells were stained with a rat anti-gp120 MAb (W#10) recognizing RIQRGPG (Y. Tanaka, unpublished data), and the amount of bound virus was determined by flow cytometry. Pseudovirions bearing WT NL4-3 Env showed comparable levels of CD4-dependent binding (about 95% of total binding) to Molt-4 cells in either the PR⁺ or PR⁻ context. Pseudovirions bearing CTdel-144 Env displayed a slight, statistically insignificant increase ($P = 0.19$, t test) in binding in the PR⁻ context (Fig. 3). These results indicate that the fusion defect displayed by PR⁻ virions is not due to differences in CD4-dependent binding to the surface of target cells.

It has been demonstrated that Env-CD4 binding triggers a series of conformational changes in gp120 and gp41 that ultimately lead to the formation of a 6HB in the ectodomain of gp41 (16). To investigate the possibility that receptor-activated formation of the 6HB is blocked in the absence of Gag pro-

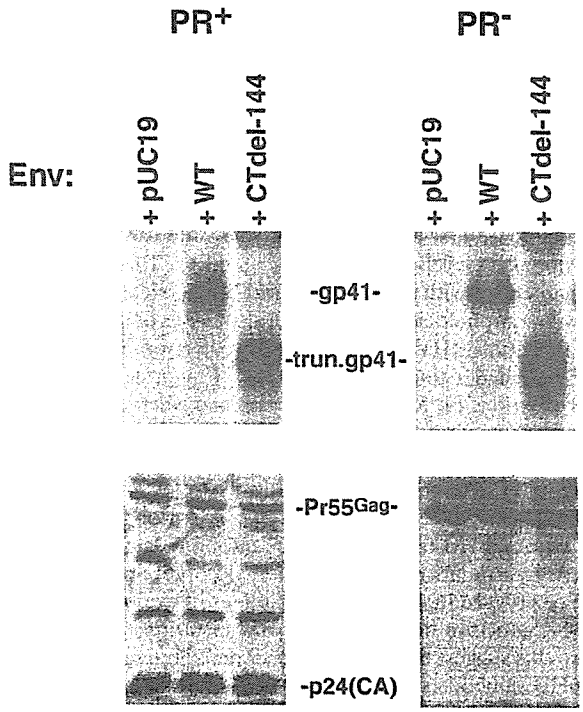


FIG. 2. PR^+ and PR^- virions incorporate comparable levels of gp41. Virion lysates, prepared from concentrated virus stocks as described in the legend of Fig. 1, were transferred to polyvinylidene difluoride membranes and immunoblotted with the anti-gp41 MAb T32 (upper panel) and AIDS patient serum (lower panel) to detect p24 (capsid) and/or $Pr55^{Gag}$. To confirm equal loading of virion-associated material, blots were reprobed with an anti-Vpr antibody (data not shown). Quantitative Western blotting was performed with a Fluor-S MAX MultiImager (Bio-Rad, Hercules, Calif.). T32 was obtained from P. Earl (14), and AIDS patient serum was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The data shown are representative of the results of at least five independent experiments.

