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Class II Integrase Mutants with Changes in Putative Nuclear Localization Signals Are Primarily Blocked at a Postnuclear Entry Step of Human Immunodeficiency Virus Type 1 Replication†

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Integrase has been implicated in human immunodeficiency virus type 1 (HIV-1) nuclear import. Integrase analyses, however, can be complicated by the pleiotropic nature of mutations: whereas class I mutants are integration defective, class II mutants display additional assembly and/or reverse transcription defects. We previously determined that HIV-1$_{V165A}$, originally reported as defective for nuclear import, was a class II mutant. Here we analyzed mutants containing changes in other putative nuclear localization signals, including ^{186}KRK^{188}/^{211}KELQKQITK^{219} and Cys-130. Previous work established HIV-1$_{K186Q}$, HIV-1$_{Q214L/Q216L}$, and HIV-1$_{C130G}$ as replication defective, but phenotypic classification was unclear and nuclear import in nondividing cells was not addressed. Consistent with previous reports, most of the bipartite mutants studied here were replication defective. These mutants as well as HIV-1$_{V165A}$ synthesized reduced cDNA levels, but a normal fraction of mutant cDNA localized to dividing and nondividing cell nuclei. Somewhat surprisingly, recombinant class II mutant proteins were catalytically active, and class II Vpr-integrase fusion proteins efficiently complemented class I mutant virus. Since a class I Vpr-integrase mutant efficiently complemented class II mutant viruses under conditions in which class II Vpr-integrases failed to function, we conclude that classes I and II define two distinct complementation groups and suggest that class II mutants are primarily defective at a postnuclear entry step of HIV-1 replication. HIV-1$_{C130G}$ was also defective for reverse transcription, but Vpr-integrase$_{C130G}$ did not efficiently complement class I mutant HIV-1. Since HIV-1$_{C130A}$ grew like the wild type, we conclude that Cys-130 is not essential for replication and speculate that perturbation of integrase structure contributed to the pleiotropic HIV-1$_{C130G}$ phenotype.

Retroviral replication requires the integration of the viral cDNA made by reverse transcription into a host cell chromosome. After entering a susceptible target cell, reverse transcriptase converts genomic RNA into linear double-stranded cDNA with a copy of the viral long terminal repeat (LTR) at each end. Integration is mediated by the viral integrase protein acting on the ends of the linear cDNA (reviewed in reference 14). Replication-defective integrase mutants of human immunodeficiency virus type 1 (HIV-1) can be grouped into one of two phenotypic classes. Class I mutants are specifically blocked at the integration step and are typified by changes in the Asp and Glu residues that constitute the enzyme active site. In contrast, class II mutants are pleiotropic, as additional defects at the postintegration step of viral particle assembly and/or at the preintegration step of reverse transcription are observed (reviewed in reference 20).

In addition to catalyzing the DNA breakage and joining steps essential for integration, integrase has been implicated in reverse transcription (33, 63, 68) and in the nuclear import of HIV-1 preintegration complexes (PICs) (reviewed in reference 53). When expressed on its own in the absence of other HIV-1 proteins, integrase localizes to cell nuclei and is therefore karyophilic (6, 12, 16, 17, 43, 47, 52, 54, 58). Despite numerous viral genetic and integrase localization analyses, the mechanism of integrase import and the role of integrase in PIC nuclear import have not been fully elucidated. Numerous human proteins interact with HIV-1 integrase, and some of these, including importin α (31), importin 7 (28), and lens epithelium-derived growth factor (LEDGF/p75) (46, 47), have been implicated in integrase and PIC nuclear import (reviewed in references 53 and 59).

One phenotype that is predicted for HIV-1 nuclear localization mutants is reduced infectivity and cDNA import specifically in nondividing compared to dividing cell types (reviewed in references 53 and 56). Since the integrase mutant HIV-1$_{Y143G}$ displayed a selective two- to fourfold defect in monocyte-derived macrophages (MDMs) compared to cycling cells, Tyr-143 contributed to PIC nuclear import in this classic nondividing cell model (58). However, the results of other mutagenic studies have likely been confounded by the pleiotropic nature of class II integrase mutant viruses (6, 18, 43). For example, a bipartite nuclear localization signal (NLS) com-

prised of NLS-proximal (NLS$_P$) (^{186}KRK^{188}) and NLS-distal (NLS$_D$) (^{211}KELQKQITK^{219}) sequences was originally described as being important for integrase-importin α interactions in vitro (31), yet HIV-1 mutated in either NLS$_P$ or NLS$_D$ residues was replication defective in both cycling and nondividing cells (9, 51, 58).

Whereas the NLS$_P$ mutant HIV-1$_{K186Q}$ (9, 51) and NLS$_D$ mutant HIV-1$_{Q214L/Q216L}$ (51) supported wild-type levels of cDNA synthesis and nuclear import in dividing cells, a separate study reported that HIV-1$_{K186Q}$ as well as a deletion of NLS$_P$ (HIV-1$_{\Delta KRK}$) caused defects in reverse transcription (58). The effects of NLS$_P$ and NLS$_D$ changes on reverse transcription and nuclear import in nondividing cells have not been reported. Although the K186Q and Q214L/Q216L changes altered the nuclear accumulation of Flag-tagged integrase in cells (51), K186Q and ΔKRK mutants were efficiently transported into cell nuclei as green fluorescent protein-integrase fusion proteins (58). Due to these conflicting reports on the phenotypes of NLS$_P$ and NLS$_D$ mutant viruses and the roles of these sequences in integrase localization, we analyzed previously described as well as novel NLS$_P$ and NLS$_D$ mutants in assays for virus replication, particle release, reverse transcription, and nuclear import. The effects of the K186Q and Q214L/Q216L changes on integrase localization were analyzed with a recently described codon-optimized expression system (43).

Residues in addition to Tyr-143 and those that constitute NLS$_P$ and NLS$_D$ have been implicated in integrase-PIC nuclear import. Among these is a noncanonical NLS comprised of Val-165 and Arg-166 (6) that was subsequently assessed as unimportant for viral import (18), as Flag-tagged IN$_{V165A}$ localized to cell nuclei and HIV-1$_{V165A}$ and HIV-1$_{R166A}$ were classified as class II integrase mutant viruses (43). However, a recent report has resurrected the notion that this region of integrase comprises a functional NLS (3). Integrase residue Cys-130 has also been implicated in HIV-1 nuclear import through affecting integrase multimerization more than functioning as an NLS per se (52). Cys-130 resides in the integrase catalytic core domain (CCD) which harbors two additional Cys residues at positions 56 and 65. To investigate the potential role(s) of CCD cysteines in HIV-1 replication and nuclear localization, HIV-1$_{C56A}$, HIV-1$_{C65A}$, and HIV-1$_{C130A}$ were constructed. In total, 10 new NLS$_P$, NLS$_D$, and Cys mutant viruses were analyzed alongside previously described HIV-1$_{K186Q}$ (9, 51, 58), HIV-1$_{Q214L/Q216L}$ (9, 51), HIV-1$_{C130G}$ (52), and HIV-1$_{V165A}$ (6, 18, 43).

Integrase comprises three functional domains, the N-terminal domain, CCD, and C-terminal domain (reviewed in reference 14). This three-domain structure was in large part inferred from the results of functional complementation assays that showed that two different catalytically inactive integrase mutant proteins restored in vitro integration activity only when the proteins were altered in different domains (22, 61). Functional complementation can also occur in cells as integrase mutant infectivity defects were overcome by incorporating wild-type integrase into assembling virus particles as fusions to the virion accessory protein Vpr (29, 64). Certain changes like V165A and R199A that by themselves rendered HIV-1 replication defective nonetheless functionally complemented class I integrase active-site mutant viruses when assayed as Vpr-integrase fusion proteins (6, 29).

