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Reassessment of the Roles of Integrase and the Central DNA Flap in Human Immunodeficiency Virus Type 1 Nuclear Import

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Human immunodeficiency virus type 1 (HIV-1) can infect nondividing cells productively because the nuclear import of viral nucleic acids occurs in the absence of cell division. A number of viral factors that are present in HIV-1 preintegration complexes (PICs) have been assigned functions in nuclear import, including an essential valine at position 165 in integrase (IN-V165) and the central polypurine tract (cPPT). In this article, we report a comparison of the replication and infection characteristics of viruses with disruptions in the cPPT and IN-V165. We found that viruses with cPPT mutations still replicated productively in both dividing and nondividing cells, while viruses with a mutation at IN-V165 did not. Direct observation of the subcellular localization of HIV-1 cDNAs by fluorescence in situ hybridization revealed that cDNAs synthesized by both mutant viruses were readily detected in the nucleus. Thus, neither the cPPT nor the valine residue at position 165 of integrase is essential for the nuclear import of HIV-1 PICs.

Human immunodeficiency virus type 1 (HIV-1) and other lentiviruses are able to infect nondividing cells productively (20, 37). This property distinguishes lentiviruses from certain other retroviruses (for example, murine leukemia virus) that require mitosis with nuclear envelope breakdown prior to viral cDNA integration (21, 29). HIV-1 infection of nondividing cell populations such as postmitotic macrophages, mucosal dendritic cells, and nondividing T cells may be critical for transmission, disease pathogenesis, and the establishment of persistent virus reservoirs.

Following virus entry, a large nucleoprotein complex is formed in the cytoplasm (hereafter called the preintegration complex [PIC]) with components from both the incoming virion core and the host cell. The ability of HIV-1 to infect nondividing cells has long been attributed to the presence of karyophilic signals within the PIC that facilitate active transport across the intact nuclear envelope via the nuclear pore complex (6). However, neither the components required for nuclear localization nor the exact mechanism of PIC nuclear uptake has been fully determined, and many conflicting reports exist in the literature. These difficulties are perhaps compounded by the fact that the composition, stoichiometry, and size of HIV-1 PICs evolve as the viral genome is reverse transcribed, transported through the cytoplasm, enters the nucleus, and reaches the final site of provirus integration (10).

Some viral proteins have been consistently identified in the PIC, including integrase, matrix ($p17^{Gag}$), nucleocapsid ($p7^{Gag}$), Vpr, and reverse transcriptase (RT) (10, 11, 24). Among the candidate karyophiles, integrase, matrix, and Vpr are reported to have nuclear localization signals (NLSs) (5, 9, 12, 14–16, 27, 36). Because the relative contributions of each of these proteins to PIC import remains controversial and viruses deficient in matrix and Vpr proteins can still infect nondividing cells with nearly wild-type efficiency under some circumstances (28), our group and others have been focusing on additional factors that may play a role in PIC trafficking.

We recently described a region within the integrase protein at residues 161 to 173 that is required for productive HIV-1 infection of both dividing and nondividing cells and was ascribed an NLS function (2). We now know that the assignment of this region as a transferable and autonomously acting NLS is incorrect (see Results). However, despite the lack of demonstrable NLS activity for this region of integrase, mutations within this conserved peptide motif have profound effects on HIV-1 replication that are due to the alteration of protein sequence. Single-amino-acid substitutions such as V165A block progression of the viral life cycle at a point prior to integration, alter the subcellular localization of infection-delivered integrase protein, and diminish the accumulation of long terminal repeat (LTR) circles.

The phenotype of the V165A virus is somewhat reminiscent of that reported for a virus with a disrupted central polypurine tract (cPPT). During reverse transcription, the plus strand of cDNA is synthesized as two segments, with the downstream segment being initiated at the cPPT. Synthesis of the upstream segment continues (with strand displacement) for 99 nucleotides, until the viral RT complex stops at the central termination sequence. As a result, HIV-1 reverse transcription produces a region of triple-stranded DNA, termed the central DNA flap. It has been reported that a virus carrying an inactivated cPPT is unable to replicate in either dividing or nondividing cells but can complete reverse transcription and give

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rise to PICs that retain integration function in vitro (39). Because the cPPT mutant virus, like the V165A mutant, is also partially deficient for the accumulation of circular viral cDNAs, we were prompted to conduct a side-by-side phenotypic comparison of these two classes of mutant virus in both X4 and R5 viral backbones.

In this article, we report that the cPPT mutant viruses can still replicate efficiently, whereas V165A integrase mutants never initiate spreading infections. We further demonstrate that cDNAs produced during challenges with both of these classes of mutant virus localized to the nucleus when assayed by fluorescence in situ hybridization (FISH). We conclude that cPPT mutant and V165A viruses are not phenotypically similar and suggest that V165A viruses are blocked at a post-nuclear entry step that may reflect an uncharacterized intranuclear targeting (or trafficking) phase of HIV-1 infection (14). However, neither mutation, in isolation, plays a significant role in preventing HIV cDNA import into the nucleus.

MATERIALS AND METHODS

Molecular clones. The wild-type and integrase-defective (V165A and D64A) HIV-1_{YU-2} proviruses have been described (2). The cPPT was inactivated by site-directed mutagenesis as described previously for the LAI isolate (39). A silent *Cla*I site was introduced at nucleotide 4684 in all YU-2-based proviruses. One set of wild-type and cPPT-D mutant LAI proviruses was provided by Marie Vodicka and Maki Imakura at the Fred Hutchinson Cancer Research Center (LAI_E), and the other was obtained from Pierre Charneau at the Pasteur Institute (LAI_C).

Three silent mutations disrupting a potential 5′ cryptic splicing site in the integrase gene (3SM) were introduced into the molecular infectious clone of HIV-1 LAI. The wild-type DNA sequence (CAG GTA AGA) coding the amino acid residues between 164 and 166 of the HIV integrase was mutated to CAA GTC CGA, which encodes the same amino acid sequence but does not retain the 5′ splice site. The presence of 5′ splice site activity in the wild-type sequence and the lack of 5′ splice site activity in the 3SM mutant were verified by assaying splicing activity when a simian virus 40 3′ splice site was positioned downstream (data not shown).