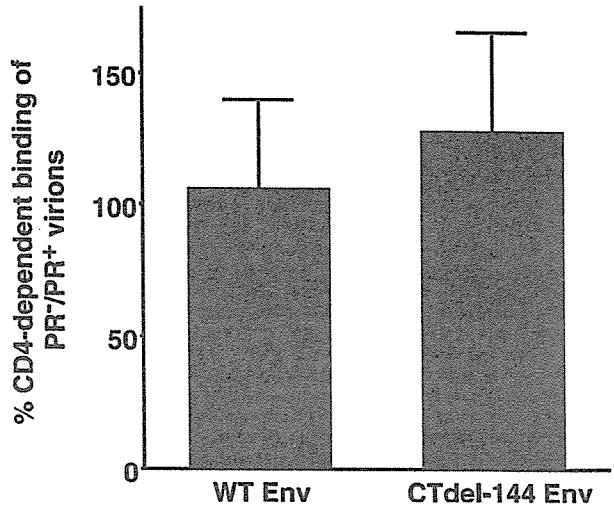
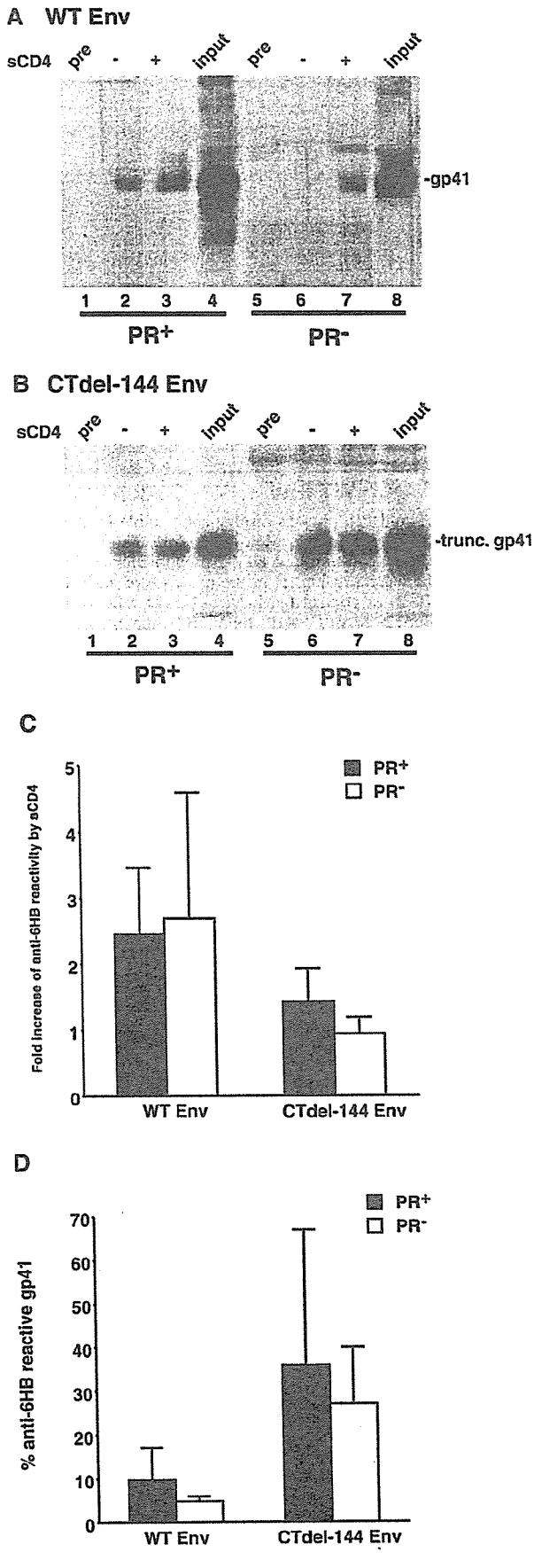


FIG. 3. PR^+ and PR^- virions display comparable levels of CD4-dependent cell surface binding. Molt-4 clone 8 cells (5×10^5) (30) were incubated with concentrated HIV-1 pseudovirions (prepared as described in the legend of Fig. 1) in the presence of anti-CD4 MAb (Beckman, 13B8.2) or control IgG1. Virus-bound cells were stained with a rat anti-gp120 MAb (W#10) followed by the addition of phycoerythrin-conjugated goat anti-rat IgG. The amount of virus bound was determined by flow cytometry. CD4-independent binding determined by measuring the amount of virus bound in the presence of the anti-CD4 MAb was subtracted from the total binding. Data are means ± standard deviations of the results of four independent experiments.

cessing, immunoprecipitation assays were performed using intact HIV-1 virions incubated with an anti-6HB antiserum (rabbit serum no. 948, kindly provided by C. Weiss, U.S. Food and Drug Administration) (12, 23) or control preimmune serum in the presence or absence of soluble CD4 (sCD4) (Fig. 4). The immunoprecipitated material was then subjected to Western blotting with the anti-gp41 MAb T32 (kindly provided by P. Earl, National Institute of Allergy and Infectious Diseases) (14). As previously reported (12), sCD4, by using intact Env-expressing cells, enhanced (by 2.5-fold) immunoprecipitation of gp41 by the anti-6HB Ab (Fig. 4A, lanes 2 and 3, and Fig. 4C). An enhanced (2.7-fold) immunoprecipitation was also induced by sCD4 in PR^- virions bearing WT NL4-3 Env (Fig. 4A, lanes 6 and 7, and Fig. 4C). For CTdel-144, reactivity to the anti-6HB Ab was enhanced slightly (1.4-fold) by sCD4 in the PR^+ context (Fig. 4B, lanes 2 and 3, and Fig. 4C), whereas there was no substantial enhancement by sCD4 in PR^- virions (Fig. 4B, lanes 6 and 7, and Fig. 4C). We also assessed the extent of 6HB formation by calculating the fraction of virion-associated gp41 detected by the anti-6HB Ab following sCD4 treatment versus total virion-associated gp41 (Fig. 4D). Again, the CTdel-144 mutant showed an increase in 6HB formation relative to WT. Reproducible but statistically insignificant reductions in 6HB formation were seen for both WT and CTdel-144 Env in the PR^- context. Together, these results suggest that the fusion defect displayed by PR^- virions is not due to a block in 6HB formation. However, at this time we cannot exclude the possibility that the anti-6HB immunoprecipitation approach used here might not detect small but meaningful differences in formation of the 6HB.