FIG. 1. Integrase domain structure and mutant viruses. (A) Schematic diagram of integrase, highlighting the three functional domains and residues and sequences pertinent to this study. The locations of active-site residues Asp-64, Asp-116, and Glu-152 in the central CCD are indicated. Residues and regions that were targeted to generate novel mutant viruses are indicated above the protein. The numbers below the protein indicate the first residue of the catalytic core and C-terminal domains (26). NTD, N-terminal domain; CTD, C-terminal domain. (B) NLS$_P$ mutants. NLS$_P$ (^{186}KRK^{188}) (31) is indicated above the integrase coding sequence, and individual mutant changes are indicated underneath the corresponding wild-type amino acid residue. (C) NLS$_D$ (^{211}KELQKQITK^{219}) (31) and NLS$_D$ mutants. (D) Cys-130 mutants.

Since both the active site and Val-165 reside in the CCD (Fig. 1A), functional complementation implied that Val-165 plays a noncatalytic role in HIV-1 infection (6, 18). Here we extended this observation by showing that Vpr fusions to CCD NLS$_P$ mutants or C-terminal domain NLS$_D$ mutants efficiently restored class I mutant infectivity under conditions in which they failed to efficiently complement class II integrase mutant function. Since a CCD class I Vpr-integrase mutant fusion protein efficiently complemented class II mutant viruses but not class I HIV-1, these results demonstrate that class I and II are two distinct complementation groups and that this grouping extends beyond the domain boundaries that were defined by studying catalytically defective integrase mutant proteins in vitro (22, 61). Consistent with these observations, recombinant integrase proteins derived from complementing class II mutant viruses displayed activity in in vitro integration assays. Based on these results and the behavior of viral and integrase mutants in nuclear import assays, we conclude that NLS$_P$, NLS$_D$, Val-165, and Cys-130 residues do not play specific roles in HIV-1 nuclear import and suggest instead that altered nuclear trafficking contributes to the pleiotropic class II mutant phenotype.

MATERIALS AND METHODS

was expressed in HeLa cells with a previously described codon-optimized vector (43). Mutagenesis was performed with QuikChange mutagenesis as recommended by the manufacturer (Stratagene, La Jolla, Calif.). Viral integrase changes were introduced into pUCWTpol (43) and 1.8-kb AgeI-PflMI *pol* fragments were swapped for the corresponding fragments in pNL43/XmaI (7) and pNLX∆env (43) to generate full-length and envelope (Env)-deleted molecular clones of pNL4-3, respectively. Integrase mutant plasmids pNL43(F185K) (35), pNLX(N/N), pNL43(1-212), (50), pNLX(V165A), and pNL43(R166A) (43) were described previously.

Plasmid pNLX(Vpr-) encoding nonfunctional Vpr protein (30) was constructed by digesting pNL43/XmaI with EcoRI, filling in with Klenow fragment, and religation. Plasmid pNLX.Luc was built by amplifying the gene for firefly luciferase (Luc) from pGL3-Basic (Promega, Madison, Wis.) with ClaI- and XhoI-tagged primers, digestion with ClaI and XhoI, and ligation to ClaI- and XhoI-digested pNLX∆envCAT (44). The 1.6-kb PflMI-NheI fragment from pNLX(Vpr-) was swapped for the corresponding fragment in pNLX.Luc to create pNLX.Luc(R−). The 1.8-kb AgeI-PflMI fragments of various integrase mutant constructs were then switched for the corresponding fragment in pNLX.Luc(R−).

The Env expression vector encoding vesicular stomatitis virus G (VSV-G) glycoprotein was described previously (66). An expression vector carrying the dualtropic HIV-1$_{89.6}$ Env was made by swapping the KpnI-BamHI fragment from p89.6 (13) for the corresponding fragment in pNLXE7 (44). The Vpr-integrase expression vector pRL2P-Vpr-IN (64) (a generous gift from J. C. Kappes, University of Alabama at Birmingham) was altered with QuikChange mutagenesis. Targeted regions of plasmid DNAs were analyzed by sequencing to confirm desired mutations and rule out unwanted secondary changes.

Cells. 293T and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented to contain 10% fetal calf serum, 100 IU of penicillin per ml, and 100 µg of streptomycin per ml. Jurkat and C8166 T cells were grown in RPMI 1640 fortified with 10% fetal calf serum, 100 IU of penicillin per ml, and 100 µg of streptomycin per ml (RPMI). MDMs were derived from HIV-1-seronegative donor blood by absorbance onto plastic as previously described (50). Cell cycle status was determined by staining ethanol-fixed cells with propidium iodide and quantification by flow cytometry with ModFit LT version 3.0 (Verity Software House Inc., Topsham, Maine).

Nuclear localization of integrase. Flag-tagged integrase in transiently transfected HeLa cells was detected by indirect immunostaining as previously described (43). Chromosomal DNA was visualized by staining with 4′,6′-diamidino-2-phenylindole as previously described (43).

Viruses and infections. Virus stocks were generated in either 293T or HeLa cells by transfection with calcium phosphate, and cell-free virus titers were determined with an exogenous ^{32}P-based reverse transcriptase assay as previously described (24, 50). Infections for virus replication assays were performed as previously described (50).

For real-time quantitative PCR (RQ-PCR) assays, 293T cells were cotransfected with a viral vector and an Env expression plasmid with FuGene 6 (Roche Molecular Biochemicals, Indianapolis, Ind.). The resulting cell-free supernatants were treated with DNase for 1 h at 37°C to degrade the bulk of residual plasmid DNA left over from transfection essentially as previously described (11, 43) with the exception that 40 U of Turbo DNase (Ambion, Austin, Tex.) per ml was used in most applications. Jurkat cells (10^6) were infected with 10^6 reverse transcriptase cpm of HIV-1$_{89.6}$ Env-containing virus by spinoculation as previously described (44, 45). For VSV-G-pseudotyped virus, Jurkat cells (4 × 10^6) were infected with 2.5 × 10^6 reverse transcriptase cpm by spinoculation, and MDMs were infected by incubation at 37°C for 2 h. Viruses for Southern blotting were derived from 293T cells as previously described (11) except that FuGene 6 was used as the transfection reagent instead of calcium phosphate.

RQ-PCR assays. DNA from infected cells was harvested with the DNeasy tissue kit as recommended by the manufacturer (Qiagen, Valencia, Calif.), and 10 µl was amplified by PCR (30-µl reaction volume) as previously described (44, 45). Two different HIV-1 amplicons, late reverse transcription products and two-LTR-containing circles, were normalized to total cellular DNA with either spectrophotometry (44) or endogenous retrovirus 3 (43, 45) as previously described. Virus particles lacking Env were used in parallel infection and RQ-PCR assays to assess residual levels of plasmid DNA that remained after transfection and DNase treatment, and these values were subtracted from values obtained with matched Env-positive infections.

Southern blot analysis. C8166 cells were infected and Southern blotting was performed essentially as previously described (7, 11) with the exception that DNA was extracted from infected cells with the Hirt protocol (34, 39). Hirt supernatant DNA digested with BsgI (6) and DpnI was recovered following extraction with phenol-chloroform and precipitation with ethanol. Viral DNA

signals were quantified by PhosphorImager with ImageQuant v1.11 (Amersham Biosciences, Inc., Piscataway, N.J.).

Purification of recombinant integrase protein and in vitro integration assay. N-terminally hexahistidine-tagged integrase expressed from pINSD.His was purified from inclusion bodies and refolded as previously described (43). The His tag was removed by cleavage with thrombin as previously described (43). C-terminally tagged integrase expressed from pKBIN6H was purified from soluble extracts of *Escherichia coli* as previously described (12).