Cells, cell lines, virus production, and single-cycle infectivity. The human cell lines 293T and CEM-SS/CCR5 have been described (13). The human $CD4^+$ T-cell line MT-4 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. The human cell line HOS-CD4-CCR5 was obtained through the National institutes of Health AIDS Research and Reference Reagent Program (courtesy of Ned Landau) and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1 μg of puromycin per ml (8). Human peripheral blood mononuclear cells were prepared following venipuncture by treatment with phytohemagglutinin and maintenance in recombinant human interleukin-2, followed by culture in RPMI 1640 medium with 10% human serum. Human monocyte-derived macrophages (MDMs) were prepared by adherence to plastic, followed by culture in RPMI 1640 medium with 10% human serum (32). Wild-type and mutant virus stocks were generated by calcium phosphate transfection of 293T or 293 cells, harvested after 24 h, and normalized for $p24^{Gag}$ content by enzyme-linked immunosorbent assay (ELISA).

The infectivity of the 3SM mutant, along with the wild-type and the V165A mutant, was assessed by using HeLa-CD4-long terminal repeat (LTR)-β-galactosidase (MAGI) reporter cells as described previously (19). The MAGI titer (infectious units per milliliter) is the number of β-galactosidase-positive cells at limiting dilution. The infectious titer was normalized to the amount of $p24^{Gag}$ (blue cells/ng of $p24^{Gag}$).

Primer extension assay. The primer extension assay for discontinuous cDNA plus-strand synthesis was performed as described previously (7). Briefly, 10^7 CEM-SS/CCR5 cells were infected with virus corresponding to ~500 ng of $p24^{Gag}$ by centrifugal inoculation (26), washed with phosphate-buffered saline (PBS), and maintained for 7 h, and low-molecular-weight DNAs were extracted (17). DNAs were then digested with *Cla*I, reprecipitated, and resuspended in 30 μl of 10 mM Tris-HCl (pH 8.5). The DNA primer 5′-GATGAGGACTTTC ATAGTGATGTCTATAAAACCATCCC-3′ was labeled at the 5′ end with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The entire 30-μl DNA sample was mixed with ~10 ng of primer, 2.5 U of AmpliTaq polymerase (Roche), 0.2 mM each of the four deoxynucleoside triphosphates, and 1× AmpliTaq buffer I. Fifty cycles of primer extension were performed (30 s at 94°C, 30 s at 52°C, and 1 min at 72°C), and half of the reaction mixture was denatured and resolved on a native 5% polyacrylamide–Tris-borate-EDTA gel.

FISH. All FISH experiments were performed as described previously (1). HOS-CD4-CCR5 cells were grown to subconfluence on glass coverslips in 24-well plates. The cells were challenged with ~200 ng of $p24^{Gag}$ by centrifugal inoculation, incubated overnight (~15 to 18 h), fixed with 4% paraformaldehyde, washed with PBS, and treated with RNase (100 μg/ml in PBS) for 30 min at 37°C. Some cultures were maintained in the presence of the reverse transcription inhibitor efavirenz (400 nM). The samples were then ethanol dehydrated, air dried, and incubated overnight at 37°C with the hybridization mix [10 ng of biotinylated probe, 100 ng of salmon sperm DNA (Gibco-BRL), 1 μg of yeast tRNA (Sigma), and 0.5 μg of human *cot-1* DNA (Gibco BRL) per μl in 50% formamide–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)].

The biotinylated probe (~100- to 400-bp fragments) was generated by nick translation of a plasmid containing a full-length HIV-1 provirus. After washing, bound probe was detected with fluorescein isothiocyanate-avidin (1:500 in 4× SSC plus 0.5% bovine serum albumin) and amplified with biotinylated antiavidin (1:250), followed by an additional fluorescein isothiocyanate-avidin step. The samples were finally equilibrated in PBS, counterstained with propidium iodide, and mounted with Vectashield (Vector Laboratories). The signals were visualized with a Leica confocal laser-scanning microscope (Leica TSC SP2 System).

Analysis of integration and replication. Experiments to analyze integration and replication were performed as described previously (2). Replication assays were initiated by infection with a normalized inoculum of virus and monitored as $p24^{Gag}$ accumulation in the supernatant. The presence of provirus in whole-cell lysates was evaluated by *Alu*-PCR with the *Alu* element primer 5′-GCCTCCCA AAGTGCTGGGATTACAG-3′. Samples were phenol extracted, ethanol precipitated, resuspended, diluted in 10-fold increments in water to achieve a semiquantitative result, and subjected to nested PCR. Because the complexity of each input sample decreased with each dilution (owing to the random nature of HIV-1 integration), the effects on band intensity were complicated. A significant difference in measurable amplicons is therefore suggested by the loss of signal at a particular dilution. Amplified products were analyzed by agarose gel electrophoresis and Southern hybridization with a random-primed ^{32}P-labeled LTR probe (*Eco*RV-*Hae*III, nucleotides 35 to 637).

Analysis of LTR circle formation. Analysis of LTR circle formation was performed as described previously (2). CEM-SS/CCR5 cells were irradiated with 1,400 rads and allowed to recover overnight. Then 2×10^7 cells were challenged with wild-type or mutant virus stocks corresponding to ~200 ng of $p24^{Gag}$ by centrifugal inoculation. Cells were washed with PBS and maintained in medium for up to 24 h, and low-molecular-weight DNAs (containing unintegrated viral DNAs) were extracted at various time points (17). These DNAs were examined for the presence of full-length linear and circular reverse transcripts by digestion with *Dpn*I (to remove residual transfected DNAs) and *Bsg*I, resolution by agarose gel electrophoresis, and Southern hybridization with a ^{32}P-labeled *env* probe (*Bsg*I-*Eco*RV, nucleotides 7722 to 9111). Linear DNA was detected as a 1,988-bp fragment, and circular DNAs containing one and two LTRs (1-LTR and 2-LTR DNAs, respectively) were detected as 2,569- and 3,199-bp fragments, respectively.

RESULTS

Silent mutagenesis of IN-V165. We previously described a transferable NLS in the integrase between residues 161 and 173 that could be abolished by mutation of either residue 165 or 166. This conclusion was based on observations that disruptions in this region inhibited the nuclear localization of integrase fusion proteins in transfection-based assays (2). More recent experiments have demonstrated that these results were obtained because the particular expression vector that we used resulted in the activation of a cryptic 5′ splice site that is coincident with residues 165 and 166 (data not shown). Splicing from this site to a downstream 3′ splice site in the plasmid backbone results in the production of fusion proteins containing an NLS encoded by the vector and not by the integrase. Mutations such as V165A therefore disrupt this splice and avert the inclusion of the unintended downstream "NLS."

