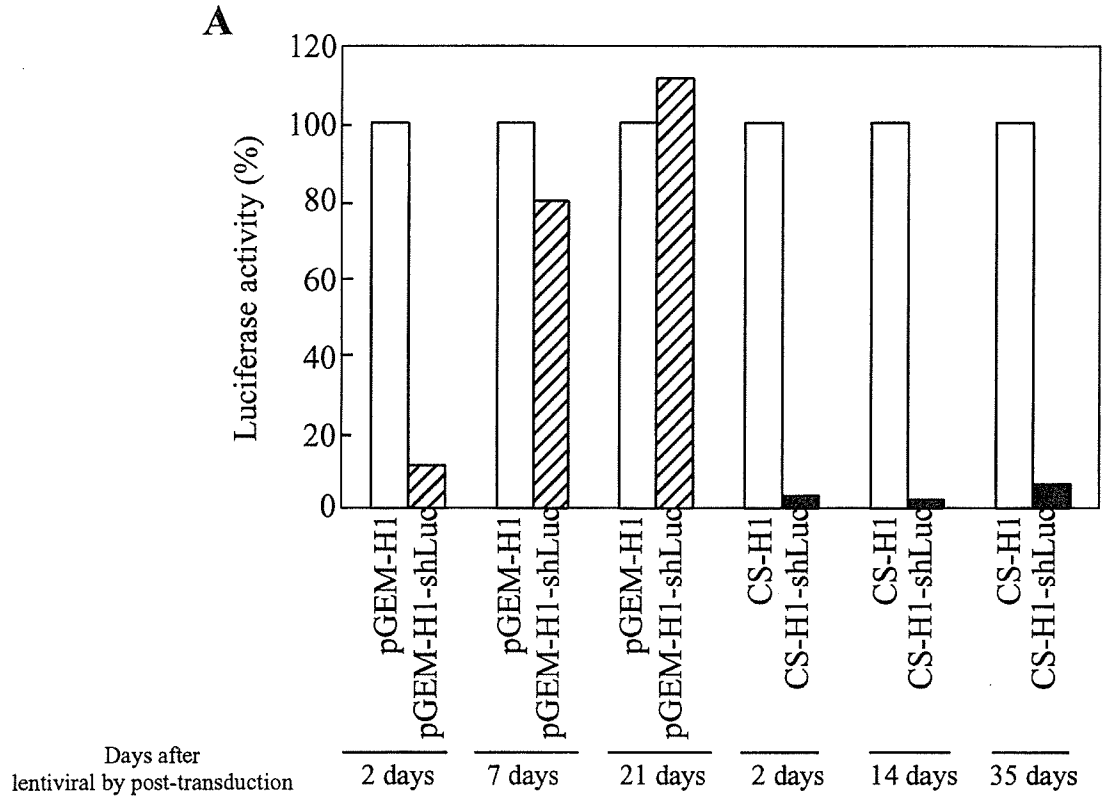
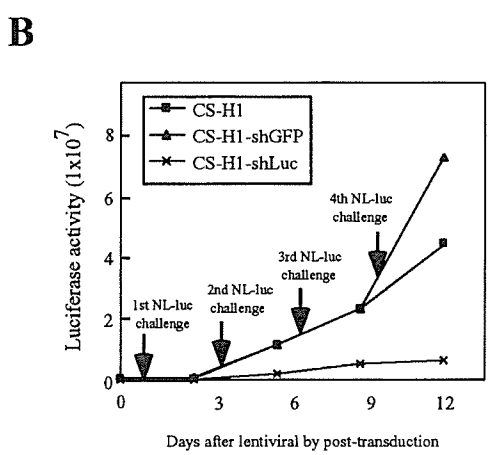
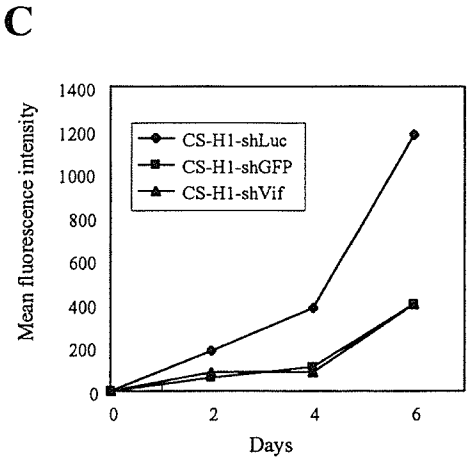
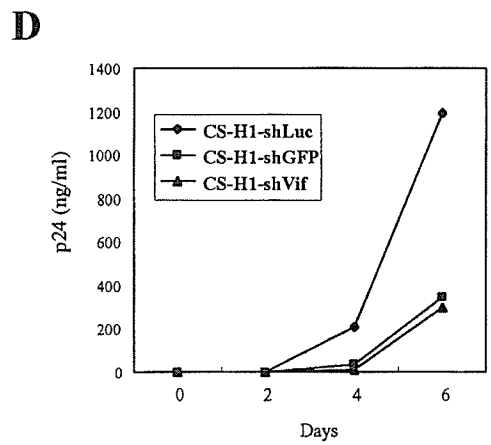

A
Luciferase activity (%)
120
100
80
60
40
20
0
pGEM-H1
pGEM-H1-shLuc
CS-H1
CS-H1-shLuc
Days after lentiviral by post-transduction
2 days
7 days
21 days
2 days
14 days
35 days


B
CS-H1
CS-H1-shGFP
CS-H1-shLuc
Luciferase activity (1x10^7)
1st NL-luc challenge
2nd NL-luc challenge
3rd NL-luc challenge
4th NL-luc challenge
Days after lentiviral by post-transduction


C
CS-H1-shLuc
CS-H1-shGFP
CS-H1-shVif
Mean fluorescence intensity
Days


D
CS-H1-shLuc
CS-H1-shGFP
CS-H1-shVif
p24 (ng/ml)
Days

shLuc on viral RNA transcripts from integrated proviral DNA, we introduced the shLuc 48 h post-infection, when most of the viral cDNAs should have completed integration into host chromosomal DNA [28]. The level of viral production was reduced to 50% by the pGEM-H1-shLuc and to 23% by the pCS-H1-shLuc compared with the level by each control vector (Fig. 2D). Taken together, these results suggested that endogenous viral RNA transcripts from integrated proviral DNAs might be the major targets for shRNA.

3.4. Expression of shRNA by lentivirus-based vector confers efficient and stable antiviral state on human cells

One of the advantages of using the lentivirus system over a plasmid-based expression system is to maintain the transferred genes stably integrated into the host chromosomal DNA without time-consuming drug-selection procedures. Antiviral effects through a plasmid-based expression system (pGEM-H1-shLuc) persisted less than 7 days (Fig. 3A, pGEM-H1-shLuc). On the other hand, the lentivirus system (CS-H1-shLuc) confers efficient antiviral status on the transduced cells for at least 35 days of culture (Fig. 3A, CS-H1-shLuc). Thus, shRNA expressed in the context of a lentivirus vector system can work more efficiently and its effects persist longer than a plasmid-based expression system. Moreover, we evaluated efficacy of shLuc introduced by lentiviral vector against multiple infection with HIV-1, by repeating challenge infections of the transduced 293T. The 293T cells transduced with lentiviral vector expressing shLuc, empty or shGFP were infected with $HIV_{NL43\text{-}luc}$ pseudotype virus every 3 days. We showed that the 293T cells transduced with lentiviral shLuc resisted (more than 86% inhibition) repeated infection with HIV at least four times (Fig. 3B). Next, we determined the extent to which lentiviral vector expressing shRNA can suppress HIV-1 replication in a multiple-infection system using replication-competent HIV-1. We constructed two shRNAs against the *GFP* gene (shGFP) or HIV-1 *vif* gene (shVif) [9] to target the HIV-1 clone carrying EGFP [25]. MT-4 cells were transduced with shGFP or shVif lentiviral vector and then challenged by replication-competent HIV-1 (NL-EGFP). The kinetics of viral replication was monitored by measuring EGFP and p24 antigen expression every 2 days (Fig. 3C,D). EGFP (Fig. 3C) and p24 (Fig. 3D) levels were significantly reduced, to less than 20%, by introduction of shGFP or shVif, compared with the level observed with irrelevant shRNA (shLuc) at 6 days post-infection (Fig. 3C). In addition, shLuc-transduced cells died at 6 days post-infection probably due to multiple replication of NL-EGFP infection, while shGFP- or shVif-transduced cells were kept alive (data not shown). Slight HIV-1 replication, however, was noticed in MT-4 cells transduced with shGFP or shVif. This might not be due to emergence of escape mutant virus, since amplified virus derived from culture supernatants of shGFP- or shVif-transduced MT-4 cells showed similar susceptibility against shGFP or shVif (data not shown). We are currently continuing to culture the escape mutant against shRNA for isolation. Taken together, the lentiviral vector system is applicable to block HIV-1 replication.

Another advantage to the lentivirus-mediated gene-delivery system is its highly efficient transduction ability, especially in post-mitotic primary non-dividing cells, such as macrophages and neurons [32]. Finally we addressed this point using primary human monocyte-derived macrophages. We introduced the shLuc into MDMs from three different donors by use of the lentiviral vector system (CS-H1-shLuc). Compared with viral gene expression in cells transduced with control lentivirus (CS-H1-shGFP), a significant reduction in viral gene expression (donor 1, 72.5%; donor 2, 86.1%; donor 3, 77.8%) was observed, when shLuc (CS-H1-shLuc) was introduced into MDMs (Fig. 4). We thus demonstrated that shLuc introduced by the lentiviral expression system (CS-H1-shLuc) also significantly suppressed viral gene expression in primary non-dividing cells.

4. Discussion

During the preparation of our manuscript, siRNA delivery systems using lentiviral vectors [22,23,33–35] have been reported. In agreement with these reports, we also observed specific and efficient inhibition of target gene expression compared with the effect with an authentic plasmid expression vector system. One of the advantages to the use of a lentiviral vector system is its ability to deliver genes efficiently in primary non-dividing cells such as neurons and macrophages. As far as we know, this is the first report to evaluate lentiviral vector-mediated shRNA in human primary non-dividing cells. We demonstrated here that an shRNA delivery system using a lentiviral vector could be useful for silencing genes specifically and efficiently, even in primary non-dividing cells.

Fig. 3. Expression of shRNA by the lentiviral vector confers an efficient and stable antiviral state on human cells. (A) 293T cells transduced with the vector (pGEM-H1, pGEM-H1-shLuc) or lentiviral vector (CS-H1, CS-H1-shLuc) were maintained in culture. Challenge infection with $HIV_{NL43\text{-}luc}$ pseudotype virus (~20 ng of p24) was carried out at each indicated time point (2, 7, 21, or 35 days after transduction). At 48 h after each infection with $HIV_{NL43\text{-}luc}$ pseudotype, the level of viral production was evaluated by measuring luciferase activity. (B) 293T cells were transduced with lentiviral vector CS-H1, CS-H1-shGFP or CS-H1-shLuc (day 0). The following day, the transduced 293T cells were infected with $HIV_{NL43\text{-}luc}$ pseudotype (~10 ng of p24). Following the first challenge infection, cells were further subjected to sequential challenge infections every 3 days (2nd- to 4th NL-luc challenge). The level of viral gene expression was monitored by measuring luciferase activity in the cell every 2 days after each challenge infection. (C, D) MT-4 cells were transduced with the indicated lentiviral vectors (CS-H1-shLuc, CS-H1-shGFP and CS-H1-shVif). At 10 days post-transduction, cells were infected with NL-EGFP (450 pg of p24). The level of virus replication was monitored by measuring EGFP (C) or p24 antigen (D) expression every 2 days.

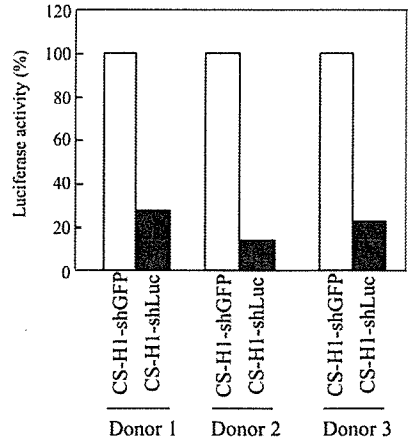


Fig. 4. Lentiviral vector expressing shRNA suppresses gene expression in MDMs. Monocyte-derived macrophages (MDM) from three different healthy donors were transduced with lentiviral vector CS-H1-shGFP or CS-H1-shLuc. At 2 days post-transduction, MDMs were infected with $HIV_{NL43\text{-}luc}$ pseudotype. The level of viral protein production in each MDM preparation was evaluated by measuring the luciferase activity at 2 days after the challenge infection.