Oligonucleotide substrate DNA representing the 21 bp of the HIV-1 U5 end was radiolabeled and annealed as previously described (43). Integration reactions (16 µl) contained 25 mM morpholinepropanesulfonic acid (pH 7.2), 0.1 mg of bovine serum albumin per ml, 10 mM β-mercaptoethanol, 10% glycerol, 25 nM labeled DNA substrate, 20 mM NaCl, 7.5 mM MnCl$_2$, and 0.4 µM integrase. Reactions were terminated after 60 min at 37°C and electrophoresed through denaturing polyacrylamide sequencing gels, and the results were quantified by PhosphorImager.

Vpr-integrase complementation. 293T cells in six-well plates were cotransfected with 2 µg of pNLX.Luc(R−), 1 µg of pRL2P-Vpr-IN, and 0.067 µg of pNLXE7 by calcium phosphate, and the resulting cell-free viruses were titered by a ^{32}P-labeled reverse transcriptase assay. Baseline integrase mutant infectivities in the absence of added Vpr-integrase were conducted by substituting pcDNA3 (Invitrogen) for pRL2P-Vpr-IN. Jurkat cells (2 × 10^6 in 0.5 ml) were infected with 5 × 10^5 reverse transcriptase cpm for 17 h at 37°C, at which time culture volumes were expanded by the addition of 5 ml of RPMI. Two days postinfection, cells were washed with phosphate-buffered saline (Mediatech, Herndon, Va.) and lysed with 75 µl of passive lysis buffer (Promega Corp.). After a freeze-thaw cycle, lysates were clarified by centrifugation at 18,730 × g for 15 min at 4°C. Supernatants (20 µl) were tested in duplicate for luciferase activity (Luciferase Assay System; Promega, Corp.) with an EG&G Berthold Microplate LB 96V luminometer and a Microlite 1 flat-bottomed microtiter plate (Thermo Labsystems, Franklin, Mass.). Luciferase activity was normalized to total protein concentration as determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.). Background values derived from infections with Env-minus HIV-1$_{NLX.Luc(R−)}$ were subtracted from Env-positive wild-type and mutant integrase activities.

RESULTS

Mutagenesis strategy. Although previous studies reported that NLS$_P$ mutant HIV-1$_{K186Q}$ (9, 51, 58), NLS$_D$ mutant HIV-1$_{Q214L/Q216L}$ (9, 51), and HIV-1$_{C130G}$ (52) were replication defective, phenotypic details of these putative NLS mutant viruses in cycling and nondividing target cells were unclear. To obtain a comprehensive picture of the roles of NLS$_P$ and NLS$_D$ in HIV-1 replication and nuclear import, seven novel mutant viruses were constructed. Whereas K186E, K186A, R187A, and K188A were designed to alter NLS$_P$ (Fig. 1B), K215A, K219A, and K215A/K219A targeted NLS$_D$ (Fig. 1C). HIV-1 integrase contains six Cys residues, two in the N-terminal domain that help coordinate zinc, three in the CCD, and one in the C-terminal domain (for a review of integrase domain structure, see references 14 and 26). Whereas zinc finger mutant HIV-1$_{C43L}$ was noninfectious (48), C-terminal domain mutant HIV-1$_{C280S}$ grew like the wild type (4). To obtain a comprehensive view of the role of each CCD cysteine in HIV-1 replication, Ala was substituted for Cys-56, Cys-65, and Cys-130 (Fig. 1A and 1D). Novel integrase changes as well as previously described K186Q (9, 51, 58), Q214L/Q216L (9, 51), and C130G (52) changes were introduced into the NL4-3 strain of HIV-1 (1).

Replication phenotypes of NLS$_P$, NLS$_D$, and Cys mutant viruses. In repeat experiments, Jurkat T cells inoculated with 10^6 reverse transcriptase cpm (an approximate multiplicity of infection of 0.04) (44, 50) supported peak levels of HIV-1$_{NL4-3}$ replication 5 to 6 days postinfection (Fig. 2). In agreement with previous reports (9, 51, 58), the K186Q change rendered HIV-
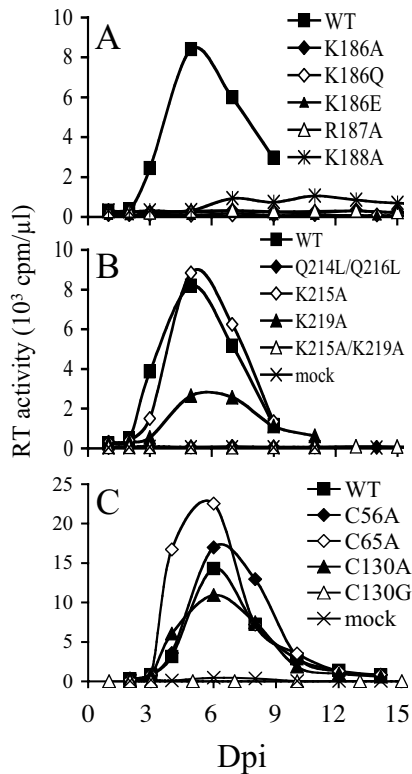
FIG. 2. Wild-type (WT) and integrase mutant replication kinetics in Jurkat T cells. (A) Supernatants of cells infected with the indicated viruses were analyzed for reverse transcriptase (RT) activity at the indicated time points. (B) Replication kinetics of NLS_D mutant viruses. (C) Reverse transcriptase profiles of Cys mutant viruses. Similar integrase mutant profiles were observed in independent sets of infections. Extended observation (2 months) of cells infected with HIV-1_{K186Q}, HIV-1_{K186A}, HIV-1_{K186E}, HIV-1_{R187A}, HIV-$1_{Q214L/Q216L}$, HIV-$1_{K215A/K219A}$, or HIV-1_{C130G} failed to reveal any evidence of virus replication. Dpi, days postinfection.

1_{NL4-3} replication defective (Fig. 2A). Integrase mutants HIV-1_{K186A}, HIV-1_{K186E}, and HIV-1_{R187A} were also replication defective. In contrast, HIV-1_{K188A} supported a low but reproducible level of virus spread (Fig. 2A and data not shown).

Akin to the previous reports (9, 51), we also found HIV-$1_{Q214L/Q216L}$ replication defective (Fig. 2B). Since NLS_D mutant HIV-$1_{G189R/K211N}$ was infectious (58) and Los Alamos sequence alignments indicated that NLS_D residues Lys-215 and Lys-219 were more highly conserved among HIV-1 isolates than Lys-211 (38), Lys-215 and Lys-219 were targeted in this study. Whereas HIV-1_{K215A} grew like HIV-1_{NL4-3}, HIV-1_{K219A} was modestly perturbed in its ability to replicate, as HIV-1_{K219A}-infected cultures yielded approximately threefold fewer total reverse transcriptase cpm than wild-type-infected cells in repeat experiments (Fig. 2B). In contrast, HIV-$1_{K215A/K219A}$ was replication defective.

Consistent with the previous report (52), HIV-1_{C130G} was replication defective (Fig. 2C). Somewhat surprisingly, HIV-1_{C130A} replicated with wild-type kinetics. HIV-1_{C56A} and HIV-1_{C65A} also yielded the wild-type replication profile in Jurkat T cells (Fig. 2C).
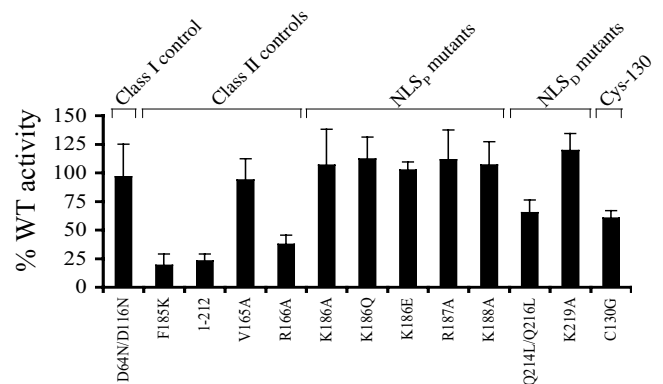

FIG. 3. Integrase mutant virus release from cells. Supernatants of HeLa cells transfected with the indicated viruses were analyzed for reverse transcriptase activity. Results are averages of duplicate reverse transcriptase assays and a minimum of four independent transfections. Virus release was expressed as a percentage of wild-type reverse transcriptase activity. Radioimmunoprecipitation analyses of cell extracts revealed wild-type levels of gene expression for HIV-1_{F185K} (25), HIV-1_{1-212}, HIV-$1_{Q214L/Q216L}$, and HIV-1_{R166A} (data not shown).