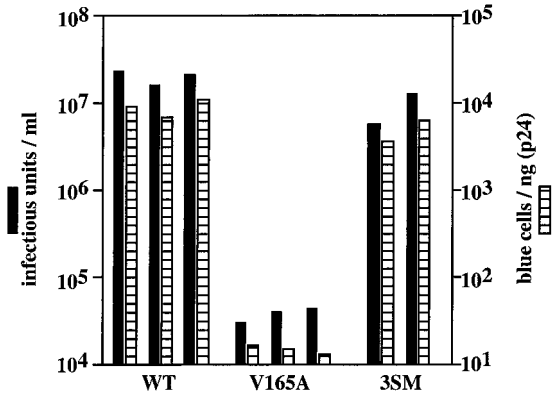


FIG. 1. Infectivity of HIV-1 lacking a potential 5′ splice site near IN-V165. Single-cycle infections of either wild-type (WT) virus or viruses carrying the V165A mutation or three silent mutations that eliminate the potential cryptic splice site at V165 (3SM) without changing the amino acid sequence of integrase (see Materials and Methods) were performed. Infectivity was measured with the MAGI assay with viruses harvested after transfection of proviral clones into 293 cells (solid bars) or normalized to $p24^{Gag}$ levels (hatched bars). These data show that the phenotype of V165A is due to a change in the amino acid sequence of the integrase rather than to a change in the cryptic splice site that overlaps the coding region of V165.

To confirm that the previously reported phenotypes for V165A viruses were due to the alteration in protein sequence, as opposed to changes in the RNA sequence, silent mutations were introduced into the LAI provirus so that the cryptic 5′ splice site was abolished but the amino acid sequence of the integrase was unchanged. Challenges of MAGI indicator cells with this virus, LAI 3SM, the LAI V165A mutant, and wild-type LAI (Fig. 1) demonstrated that these silent changes had no impact on virus infectivity, whereas the V165A mutation reduced infectivity ~1,000-fold (as reported previously). Thus, although the V165A mutation does not affect a transferable NLS in the integrase, it does block virus replication, and this is attributable to the alteration in protein sequence. We believe that this defect is not due to the loss of integrase catalytic functions, since delivery of the V165A integrase protein in *trans* restored a high level of infectivity to virions carrying a catalytically defective integrase protein. This suggests that the V165A protein is competent to mediate integration in vivo and, presumably, that it is present in PICs in a biologically active conformation (2).

Mutation of cPPT in the YU-2 viral backbone. The cPPT and the downstream polypurine tract (PPT) that abuts the 3′ LTR are identical over their 19-nucleotide span. Resistant to the RNase H activity of HIV-1 RT, these regions serve as the primers for plus-strand DNA synthesis. The plus strand of the viral cDNA is therefore synthesized as two subgenomic, partially overlapping sections. While the integrity of the downstream PPT is essential for plus-strand synthesis, disruption of the cPPT still allows completion of reverse transcription, but with the plus strand being produced as a single, full-length molecule. Recently, Zennou and colleagues described 10 nucleotide changes within the cPPT (the cPPT-D virus) that, in combination, completely disrupted cPPT activity. A mutated virus carrying these changes was shown to be noninfectious and to be deficient for PIC nuclear import. To help the comparison of cPPT-D viruses with V165A integrase mutant viruses, the same 10 point mutations were introduced into the provirus of the R5 strain, YU-2, to create YU-2 cPPT-D (Fig. 2A).

As shown previously, wild-type and cPPT-D viruses were produced at similar levels following provirus transfections of 293T cells, as judged by $p24^{Gag}$ yields in culture supernatants (data not shown). To evaluate cPPT disruption, CEM-SS/CCR5 cells were challenged with YU-2 cPPT-D, YU-2 V165A, and wild-type YU-2, and low-molecular-weight DNAs were extracted after 6 h and analyzed for cPPT function by primer extension (Fig. 2B). With the wild-type virus, primer extension terminated when the polymerase reached the 5′ end of the downstream plus strand (370-nucleotide product). In contrast, the products from cPPT mutant viruses extended to the position of the restricted *Cla*I site. As shown in Fig. 2C, the wild-type and V165A viruses produced (predominantly) the expected, shorter 370-nucleotide product, whereas the products from the cPPT-D-derived sample were all 490 nucleotides in length. Thus, the YU-2 cPPT-D virus does not generate the central DNA flap during reverse transcription. As expected, the cPPT activity of the YU-2 V165A virus was unchanged.

Inactivation of cPPT does not prevent HIV-1 replication. To compare the effects of cPPT inactivation and the V165A mutation on HIV-1 replication, two different sets of viruses were compared, an R5-tropic set and an X4-tropic set. The proliferating T-cell line CEM-SS/CCR5 was initially challenged with the R5 viruses (wild-type YU-2, YU-2 V165A, and YU-2 cPPT-D), and replication was monitored by measuring $p24^{Gag}$ accumulation in the culture supernatants. Surprisingly, and as shown in Fig. 3A, the wild-type and cPPT-D mutant viruses replicated with similarly robust kinetics, while the V165A virus failed to establish a spreading infection (as reported previously).

To date, the effects of cPPT mutations on viral replication have been reported for the X4-tropic viral isolate LAI. To generate a complete set of X4-tropic viruses, the V165A integrase mutation was introduced into the closely related HXB-3 isolate, and wild-type and cPPT-D versions of LAI were created (LAI_E) or provided (LAI_C). Upon challenge of CEM-SS/CCR5 cells, the wild-type and cPPT mutant viruses again replicated with matched kinetics, whereas the IIIB V165A virus failed to grow (data not shown). To rule out the possibility that the unanticipated growth of cPPT-D mutant strains was attributable to the cell line used, the X4-tropic virus set was next used to challenge a second human T-cell line, MT-4. Although these cells require higher inocula to establish a spreading infection, the relative behavior of the different viruses was the same: the wild-type and cPPT-D viruses grew, whereas the V165A mutant did not (Fig. 3B).