HIV-1 uses viral RNA transcript as its replication intermediate. As described in previous reports, viral genomic RNA originally packaged in viral particles can also be targeted by chemically synthesized siRNA during its entry into cells. [9,10,30]. In contrast to these previous reports, we failed to detect shRNA-mediated degradation of HIV-1 genomic RNA in our shRNA expression system. This difference may account for the difference in intracellular localization of chemically synthesized siRNAs and shRNAs transcribed from pol III promoters. shRNAs driven by pol III promoter are primarily localized in the nucleus [16,36]. Since HIV-1 reverse transcription occurs in the cytoplasm, chemically synthesized siRNAs, but not shRNA transcribed from the pol III promoter have access to HIV-1 genomic RNA before reverse transcription. This explanation, however, might be less likely, since chemically synthesized siLuc targeting the same sequences of luc did not reduce the cDNA synthesis of HIV-1 in acute infection (Fig. 2C). Alternatively, the site recognized by shLuc might be less accessible in the genomic RNA than it is in the spliced mRNA transcribed from integrated proviral DNA.

As has been reported, synthetic siRNAs specific to HIV-1 genome sequences and/or cellular co-factors [9–12] could be possible therapeutic agents against HIV-1. As presented in the recent paper by Qin et al. [23] and our present study, a lentiviral shRNA delivery system could be a useful vehicle in gene therapy to efficiently and stably confer to cells a resistant status against HIV-1 replication.

Acknowledgements

We thank I.S.Y. Chen, T. Dull and Y.C. Sung for providing plasmids, and M. El-Farrash for critical reading of the manuscript. This work was supported by Grant-in-Aid for scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and grants of Research on HIV/AIDS from the Ministry of Health and Welfare of Japan.

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JOURNAL OF VIROLOGY, Nov. 2004, p. 11563–11573 Vol. 78, No. 21
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.21.11563–11573.2004

Evaluation of the Functional Involvement of Human Immunodeficiency Virus Type 1 Integrase in Nuclear Import of Viral cDNA during Acute Infection

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Received 23 April 2004/Accepted 18 June 2004

Nuclear import of viral cDNA is a critical step for establishing the proviral state of human immunodeficiency virus type 1 (HIV-1). The contribution of HIV-1 integrase (IN) to the nuclear import of viral cDNA is controversial, partly due to a lack of identification of its bona fide nuclear localization signal. In this study, to address this putative function of HIV-1 IN, the effects of mutations at key residues for viral cDNA recognition (PYNP at positions 142 to 145, K156, K159, and K160) were evaluated in the context of viral replication. During acute infection, some mutations (N144Q, PYNP>KL, and KKK>AAA) severely reduced viral gene expression to less than 1% the wild-type (WT) level. None of the mutations affected the synthesis of viral cDNA. Meanwhile, the levels of integrated viral cDNA produced by N144Q, PYNP>KL, and KKK>AAA mutants were severely reduced to less than 1% the WT level. Quantitative PCR analysis of viral cDNA in nuclei and fluorescence in situ hybridization analysis showed that these mutations significantly reduced the level of viral cDNA accumulation in nuclei. Further analysis revealed that IN proteins carrying the N144Q, PYNP>KL, and KKK>AAA mutations showed severely reduced binding to viral cDNA but kept their karyophilic properties. Taken together, these results indicate that mutations that reduced the binding of IN to viral cDNA resulted in severe impairment of virus infectivity, most likely by affecting the nuclear import of viral cDNA that proceeds integration. These results suggest that HIV-1 IN may be one of the critical constituents for the efficient nuclear import of viral cDNA.

Human immunodeficiency virus type 1 (HIV-1) and other lentiviruses efficiently establish in nondividing as well as dividing cells a proviral state in which a double-stranded DNA copy of the viral genomic RNA (viral cDNA) is stably integrated into a chromosome of the host cell (5, 41, 64). This property distinguishes lentiviruses from certain other retroviruses that require mitosis with nuclear envelope breakdown prior to viral cDNA integration (42, 56).

The proviral state is established through several steps following binding to and entry into the target cell, including uncoating, reverse transcription, nuclear transport, and integration of the viral genome. These early events are mediated through the interactions of several viral proteins and host factors with the viral genome, often referred to as the reverse transcription complex and the preintegration complex (PIC) (reviewed in references 3, 13, and 29). The average diameter of HIV-1 PIC has been estimated to be ~56 nm (50), precluding its passive nuclear import through intact nuclear pore complexes on the nuclear envelope of host cells (reviewed in reference 13).

To facilitate the efficient nuclear import of HIV-1 cDNA, HIV-1 PIC may contain karyophiles with nuclear localization signal (NLS) sequences. Among the HIV-1 PIC constituents, matrix (MA) protein, viral protein R (Vpr), and integrase (IN) have been reported to have karyophilic properties (4, 14, 24, 26, 27, 32, 37, 54). However, several contradictory results have also been reported, arguing against this putative role for NLS sequences within the MA protein (25, 55) or Vpr (2) in the nuclear transport of viral cDNA. In addition, the *cis*-acting viral cDNA structure, the central DNA flap, generated during lentivirus-specific reverse transcription, has also been reported to play an important role in the nuclear import of the HIV-1 genome (68). However, recent studies have shown that the effect of the central DNA flap appears to be virus strain and host cell dependent (17, 44). Thus, key factors that facilitate the nuclear import of viral cDNA in the context of the viral replication cycle still remain to be determined.

Among the HIV-1 PIC constituents described above, IN is a logical and highly probable candidate for facilitating the efficient nuclear import of HIV-1 cDNA, since it has karyophilic properties (2, 14, 26, 43, 54, 60) and mediates the integration of viral cDNA into the host chromosome in nuclei. Previously, atypical bipartite NLS sequences within HIV-1 IN were reported to be recognized by the importin/karyopherin pathway (26). However, several studies showed that these NLS sequences did not mediate nuclear import but were instead required for reverse transcription of the viral genome or other steps in the viral replication cycle (53, 60). Recently, the Val and Arg residues at positions 165 and 166 (V165/R166) in HIV-1 IN were shown to be critical for its NLS function in mediating the nuclear import of viral cDNA (2). However, reassessments of V165/R166 functions have failed to confirm this conclusion (17, 43). Thus, despite many experimental at-

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TABLE 1. Mutations in HIV-1 IN

Mutant	Changes in:	
	Nucleotide[a]	Amino acid[b]
P142F	4654-CCC → *TTC*	P142 → F
Y143G	4657-TAC → *GGG*	Y143 → G
N144Q	4660-AAT → *CAA*	N144 → Q
P145F	4663-CCC → *TTC*	P145 → F
PYNP>KL	4654-CCC. . .4666-GTA → *AAGCTT*	PYNP (positions 142–145) → KL
K156A	4696-AAA → *GCA*	K156 → A
K159A	4705-AAG → *GCG*	K159 → A
K160A	4708-AAA → *GCA*	K160 → A
KKK>AAA	4696-AAA → *GCA*/4705-AAG → *GCG*/4708-AAA → *GCA*	KKK (positions 156, 159, and 160) → AAA

[a] Altered nucleotides are indicated by italic type. The nucleotide positions are numbered according to the NL43 sequence.
[b] Numbers are the amino acid residues of NL43 IN.

tempts, the contribution of HIV-1 IN to the nuclear import of viral cDNA remains controversial, partly due to a lack of identification of bona fide NLS sequences within IN. More recently, it was reported that the nuclear import of HIV-1 cDNA was mediated through the interaction of IN and importin 7, one of the host import factors for the nuclear import of ribosomal proteins and histone H1 (23). In addition, human lens epithelium-derived growth factor/transcription coactivator p75 (LEDGF/p75) was identified as an essential cellular factor for the chromosomal targeting of HIV-1 IN (47).

On the other hand, a detailed mechanism of retroviral integration was elucidated from in vitro studies with recombinant IN protein (reviewed in references 35 and 38). For the integration of viral cDNA, IN reacts with the attachment (*att*) site located at the U3 and U5 termini of the viral cDNA (12, 39, 48, 58, 61, 62). These studies of HIV-1 IN identified three functional domains: an N-terminal zinc binding domain (6–8), a central catalytic core domain (7, 11, 18, 57, 63), and a C-terminal nonspecific DNA binding domain (11, 19, 46). The central core domain contains the highly conserved D,D35E motif, which is directly involved in the catalytic activities of IN (7, 20, 30, 57). Tsurutani et al. previously reported that a single amino acid substitution of the Tyr residue at position 143 with Gly (Y143G) in HIV-1 IN significantly reduced the level of stably integrated proviral cDNA during acute infection of human primary cells (60). This reduction, concomitant with a reduction in the level of the two-long-terminal-repeat (2-LTR) circular form of viral cDNA, was evident in nondividing cells such as monocyte-derived macrophages (MDMs). Since circular forms of viral cDNA are produced in the nucleus, the Y143G mutation may affect the nuclear import of viral cDNA. The Y143 residue is located within the highly conserved core domain containing the D,D35E motif. The central core domain also contains several highly conserved residues critical for specific binding to viral cDNA (28, 33, 34, 40). These include Y143 (22, 45) and Lys residues at positions 156 (K156) (36, 67) and 159 (K159) (16, 36, 67). Thus, Y143 has been suggested to be one of the key residues for specific binding to viral cDNA.

In this study, we generated HIV-1 IN mutants carrying mutations at these key residues for viral cDNA recognition. Then, as an alternative approach to addressing the functional involvement of HIV-1 IN in the nuclear import of viral cDNA, the effects of these mutations were evaluated in the context of viral replication and the biochemical properties of the recombinant protein forms. Of note, mutations that reduced the binding of IN to viral cDNA resulted in the inefficient nuclear import of viral cDNA during acute infection. These results strongly suggest that HIV-1 IN may be a critical factor for the efficient transport of viral cDNA into nuclei.

MATERIALS AND METHODS

Construction of mutant DNA. DNA fragments for the mutagenesis of HIV-1 IN were derived from the HIV-1 pNL43lucΔenv vector (49), in which the *env* gene is defective, allowing the formation of pseudotypes, and the *nef* gene is replaced with the firefly luciferase gene. For the mutagenesis of IN mutants [P142F, Y143G, N144Q, P145F, PYNP>KL, K156A, K159A, K160A, and KKK (156/159/160) >AAA], a 1.6-kb fragment of the pNL43lucΔenv vector spanning the KpnI and SalI sites (nucleotides [nt] 4154 to 5785) was subcloned into pBluescript SKII(+) (Stratagene, La Jolla, Calif.) (pSKnK/S). To introduce mutations, all mutagenic primers were designed to span the NsiI and AflII sites (nt 4377 to 4743). PCR products amplified with each mutagenic primer pair (Table 1) and the pNL43lucΔenv vector as a template were digested with NsiI and AflII. The mutant fragments were ligated to NsiI-AflII-digested pSKnK/S. To generate some mutations with the backbone of the D116G mutation (D116G-ΔPYNP and D116G-KKK>AAA), mutagenic PCR was performed with each mutagenic primer pair and the pNL43lucΔenv vector carrying the D116G mutation (49) as a template. After confirmation of each mutation by DNA sequence analysis, KpnI-SalI fragments (nt 4154 to 5785) containing the mutations were ligated to the corresponding region in the 4.2-kbp SpeI-SalI fragment (nt 1507 to 5785). SpeI-SalI fragments containing the mutations were inserted back into the pNL43lucΔenv vector. The amplified region and cloning junctions were confirmed by DNA sequencing.

Cells. COS-7, 293T, HeLa, and RD cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human MDMs and peripheral blood lymphocytes (PBLs) 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-Supply Center, Tokyo, Japan) for 2 h. Nonadherent cells (PBLs) were grown in RPMI 1640 medium containing 10% FBS and 2 ng of recombinant interleukin-2 (Shionogi, Osaka, Japan)/ml. Adherent cells were detached with cell dissociation solution (Sigma) and cultured in RPMI 1640 containing 5% human AB serum for 5 to 7 days. More than 98% of the cells that were shown to be positive for CD14, CCR5, HLA-DR, and mannose receptors were used as MDMs.