Conclusions. In the present study, we hypothesized that the MA-gp41 CT interaction may be altered by Gag processing, perhaps affecting Env function. Indeed, we found that inactivating HIV-1 PR suppresses Env-mediated cell-cell fusion from without. The fact that the fusion defect is rescued by truncating the gp41 CT suggests that interactions between unprocessed Gag and the gp41 CT inhibit fusion. These results demonstrate a heretofore unappreciated role of the viral PR in activating infectivity and suggest that a fusion defect imposed by unprocessed Gag may contribute to the high degree of potency displayed by PR inhibitors.

How might MA-gp41 interactions suppress membrane fusion? We and others have observed a detergent-resistant association between $Pr55^{Gag}$ and gp41 in immature (PR^-) HIV-1 virions (56; Murakami and Freed, unpublished). This detergent-resistant Gag-gp41 interaction is not detected in mature (PR^+) particles. We speculate that a physical association between Gag and the gp41 CT locks the Env glycoprotein complex into a nonfusogenic conformation that is reversed by PR-mediated Gag processing or by gp41 mutations that eliminate the Gag-gp41 interaction. Interestingly, previous reports



suggested that the association between matrix proteins and the CT of envelope proteins may negatively regulate the fusogenicity of measles and Newcastle disease viruses (7, 8, 47). Taken together, it can be postulated that a variety of enveloped viruses have evolved mechanisms, including MA-Env interactions and PR-mediated Env CT cleavage (5, 42, 43), to suppress Env-induced membrane fusion until virus budding has been completed. Such mechanisms would limit fusion-induced cytopathicity and abortive reinfections.

To define the step in the fusion process inhibited by unprocessed Gag, we first determined that the state of the viral PR does not affect levels of virion-associated Env. This conclusion is in agreement with that of a previous biochemical report (35) and with morphological and structural analyses of both mature and immature HIV-1 virions by cryoelectron microscopy, which revealed a comparable density of Env proteins on both types of virions (4, 53). Next, we examined whether Gag processing affects virus binding to target $CD4^+$ T cells. We observed that Env proteins on PR^- virions are functionally competent in the attachment step. Finally, we tested whether 6HB formation takes place to a comparable extent on PR^+ versus PR^- virions. 6HB formation occurs during membrane fusion induced by a number of different viral glycoproteins, including those of HIV-1 (16, 48), and several lines of evidence suggest that this structure directly participates in fusion (29, 36, 52). Our results suggest that the enhanced immunoprecipitation by anti-6HB antibodies induced by sCD4 treatment in virions bearing WT NL4-3 Env is not affected by the state of Gag processing, though the inherent variability in the detection assay would make subtle differences difficult to measure. Interestingly, relative to WT Env, CTdel-144 mutant Env appears to be highly reactive with the anti-6HB Ab even without sCD4 treatment. This phenomenon may in part explain why CD4-independent isolates often acquire CT truncations (17, 26, 34). Indeed, Edwards et al. recently reported that truncation of the gp41 CT enhances not only binding of monoclonal antibodies to CD4-induced epitopes in the ectodomain of gp120 but also neutralization sensitivity to HIV-1-positive serum (18). Thus, our results suggest that the fusion defect observed with PR^- virions is elicited at a step following 6HB formation, e.g., hemifusion or the initiation and enlargement of the fusion pore. Gag processing may be necessary for the