It is possible that the reported defective phenotype of the cPPT mutant virus could be masked or ameliorated by target cell type or proliferation. To assess replication in primary cells, the YU-2-based viruses were used to challenge peripheral blood mononuclear cells (Fig. 3C) and MDMs (Fig. 3D). Although the YU-2 cPPT-D virus was able to establish spreading infections in both dividing peripheral blood mononuclear cells and nondividing MDMs, analyses with cells obtained from multiple different donors consistently showed that replication of the cPPT-D mutant was modestly attenuated relative to that of the wild-type virus. Thus, while it is clear that the cPPT

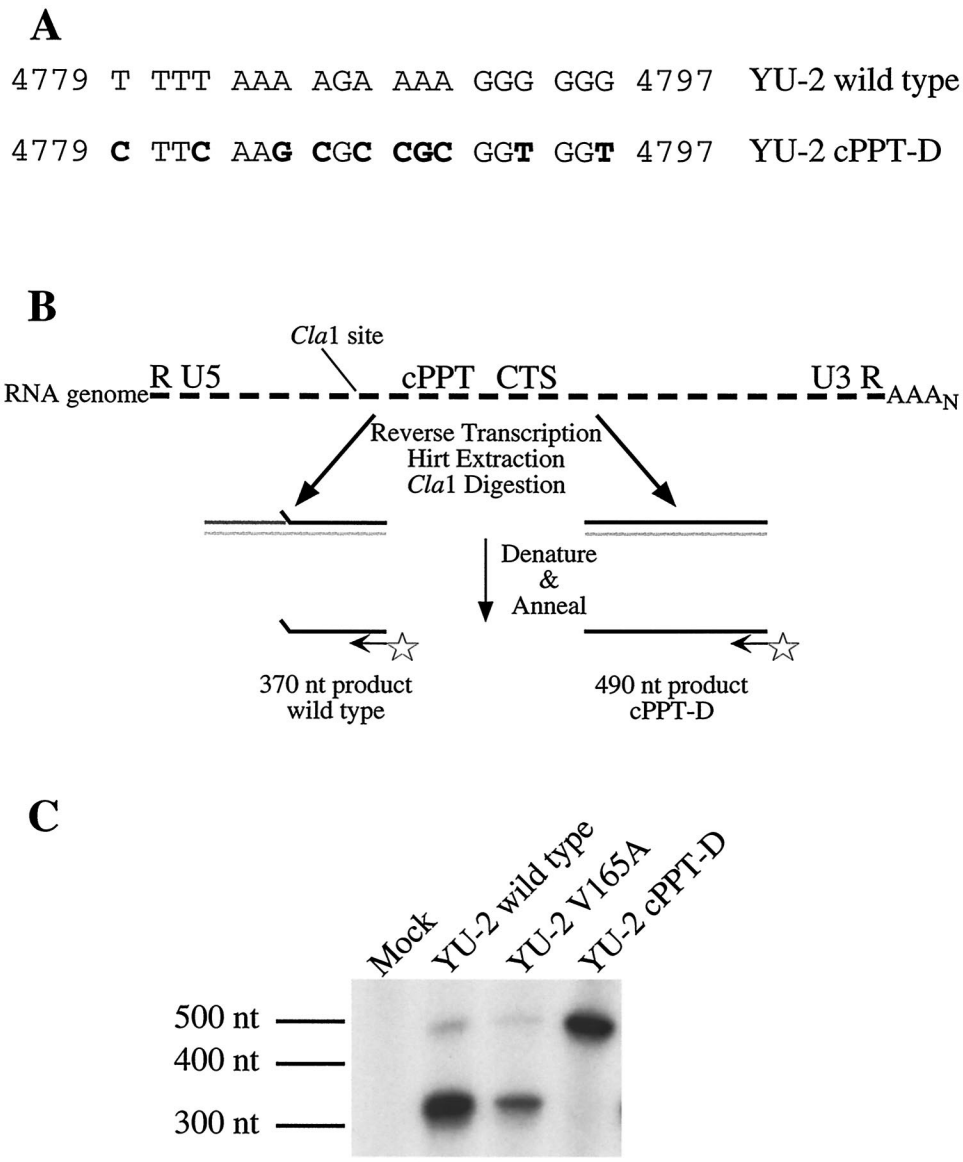


FIG. 2. Disruption of cPPT. (A) Nucleotide sequence of the wild-type and mutant cPPTs. Ten nucleotide changes were introduced into the wild-type cPPT (mutations shown in bold) to form the cPPT-D mutant. The 10 mutations resulted in a single amino acid change in the coding region for integrase (lysine at position 188 was replaced with arginine). (B) Diagram of primer extension assay to measure cPPT activity. Following infection, the RNA genome (dashed line) was reverse transcribed to create the viral cDNA. The plus strand of cDNA was synthesized either as two subgenomic, partially overlapping segments (wild-type virus, left side) or as a single full-length molecule (cPPT-D virus, right side). Following restriction with *Cla*I, the extracted DNAs were denatured in the presence of a 5′-end-labeled, plus-strand-specific primer (arrow with a star at one end). The primer was extended with *Taq* polymerase until the enzyme reached either the 5′ end of the downstream plus strand (wild-type virus) or the restricted site (cPPT-D virus). CTS, central termination sequence. (C) Polyacrylamide gel electrophoresis of primer extension products. CEM-SS/CCR5 cells (10^7) were infected with virus (mock, YU-2 wild type, YU-2 V165A, or YU-2 cPPT-D) corresponding to ~500 ng of $p24^{Gag}$ and incubated for 6 h, and low-molecular-weight DNAs were extracted. Primer extension was performed as in panel B, and the products were denatured and resolved on a native 5% polyacrylamide–Tris-borate-EDTA gel. The major product from YU-2 wild-type and YU-2 V165A (lanes 2 and 3) was 370 nucleotides (nt), whereas the only product from YU-2 cPPT-D (lane 4) was 490 nucleotides.

mutations do not prevent replication, modest reductions in replicative capacity are evident in cultures of primary cells.

Efficiency of integration is modestly decreased by cPPT mutations. Alterations in the efficiency of the early steps of infection may be masked when measuring virus replication because of possible effects on other stages of the life cycle. To address the efficiency of provirus formation, we used *Alu*-PCR to test the ability of wild-type, V165A, cPPT-D, and integrase D64A (a catalytically inert integrase mutant) viruses to mediate viral DNA integration. By using a primer set in which one primer binds the repetitive *Alu* element present throughout the human genome and the other binds HIV-1 cDNA, only viral DNA that has been covalently joined to the host cell genome is amplified. The assay was made semiquantitative by serial dilution of the input DNAs (see Materials and Methods).