Virus preparation and infection. Pseudotype viruses were generated by cotransfection of COS-7 cells or 293T cells with the pNL43lucΔenv vector containing each IN mutation and an amphotropic Moloney murine leukemia virus (MuLV) envelope expression vector, pJD-1 (a kind gift from Irvin S. Y. Chen, University of California at Los Angeles), or a macrophage-tropic HIV-1 envelope vector, pJR-FL (a kind gift from Yoshio Koyanagi, Tohoku University), by using Lipofectamine (Invitrogen, Carlsbad, Calif.) or a calcium phosphate precipitation method. The culture supernatants (4 ml) of the transfected COS-7 cells

were harvested at 48 h posttransfection, filtered though 0.45-μm-pore-size filters, and used as the virus preparations. Each virus preparation was treated with DNase I (40 U/ml; Takara, Kyoto, Japan) in the presence of 10 mM $MgCl_2$ at 37°C for 1 h. To monitor residual contamination of the plasmid DNA, an aliquot of each virus preparation was incubated at 65°C for 1 h and used as a heat-inactivated control. To monitor the amount of virus in each preparation, the levels of HIV-1 p24 antigen were determined with an enzyme immunoassay system (RETRO-TEK; ZeptoMetrix Corp., Buffalo, N.Y.). To monitor viral gene expression from each plasmid vector, the luciferase activity in transfected COS-7 cells was also measured. At 48 h posttransfection, COS-7 cells were lysed with 1 ml of cell lysis buffer (Promega, Madison, Wis.), and then 1 μl of each cell lysate was subjected to a luciferase assay with Lumat LB 9507 (EG & G Berthold, Bad Wildbad, Germany). For virus infection, an aliquot (corresponding to ~20 ng of p24) of DNase I-treated virus was inoculated into RD cells (5×10^4), MDMs (5×10^5), or PBLs (1×10^6) in the presence of Polybrene (10 μg/ml). After incubation for 6 h at 37°C, the virus-containing medium was removed and replaced with fresh medium. At 4 days postinfection, the cells were harvested, washed twice with phosphate-buffered saline (PBS), and then lysed with 200 μl of cell lysis buffer. An aliquot (10 μl) of each lysate was subjected to the luciferase assay.

Western blot analysis. Viruses were concentrated by ultracentrifugation (1 h at 315,000 × *g* in a Beckman TLX-100 centrifuge with a TLA-100.4 rotor), and the pellets were resuspended in PBS. Viral proteins containing approximately 10 ng of p24 were subjected to sodium dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE). Following blotting of proteins onto a nitrocellulose membrane (ATTO, Tokyo, Japan), the membrane was incubated with antiserum from AIDS patients, anti-HIV-1 IN antibody (kindly provided by Duane Grandgenett, St. Louis University Health Sciences Center, Institute for Molecular Virology), or anti-HIV-1 p24 antibody (Chemicon International, Temecula, Calif.) followed by horseradish peroxidase-conjugated anti-human, anti-rabbit, or anti-mouse immunoglobulin. HIV-1 proteins were visualized by using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Tokyo, Japan).

Analysis of de novo-synthesized HIV-1 cDNA during acute infection. Total cells were harvested from each well at 1 or 2 days postinfection. After the cells were washed with PBS, nucleic acids were extracted as described previously (66). Briefly, the cells were disrupted in urea lysis buffer (4.7 M urea, 1.3% SDS, 0.23 M NaCl, 0.67 mM EDTA [pH 8.0]) and subjected to phenol-chloroform extraction and ethanol precipitation. The resulting DNA pellet was resuspended in 100 μl of distilled deionized water (ddw). An aliquot (5 μl) of each sample was subjected to PCR with a primer pair specific for the R/U5 region of HIV-1 (M667-AA55) or the R/gag region (M667-M661) (66). Detection of HIV-1 DNA sequences with each primer pair was performed by using PCR conditions of 30 cycles of 94°C for 1 min, 65°C for 2 min, and 72°C for 2 min. For DNA standards, 8 to 25,000 copies of linearized HIV-1 pNL43lucΔenv DNA were amplified in parallel. Quantitative analysis of the amplified products was performed by using a real-time Light Cycler detection system (Light Cycler Instrument; Roche, Mannheim, Germany). To normalize the amount of cellular DNA in the samples, a primer pair complementary to the first exon of the human β-globin gene was used. For the detection of human β-globin DNA, amplification was performed by using PCR conditions of 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. A standard curve for human β-globin DNA was obtained by amplifying known amounts of cellular DNA from RD cells or MDMs in parallel. The 2-LTR circular DNA in nuclei was detected with specific primers as described previously (21). For detection of the integrated form of HIV-1 DNA, we used the Alu PCR method with an HIV-1-specific primer (M661) and an Alu-specific primer (5′-TCCCAGCTACTCGGGAGGCTGAGG-3′) (59). The cycling conditions were 94°C for 3 min, followed by 22 cycles of 94°C for 30 s, 66°C for 30 s, 70°C for 10 min, and then 72°C for 10 min. PCR products were purified and diluted 100- to 10,000-fold. The diluted samples were subjected to real-time PCR with the Light Cycler Instrument and an R/U5-specific primer pair (M667-AA55).

Confocal microscopic analysis of GFP-IN. HeLa cells (4×10^3) were seeded onto glass slides (Cel-Line; Erie Scientific Co, Portsmouth, N.H.) and transfected with a plasmid expressing green fluorescent protein (GFP) fused to full-length IN (60). At 24 h posttransfection, the cells were washed once with PBS and then fixed with 4% paraformaldehyde (Wako, Osaka, Japan) for 10 min. Confocal microscopy was performed with an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan). Representative medial sections were mounted by using Adobe Photoshop software.

In vitro GST pull-down assay. DNA fragments encoding the entire HIV-1 IN were amplified by PCR with wild-type (WT) or each mutant plasmid DNA (pNL43lucΔenv) as a template. The primers used for the amplification of HIV-1 IN were as follows: GST-IN sense, 5′-GCGGATCCTTTTTAGATGGAATAG ATAAGGCCC-3′, and GST-IN end antisense, 5′-CCGGAATTCAATCCTCA TCCTG-3′; the sequences for BamHI or EcoRI recognition in each primer are underlined. Amplified products were digested with BamHI and EcoRI. Fragments with or without mutations were ligated to BamHI-EcoRI-digested vector pGEX-2T (Amersham Pharmacia Biotech). To express glutathione *S*-transferase (GST)–IN, *Escherichia coli* strain BL21(DE3) cells which had been transformed with pGEX-IN were grown in 16% Bacto Tryptone–10% yeast extract–5% NaCl–50 mg of carbenicillin/ml at 37°C to an optical density at 600 nm of 0.7. Expression was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) for 17 h at 20°C. Cells were harvested by centrifugation and resuspended at a 1/10 volume of the original cell culture in high-salt buffer (1 M NaCl in PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and a proteinase inhibitor mixture (1 μg of pepstatin A/ml, 2 μg of aprotinin/ml, and 0.5 μg of leupeptin/ml). Cells were lysed by sonication (Sonifier; Branson, Danbury, Conn.) followed by centrifugation at 19,000 × *g* for 30 min. The supernatant fraction of the cell lysate was incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) at 4°C overnight. The recombinant protein-bound beads were washed extensively with a wash buffer (1 M NaCl–1% Tween 20 in PBS) and then eluted with elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM reduced glutathione) containing the proteinase inhibitor mixture at 25°C for 2 h. The eluted fraction containing the recombinant GST-IN protein was dialyzed against IN storage buffer {20 mM HEPES [pH 7.5], 0.1 mM EDTA, 0.3 M NaCl, 20% glycerol, 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 10 mM DTT}. The purity of each recombinant GST-IN protein was confirmed by PAGE, and the protein concentrations were determined by the Bradford method.

The HIV-1 cDNA for the pull-down assay was prepared from crude PIC fractions (31). Briefly, MOLT-4/IIIB cells were cocultured with MOLT-4 cells for 6 h. Cells were harvested and permeabilized with 0.025% digitonin in buffer K (20 mM HEPES [pH 7.4], 150 mM KCl, 5 mM $MgCl_2$) followed by sequential centrifugations at 1,000 × *g* for 10 min and 12,000 × *g* for 20 min. The supernatants were used as crude PIC fractions. Viral cDNA was extracted from the crude PIC fractions by the urea lysis method as described above. The amount of viral cDNA was determined by real-time PCR with R/U5- and R/gag-specific primers. Viral cDNA (~10^5 copies) was incubated with each GST-IN protein (200 nM) immobilized on glutathione-Sepharose beads in binding buffer (20 mM HEPES [pH 7.3], 7.5 mM $MnCl_2$, 1 mM DTT, 1 mM PMSF, 10% polyethylene glycol) for 15 min at 4°C. The beads were washed five times with a wash buffer (1.0% Triton X-100 in PBS) and eluted with elution buffer. Viral cDNA bound to each GST-IN protein was extracted by phenol-chloroform treatment followed by ethanol precipitation with a carrier (Ethachinmate; Wako/Nippon Gene Co., Tokyo, Japan). The resulting DNA pellet was suspended in ddw. The amount of viral cDNA in each preparation was determined by real-time PCR with the M667-AA55 (R/U5-specific) or the M667-M661 (R/gag-specific) primer pair. The background level due to nonspecific binding of the input viral cDNA to GST protein and glutathione-Sepharose beads was determined by carrying out the same experiment with GST only in parallel. After subtraction of the background level (~5,000 copies per total input of ~10^5 copies), the relative copy number (R/gag) for each mutant as a percentage of that for the WT was calculated.

Isolation of DNA from the nuclear fraction. RD or 293T cells (5×10^6) were infected with each pseudotype virus and harvested at 24 h postinfection. For the isolation of nuclei, cells were resuspended in buffer A (10 mM HEPES [pH 7.3], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2% NP-40), homogenized by 20 strokes with an all-glass Dounce homogenizer (Kontes, Vineland, N.J.), and centrifuged at 500 × *g* for 5 min at 4°C. The pellet of the cell lysate was washed twice with Nuclei EZ Lysis Buffer and then with Nuclei Storage Buffer (Sigma). Nuclei were collected by centrifugation at 25,000 × *g* for 20 min at 4°C. DNA samples from each nuclear fraction were subjected to real-time PCR with the M667-AA55 (R/U5-specific) or the M667-M661 (R/gag-specific) primer pair as described above.

FISH analysis with HIV-1-specific PNA probes. For fluorescence in situ hybridization (FISH) analysis with HIV-1-specific peptide nucleic acid (PNA) probes (51), glass slides (Cel-Line) were coated with 3-aminopropyl triethoxysilane (silane; Aldrich). HeLa cells (4×10^3) seeded on the silane-coated slides were cultured overnight. Pseudotype viruses were prepared by cotransfection of 293T cells with the pNL43lucΔenv vector together with the MuLV envelope expression vector (pJD-1). After treatment with DNase I (40 μg/ml; Worthington), each virus (70 ng of p24) was inoculated into HeLa cells and cultured at 37°C for 6 h. The cells were washed with PBS and resuspended in fresh medium (DMEM plus 10% FBS). After 18 h, the cells were harvested and fixed with 4% paraformaldehyde for 30 min, and the slides were treated with proteinase K (2 ng/ml) for 8 min at room temperature and then with RNase A (10 ng/ml) for 10

min at room temperature. Endogenous biotin reactivity was blocked by the addition of 0.3% H_2O_2 in methanol. The slides were dehydrated in absolute ethanol.

Fluorescein isothiocyanate (FITC)-conjugated PNA probes were synthesized by Fasmac Co. (Kanagawa, Japan). The structure of the antisense probe was as follows: FITC-5′-GCACATTGTACTGATA-3′; this sequence corresponded to nt 2878 to 2994 of the *pol* region of pNL43 (1). The hybridization solution (Dako, Carpinteria, Calif.) containing the FITC-conjugated PNA probes was mounted on the slides, boiled for 5 min at 93°C, and then incubated for 17 h at 57°C. The slides were washed twice with a stringent wash solution (Dako) at 45°C for 20 min. After being washed with Tris-buffered saline containing 0.1% Tween 20 (TBST), the slides were reacted with 63 pg of a horseradish peroxidase (HRP)-conjugated anti-FITC antibody (Vector, Burlingame, Calif.)/ml for 30 min at 37°C. The slides were washed with TBST and reacted with biotinyl-tyramide (Dako) for 15 min at room temperature. After being washed with TBST, the slides were reacted with HRP-conjugated streptavidin (Dako). The slides were washed with TBST and reacted with biotinyl-tyramide for 15 min. Finally, the slides were reacted with 250 ng of Alexa 488-conjugated streptavidin/ml to detect the hybridized probes. Nuclei were stained with 125 ng of 4′,6′-diamidino-2-phenylindole (DAPI; Wako)/ml. The signals were observed with a confocal microscope (Olympus BX50). We counted the signal dots inside the nuclei of 20 to 50 cells from three independent hybridizations. Representative medial sections were mounted by using Adobe Photoshop software.