FIG. 4. PR^+ and PR^- virions display comparable levels of 6HB formation. Concentrated HIV-1 virions (prepared as described in the legend of Fig. 1, except that in this case NL4-3 [1] and CTdel-144 [39] and their PR^- counterparts were used) were incubated with an anti-6HB antiserum (rabbit serum no. 948) or preimmune serum (pre) in the presence or absence of 1 μg of sCD4 (Immuno Diagnostics) at 37°C for 2 h. Virions were then pelleted by centrifugation (20,000 × *g* for 2 h) prior to lysis and immunoprecipitation with protein G Sepharose beads. The immunoprecipitated material was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to quantitative Western blotting with the anti-gp41 MAb T32. An amount of viral lysate equivalent to input virus was run as a positive control. Gels were run for WT Env (A) and CTdel-144 Env (B). The increase in anti-6HB reactivity (*n*-fold) induced by sCD4 in the PR^+ (solid bar) and PR^- (open bar) context (C) is shown. Also shown is the percentage of anti-6HB-reactive gp41, calculated by dividing the amount of 6HB-reactive gp41 following sCD4 addition by input gp41 in PR^+ (solid bar) and PR^- (open bar) virions (D). Rabbit serum no. 948 was obtained from C. Weiss (12, 23). Data are means ± standard deviations of the results of at least three independent experiments.

assembly of the higher-order Env oligomers that are postulated to be required for fusion (9, 19, 33). Given the low level of gp120 and gp41 on the surface of HIV-1 virions (10), Env trimers must presumably be free to diffuse and cluster in the plane of the lipid bilayer in order to form fusion-active, higher-order complexes. Interactions between the gp41 CT and unprocessed Gag may limit the ability of Env trimers to diffuse in the plane of the lipid bilayer, thereby inhibiting fusion pore formation. It is also possible that PR-mediated Gag processing and concomitant reorganization of the structure of the mature virion may affect the interaction of Env with lipids or other components of the viral (or cell) membrane, thereby in some manner activating membrane fusion. Alternatively, one of the mature Gag proteins could directly or indirectly influence the fusion reaction.

In summary, we describe here a fundamentally novel observation regarding the functional relationship between the HIV-1 Gag and Env proteins. Work is ongoing in our laboratories to further define the ability of Gag to regulate Env function and the implications of this regulation to our understanding of HIV-1 replication and the development of antiviral agents.

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

Virus preparation. 293T cells (4×10^6) 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

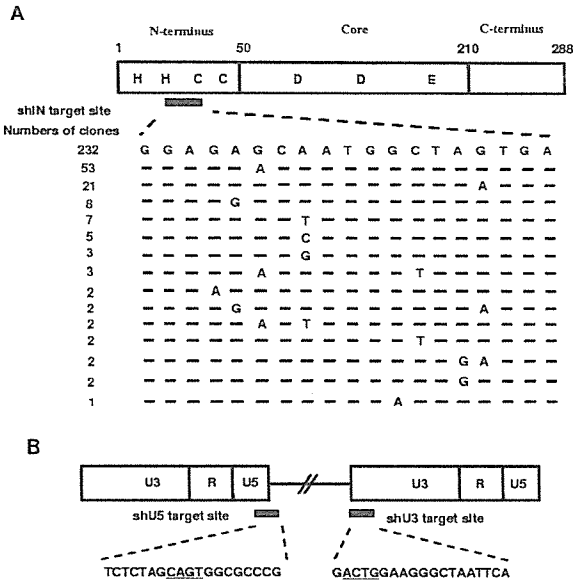

FIG. 1. shRNA target sequences of HIV-1 *int* and the U3 and U5 *att* sites. (A) Schematic representation of HIV-1 integrase (top squares) and shIN target region (bold bar). The conserved HHCC residues in the zinc-binding motif and enzyme active-site residues (DDE) located in the N terminus of the core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