The results obtained with DNAs extracted from proliferat-

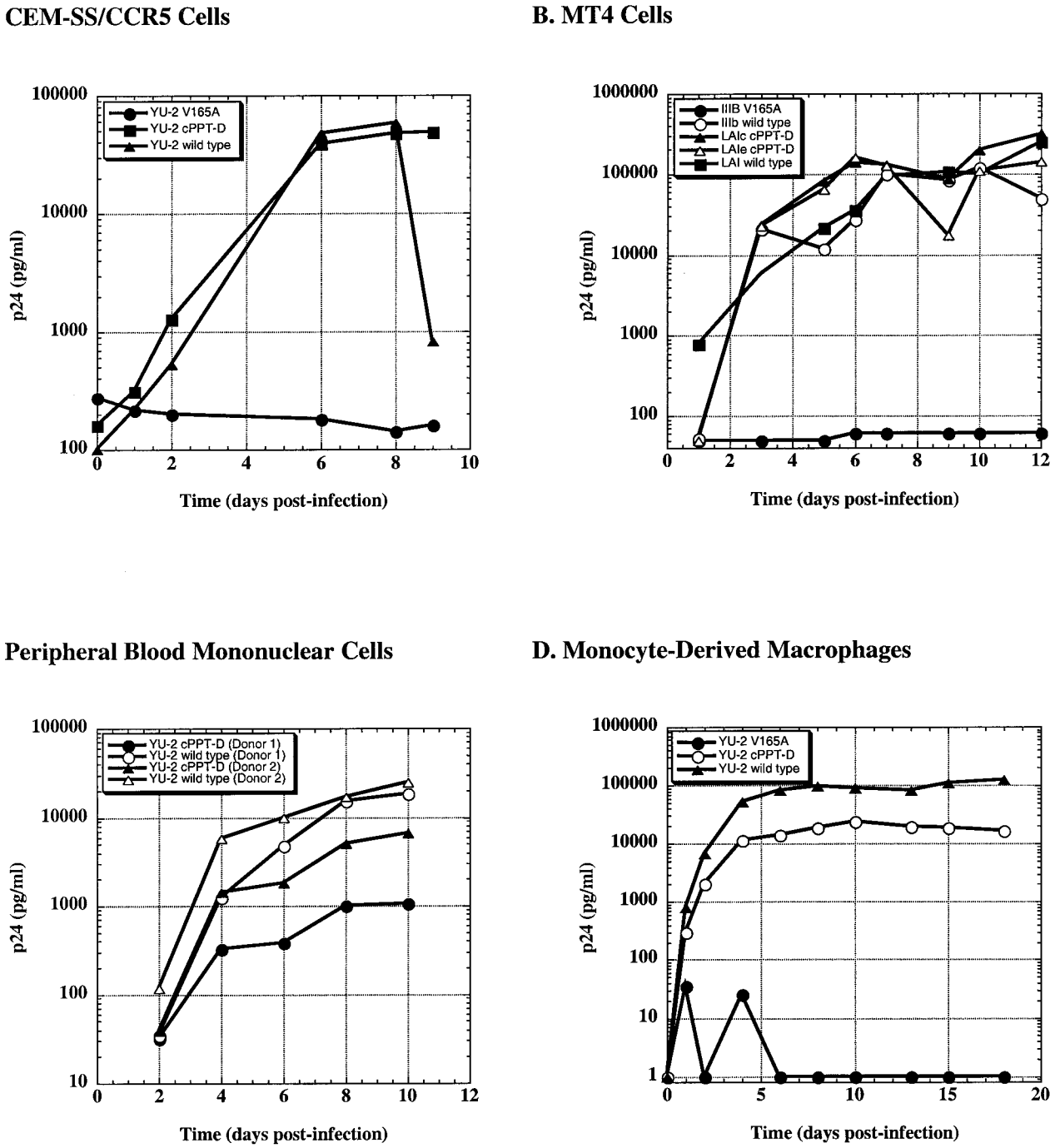


FIG. 3. Analysis of HIV-1 replication. Cells were challenged with 293T-derived virus stocks, and replication was measured as the accumulation of $p24^{Gag}$ in the supernatant by ELISA. (A) CEM-SS/CCR5 cells were challenged with inocula corresponding to 10 ng of $p24^{Gag}$ of the R5 set of viruses: YU-2 wild type (solid triangles), YU-2 V165A (solid circles), or YU-2 cPPT-D (solid squares). (B) As in panel A, MT-4 cells were challenged with 100 ng of $p24^{Gag}$ of the X4 set of viruses: IIIB wild type (open circles), LAI wild type (solid squares), LAI_E cPPT-D (open triangles), LAI_C cPPT-D (solid triangles), or IIIB V165A (solid circles). (C) Peripheral blood mononuclear cells from donor 1 and donor 2 were challenged with 1 and 10 ng, respectively, of $p24^{Gag}$ of the R5 viruses: YU-2 wild type [donor 1 (open circles), donor 2 (open triangles)] or YU-2 cPPT-D [donor 1 (solid circles), donor 2 (solid triangles)]. (D) MDMs (in 48-well plates) that had been maintained for 8 days were challenged with 20 ng of $p24^{Gag}$ of the R5 viruses: YU-2 wild type (solid triangles), YU-2 cPPT-D (open circles), or YU-2 V165A (solid circles).

ing CEM-SS/CCR5 cells at 18 h postchallenge are shown in Fig. 4A. The efficiencies of integration were similar for the R5-tropic wild-type and cPPT-D viruses, as judged by the broad smear observed at all dilutions (compare lanes 1 to 4 with lanes 9 to 12). Analogous analyses were also performed with the X4-tropic set of viruses; here, the 1,000-fold dilution samples revealed a minor decrease in the integration efficiency for the cPPT-D virus compared to the wild type (compare