RESULTS

Construction of HIV-1 IN mutants. We introduced single amino acid substitutions or deletions at highly conserved Pro-Tyr-Asn-Pro residues (PYNP) spanning positions 142 to 145 or the three Lys residues at positions 156, 159, and 160 (K156, K159, and K160) in HIV-1 IN (Table 1), which are regions that contain several key residues critical for viral cDNA recognition (16, 22, 36, 45, 67). The mutations (Table 1) included a Pro-to-Phe substitution at position 142 (P142F), a Tyr-to-Gly substitution at position 143 (Y143G), an Asn-to-Gln substitution at position 144 (N144Q), a Pro-to-Phe substitution at position 145 (P145F), deletion of the PYNP motif at positions 142 to 145 by replacement with Lys-Leu (PYNP>KL), and Ala substitution(s) at each or all of the Lys residues at positions 156, 159, and 160 (K156A, K159A, K160A, and KKK>AAA). Due to the lack of identification of bona fide NLS sequences within IN, the effects of these mutations, which were expected to be defective at binding to viral cDNA, were evaluated in the context of viral replication, as an alternative approach to addressing the functional involvement of HIV-1 IN in the nuclear import of viral cDNA.

Infectivity of each HIV-1 IN mutant. A single-round infection system that uses an HIV-1 (pNL43lucΔBg) pseudotype virus is useful for estimating reverse transcription and integration efficiency in vivo by monitoring the levels of de novo-synthesized viral cDNA and luciferase activity produced in infected cells (48, 49, 60). Therefore, we examined the effect of each mutation on early events in viral replication by using the single-round infection system. Briefly, pseudotype viruses were generated by cotransfection of COS-7 cells with pNL43lucΔenv containing the WT or each IN mutation together with an expression vector for the amphotropic Moloney MuLV envelope (pJD-1) or the macrophage-tropic HIV-1 envelope (pJR-FL). At 48 h posttransfection, all mutants had comparable levels of p24 in culture supernatants harvested from transfected COS-7 cells (Fig. 1A) and comparable levels of luciferase activity in lysates of transfected COS-7 cells (data not shown). Thus, none of the mutations had a significant effect on transfected proviral gene expression or virus release.

In order to verify the Gag-Pol polyprotein processing in mutant virus particles, we performed Western blotting analyses of viral proteins contained in the virus particles with serum from an AIDS patient or antibodies to HIV-1 IN or p24. No apparent differences between the parental (WT) and mutant viruses were observed in the profiles (Fig. 1B and C). These

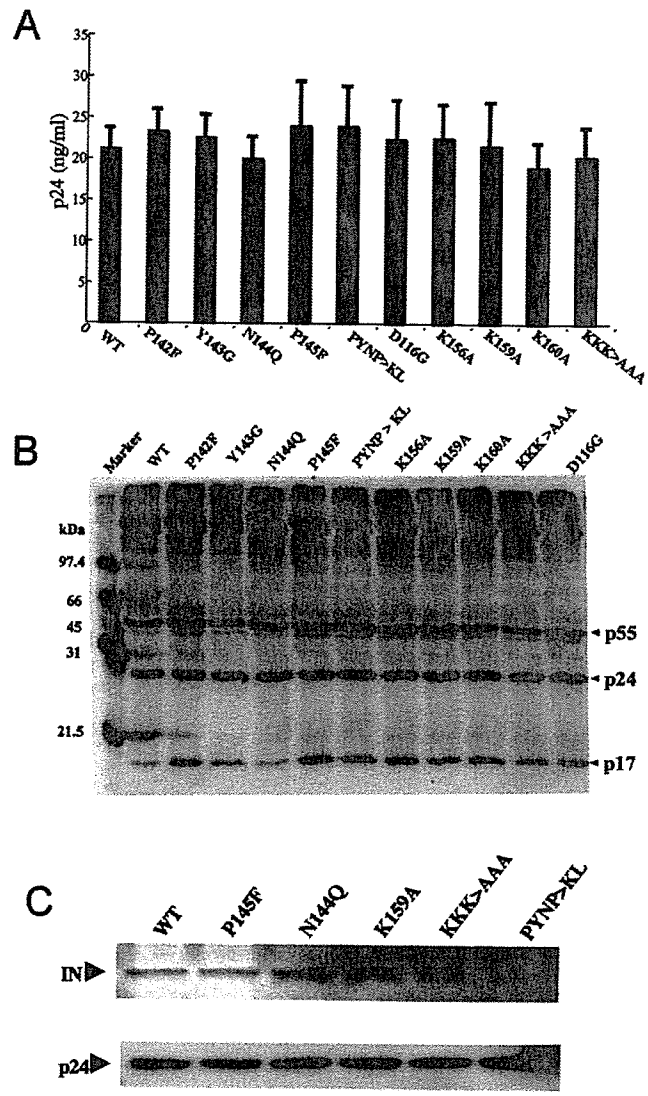

FIG. 1. Gene expression of each mutant proviral DNA after transfection of COS-7 cells and viral protein profiles. Pseudotype viruses were generated by cotransfection of COS-7 cells with the pNL43lucΔenv vector containing mutations in IN and an amphotropic Molony MuLV envelope expression vector (pJD-1) by using Lipofectamine. Culture supernatants (5 ml) of the transfected COS-7 cells were harvested at 48 h posttransfection. (A) p24 levels in culture supernatants were determined with the RETRO-TEK enzyme immunoassay system. (B and C) Virus particles in culture supernatants (5 ml) of COS-7 cells were precipitated at 48 h posttransfection by ultracentrifugation (1 h at 315,000 × *g* in a Beckman TLX-100 centrifuge). Viral proteins were separated by SDS–12% PAGE. After blotting of proteins onto a nitrocellulose membrane, the membrane was reacted with serum from an AIDS patient (B) or anti-HIV-1 IN or anti-p24 antibody (C) and then incubated with horseradish peroxidase-conjugated anti-human, anti-rabbit, or anti-mouse immunoglobulin. Viral proteins were visualized by using the enhanced chemiluminescence detection system. The positions of the major viral proteins are indicated.

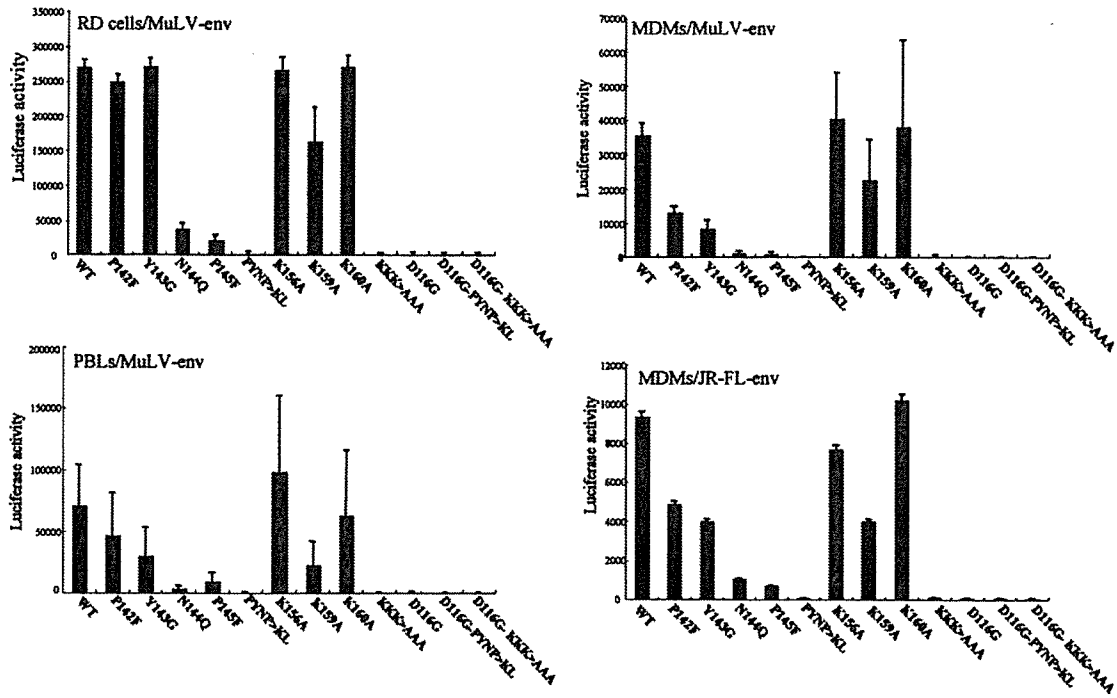

FIG. 2. Effects of HIV-1 IN mutations on viral infectivity. Viruses were prepared by cotransfection of COS-7 cells with the pNL43lucΔenv vector containing either WT IN or mutant IN together with an amphotropic Moloney MuLV envelope expression vector (pJD-1) or a macrophage-tropic HIV-1 envelope vector (pJR-FL) by using Lipofectamine. At 48 h posttransfection, culture supernatants of the transfected COS-7 cells were harvested. DNase I-treated supernatants were inoculated into 10^5 RD cells, PBLs, and MDMs. At 4 days postinfection, the cells were washed with PBS and lysed with 200 μl of cell lysis buffer. Ten microliters of each cell lysate was subjected to the luciferase assay. Mean values from five independent experiments are shown with the error bars.

results showed that none of the mutations significantly affected the late stage of viral replication from proviral gene expression to virus particle release, Gag-Pol polyprotein processing, and IN incorporation into the virus particles.

For virus infection, a DNase I-treated preparation of each pseudotype virus was inoculated into RD cells, MDMs, or PBLs. To examine the level of viral gene expression for each HIV-1 IN mutant, the luciferase activity expressed in infected cells was measured at 4 days postinfection. In this experiment, an IN mutant carrying a single amino acid substitution at one of the catalytic sites (D116G) was used as a control for integration-defective mutants (49). We repeated this experiment more than 10 times with independently prepared viruses (Fig. 2). In RD cells, the PYNP>KL mutation or the KKK>AAA mutation severely reduced the luciferase activity to less than 1% the WT level, which was similar to the level seen with the catalytic site mutation (D116G). The severe effects induced by these mutations were also observed after acute infection of human primary PBLs and MDMs. A significant reduction in the level of luciferase gene expression was also detected with some mutants with single amino acid substitutions in the PYNP motif (residues 142 to 145) or at K156, K159, and K160. The levels of luciferase activity produced by N144Q and P145F were significantly lower in RD cells, PBLs, and MDMs and ranged from 3 to 15% the WT level. As reported in a previous study (60), the effect of the Y143G mutation in the PYNP motif was more evident in primary cells (PBLs and MDMs) than in RD cells. Like Y143G, P142F (a single amino acid substitution in the same PYNP motif) produced lower levels of luciferase activity (24 to 65% the WT level) in PBLs and MDMs but produced a high level (90 to 100% the WT level) in RD cells. Thus, the integrity of the PYNP motif is more rigidly required for virus infection in primary cells (PBLs and MDMs) than in an in vitro-adapted cell line (RD cells). In addition, K159A produced a modest but consistent reduction in luciferase activity (42 to 60% the WT level), while other point mutations in the K residues (K156A and K160A) produced levels of luciferase activity comparable to that produced by the WT in all cell types. Since the KKK>AAA mutation produced a severe reduction in luciferase activity (<1% the WT level), the three Lys residues may function in a compensatory manner. Furthermore, these severe defects were efficiently complemented when WT IN was supplied in *trans* (data not shown), thus excluding possible effects of the mutations on *cis*-acting functions of the central polypurine tract (10, 68). Taken together, these results suggested important roles of PYNP at residues 142 to 145 and of K at residues 156, 159, and 160 in *trans*-acting functions of IN in the early events of the HIV-1 replication cycle.