Integrase mutants and HIV-1 release. Having determined that a subset of NLS_P, NLS_D, and Cys-130 mutant viruses were replication defective, we next investigated the step(s) in the HIV-1 life cycle where these viruses were blocked. Defective particle assembly and release is one of the phenotypes associated with pleiotropic class II mutants (20). Particle release was assayed by transfecting CD4$^-$ cells and measuring HIV-1 in the resulting cell-free supernatants. This assay is well suited for quantifying the effects of integrase mutations on virus release because (i) it bypasses the requirement for integration as a prerequisite for gene expression and (ii) receptor-minus cells afford comparison of replication-defective and replication-competent strains. As controls we included wild-type HIV-1_{NL4-3}, active-site class I mutant HIV-$1_{D64N/D116N}$ (50), assembly-defective class II mutants HIV-1_{F185K} (35) and HIV-1_{1-212} (50), and class II mutants HIV-1_{V165A} and HIV-1_{R166A} which we and others previously determined were released from 293T and COS-7 cells at the wild-type level (6, 43, 62). We note that previous analyses of virus-expressing cells revealed that wild-type and assembly-defective class II integrase mutants supported similar levels of HIV-1 gene expression (2, 8, 24, 25).

Whereas the class I HIV-$1_{D64N/D116N}$ mutant released from HeLa cells at the wild-type level, class II assembly-defective controls HIV-1_{F185K} and HIV-1_{1-212} were released at 20% to 25% of the wild-type level (Fig. 3). Consistent with previous reports (6, 43), HIV-1_{V165A} was released as efficiently as the wild type. In contrast, HIV-1_{R166A} release was reduced approximately threefold from that of the wild type (Fig. 3). This result agrees with previous findings that a class II integrase deletion mutant was released more efficiently from 293T and COS-7 cells than from HeLa cells (8). Each of the replication-defective NLS_P mutants released with wild-type efficiency. In contrast, HIV-1_{C130G} and HIV-$1_{Q214L/Q216L}$ release was reduced to 60% to 70% of the wild-type level (Fig. 3). In separate experiments, HIV-1_{K215A} and HIV-$1_{K215A/K219A}$ behaved like HIV-1_{K219A} and thus did not reveal any release defect in this assay (data not shown).

Reverse transcription defect of HIV-1$_{V165A}$. In addition to particle release, integrase mutations can affect reverse transcription (20). Reverse transcription is a multistep process requiring two template switches to generate full-length duplex cDNA (reviewed in reference 57), and PCR primers can be designed to quantify specific steps in the sequential process (36, 67). We previously devised an RQ-PCR assay (43, 44) with LTR- and *gag*-specific primers to quantify levels of full-length and nearly full-length late reverse transcription products. A second set of primers was designed to amplify two-LTR circles, a ligation product formed by host-mediated nonhomologous DNA end joining (42). Since two-LTR circles form in the nuclei of HIV-1-infected cells, the two-LTR/late reverse transcription ratio can be used as an indicator of HIV-1 nuclear import (reviewed in reference 56).

Previous results established that class II integrase mutant viruses were defective for two-LTR circle formation (2, 6, 24, 25, 40, 43). Although we previously proposed that defective nuclear import might be a phenotype common to all class II mutant viruses (43), other results indicated that reduced levels of reverse transcription might in large part account for the observed reductions in two-LTR circle formation (40). Based on these observations as well as other discrepancies, for example, whether HIV-1$_{K186Q}$ synthesized wild-type (9, 51) or diminished (58) cDNA levels, we reevaluated our RQ-PCR assay to ensure that it detected bona fide levels of wild-type and integrase mutant reverse transcription.

Since class II integrase mutants are replication defective, virus stocks for RQ-PCR assays must be generated by transfection. Supernatants derived from transiently transfected cells contain residual plasmid DNA, and it is therefore important to treat such viruses with a DNA-degrading enzyme such as DNase I prior to infection. We originally determined by Southern blotting (11) and RQ-PCR (44) that 2 U of RQ1 DNase I (Promega Corp.) per ml reduced plasmid DNA signals to background levels.

To investigate whether residual plasmid might in some instances survive this treatment, single-round Env-positive and Env-negative HIV-1$_{NLX.Luc}$ was treated with various concentrations of DNase I prior to infection, and cell lysates prepared at 3 h, 6 h, and 24 h postinfection were analyzed by RQ-PCR as previously described (44). Although Env-positive virus treated with 2 U of DNase I per ml yielded more HIV-1 DNA than the Env-negative control, the Env-negative signal was significantly above the background level of detection at 3 h postinfection (Fig. S1 in the supplemental material). Whereas the Env-positive signal increased from 3 to 6 h postinfection, the Env-negative signal decreased during this time frame and approached the detection limit of the assay by 24 h postinfection (Fig. S1). By increasing the concentration of DNase I in the pretreatment, the levels of DNA detected after mock infection were reduced to near background levels without affecting the level of Env-dependent reverse transcription (Fig. S1). These results confirmed our suspicion that plasmid DNA in viral stocks could in some instances survive DNase I treatment. Based on these results, we shifted to treating viruses for RQ-PCR assays with relatively high concentrations of DNase I. The results of additional preliminary experiments revealed that Turbo DNase reproducibly yielded lower levels of RQ-PCR
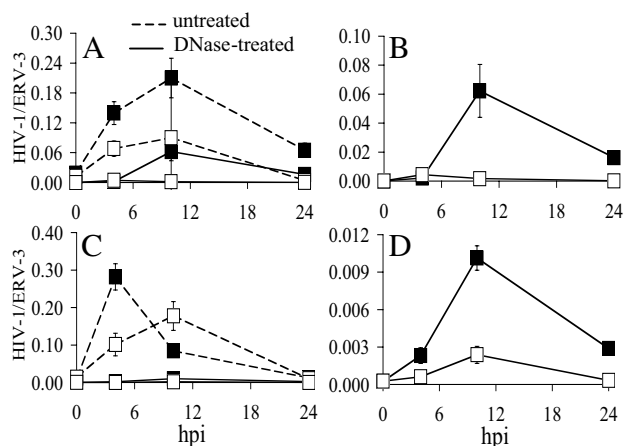

FIG. 4. Analysis of HIV-1$_{V165A}$ reverse transcription by RQ-PCR. (A) Jurkat cells were infected with untreated (dashed line) or DNase-treated (solid line) Env-positive (■) or Env-negative (□) HIV-1$_{NLX.luc}$. Cellular DNA prepared at the indicated times was evaluated for HIV-1 by RQ-PCR as previously described (43, 45). (B) Same as in panel A except that DNase-treated samples were regraphed to highlight the level of HIV-1$_{NLX.Luc}$ reverse transcription (■) over that of residual plasmid DNA (□). (C and D) Same as in panels A and B except that integrase mutant V165A was analyzed. Error bars represent variations between duplicate RQ-PCR assays. ERV-3, endogenous retrovirus 3; hpi, hours postinfection.

signals after mock infection than did RQ1 DNase I (data not shown).