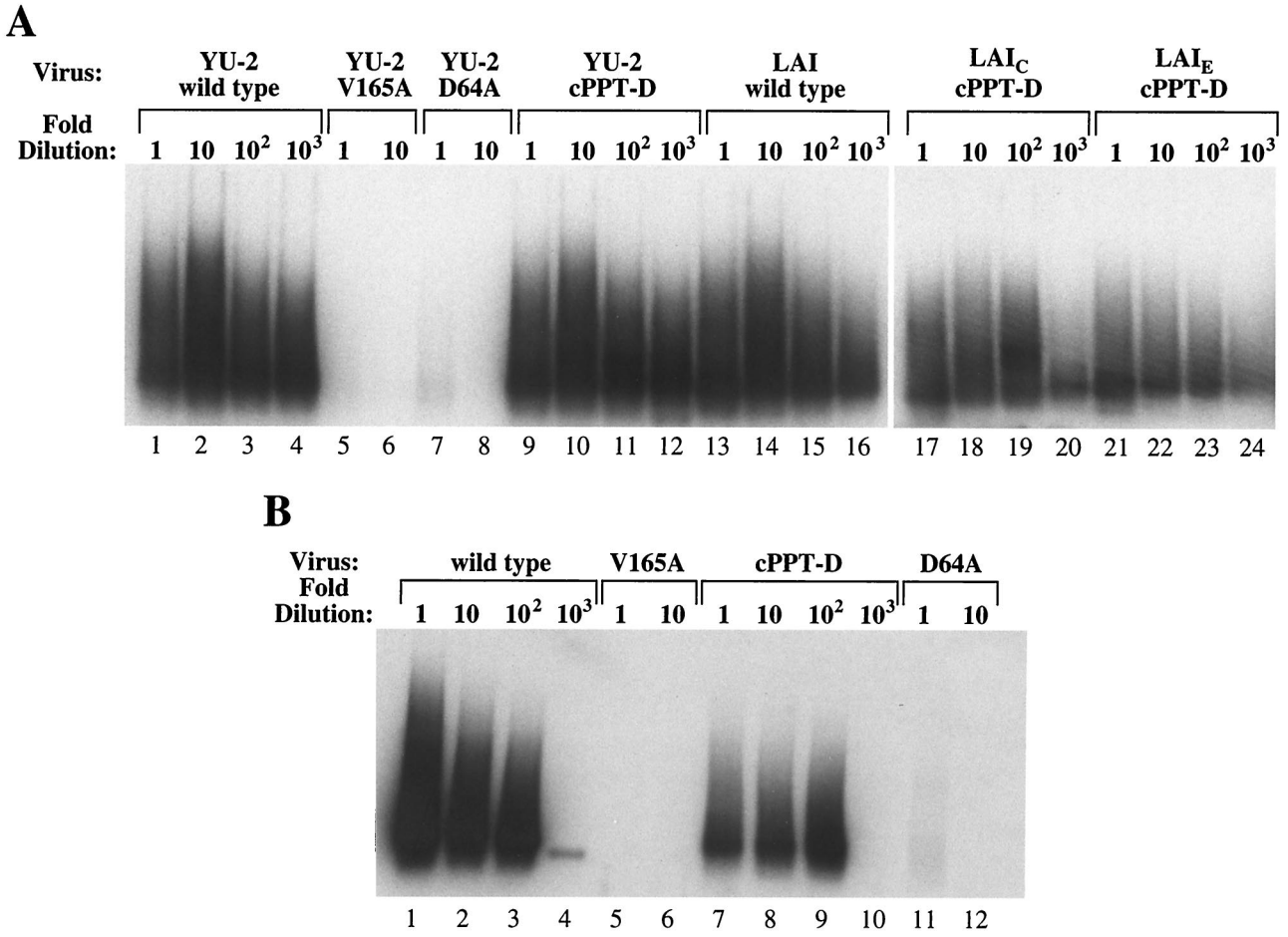


FIG. 4. Analysis of integration efficiency in vivo. (A) Provirus formation by R5 isolates. Cell lysates obtained 15 to 18 h after infection of CEM-SS/CCR5 cells were serially diluted and subjected to nested *Alu*-PCR. Amplified products were resolved by agarose gel electrophoresis and analyzed by Southern hybridization with an LTR-specific probe, followed by autoradiography. Integrated HIV-1 DNAs were detected as a broad smear (lanes 7 to 11). (B) Provirus formation in MDMs. As in panel A, lysates obtained 18 h after infection of MDMs were subjected to *Alu*-PCR analysis.

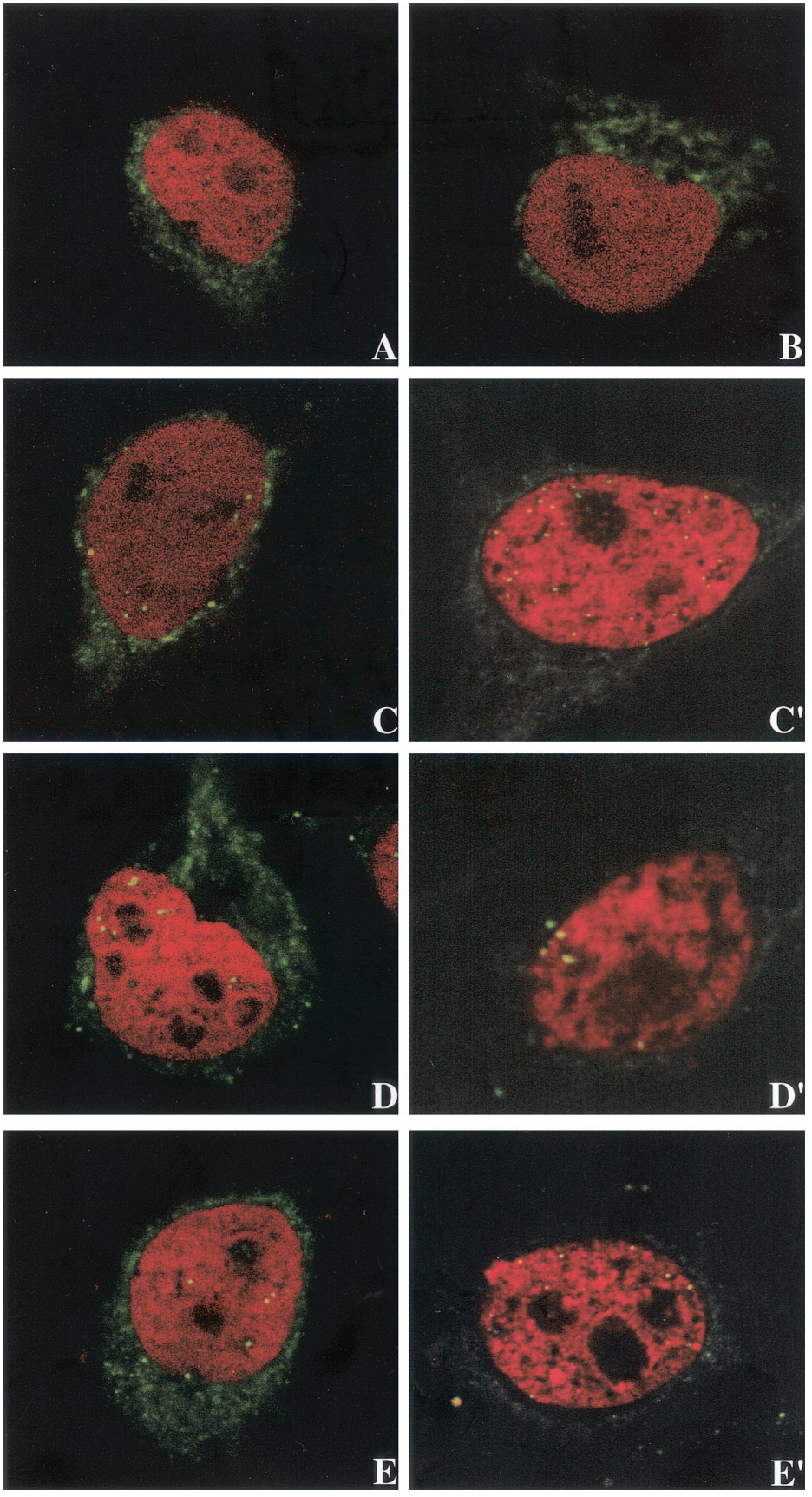
lanes 20 and 24 with lane 16). Consistent with previous results, the viral cDNAs from R5 and X4 V165A viruses, as well as a D64A virus, failed to integrate to a significant degree (Fig. 4A, lanes 5 to 8, and data not shown).