Quantitative analysis of viral cDNA after infection with HIV-1 IN mutants. Next, we assessed the ability of each mutant to synthesize viral cDNA and to form integrated proviral cDNA following infection of RD cells (Fig. 3). At 24 h after infection with DNase I-treated virus, total DNA was harvested from infected RD cells, and an aliquot of each DNA sample was subjected to real-time quantitative PCR analysis. First, we monitored the formation of various species of viral cDNAs by using the M667-AA55 (R/U5-specific) primer pair for early

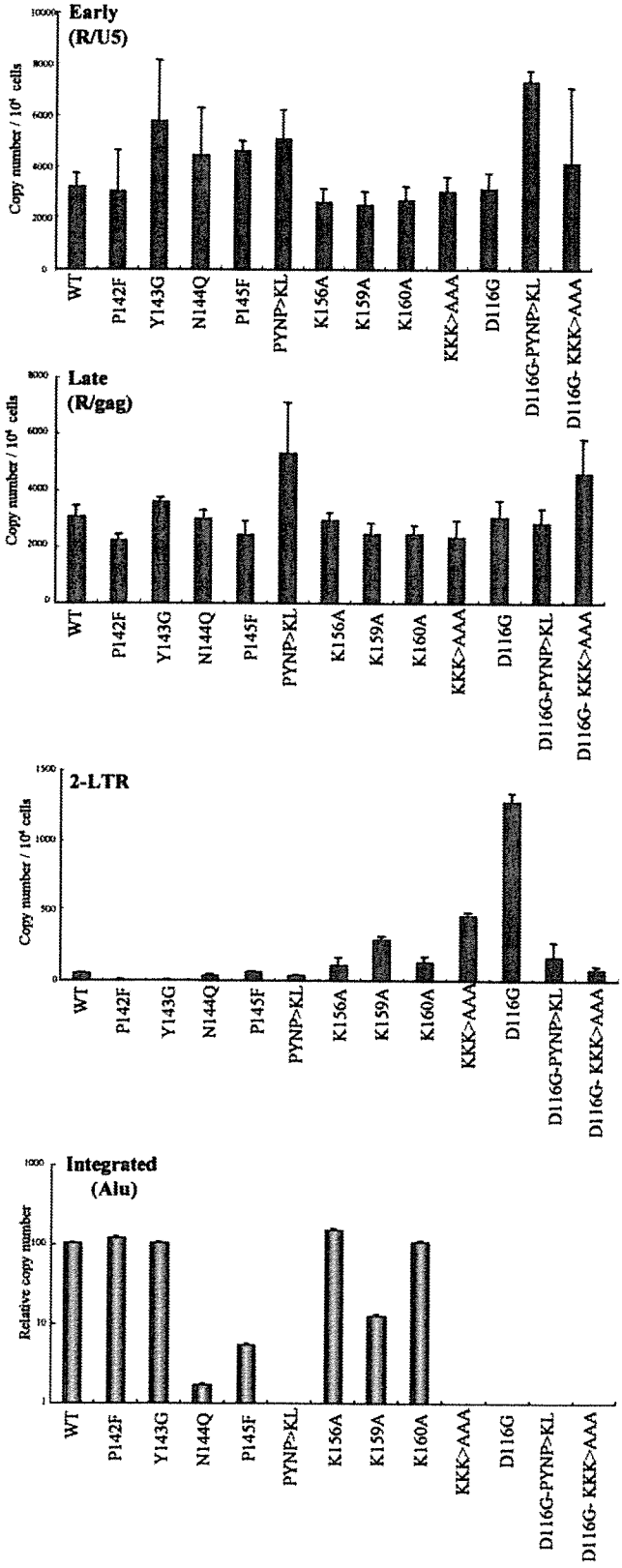

FIG. 3. Analysis of viral cDNA synthesis and proviral DNA formation. Each virus was prepared by cotransfection of COS-7 cells with pNL43lucΔenv (WT or mutant IN) together with pJD-1. DNase I-treated supernatants were inoculated into RD cells as described in the legend to Fig. 2. Viruses treated at 65°C for 1 h prior to inoculation were used as a heat-inactivated control. At 1 day postinfection, the entire cell culture was harvested, and total DNAs were extracted from infected RD cells. Each DNA sample was subjected to real-time quantitative PCR analysis with primer pairs specific for early (R/U5), late (R/gag), 2-LTR circular (2-LTR), or integrated (Alu) forms of viral cDNAs. The estimated copy number of each viral cDNA (R/U5, R/gag, 2-LTR, and Alu) per 10^5 cell equivalents is shown. For estimation of integrated viral cDNA (Alu), each DNA sample was subjected to PCR with the Alu- and R/gag-specific primer pair. Serial dilutions of each PCR product (Alu and R/gag) were subjected to real-time quantitative PCR with the R/U5-specific primer pair. Values were calculated as the copy number (R/U5) of each mutant relative to that of the WT, taken as 100%. Mean values from five independent experiments are shown with the error bars.

viral cDNA (early) and the M667-M661 (R/gag-specific) primer pair for complete or nearly complete viral cDNA (late). Relative to the WT, all PYNP motif mutants (P142F, Y143G, N144Q, P145F, and PYNP>KL) as well as K mutants K156A, K159A, K160A, and KKK>AAA produced comparable levels of early (R/U5) and complete or nearly complete (R/gag) forms of viral cDNA products. These results showed that none of these mutations affected reverse transcription.

Since all of the mutations in the PYNP and K residues produced comparable levels of complete or nearly complete viral cDNA products (R/gag), we next examined the fate of each de novo-synthesized viral cDNA in RD cells. To this end, we monitored the formation of 2-LTR circular DNA (Fig. 3, 2-LTR) by using a primer pair which amplified a sequence unique to the 2-LTR DNA junction (21). We also monitored the level of the integrated form of each viral cDNA by the Alu PCR method (59) with an HIV-1-specific primer (M661) and an Alu-specific primer (Fig. 3, Integrated). Products of 2-LTR DNA have been used as markers for its presence in the nucleus, since the enzymes responsible for its formation are thought to be located within the nucleus. In addition, 2-LTR DNA is formed in the absence of the catalytic function of IN (60). PCR amplification of this structure therefore confirms the specific inhibition of integration. An HIV-1 IN catalytic site mutant (D116G) (49) was used as a control for integration-defective mutants. As in a previous study (60), we clearly detected an amplified fragment corresponding to the 2-LTR circular junction in DNA samples from RD cells infected with integration-defective mutant D116G. Meanwhile, the P142F, Y143G, K156A, and K160A mutants as well as the WT produced much lower levels of 2-LTR DNA (2 to 9% the level produced by D116G), suggesting successful integration of viral cDNA. These conclusions were consistent with the level of expression of each viral gene (luciferase activity) shown in Fig. 2 and were further confirmed by Alu PCR analysis (Fig. 3, Integrated). On the other hand, the N144Q, P145F, PYNP>KL, K159A, and KKK>AAA mutants produced 2-LTR DNA at various levels—3, 5, 3, 23, and 36% the level produced by D116G, respectively (Fig. 3, 2-LTR). Of note, the levels of 2-LTR produced by these mutants were not always correlated with the reduction in luciferase activity (Fig. 2) or the integration level (Fig. 3, Integrated). In particular, the PYNP>KL mutant showed significant reductions in the level of luciferase activity (<2% the WT level) and integration (below the detection level). These phenotypes of PYNP>KL were almost iden-

tical to those of D116G. However, the level of 2-LTR DNA produced by PYNP>KL was only ~3% the level produced by D116G. Similar discrepancies were also noted for the N144Q and KKK>AAA mutants. In addition, a similar reduction in the level of 2-LTR DNA caused by the PYNP>KL and KKK>AAA mutations was reproduced on the backbone of the D116G mutation (Fig. 3, D116G-PYNP>KL and D116G-KKK>AAA). Taken together, these data suggested that the reduced levels of integration and subsequent gene expression seen with PYNP>KL, N144Q, and KKK>AAA might have been partly due to a reduction in the nuclear import of viral cDNA that precedes integration.

Interaction of IN protein with viral cDNA. Some of the IN mutants (PYNP>KL, N144Q, and KKK>AAA) showed a severe integration-defective phenotype, partly due to a failure to transport the viral cDNA into the nucleus. These mutants were generated by introducing mutations at critical residues in IN for viral cDNA recognition (16, 22, 36, 45, 67). Therefore, we next examined the binding of these IN proteins to viral cDNA. Viral cDNA (~10^5 copies) extracted from cells acutely infected with HIV-1 was incubated with 200 nM each GST-IN protein (WT, N144Q, PYNP>KL, or KKK>AAA) immobilized on glutathione-Sepharose beads. After extensive washing, the viral cDNA bound to each GST-IN protein was extracted. The amount of viral cDNA in each preparation was determined by real-time PCR with a primer pair specific for late forms (R/gag) of viral cDNA products (Fig. 4A). The levels of viral cDNA pulled down by GST-IN protein containing N144Q, PYNP>KL, and KKK>AAA were 2.1, 3.8, and 11.7% the WT level, respectively (Fig. 4A). Whether these in vitro data can be extrapolated to biological relevance in vivo still remains to be determined, but the PYNP>KL, N144Q, and KKK>AAA mutations reduced the binding of IN to viral cDNA at least in our in vitro assay.

Nuclear localization of IN fused to GFP. Tsurutani et al. previously reported that GFP-IN fusions efficiently localized to the nuclei of transiently transfected cells (60). We next examined the effects of the N144Q, PYNP>KL, K159A, and KKK>AAA mutations on the karyophilic properties of IN. Briefly, HeLa cells were transfected with GFP-IN expression vectors and subjected to analysis by confocal microscopy at 24 h posttransfection. In agreement with the previous report (60), GFP-IN accumulated predominantly in the nucleus [Fig. 4B, GFP-IN (WT)], while the GFP control protein was uniformly scattered throughout both the cytoplasm and the nucleus (Fig. 4B, GFP). Under the same conditions, IN mutants (N144Q, PYNP>KL, and KKK>AAA) were efficiently localized to the nucleus (Fig. 4B), suggesting that none of these mutations affected the karyophilic properties of IN.

Localization of HIV-1 cDNA during acute infection. The experimental data described above indicated that the loss of binding to viral cDNA seen with the IN mutations resulted in the decreased accumulation of viral cDNA in the nuclei. To confirm these observations more directly, we first determined the levels of viral cDNA in nuclear fractions isolated from acutely infected cells. 293T cells were infected with a DNase I-treated preparation of each pseudotype virus. At 24 h postinfection, nuclei were isolated from the cells. The levels of viral cDNA in the nuclei were determined by real-time PCR with a primer pair specific for R/U5 to monitor the total viral cDNA

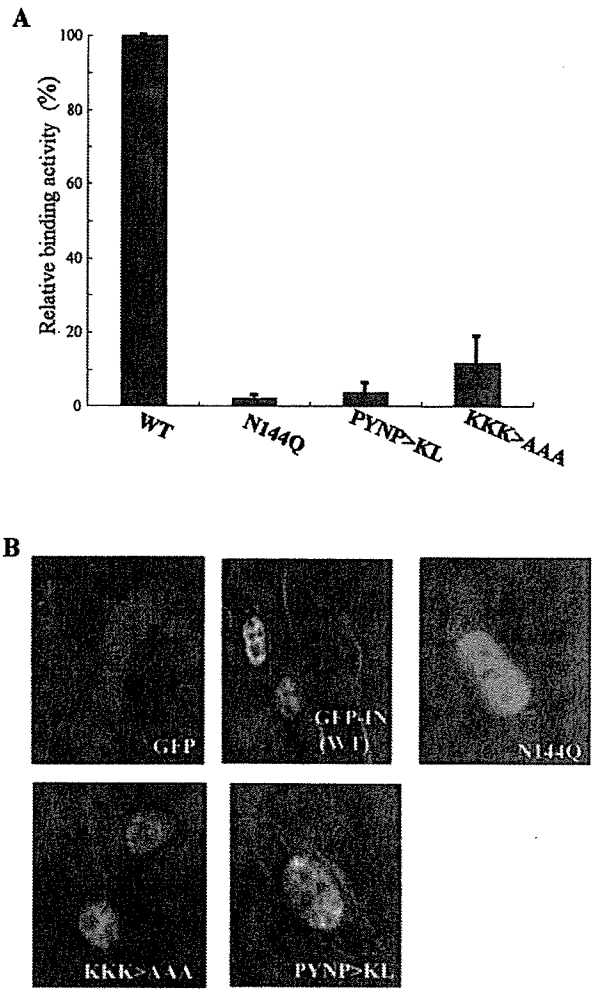

FIG. 4. Properties of HIV-1 IN mutant proteins. (A) Interactions of IN proteins with HIV-1 cDNA. Viral cDNA (~10^5 copies) extracted from a crude PIC fraction (see Materials and Methods) was incubated with 200 nM each GST-IN protein (WT, N144Q, PYNP>KL, and KKK>AAA) immobilized on glutathione-Sepharose beads in binding buffer. The beads were washed five times with a wash buffer (1.0% Triton X-100 in PBS) and eluted with elution buffer. Viral cDNA bound to each GST-IN protein was extracted by phenol-chloroform treatment followed by ethanol precipitation. The resulting DNA pellet was resuspended in ddw. The amount of viral cDNA in each preparation was measured by a real-time PCR system with a primer pair specific for the late product (R/gag) of viral cDNA. The values shown are the copy number (R/gag) of each mutant relative to that of the WT, taken as 100%. Mean values from three independent experiments are shown with the error bars. (B) Confocal microscopic analysis of GFP-IN. HeLa cells were transfected with a plasmid expressing GFP only, GFP fused to full-length IN (WT), or IN carrying the PYNP>KL, N144Q, or KKK>AAA mutation by using Lipofectamine. At 24 h posttransfection, the cells were fixed and examined with a confocal fluorescence microscope.