The kinetics of HIV-1$_{NLX.Luc}$ and HIV-1$_{V165A.Luc}$ reverse transcription were compared with and without Turbo DNase pretreatment. Untreated Env-positive and Env-negative HIV-1$_{NLX.Luc}$ yielded surprisingly similar RQ-PCR profiles (Fig. 4A, dashed lines). Whereas similarly low levels of DNA were detected at time zero, both signals rose until 10 h postinfection and subsided thereafter (Fig. 4A). DNase treatment collapsed the Env-negative profile nearly to background levels, revealing the kinetics of HIV-1$_{NLX.Luc}$ reverse transcription under these conditions (Fig. 4A and B). Similar to the wild type, untreated HIV-1$_{V165A.Luc}$ yielded robust RQ-PCR signals independent of Env function (Fig. 4C, dashed lines). In this case, DNase significantly reduced both Env-positive and Env-negative signals (Fig. 4C). Rescaling unmasked the level of integrase mutant reverse transcription which, after correcting for the residual Env-negative signal, revealed approximately eightfold defective cDNA synthesis for HIV-1$_{V165A.Luc}$ (compare Fig. 4D to 4B).

We note that the class II mutation reduced the quantity of late reverse transcription products without detectably altering the kinetics of cDNA synthesis or the stability of completed reverse transcripts (Fig. 4B and D). HIV-1$_{NL4-3}$ and HIV-1$_{V165A}$ reverse transcription was also analyzed by Southern blotting. Hirt supernatant DNAs were treated with BsgI to yield 1.2-kb and 1.9-kb products representing the left and right ends of linear HIV-1 cDNA, respectively (Fig. 5A). HIV-1$_{V165A}$ yielded about fivefold less cDNA than HIV-1$_{NL4-3}$ at 7 h postinfection (Fig. 5B, lanes 1 and 2). Since Southern blotting confirmed a reverse transcription defect for HIV-1$_{V165A}$, we concluded that the modified RQ-PCR assay (Fig. 4)
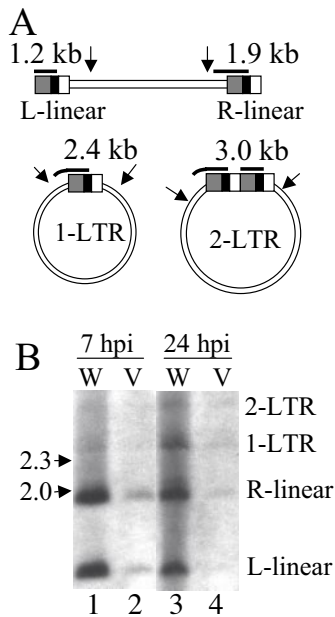
FIG. 5. Southern blot analysis of HIV-1$_{NL4-3}$ and HIV-1$_{V165A}$ DNA. (A) Major forms of unintegrated HIV-1 cDNA in infected cells. Arrows denote the approximate locations of relevant BsgI sites. Bold horizontal lines represent the *nef*/U3 minus-strand-specific probe used for blotting (7, 11), with the predicted size of each hybridizing fragment indicated above the probe. (B) Wild-type (W) and HIV-1$_{V165A}$ (V) DNA synthesis. The migration positions of DNA standards are indicated to the left of the blot (in kilobases), and the identities of HIV-1 DNA species are indicated on the right. hpi, h postinfection.

accurately determined levels of integrase mutant reverse transcription.

Reverse transcription and nuclear import profiles of integrase mutant viruses. We next analyzed the cDNA synthesis and nuclear import profiles of a subset of replication-defective integrase mutant viruses in cycling and nondividing cells. Since each NLS$_P$ mutant was released from HeLa cells at the wild-type level (Fig. 3), HIV-1$_{K186Q}$ was chosen as a representative mutant. As the replication-defective HIV-1$_{Q214L/Q216L}$ and HIV-1$_{K215A/K219A}$ NLS$_D$ mutants displayed slightly different

release profiles, both of these were selected for study. HIV-1$_{V165A}$ and HIV-1$_{C130G}$ were also analyzed.

Infectivity and reverse transcription profiles were initially tested in cycling T cells. As anticipated from the results of spreading infection assays (Fig. 2), the single-round infectivities of these replication-defective integrase mutants were reduced to near the detection limit of the luciferase assay (approximate 400- to 10,000-fold reductions compared to HIV-1$_{NLX.Luc[R-]}$; Table 1). Since wild-type and integrase mutant reverse transcription levels peak approximately 7 to 8 h postinfection (10, 11, 37) (Fig. 4), late reverse transcription values were determined at 7 h postinfection. HIV-1$_{V165A.Luc}$ and HIV-1$_{K186Q.Luc}$ synthesized about fourfold less cDNA than HIV-1$_{NLX.Luc}$ (Fig. 6A). HIV-1$_{Q214L/Q216L.Luc}$ and HIV-1$_{K215A/K219A.Luc}$ yielded similar low levels of reverse transcripts, while HIV-1$_{C130G.Luc}$ yielded even lower cDNA levels, which amounted to approximately 10% of the wild-type level in repeat experiments (Fig. 6A and data not shown).

The fraction of wild-type and integrase mutant cDNA converted into two-LTR circles at 24 h postinfection is shown in Fig. 6C. Although each integrase mutant formed a lower absolute number of DNA circles, mutant two-LTR/late reverse transcription ratios were equal to or greater than the wild-type value (Fig. 6C). Thus, none of the class II integrase mutants was defective for nuclear import under these infection conditions. Southern blotting confirmed that a normal fraction of HIV-1$_{V165A}$ cDNA was converted to LTR circles at 24 h postinfection (Fig. 5B).

Terminally differentiated MDMs present a paradigm for active HIV-1 nuclear import (53, 56). Thus, for the purpose of this work, it was important to address integrase mutant nuclear import in this cell type. Preliminary experiments, however, revealed that HIV-1$_{NLX.Luc}$ pseudotyped with the dual-tropic HIV-1$_{89.6}$ Env failed to yield detectable levels of two-LTR circles in MDMs derived from most blood donors (data not shown). Furthermore, class II integrase mutant viruses yielded less two-LTR circle DNA than wild-type virus under the favorable condition of dividing cell infection (2, 24, 25, 40, 43) (Fig. 5B, 6B, and 6C). For these reasons, the VSV-G Env glycoprotein was used to enhance levels of HIV-1 entry. Class II integrase mutant reverse transcription (Fig. 6B) and infec-

TABLE 1. Complementation of mutant viruses with Vpr-integrase fusions[a]

Integrase mutant	% of wild-type activity[b]	Luciferase activity of Vpr-integrase fusions							
		Wild-type activity[c]	Complementation relative to Vpr-IN$_{WT}$[d]						
			V165A	C130G	K186Q	K215A/K219A	Q214L/Q216L	Q62K	R199A
V165A	0.00 (0.01)	26.5 (3.0)	−	−	+/−	+/−	+/−	++[e]	+
C130G	0.02 (0.04)	71.3 (28.2)	−	−	+/−	+/−	+/−	+/−	+/−
K186Q	0.23 (0.28)	31.4 (8.4)	−	−	+/−	+/−	+/−	++	+
K215A/K219A	0.02 (0.02)	27.9 (4.3)	−	−	+/−	+/−	+/−	++	+
Q214L/Q216L	0.11 (0.05)	36.1 (12.0)	−	−	+	+	+/−	++	+
D64N/D116N	0.07 (0.03)	18.2 (9.7)	++	+/−	++	++	++	−	++
R199A	0.39 (0.04)	37.5 (18.5)	−	−	+/−	+	+/−	+	+