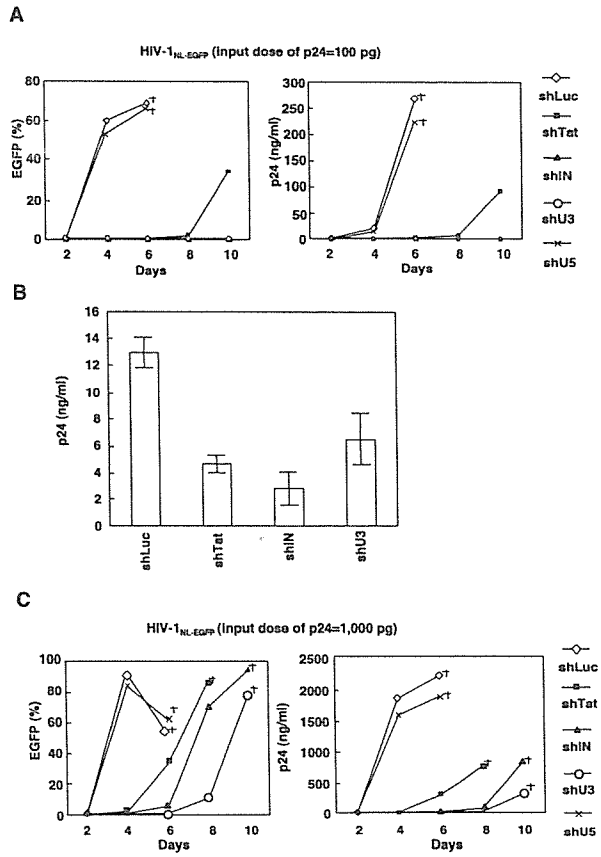

FIG. 2. HIV-1 replication in shRNA-transduced MT-4 or primary $CD4^+$ T cells. (A) MT-4 cells were transduced by the indicated shRNA using a lentivirus vector system (shLuc, shTat, shIN, shU3, and shU5). Seven days later, transduced MT-4 cells were infected by $HIV\text{-}1_{NL\text{-}EGFP}$ at an input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

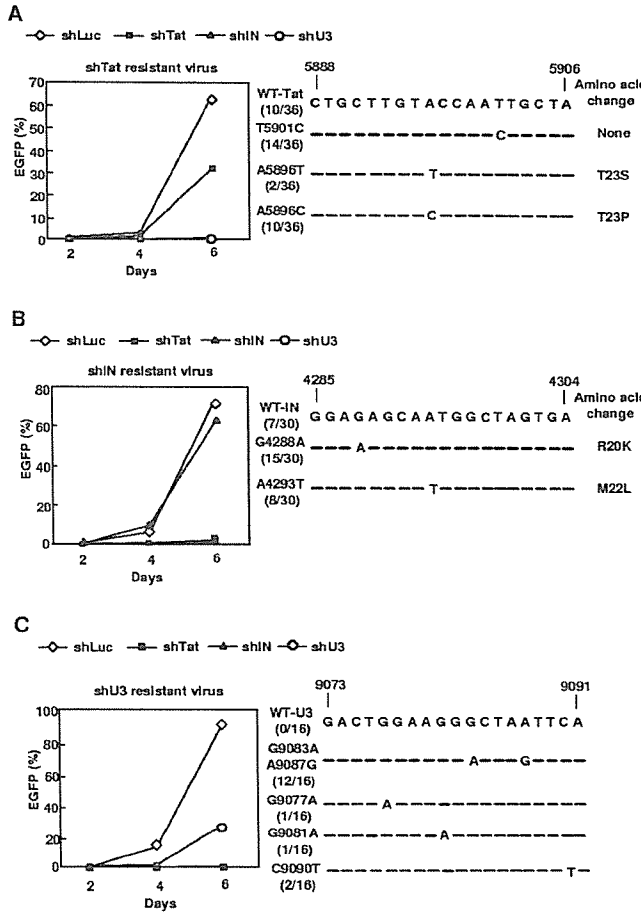

FIG. 3. shRNA-specific resistance of HIV-1 escape mutants in shRNA-transduced MT-4 cells. Culture supernatants of shTat-transduced MT-4 cells infected with a low dose of HIV-1 (100 pg of p24) (A) or shIN (B)- or shU3 (C)-transduced MT-4 cells infected with a high dose of HIV-1 (1,000 pg of p24 per 10^6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