Following challenge of nondividing MDMs with the R5-tropic set of viruses, *Alu*-PCR analysis demonstrated that both the wild-type and cPPT-D viruses formed proviruses efficiently (Fig. 4B). At a 1,000-fold dilution, the signal for the cPPT-D was lost but was still weakly present for wild-type virus (Fig. 4B, compare lane 10 with lane 4). This indicates that integration efficiency is modestly decreased for cPPT-D viruses, and this is consistent with the replication data (Fig. 3). In summary, there does not appear to be a significant barrier to viral cDNA nuclear import or integration for R5 or X4 viruses harboring inactivated cPPTs.

Viral cDNA from V165A and cPPT-D viruses is nuclear. Historically, the nuclear import and accumulation of HIV-1 PICs has been assessed with two surrogates. First, the ability of full-length reverse transcripts to form 1-LTR and 2-LTR circles (collectively termed LTR circles) has been used as a marker of presence in the nucleus, since the enzymes responsible for their formation are thought to be nuclear (3). Second, biochemical separations of infected cells have been used to indicate that infection-delivered viral proteins can accumulate in nucleus-derived fractions (2, 4, 15).

We wished to apply a third and more direct methodology to

FIG. 5. FISH. HOS-CD4-CCR5 cells were challenged with inocula corresponding to 200 to 400 ng of $p24^{Gag}$ by centrifugal inoculation, incubated for ~18 h, fixed, treated with RNase, and ethanol dehydrated. The samples were hybridized overnight at 37°C with an HIV-1-specific, biotinylated probe, followed by fluorescein isothiocyanate-avidin and an additional amplification step. The nuclei were counterstained with propidium iodide. The signals were visualized with a Leica confocal laser scanning microscope. Mock-infected cells (A) and cells challenged in the presence of efavirenz (B) served as negative controls. Representative data from two independent experiments are shown for YU-2 wild type (panels C and C′), YU-2 cPPT-D (panels D and D′), and YU-2 V165A (panels E and E′).


A
B
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D
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E
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the analysis of the cPPT-D and V165A virus challenges, specifically, FISH. Accordingly, cultures of the adherent cell line HOS-CD4-CCR5 were challenged with the R5-tropic set of viruses, incubated for ~18 h, fixed, and analyzed with a biotinylated HIV-1-specific probe and confocal microscopy (Fig. 5; representative cells are shown for each viral challenge). One important point to note is that our analyses were designed to be qualitative rather than quantitative; hence, the location of viral cDNA is more informative than the number of cDNAs detected within each cell. For instance, in a typical experiment, we saw substantial variations in the number of FISH signals per individual cell. We suspect that this may be due to single-cell variations in susceptibility to infection, perhaps as a consequence of variations in CD4 and coreceptor expression.

When cells were mock infected (Fig. 5A) or infected in the presence of the RT inhibitor efavirenz (Fig. 5B), no FISH signals were detected. As expected, signals seen in a wild-type infection (Fig. 5C and C′) were almost entirely nuclear and, presumably, represented both unintegrated and integrated DNA. Also, consistent with our finding that cPPT-D viruses established proviruses efficiently, the DNAs from this challenge were mostly nuclear (Fig. 5D and D′). A significant result of this experiment was that viral cDNAs from the V165A challenge were also mainly nuclear and present in a pattern that was indistinguishable from that noted for the wild-type virus (compare Fig. 5C and C′ with Fig. 5E and E′). Even though the V165A virus was originally described as being partially deficient for LTR circle formation, this result was perhaps not entirely surprising, since LTR circles did still constitute the majority of V165A reverse transcripts 24 h after infection of CEM-SS/CCR5 cells (2).

These FISH data therefore demonstrate that the phenotypic deficiencies of the V165A virus do not reflect a lack of viral DNA nuclear localization. As will be discussed at greater length below, this finding, together with the fact that the V165A integrase protein is catalytically active but unable to mediate provirus formation, leads us to hypothesize that the V165A mutation may be interfering with a critical post-nuclear entry targeting step that is essential for progression of the viral life cycle.

Because FISH experiments are inherently qualitative, the efficiency of cPPT-D nuclear import was further evaluated by analysis of LTR circle formation in nondividing cells (Fig. 6). This was accomplished by extraction of low-molecular-weight DNAs during the first 24 h of infection, followed by strategic restriction digestion and Southern hybridization. Irradiated CEM-SS/CCR5 cells were exposed to wild-type or cPPT-D YU-2 virus, and DNAs were harvested at 7 and 24 h postchallenge. The efficiency of LTR circle formation was similar for both viruses (Fig. 6, lanes 2 and 4), indicative of similar nuclear import efficiencies and kinetics. The results of similar analyses for the V165A virus have been reported previously (2, 22). As a control, cells were infected with murine leukemia virus. As expected, the amount of LTR circle formation for murine leukemia virus was significantly reduced in γ-irradiated cells compared with that in dividing cells (data not shown).