[a] Values represent averages of a minimum of two infections.
[b] Luciferase activity of the indicated mutant virus relative to HIV-1$_{NLX.Luc(R-)}$, with standard deviations in parentheses.
[c] Activity of the indicated mutant viruses complemented with Vpr-IN$_{WT}$ relative to WT HIV-1$_{NLX.Luc(R-)}$, with the standard deviations in parentheses.
[d] ++, ≥50% of Vpr-IN$_{WT}$ complementation; +, 10 to 50%; +/−, 1 to 10%; −, <1%.
[e] Whereas Vpr-IN$_{Q62K}$ displayed 51% of Vpr-IN$_{WT}$ complementation activity, the class I active-site Vpr-IN$_{D116A}$ mutant protein (29) displayed about 4% activity. Thus, we did not observe the same pattern of efficient reciprocal complementation between active-site and V165A mutant viruses and Vpr-integrase proteins as noted in reference 6.
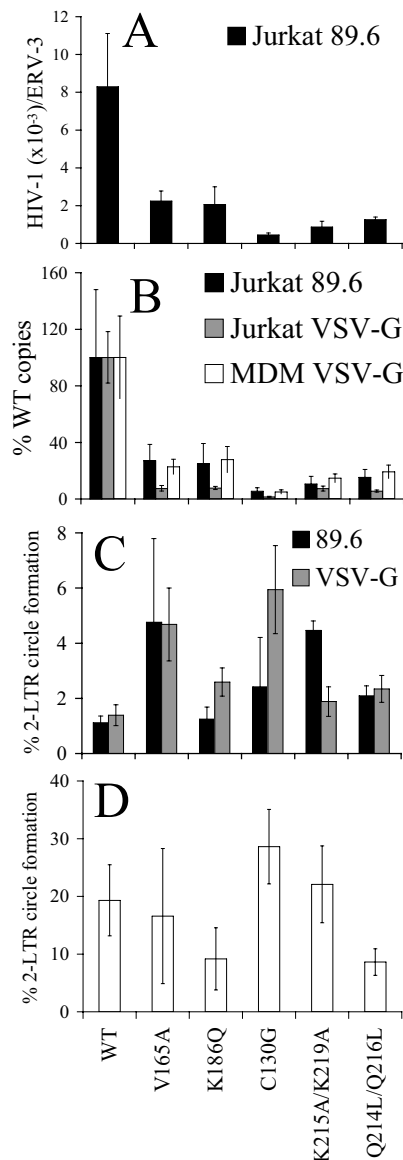
FIG. 6. DNA synthesis and nuclear import profiles of integrase mutant viruses. (A) Jurkat cells were infected with the indicated viruses, and DNA prepared 7 h postinfection was analyzed for total HIV-1 DNA by RQ-PCR. (B) Same as in panel A except VSV-G pseudotypes were used to infect Jurkat cells (grey bars) or MDMs (white bars). Integrase mutant DNA levels were normalized to the wild-type (WT) level, which was set to 100%. DNA levels from panel A are included for comparison. (C) Percentage of total HIV-1 DNA converted to two-LTR circles, determined by dividing the two-LTR circle value obtained at 24 h postinfection by the total DNA level from 7 h postinfection. Black bars, Jurkat cells infected via HIV-1$_{89.6}$ Env; grey bars, infections mediated via VSV-G. (D) Percentage of total HIV-1 DNA converted to two-LTR circles in MDMs infected by the indicated VSV-G-pseudotypes. Values were determined as in panel C except that two-LTR circle levels peaked 48 h postinfection in MDMs. Cell cycle analysis revealed that the MDM cultures used in these experiments comprised approximately 2% cells in S phase. The error bars in panels A through D represent variation between duplicate RQ-PCR assays. Similar results were determined in duplicate sets of infections. VSV-G-pseudotyped HIV-1$_{K188A}$, HIV-1$_{K215A}$, and HIV-1$_{K219A}$ yielded two-LTR/total cDNA fractions in MDMs that were greater than or equal to the wild-type value (data not shown). ERV-3, endogenous retrovirus 3.

tivity (data not shown) defects were observed in Jurkat T cells following VSV-G-mediated entry. Similar to HIV-1 Env-mediated infections, the percentages of integrase mutant cDNAs converted into two-LTR circles were at or above the wild-type level following VSV-G-mediated entry (Fig. 6C).

The reverse transcription profiles of integrase mutant viruses in MDMs mirrored their profiles in Jurkat T cells (Fig. 6B). The frequencies of integrase mutant two-LTR circle formation in MDMs were again quite similar to that of HIV-1$_{NLX.Luc}$, with HIV-1$_{Q214L/Q216L.Luc}$ and HIV-1$_{K186Q.Luc}$ revealing minor defects (approximately twofold) in repeat experiments (Fig. 6D and data not shown). However, since the partially defective NLS$_P$ mutant HIV-1$_{K188A.Luc}$ (data not shown) and replication-defective NLS$_D$ mutant HIV-1$_{K215A/K219A.Luc}$ (Fig. 6D) supported the wild-type frequency of two-LTR circle formation in MDMs, we conclude that neither NLS$_P$, NLS$_D$, Cys-130, nor Val-165 plays a critical role in HIV-1 nuclear import in cycling cells or in primary MDMs (Fig. 6).

Cellular localization of integrase mutant proteins. The results of the previous section discounted significant roles for NLS$_P$, NLS$_D$, Val-165, and Cys-130 in PIC nuclear import and instead highlighted diminished levels of cDNA synthesis as the primary reason for reduced levels of nuclear HIV-1. We next tested the roles of NLS$_P$ and NLS$_D$ in the nuclear import of integrase protein. This interest stemmed from inconsistencies in the literature; whereas one study reported that Flag-tagged NLS$_P$ and NLS$_D$ mutants mislocalized and were dispersed throughout transfected cells (51), a separate study reported wild-type localization properties for green fluorescent protein-integrase fusion proteins (58).

The effects of K186Q and Q214L/Q216L changes on integrase localization were assayed in the context of a recently developed codon-optimized Flag-tagged expression system (17, 43). As expected, wild-type integrase efficiently accumulated in cell nuclei (Fig. 7). The integrase mutants IN$_{K186Q}$ and IN$_{Q214/Q216L}$ also localized to cell nuclei under these experimental conditions (Fig. 7). Since the C130G change was also reported to alter the cellular distribution of Flag-tagged integrase (52), we also tested this mutant in our expression system. Although IN$_{C130G}$ was predominantly nuclear, it displayed substantially more cytoplasmic staining than the wild-type, NLS$_P$, or NLS$_D$ mutant proteins (Fig. 7). Our results indicate that neither K186Q, Q214L/Q216L, nor C130G dramatically altered the nuclear accumulation of Flag-tagged integrase, although, similar to the previous report (52), IN$_{C130G}$ in part localized to the cell cytoplasm.

Analyses of mutant catalytic activities. (i) In vitro integration. Our results highlighted that replication-defective integrase mutants HIV-1$_{V165A}$, HIV-1$_{K186Q}$, HIV-1$_{Q214L/Q216L}$, HIV-1$_{K215A/K219A}$, and HIV-1$_{C130G}$ were 4- to 10-fold defective in their ability to synthesize HIV-1 cDNA. Since single-round mutant infectivities were reduced approximately 400- to 10,000-fold from the wild-type level, we reasoned that the primary replication defect(s) of these viruses was at a step downstream of reverse transcription and nuclear localization. We reasoned that the defect was likely to be at integration because (i) mutant virus assembly and release were comparable to those of the wild type (Fig. 3), (ii) mutant virus was transported into the nucleus with nearly wild-type efficiency, as judged from two-LTR/late reverse transcription ratios (Fig. 6C
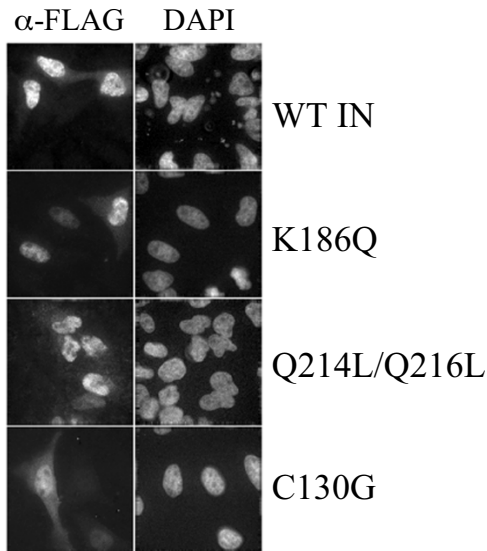
FIG. 7. Distribution of Flag-tagged wild-type (WT) and mutant integrase (IN) proteins in HeLa cells. Cells transfected with codon-optimized Flag-tagged expression vectors were fixed and mounted in 4′,6′-diamidino-2-phenylindole (DAPI)-containing medium to visualize nuclear DNA. Integrase was visualized by indirect immunofluorescence microscopy with the anti-Flag M2 antibody.