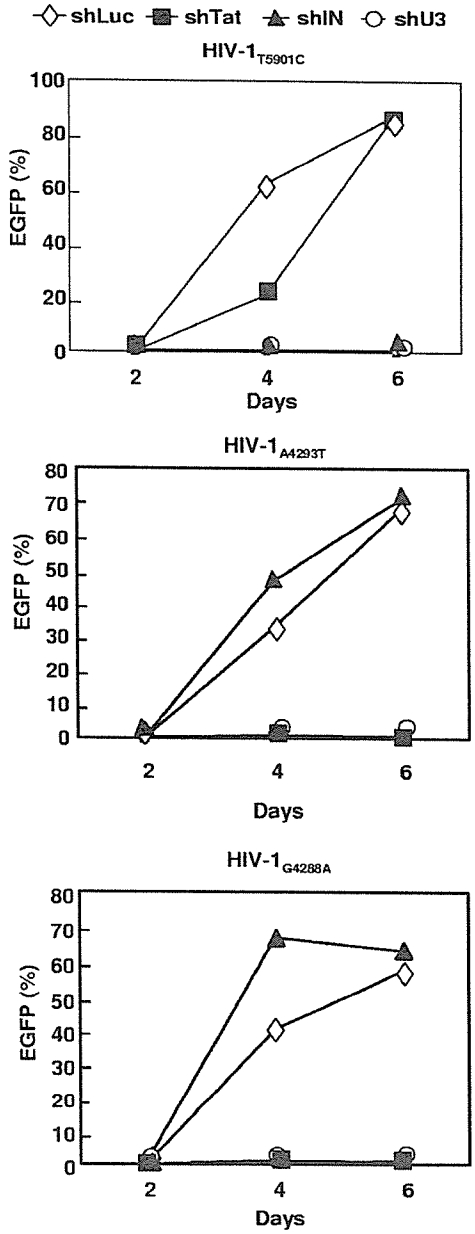

FIG. 4. shRNA-specific resistance of HIV-1 molecular clones carrying point mutations within each shRNA target site. Point mutations within the shTat target site (Tat-T5901C) or shIN target site (IN-G4288A or IN-A4293T) were introduced into the parental HIV-1 clone (pNL-EGFP) through mutagenesis. Each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

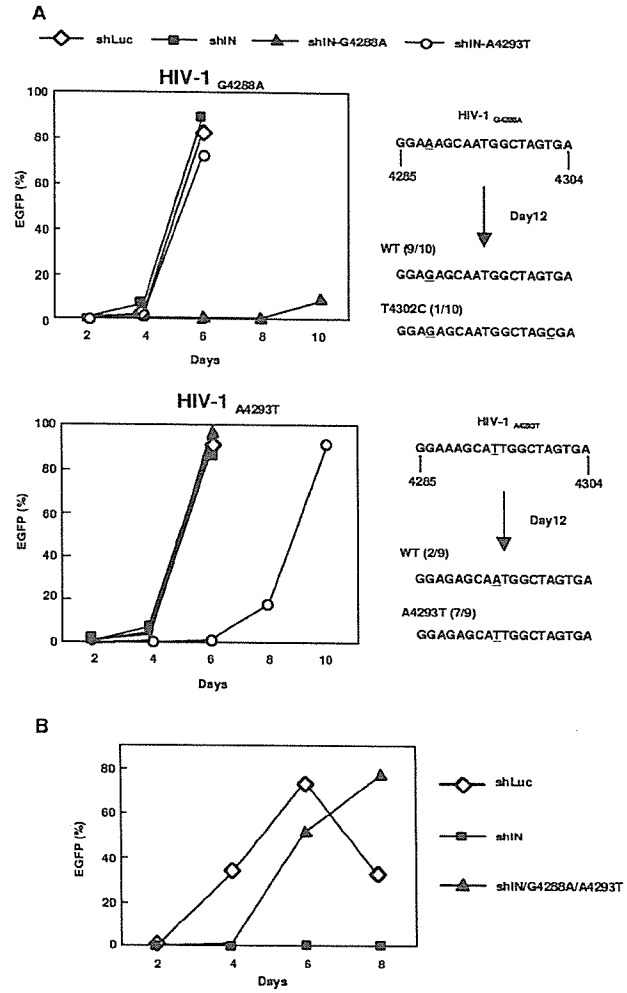

FIG. 5. Reversion of escape mutants in the presence of modified shRNAs targeting mutant sequences. (A) MT-4 cells were transduced with shIN or its modified shIN, which targeted escape mutant sequences (shIN-G4288A or shIN-A4293T). As a negative control, shLuc was introduced into MT-4 cells as well. The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