in the nuclei. The levels of viral cDNA in the nuclei after infection with the N144Q, PYNP>KL, KKK>AAA, and D116G IN mutants were 50.3, 38.9, 43.6, and 90.3% the WT level, respectively (Fig. 5A). These data indicated that the nuclear import of viral cDNA was significantly decreased by the N144Q, PYNP>KL, and KKK>AAA mutations ($P < 0.005$) but not by the D116G mutation ($P = 0.14$). Similar significant reductions in the levels of viral cDNA in the nuclei seen with

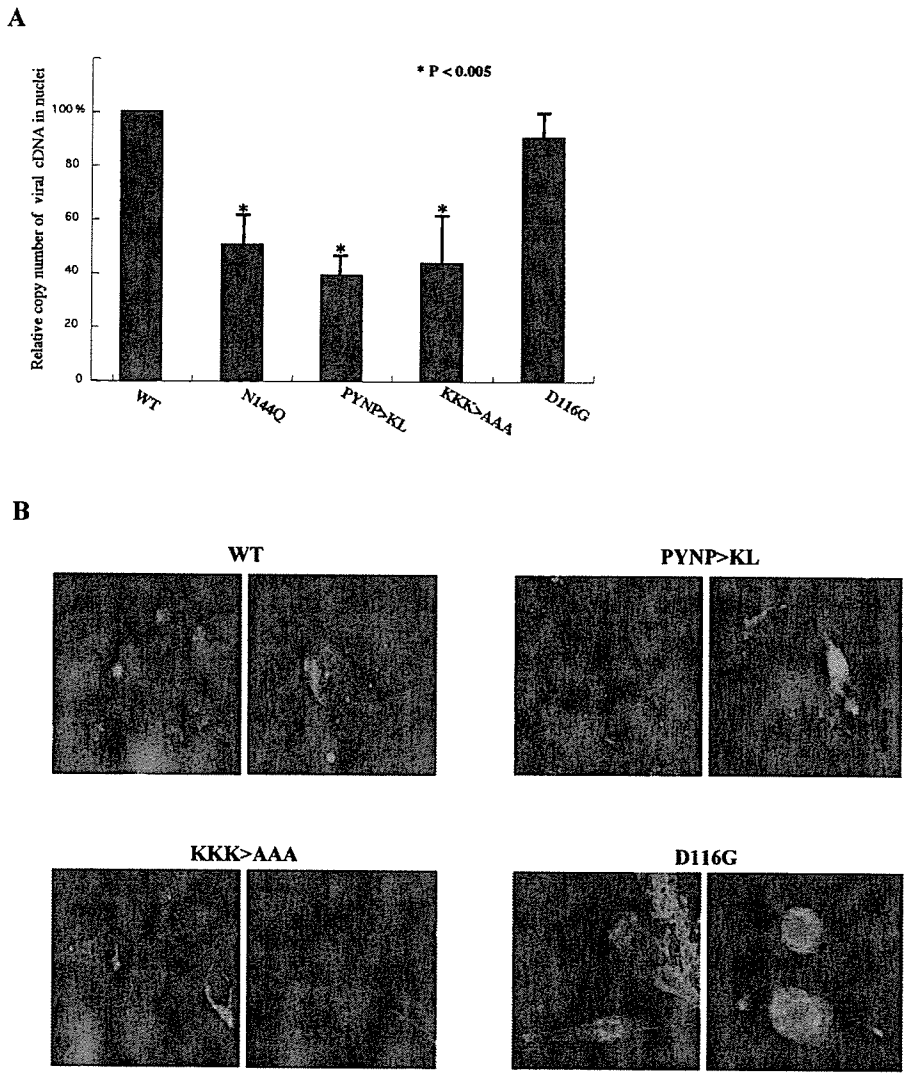

FIG. 5. Localization of HIV-1 cDNA after infection with each mutant. (A) Quantification of HIV-1 cDNA in the nuclear fraction. 293T cells were infected with each virus (WT, N144Q, PYNP>KL, KKK>AAA, or D116G). At 24 h postinfection, nuclei were isolated from the infected cells as described in Materials and Methods. A DNA sample from each nuclear fraction was subjected to real-time quantitative PCR with the M667-AA55 (R/U5-specific) or the M661-M667 (R/gag-specific) primer pair. The values shown are the copy number (R/U5) of each mutant per 10^4 nuclei relative to that of the WT, taken as 100%. Means and standard deviations from three independent experiments are shown ($n = 5$). (B) HeLa cells (4×10^3) were seeded on silane-coated glass slides. Cells were infected with each IN mutant (PYNP>KL, KKK>AAA, or D116G) or with the WT (~70 ng of p24). At 6 h postinfection, the cells were fixed with 4% paraformaldehyde and subjected to FISH analysis with an FITC-labeled HIV-1-specific PNA probe corresponding to nt 2878 to 2994 of the *pol* region of pNL43 (1). The signals were observed with a confocal microscope. Representative medial sections were mounted by using Adobe Photoshop software. The representative cells in each slide are shown with lower (left) and higher (right) magnification scales.

the N144Q, PYNP>KL, and KKK>AAA mutations were reproduced when a primer pair specific for R/gag was used (data not shown).

Finally, we analyzed the subcellular localization of de novo-synthesized viral cDNA during acute infection by FISH. Briefly, HeLa cells were infected with a DNase I-treated pseudotype virus of each IN mutant, incubated for 6 or 24 h, fixed, and subjected to FISH with an FITC-tagged HIV-1-specific PNA probe. Hybridized probe signals were detected by using a CAS system (Dako) with Alexa 488 and were visualized with an Olympus confocal laser scanning microscope (Fig. 5B). The majority (~78%) of the signals produced by the WT or the D116G mutant were in the nuclei at 6 h postinfection. In contrast, under the same conditions, markedly fewer signals were detected in the nuclei after infection with the PYNP>KL and KKK>AAA mutants. These signals in the cytoplasm tended to be weakened at 24 h postinfection (data not shown), suggesting that PYNP>KL and KKK>AAA might reduce the nuclear accumulation of viral cDNA partly because these mutant PICs were more susceptible to degradation. Although we cannot rule out the partial contribution of the effect of these mutations on the stability of viral cDNA and/or the PIC, these results indicated that the nuclear transport that precedes the integration of viral cDNA was significantly reduced by muta-

tions that reduced the binding affinity of HIV-1 IN for viral cDNA.

DISCUSSION

In this study, we generated HIV-1 IN mutants carrying mutations at key residues for viral cDNA recognition (16, 22, 36, 45, 67) and evaluated the effects of these mutations in the context of viral replication and the biochemical properties of the protein forms. Our major finding was that mutations reducing the interaction of IN with viral cDNA caused a severe impairment of integration, most likely by affecting the efficient nuclear import of viral cDNA.

Despite many experimental data indicating the karyophilic properties of HIV-1 IN (2, 14, 26, 43, 54, 60), the contribution of IN to the nuclear import of viral cDNA remains controversial. This situation may be partly due to a lack of identification of bona fide NLS sequences within IN. We also generated IN mutants by amino acid substitution of the V165/R166 residues that have been reported to be critical residues for an NLS within HIV-1 IN (2). However, our mutant (V165R/R166L) showed a complete lack of viral infectivity due to a failure to complete viral cDNA synthesis. Moreover, this IN mutant protein was unstable when expressed in the form of a GFP-IN fusion (data not shown). Thus, we cannot evaluate the importance of the V165/R166 residues in the karyophilic properties of IN or in the nuclear transport of viral cDNA in the context of viral replication. Recently, reassessments of these V165/R166 functions by use of IN mutants with Ala substitutions at the V165/R166 residues were reported by two different laboratories (17, 43). Both of these reports failed to confirm the NLS functions. Instead, V165A/R166A mutants were shown to be pleiotropic IN mutants primarily defective in integration. These conclusions are partly consistent with our data.

More recently, Devroe et al. reported that HIV-1 IN is unable to access the nucleus when expressed in the form of a large fusion with GFP and pyruvate kinase, suggesting that IN may lack a transferable NLS (15). Alternatively, they showed that cytoplasmic IN is highly unstable and prone to degradation and that the karyophilic properties can be attributed to an interaction with a cellular component. Interestingly, one of the nuclear factors, LEDGF/p75, has been identified as being essential for the karyophilic properties of HIV-1 IN, although the functional role of cytoplasmic LEDGF/p75 in the nuclear import of IN remains to be determined (47).

Meanwhile, genetic analyses of HIV-1 IN (9, 21, 49, 52, 60, 65) have suggested putative roles for IN at steps prior to integration, such as uncoating, reverse transcription, and nuclear import of viral cDNA. It was previously reported that a single amino acid substitution of the Tyr residue at position 143 with Gly (Y143G) in HIV-1 IN significantly reduced the level of stably integrated proviral DNA during acute infection of human primary cells. This reduction, concomitant with a reduction in the level of the 2-LTR circular form of viral cDNA, was evident in primary PBLs and MDMs (60); these data suggest that Y143 is a critical residue for efficient proviral DNA formation at steps prior to integration, most likely the nuclear import of viral cDNA. Y143 is located in the central catalytic domain of IN and is suggested to be one of the critical residues for specific binding to viral cDNA (22, 45). Thus, we reasoned that we could address the functional involvement of HIV-1 IN in the nuclear import of viral cDNA by using IN mutants that show reduced binding to viral cDNA.

Structural and biochemical analyses of HIV-1 IN identified three functional domains (reviewed in references 35 and 38): an N-terminal zinc binding domain, a central catalytic core domain, and a C-terminal nonspecific DNA binding domain. The central core domain contains the highly conserved D,D35E motif, which is directly involved in the catalytic activities of IN. The central core domain also contains several highly conserved residues critical for specific binding to viral cDNA. These include Y143, K156, K159, and K160 (16, 22, 36, 45, 67). In this study, we generated IN mutants by introducing single or triple amino acid substitutions or deletions of the highly conserved Pro-Tyr-Asn-Pro residues (PYNP) spanning positions 142 to 145 or three Lys residues (K156, K159, and K160) of HIV-1 IN (Table 1). We found that some of the IN mutant proteins carrying single amino acid substitutions in or deletions of the PYNP motif at positions 142 to 145 (N144Q and PYNP>KL) or a triple amino acid substitution for the three conserved Lys residues at positions 156, 159, and 160 (KKK>AAA) showed a severe reduction in binding to viral cDNA but retained their efficient karyophilic properties (Fig. 4B). However, whether these in vitro data can be extrapolated to biological relevance in vivo still remains to be determined. In the context of viral replication, HIV-1 carrying these IN mutations showed viral gene expression severely reduced to less than 1% the WT level, similar to the effects of a catalytic site mutation of IN (D116G) (Fig. 2). These defects were efficiently complemented by supplying WT IN in *trans* (data not shown), excluding the possible effects of these mutations on *cis*-acting functions of the central polypurine tract (10, 68).

Quantitative analysis of viral cDNA after infection with these HIV-1 IN mutants indicated that these mutations did not affect the de novo synthesis of viral cDNA products (Fig. 3, R/U5 and R/gag) but did severely reduce integration (Fig. 3, Integrated). Of note, the level of 2-LTR DNA, a surrogate marker for nuclear import, produced by the PYNP>KL mutant was only ~3% that produced by the catalytic site mutant D116G. A similar reduction in the level of 2-LTR DNA was also noted for the N144Q and KKK>AAA mutants, even on the backbone of the D116G mutation (Fig. 3, 2-LTR), suggesting that inhibition of the binding of IN to viral cDNA may affect the nuclear import of viral cDNA that precedes integration. This conclusion was further confirmed by more direct methods quantifying viral cDNA in isolated nuclei and FISH analyses of acutely infected cells (Fig. 5). However, although the signals of viral cDNA for the KKK>AAA and D116G-PYNP>KL mutants were evident at 6 h postinfection (Fig. 5B), they tended to be weakened at 24 h postinfection (data not shown), suggesting that PYNP>KL and KKK>AAA may reduce the nuclear accumulation of viral cDNA partly because these mutant PICs are more susceptible to degradation. Thus, an alternate explanation for the results is that if IN does not bind to viral cDNA, then the PIC falls apart and is nonfunctional. Although we cannot rule out the partial contribution of the effect of these mutations on the stability of viral cDNA and/or the PIC, our results indicate that the nuclear transport that precedes the integration of viral cDNA was significantly

reduced by mutations that reduced the binding affinity of HIV-1 IN for viral cDNA.