FIG. 8. Catalytic activity of class II integrase mutant proteins. (A) Activities of C-terminally His$_6$-tagged proteins. Integrase (IN) was omitted from the reaction in lane 1. (B) Effect of His$_6$ tag on the 3′-processing activity of IN$_{V165A}$. Whereas the protein in lane 2 retained the tag, the tag was removed from the protein in lane 3. The tag was also removed from wild-type integrase (lane 1). 21, migration position of the 21-base labeled strand in substrate DNA; 19, position of cleaved DNA product.

and D), and (iii) the viruses were altered in integrase. To further dissect the replication blocks of these viruses, the catalytic activities of the mutant integrase proteins were analyzed.

In cells, integrase catalyzes two distinct activities: 3′ processing of newly synthesized reverse transcripts, which is followed by the transfer of the nicked viral ends to the 5′ phosphates of a double-stranded cut in chromosomal DNA at the site of integration (reviewed in reference 14). Recombinant integrase proteins were expressed as fusions to a noncleavable C-terminal hexahistidine tag to enable purification by nickel nitrilotriacetate chromatography from soluble extracts of E. coli (12, 47). To assess catalytic activity, purified proteins were reacted with an oligonucleotide substrate that modeled the U5 end of HIV-1 cDNA. Wild-type integrase converted about 43% of the substrate to the nicked 3′-processing product (Fig. 8A, lane 2). The IN$_{D64N}$ active-site mutant protein was predictably inactive (23) under these reaction conditions (<0.2% of substrate converted to product; Fig. 8A, lane 3). In stark contrast, the integrases derived from replication-defective HIV-1$_{V165A}$, HIV-1$_{K186Q}$, HIV-1$_{Q214L/Q216L}$, and HIV-1$_{K215A/K219A}$ were catalytically active (Fig. 8A, lanes 4 to 7). Of note, IN$_{V165A}$ displayed about 60% of wild-type integrase activity under these conditions (Fig. 8A, lane 4, and data not shown).

Using similar reaction conditions, we noted previously that IN$_{V165A}$ displayed only about 3% of wild-type integrase activity (43). IN$_{V165A}$ was previously solubilized with guanidine-HCl, purified in denatured form, and refolded, and the N-terminal His$_6$ tag was removed by protease cleavage. To see if the His$_6$ tag and/or purification scheme affected IN$_{V165A}$ activity, the activity of refolded and tagged protein was compared to that of refolded and thrombin-cleaved IN$_{V165A}$. The tagged protein supported about 45% of wild-type integrase activity (Fig. 8B, compare lane 2 to lane 1). Removing the tag reduced IN$_{V165A}$
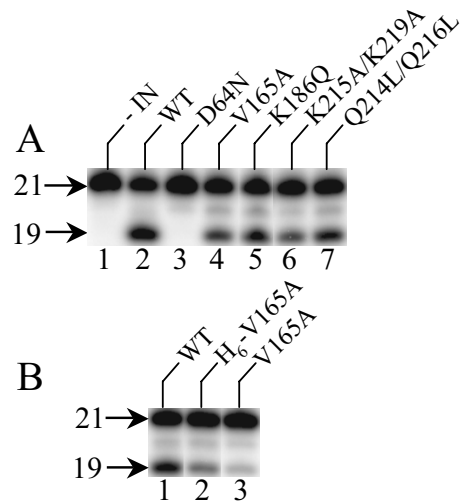
activity approximately threefold, to about 14% of wild-type integrase activity (Fig. 8B, lanes 1 and 3). Based on this, we conclude that although the His$_6$ tag can influence the activity of recombinant integrase protein derived from replication-defective class II integrase mutant viruses, these enzymes posses significantly more in vitro integration activity than active-site mutant proteins derived from replication-defective class I mutant viruses (Fig. 8).

(ii) Vpr-integrase trans-complementation. Vpr-integrase complementation was next used to investigate the infectivity defects and catalytic activities of class I and class II integrase mutants. The infectivity of integration-defective HIV-1 can be restored by incorporating wild-type integrase into assembling particles as a Vpr-integrase fusion protein (29, 64). In addition, certain replication-incompetent integrase mutants were shown to complement class I integrase mutant virus when expressed as Vpr-integrase fusions. Three different noninfectious CCD mutants were previously shown to restore function to class I integrase mutants as Vpr-integrase fusions: V165A (6), L172A/K173A (55), and R199A (29).

Based on these observations, the following changes were incorporated into Vpr-integrase alongside V165A and R199A controls: C130G, K186Q, K215A/K219A, and Q214L/Q216L. The Q62K change, which perturbs integrase-cDNA binding (11, 27, 32) and causes an integration-specific defect (11; R. Lu, A. Limón, H. Ghory, and A. Engelman, submitted for publication), was engineered as a CCD class I Vpr-integrase mutant protein, and HIV-1$_{D64N/D116N}$ (50) was used as a CCD class I mutant virus. Functional Vpr was deleted from the single-round luciferase-expressing viruses used in these assays to enhance trans-incorporation of Vpr-integrase during particle assembly. Table 1 reports the levels at which Vpr-wild-type integrase fusions complemented each integrase mutant virus as

a percentage of wild-type HIV-1$_{\text{NLX.Luc(R-)}}$ infectivity. The level at which each Vpr-integrase mutant protein functioned was then expressed as the percentage of Vpr-wild-type integrase activity.

Consistent with previous reports, Vpr-IN$_{\text{R199A}}$ (29) and Vpr-IN$_{\text{V165A}}$ (6) efficiently complemented HIV-1$_{\text{D64N/D116N.Luc(R-)}}$ (Table 1). Vpr-IN$_{\text{K186Q}}$, Vpr-IN$_{\text{K215A/K219A}}$, and Vpr-IN$_{\text{Q214L/Q216L}}$ behaved like Vpr-IN$_{\text{R199A}}$ and Vpr-IN$_{\text{V165A}}$ in that efficient complementation of HIV-1$_{\text{D64N/D116N.Luc(R-)}}$ was observed (Table 1). In contrast, the class I mutant Vpr-IN$_{\text{Q62K}}$ protein failed to complement HIV-1$_{\text{D64N/D116N.Luc(R-)}}$. The inability of Vpr-IN$_{\text{Q62K}}$ to complement HIV-1$_{\text{D64N/D116N.Luc(R-)}}$ is consistent with the results of in vitro assays that demonstrated that different catalytically inactive CCD integrase mutants failed to complement each other (61). Thus, these results define Vpr-IN$_{\text{Q62K}}$ and HIV-1$_{\text{D64N/D116N.Luc(R-)}}$ as belonging to the same complementation group and Vpr-IN$_{\text{V165A}}$, Vpr-IN$_{\text{R199A}}$, Vpr-IN$_{\text{K186Q}}$, Vpr-IN$_{\text{K215A/K219A}}$, and Vpr-IN$_{\text{Q214L/Q216L}}$ as belonging to a different group. Of note, V165A, R199A, and K186Q are CCD changes and K215A/K219A and Q214L/Q216L are C-terminal domain changes (Fig. 1A). We also note that Vpr-IN$_{\text{C130G}}$ displayed an intermediately low level of activity (about 3% of that of Vpr-wild-type integrase) when tested in trans to HIV-1$_{\text{D64N/D116N.Luc(R-)}}$ (Table 1).