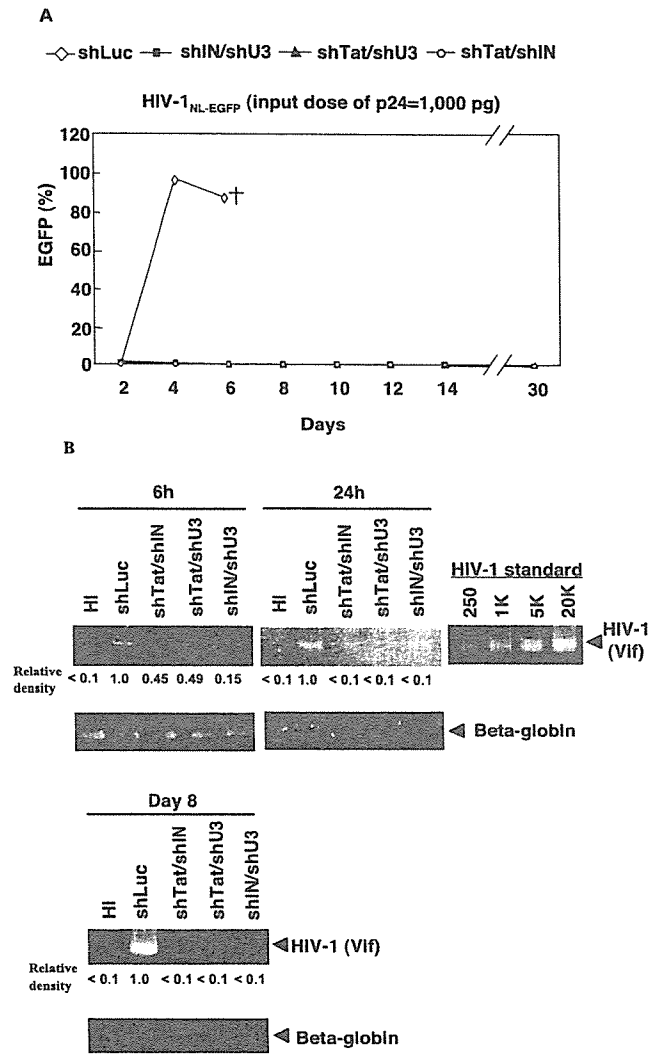

FIG. 6. Efficient antiviral activity with a combination of shRNAs targeting different sites in the HIV-1 genome. (A) MT-4 cells were transduced with combinations of shIN and shU3 (shIN/shU3), shTat and shU3 (shTat/shU3), or shTat and shIN (shTat/shIN). The dual-transduced cells were infected with DNase 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-