In summary, our findings indicate that IN mutants that were defective in viral cDNA recognition lost viral infectivity, thereby partly affecting the nuclear import of viral cDNA that precedes integration during acute infection with HIV-1. Taken together, these results suggest that HIV-1 IN may be one of the critical constituents for the efficient nuclear import of viral cDNA.

ACKNOWLEDGMENTS

We thank I. S. Y. Chen and Y. Koyanagi for providing plasmids, D. P. Grandgenett for providing anti-IN antibodies, and R. Yoshinari and S. Nishino for technical assistance.

This work was supported by a grant-in-aid for scientific research on priority areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and a grant from the Ministry of Health, Labor and Welfare, Japan (Research on prevention of HIV replication and mutation 16150301).

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JOURNAL OF VIROLOGY, Aug. 2005, p. 10088–10092
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.15.10088–10092.2005

Vol. 79, No. 15

Identification of Two New HLA-A*1101-Restricted Tax Epitopes Recognized by Cytotoxic T Lymphocytes in an Adult T-Cell Leukemia Patient after Hematopoietic Stem Cell Transplantation

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Received 26 January 2005/Accepted 26 April 2005

We previously reported that Tax-specific CD8$^+$ cytotoxic T lymphocytes (CTLs), directed to single epitopes restricted by HLA-A2 or A24, expanded in vitro and in vivo in peripheral blood mononuclear cells (PBMC) from some adult T-cell leukemia (ATL) patients after but not before allogeneic hematopoietic stem cell transplantation (HSCT). Here, we demonstrated similar Tax-specific CTL expansion in PBMC from another post-HSCT ATL patient without HLA-A2 or A24, whose CTLs equally recognized two newly identified epitopes, Tax88-96 and Tax272-280, restricted by HLA-A11, suggesting that these immunodominant Tax epitopes are present in the ATL patient in vivo.

Adult T-cell leukemia (ATL) caused by human T-cell leukemia virus type I (HTLV-I) is characterized by poor prognosis following chemotherapy (7, 18, 20, 23). However, the results of recent allogeneic hematopoietic stem cell transplantation (HSCT) for ATL patients are encouraging (9, 24). This indicates that a graft-versus-leukemia (GVL) response may be effective for ATL as well as other types of leukemia, although there is a risk of graft-versus-host (GVH) diseases (GVHD).

We previously found that peripheral blood mononuclear cells (PBMC) from ATL patients after but not before HSCT from HLA-identical donors exhibited vigorous HTLV-I-specific cytotoxic T lymphocyte (CTL) responses that were directed to a limited number of Tax epitopes, i.e., an HLA-A2-restricted Tax11-19 epitope in one patient and an HLA-A24-restricted Tax301-309 epitope in another (6). These patients have now been in complete remission for more than 3 years.

Since HTLV-I Tax is the dominant target antigen recognized by HTLV-I-specific CTLs (8, 10, 16), which are thought to be responsible for in vivo immune surveillance for HTLV-I leukemogenesis (11), the positive conversion of Tax-specific CTL responses in post-HSCT ATL patients suggested that these CTLs might be involved in a GVL response. In a rat model of HTLV-I-infected T-cell lymphomas, Tax oligopeptide at a dominant CTL epitope successfully induced antitumor immunity, implying that the dominant CTL epitope identified in ATL patients may also be a potential candidate for a tumor vaccine (5, 15).

In the present study, we analyzed T-cell responses in another post-HSCT ATL patient without HLA-A2 or A24 and identified two new HLA-A11-restricted epitopes.

PBMC from an acute-type ATL patient (patient 156, a 51-year-old male) at 145 days after HSCT and from his HLA-identical (HLA-A11/A26, B52/B61 DR6/DR15) sibling donor (donor 167, a 55-year-old male) were collected after signed informed consent. Patient 156 obtained complete remission within 2 months after HSCT and sustained remission for longer than 15 months, although chronic GVHD was observed from 8 months after HSCT. Donor 167 was negative for HTLV-I.

A spontaneously HTLV-I-infected T-cell line (ILT-156) established from the PBMC of patient 156 before HSCT and an exogenously HTLV-I-infected T-cell line (ILT-167) established from PBMC of the seronegative donor 167 were maintained in the presence of interleukin-15. An Epstein-Barr virus-transformed lymphoblastoid cell line (LCL-156) was established from PBMC of patient 156 before HSCT, as described elsewhere (6, 22).

CD8$^+$ PBMC isolated from post-HSCT patient 156 at 147 days after HSCT were cocultured with 1% formalin-treated ILT-156 cells, derived from pre-HSCT patient 156, twice with a 14-day interval in the presence of interleukin-2. The responder PBMC vigorously proliferating in culture at 17 days after initiation of culture produced significant levels of gamma interferon (IFN-γ) against ILT-156, but not against LCL-156, following overnight incubation (Fig. 1A). Cytotoxicities of the CTLs against ILT-156 cells were confirmed by ^{51}Cr release assay. Significant levels of IFN-γ response were observed against allogeneic HTLV-I-infected cells sharing only HLA-A11 (TCL-Kan) but not against the ones sharing only HLA-A26 (ILT-Nkz-2). IFN-γ production of the responder cells against ILT-156 cells was significantly inhibited by treatment of responder cells with anti-CD8 monoclonal antibody (MAb) or

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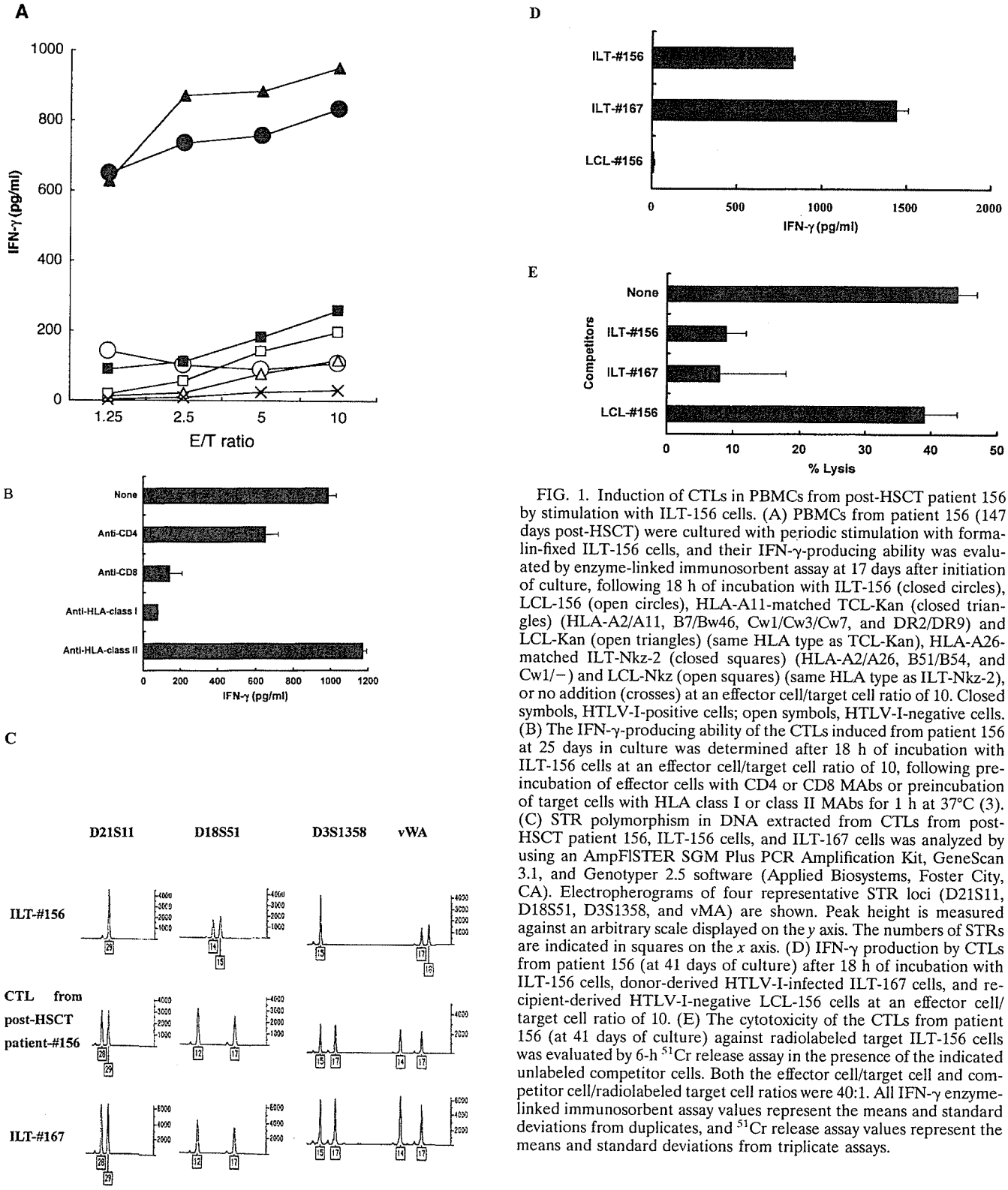

FIG. 1. Induction of CTLs in PBMCs from post-HSCT patient 156 by stimulation with ILT-156 cells. (A) PBMCs from patient 156 (147 days post-HSCT) were cultured with periodic stimulation with formalin-fixed ILT-156 cells, and their IFN-γ-producing ability was evaluated by enzyme-linked immunosorbent assay at 17 days after initiation of culture, following 18 h of incubation with ILT-156 (closed circles), LCL-156 (open circles), HLA-A11-matched TCL-Kan (closed triangles) (HLA-A2/A11, B7/Bw46, Cw1/Cw3/Cw7, and DR2/DR9) and LCL-Kan (open triangles) (same HLA type as TCL-Kan), HLA-A26-matched ILT-Nkz-2 (closed squares) (HLA-A2/A26, B51/B54, and Cw1/−) and LCL-Nkz (open squares) (same HLA type as ILT-Nkz-2), or no addition (crosses) at an effector cell/target cell ratio of 10. Closed symbols, HTLV-I-positive cells; open symbols, HTLV-I-negative cells. (B) The IFN-γ-producing ability of the CTLs induced from patient 156 at 25 days in culture was determined after 18 h of incubation with ILT-156 cells at an effector cell/target cell ratio of 10, following preincubation of effector cells with CD4 or CD8 MAbs or preincubation of target cells with HLA class I or class II MAbs for 1 h at 37°C (3). (C) STR polymorphism in DNA extracted from CTLs from post-HSCT patient 156, ILT-156 cells, and ILT-167 cells was analyzed by using an AmpFlSTER SGM Plus PCR Amplification Kit, GeneScan 3.1, and Genotyper 2.5 software (Applied Biosystems, Foster City, CA). Electropherograms of four representative STR loci (D21S11, D18S51, D3S1358, and vMA) are shown. Peak height is measured against an arbitrary scale displayed on the *y* axis. The numbers of STRs are indicated in squares on the *x* axis. (D) IFN-γ production by CTLs from patient 156 (at 41 days of culture) after 18 h of incubation with ILT-156 cells, donor-derived HTLV-I-infected ILT-167 cells, and recipient-derived HTLV-I-negative LCL-156 cells at an effector cell/target cell ratio of 10. (E) The cytotoxicity of the CTLs from patient 156 (at 41 days of culture) against radiolabeled target ILT-156 cells was evaluated by 6-h ^{51}Cr release assay in the presence of the indicated unlabeled competitor cells. Both the effector cell/target cell and competitor cell/radiolabeled target cell ratios were 40:1. All IFN-γ enzyme-linked immunosorbent assay values represent the means and standard deviations from duplicates, and ^{51}Cr release assay values represent the means and standard deviations from triplicate assays.

by treatment of target cells with anti-HLA-class I MAb (Fig. 1B), confirming that the responder cells induced from post-HSCT patient 156 contained CD8-positive, HLA-A11-restricted, HTLV-I-specific CTLs.