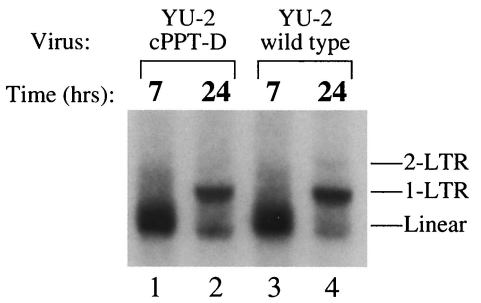

FIG. 6. Nuclear accumulation of viral cDNAs: DNA synthesis and nuclear accumulation. Irradiated CEM-SS/CCR5 cells were challenged with wild-type or cPPT-D mutant virus, and low-molecular-weight DNAs were isolated at 7 and 24 h. Low-molecular-weight DNAs were digested with *Bsg*I and subjected to Southern analysis. The bands corresponding to the linear, 1-LTR circle, and 2-LTR circle forms of viral DNA are indicated.

DISCUSSION

In this article, we describe a side-by-side comparison of V165A integrase and cPPT-D mutant viruses. In contrast to previous data (39), we found that disruption of the cPPT does not significantly affect the viral life cycle in immortalized cell lines but does delay replication modestly in primary cell cultures (Fig. 3). While this defect could represent a decrease in the rate of PIC nuclear import in these cells, we propose that disruption of the cPPT may render reverse transcription less efficient, leading to slight delays in replication. Evidently, these findings are very different from previously published work; since the basis for these discrepancies is not understood, further experimentation in this area is required. Studies from multiple laboratories have shown that inclusion of the cPPT in lentivirus-based gene transfer vectors increases transduction efficiency ~3- to 10-fold, depending on the cell type infected (25, 33, 39). Our experiments with primary cells and full-length infectious clones of HIV-1 appear to be consistent with these stimulatory effects.

The V165A integrase mutation blocked infection prior to integration for both X4 and R5 viruses (Fig. 4). However, FISH analyses revealed that the cDNAs from these viruses were located within the nucleus by 18 h postinfection (Fig. 5). Therefore, even though previous work had indicated that this mutant virus was partially deficient in PIC nuclear import (2), the accumulation of viral DNA in the nucleus was substantial by 18 h. Given that we no longer consider the 161 to 173 region of integrase to be a discrete NLS, this result is perhaps not so surprising. It does, however, leave the question of the nature of the infection defect for the V165A virus unanswered. Because the V165A integrase protein is catalytically active in the setting of virus infection (2) but is unable to mediate integration of cDNAs that are demonstrably nuclear (Fig. 3 and 4), we suggest that this region of integrase may be important for an uncharacterized post-nuclear entry event that positions PICs in an environment that is permissive for integration.

While the features of this hypothesized step are unknown, analogy with other targeting signals would suggest that the 161 to 173 region of integrase may interact with host cell nuclear factors and that these may help direct PICs to chromatin. Alternatively, it is also possible that this region of integrase

might be important for proper association of integrase with the PIC. We also do not know why this mutation results in a modest decrease in LTR circle formation (a result that has been independently confirmed) (22). One might imagine that mislocalization of PICs or a failure to recruit important cofactors could result in structural or stability changes in the PIC that negatively impact the juxtapositioning of LTRs and, consequently, the efficiency of recombination, autointegration, and/or LTR-LTR junction formation. Finally, it is possible that the V165A mutation may impair an aspect of enzymatic activity in vivo that we are not able to evaluate with current assay systems.

The mechanism of HIV-1 PIC nuclear import still requires further investigation, and these studies may be obscured by several potential complications. First, the PIC may contain NLSs that await identification, and it is possible that these may be formed by multiple PIC components so that they cannot be defined in terms of short, linear peptide motifs. Second, the HIV-1 PIC may achieve import by recruiting cellular NLS-containing proteins rather than by providing the signal(s) itself. In this regard, a recent report has shown that the nuclear import of the DNA genome of adenovirus 2 is dependent on both the interaction between disassembling capsids and histone H1 and the subsequent action of the importin-β/importin-7 H1 import receptor complex (34). The third possibility is that viral nucleic acid (DNA and/or RNA) can be imported into the nucleus without the aid of virus-derived signals, hypothetically as complexes with cellular karyophilic proteins. While this process would intuitively be an inefficient way of delivering PICs to the nucleus, we have found that transfected linear DNA can be end ligated in growth-arrested fibroblasts as efficiently as in proliferating cells (23), an observation consistent with DNA's entering the nucleus in the absence of nuclear envelope breakdown (data not shown).

Importantly, retroviral DNA in freshly infected cells is not naked but is contained in large, multicomponent PICs. Nevertheless, these observations with transfected DNA suggest, perhaps, that the use of LTR circle formation as a marker of virus-mediated nuclear import may be less definitive than was previously thought. In other words, while LTR circles likely represent a "nucleus-only" form of the viral cDNA, DNA import and circularization may not be dependent on the provision of specific viral nuclear targeting signals. For instance, if the PIC disassembles prematurely in the cytoplasm, the released viral cDNA could still enter the nucleus and LTR circles may still be formed. If there is validity to this notion, it will be interesting to determine why murine leukemia virus DNA is excluded from the nucleus in the absence of cell division.

It is evident not only that relatively little is understood about how HIV-1 PICs enter the nucleus, but also that current work suffers from technical limitations. For instance, while FISH is an accurate way of defining nuclear accumulation of HIV-1 cDNAs, samples must be examined at least 6 h after infection to ensure that reverse transcription has been completed. The percentage of cells with detectable FISH signals increases significantly by 15 to 18 h postchallenge (1). As a result, kinetic effects on subcellular trafficking and/or nuclear import that are manifested on a shorter time scale cannot be examined by this method. This represents a significant practical obstacle for this field, since earlier subcellular fractionation studies suggested that virion proteins can be detected in the nucleus within 2 h of viral challenge (2, 4, 15). In addition, even though such observations indicate that the import of PICs, like that of the DNA genomes of adenovirus, herpes simplex virus, and adeno-associated virus (18, 31, 38), may be rapid, it is also possible that the HIV-1 proteins being assayed in these experiments are no longer associated with functional PICs. To study these processes during virus infection, it is critical that appropriately sensitive methods be developed for visualizing incoming viral proteins and nucleic acids (and hence PICs) at the same time (30, 35).

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ADDENDUM IN PROOF

Limón and coworkers have also demonstrated that the HIV-1 cPPT is not required for virus replication (A. Limón, N. Nakajima, R. Lu, H. Z. Ghory, and A. Engelman, J. Virol. **76:**12078–12086, 2002).

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