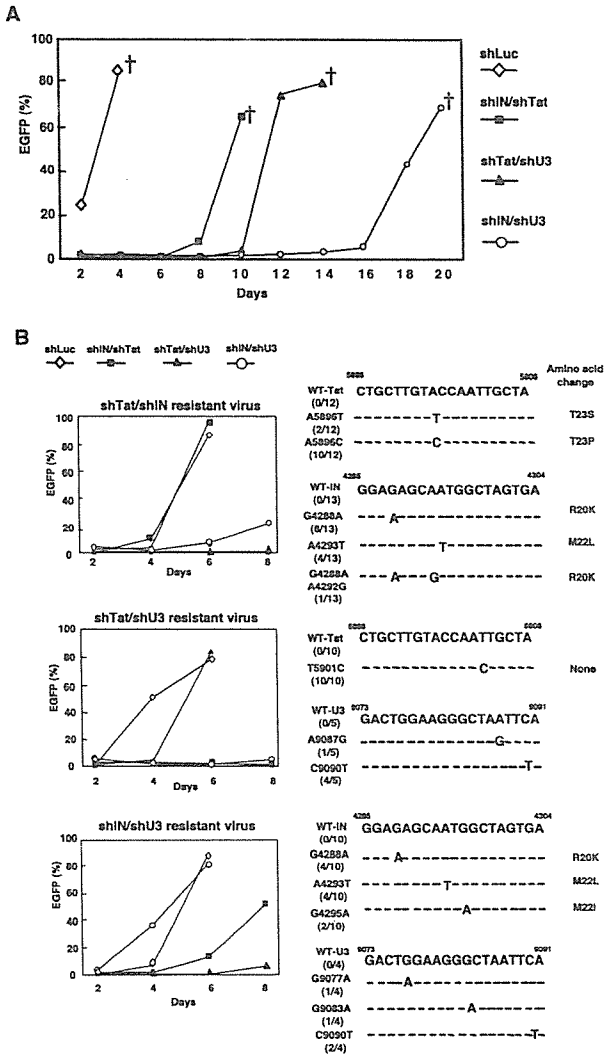

FIG. 7. Emergence of escape mutants from combinations of shRNAs targeting different sites following a high dose of HIV-1 infection. (A) MT-4 cells were transduced with combinations of two shRNAs as described for Fig. 6. The dual-transduced cells were infected with HIV-$1_{NL\text{-}EGFP}$ at 10,000 pg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

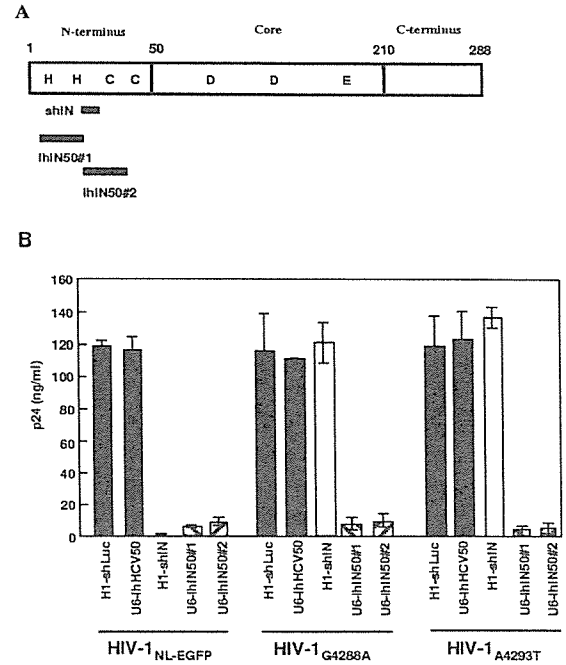

FIG. 8. Inhibitory effect of lhRNA on wild-type or shIN-resistant viral clones. (A) The target sites of lhRNAs against the HIV-1 *int* gene (lhIN). The target sites of two lhINs (lhIN50#1 and lhIN50#2) are indicated by bold bars. lhIN#1 was designed to target the 50 nucleotides upstream of the shIN target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

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

DISCUSSION

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

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

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

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

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

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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

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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

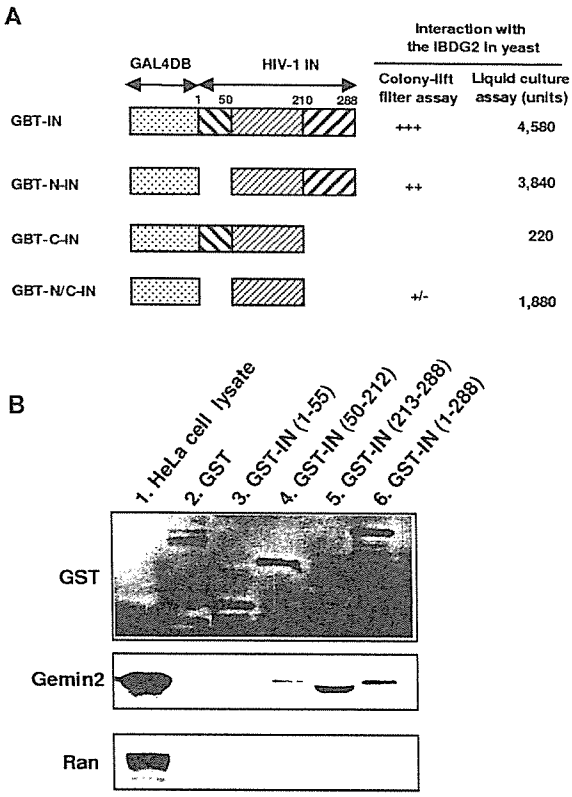

FIG. 1. Interaction of IN with Gemin2. (A) Yeast AH109 or HF7c cells were cotransformed with the pGAD-IBDG2 vector carrying the IBDG2 domain of Gemin2 together with various pGBT9 plasmids carrying full-length (pGBT-IN) or truncated (pGBT-DN-IN, pGBT-DC-IN, and pGBT-DN/DC-IN) forms of HIV-1 IN. Dashed 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-