The hematopoietic system in post-HSCT patient 156 when tested had been reconstituted by that derived from donor 167, as determined by short tandem repeat (STR) polymorphism. By using similar methods, we assessed the origin of the CTLs from post-HSCT patient 156. As shown in Fig. 1C, the pattern of STRs of the CTLs was identical to that of ILT-167 cells but

not ILT-156 cells, clearly indicating that CTLs from post-HSCT patient 156 were derived from donor 167. We then examined whether the CTLs from post-HSCT patient 156 recognized potential GVH antigens expressed in ILT-156 cells but not in ILT-167 cells, besides HTLV-I. As shown in Fig. 1D, the CTLs induced from post-HSCT patient 156 equally recognized ILT-167 and ILT-156 but not LCL-156. Furthermore, cytotoxicity of the CTLs against radiolabeled ILT-156 was competed with unlabeled ILT-167 cells as well as ILT-156 cells significantly and to similar extents but was not competed with LCL-156 cells (Fig. 1E). These results indicated that CTLs from post-HSCT patient 156 were directed mainly to HTLV-I antigens commonly expressed in ILT-156 and -167 cells but not to potential GVH antigens expressed only in ILT-156 cells.

We next performed mapping analysis on the epitopes recognized by CTLs from post-HSCT patient 156 by using a panel of oligopeptides of HTLV-I Tax (6, 12), the major target antigen for HTLV-I-specific CTLs. LCL-156 cells, pulsed with a series of 15- to 24-mer oligopeptides corresponding to the amino acid sequence of the whole region of Tax, were incubated with CTLs from patient 156. Among 28 oligopeptides used, Tax81-104 (Fig. 2A) and Tax271-285 (Fig. 2B) selectively sensitized CTLs to produce IFN-γ. We then prepared five 9-mer peptides inside Tax81-104 and Tax271-285 sequences, which were predicted by computer analysis to bind HLA-A*1101 based on the anchor motifs in two databases (the BIMAS and SYFPEITHI databases) (14, 17). Among these peptides, we found that Tax88-96 (KVLTPPITH) and Tax272-280 (QSSSFIFHK) (Table 1) were dominantly recognized by the CTLs from post-HSCT patient 156.

Finally, we used phycoerythrin (PE)-conjugated HLA-A*1101/Tax88-96 and HLA-A*1101/Tax272-280 tetramers, which were prepared through the NIAID Tetramer Facility (Atlanta, GA), to directly detect HLA-A11-restricted Tax-specific CTLs. As shown in Fig. 3A, in the PBMC culture from post-HSCT patient 156 at 41 days from the initiation of culture, 5.7% of cells were positive for HLA-A*1101/Tax88-96 tetramer and CD8 and 5.8% of cells were positive for HLA-A*1101/Tax272-280 tetramer and CD8. When a mixture of both tetramers was used, 10.3% of the cells bound to these tetramers. These data clearly indicate that CTLs recognizing each epitope equally expanded in the PBMC culture derived from post-HSCT patient 156 in response to stimulation with pre-HSCT cell line ILT-156.

We further applied tetramer staining for uncultured PBMCs from post-HSCT patient 156 (Fig. 3B) and donor 167 (Fig. 3C). Although low levels of nonspecific stain were observed in the PBMCs of the seronegative donor 167, significantly higher percentages of the PBMCs of post-HSCT patient 156 were stained with CD8 MAbs and the tetramers: 0.48% for HLA-A*1101/Tax88-96 tetramer, 0.71% for HLA-A*1101/Tax272-280 tetramer, and 1.87% for both tetramers.

A number of CTL epitopes restricted by HLA-A2, -B14, and -B15 have been identified in HTLV-I Tax; most were found in the context of HTLV-I-specific CTLs derived from HTLV-I-associated myelopathy/tropical spastic paraparesis patients and asymptomatic HTLV-I carriers (2, 12, 13, 16, 25). However, to our knowledge this is the first report demonstrating HLA-A*1101-restricted Tax epitopes recognized by HTLV-I-specific CTLs. The phenotypic frequencies of HLA-A11 are 10% in

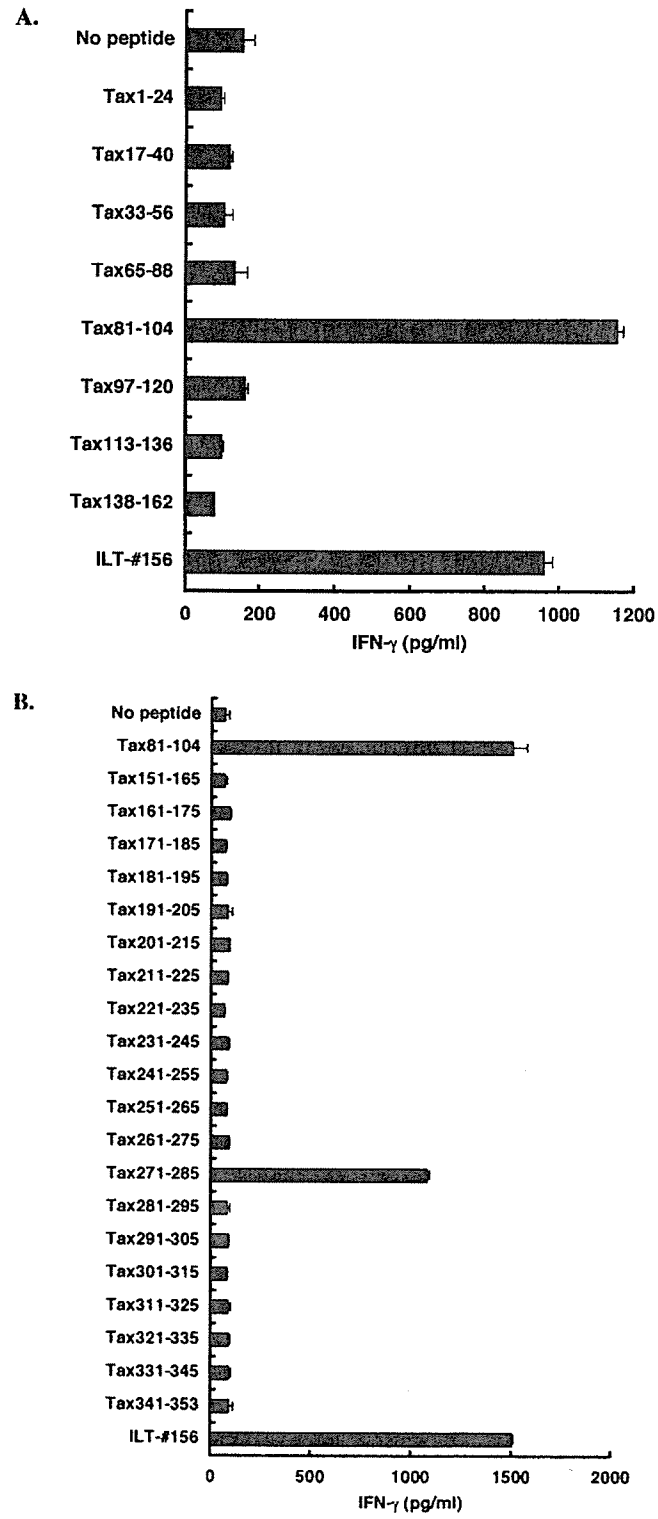

FIG. 2. Mapping of HTLV-I Tax epitopes recognized by CTLs from post-HSCT patient 156. LCL-156 cells were pulsed with 10 μM of a series of 24-mer synthetic oligopeptides covering the N-terminal half (A) and a series of 15-mer oligopeptides covering the C-terminal half (B) of the Tax amino acid sequence, and their susceptibility to CTLs of post-HSCT patient 156 was measured by IFN-γ enzyme-linked immunosorbent assay following 18 h of incubation at an effector cell/target cell ratio of 10. Values represent the means and standard deviations from duplicate assays.

TABLE 1. Reactivity of the Tax-specific CTLs to 9-mer oligopeptides with binding motifs to HLA-A*1101 within Tax81-104 and Tax271-285[a]

Peptide	Sequence	IFN-γ production (pg/ml)[b]
None		13.5 ± 4.4
Tax81-104	Q R T S K T L K V L T P P I T H T T P N I P P S	1,080.2 ± 92.6
Tax82-90	R T S K T L K V L	19.6 ± 8.1
Tax88-96	K V L T P P I T H	1,201.6 ± 55.9
Tax271-285	L Q S S S F I F H K F Q T K A	957.2 ± 47.0
Tax270-278	V L Q S S S F I F	104.4 ± 6.4
Tax272-280	Q S S S F I F H K	1,255.3 ± 13.4
Tax276-284	F I F H K F Q T K	79.1 ± 46.8

[a] CTLs induced from post-HSCT patient 156 at 41 days after initiation of culture were examined for IFN-γ-producing ability by enzyme-linked immunosorbent assay against LCL-156 cells pulsed with 10 μM of indicated Tax peptides (>80% purity) at an effector cell/target cell ratio of 10. All 9-mer peptides within Tax81-104 and Tax271-285 listed were selected based on the binding motif for HLA-A*1101 by using two databases (the BIMAS and SYFPEITHI databases) (14, 17) for HLA binding peptide prediction.

[b] Values represent the means and standard deviations from duplicate assays.

Caucasians, 33% in Chinese, 20% in Japanese, and 4% in black North Americans (19, 21). The newly identified HLA-A11-restricted epitopes together with previously identified epitopes can thus be applied to a large portion of the world's population.

A major challenge in the field of allogeneic HSCT is to prevent the alloreactivity that leads to GVHD while preserving a GVL effect (4). Although it is still not clear whether Tax could be a GVL target in ATL, our present study and earlier studies (6) suggest the presence of Tax antigen presentation in vivo in ATL patients and the potential contribution of these CTLs to GVL effects.

In summary, we identified two HLA-A*1101-restricted HTLV-I-specific CTL epitopes that were recognized by CTLs induced from an ATL patient after HSCT. The identified epitopes broaden the adaptable population for potential immunotherapy for ATL as well as for the monitoring of HTLV-I-specific CTL responses.

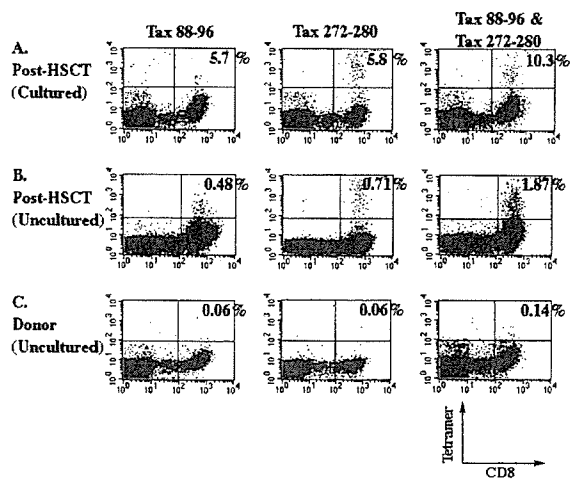

FIG. 3. Detection of Tax88-96 and Tax272-280-specific CTLs by tetramers in PBMCs from post-HSCT patient 156. CTLs from post-HSCT patient 156 at 41 days after initiation of culture (A), uncultured PBMCs from post-HSCT patient 156 (B), and uncultured PBMCs from donor 167 (C) were stained with PE-Cy5-labeled CD8 MAbs (HIT8a; BD PharMingen) together with PE-conjugated HLA-A*1101/Tax88-96 (left), HLA-A*1101/Tax272-280 (center), or a mixture of both tetramers (right). Both tetramers were provided by the National Institute of Allergy and Infectious Diseases Tetramer Facility, Emory University, and were used at a dilution of 1:800. Numbers in the upper right corners indicate percentages of CD8-positive cells bound to the tetramer as analyzed on a flow cytometer (1). A total of 100,000 events were collected in each case.

We thank Kyogo Itoh (Kurume University, Fukuoka, Japan) and SRL, Inc. (Tokyo, Japan), for providing anti-HLA MAbs and for performing STR analysis, respectively. We also thank the NIAID Tetramer Facility, Emory University Vaccine Center at Yerkes (Atlanta, GA), for providing HLA-A*1101/Tax88-96 and HLA-A*1101/Tax272-280 tetramers.

This work was supported by grants from the Ministry of Health, Welfare, and Labor of Japan and the Ministry of Education, Science, Culture and Sports of Japan.

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