Each of the class II Vpr-integrase fusions was next assayed in trans to its respective virus as well as the other class II mutant viruses. Unexpectedly, none of the class II Vpr-integrase mutants complemented a class II mutant virus as well as they complemented HIV-1$_{\text{D64N/D116N.Luc(R-)}}$. This was especially evident with Vpr-IN$_{\text{V165A}}$ (Table 1). Although Vpr-IN$_{\text{K186Q}}$, Vpr-IN$_{\text{K215A/K219A}}$, and Vpr-IN$_{\text{Q214L/Q216L}}$ displayed partial activities with class II mutant viruses, we note that each of these Vpr-integrase mutants displayed similar low levels of activity when tested against their cognate mutant viruses (Table 1). The ability of a particular Vpr-integrase mutant protein to functionally complement its own otherwise defective virus has been reported previously (55). Whereas Vpr-IN$_{\text{R199A}}$ also displayed this phenotype (Table 1), Vpr-IN$_{\text{Q62K}}$ did not (data not shown). These results suggest that HIV-1$_{\text{R199A}}$ is a class II mutant virus, which is consistent with previous findings that strains carrying this change (i) failed to yield detectable levels of two-LTR circles (62) and (ii) were approximately 1,000-fold less infectious than active-site class I mutants in the multinuclear activation of galactosidase indicator assay (62), a single-round infectivity assay that is particularly sensitive to transient levels of class I mutant gene expression (50; reviewed in reference 20). In contrast to the results obtained with class II Vpr-integrase mutants, Vpr-IN$_{\text{Q62K}}$ efficiently complemented class II mutant viruses HIV-1$_{\text{V165A.Luc(R-)}}$, HIV-1$_{\text{K186Q.Luc(R-)}}$, HIV-1$_{\text{K215A/K219A.Luc(R-)}}$, and HIV-1$_{\text{Q214L/Q216L.Luc(R-)}}$ (Table 1).

DISCUSSION

Integrase plays a key role during the early phase of retroviral replication by catalyzing the integration of the cDNA made by reverse transcription into a cell chromosome (14). However, mutations in integrase can cause a range of pleiotropic phenotypes. We previously defined two classes of replication-defective HIV-1 integrase mutant viruses (20). Whereas class I mutants are specifically blocked at the integration step, the

mutations in class II viruses are pleiotropic, as they display defects at the relatively late step of particle assembly and/or at the early preintegration step of reverse transcription. Whereas class I mutants are typified by changes in the integrase active site, integrase deletion mutants as well as point mutants with mutations in the N-terminal zinc finger domain are typical class II mutant viruses (2, 20, 24, 40, 48).

The results presented here clarify that replication-defective class II integrase mutant viruses can encode catalytically active integrase. This interpretation was based on the in vitro integration activities of purified recombinant proteins (Fig. 8) and efficient complementation of class I integrase mutant HIV-1$_{\text{D64N/D116N.Luc(R-)}}$ with class II Vpr-integrase mutant proteins (Table 1). Of note, a subset of these proteins, including Vpr-IN$_{\text{V165A}}$, Vpr-IN$_{\text{K186Q}}$, and Vpr-IN$_{\text{R199A}}$, carried alterations of CCD residues (Fig. 1A). These results highlight that class II Vpr-integrase mutant proteins can functionally complement class I integrase mutant viruses even when the mutations reside in the same functional domain. This unanticipated finding confirms and extends the results of Fletcher et al. (29), Bouyac-Bertoia et al. (6), and Priet et al. (55), who reported similar phenomena with Vpr-IN$_{\text{R199A}}$, Vpr-IN$_{\text{V165A}}$, and Vpr-IN$_{\text{L172A/K173A}}$, respectively, in trans to active-site mutant viruses.

Although we previously reported that PICs purified from HIV-1$_{\text{V165A}}$-infected cells failed to support detectable levels of in vitro integration activity, recombinants IN$_{\text{V165A}}$ (Fig. 8), IN$_{\text{K186Q}}$ (Fig. 8) (60), IN$_{\text{R199S}}$ (60), IN$_{\text{R199C}}$ (41), and IN$_{\text{L172A/K173A}}$ (55) supported 15 to 100% of wild-type integrase activity. Additional experiments will be required to determine if other replication-defective class II integrase mutant viruses that encode functional integrase proteins and Vpr-integrase fusions (Table 1) yield active or defective PICs.

Integrase and PIC nuclear import. HIV-1$_{\text{V165A}}$ was originally reported as defective for nuclear import (6). The results reported here highlight that although fewer HIV-1$_{\text{V165A}}$ two-LTR circles formed in cell nuclei, this was due to an overall decrease in the level of reverse transcription rather than a defect in nuclear import (Fig. 5 and 6). We had previously proposed that defective nuclear import was a phenotype common to many replication-defective integrase mutant viruses (43). In light of our current results, we now believe that this interpretation was premature and that it was likely due to residual levels of plasmid DNA that resisted DNase I digestion (supplemental Fig. S1). Our experience highlights that caution should be exercised when using sensitive RQ-PCR assays to quantify levels of retroviral reverse transcription, especially when transient transfection is used to generate virus stocks that may be defective for reverse transcription (Fig. 4).

When expressed in human cells, HIV-1 integrase localizes to cell nuclei (6, 12, 16, 17, 43, 47, 52, 54, 58). These observations, coupled with prior results that NLS$_P$ change K186Q and NLS$_D$ change Q214L/Q216L reduced the affinity of integrase for importin α in vitro, led to suggestions that integrase plays a role in PIC nuclear import (reviewed in references 53 and 56). Although the NLS$_P$ and NLS$_D$ mutant viruses were replication defective, they failed to reveal evidence of altered nuclear localization in dividing cells (9, 51, 58). Since active nuclear import of the HIV-1 PIC might play a particularly important role in nondividing cells (53, 56), reverse transcription and

two-LTR-circle formation were analyzed in MDMs. Similar to the results observed in dividing T cells, VSV-G-pseudotyped HIV-1$_{V165A}$, HIV-1$_{K186Q}$, HIV-1$_{C130G}$, HIV-1$_{K215A/K219A}$, and HIV-1$_{Q214L/Q216L}$ formed fewer two-LTR circles than HIV-1$_{NL4-3}$ in MDMs, but this was primarily due to lowered levels of reverse transcription rather than defective nuclear import (Fig. 6). These results confirm those of Tsurutani et al. (58), who reported that NLS$_P$ mutants HIV-1$_{K186Q}$ and HIV-1Δ_{KRK} were defective for reverse transcription, and extend those findings to include the physiologically relevant MDM cell type. Considered alongside the results that NLS$_P$ and NLS$_D$ mutant proteins effectively localize to cell nuclei (Fig. 7) (58), we conclude that the basic amino acid residues of the NLS$_P$ and NLS$_D$ do not play a discernible role in integrase or HIV-1 nuclear import. Analyses of peptide-based nuclear import in semipermeabilized cells similarly discounted functional NLS roles for NLS$_P$ and NLS$_D$ (15).

Cys-130 and HIV-1 replication. Our results confirmed the previous finding that HIV-1$_{C130G}$ was replication defective (52). Because of this, we were somewhat surprised to discover HIV-1$_{C130A}$ grew like wild-type HIV-1$_{NL4-3}$ (Fig. 2C). These results demonstrate that Cys-130 is not essential for HIV-1 replication and highlight that HIV-1$_{C130G}$ was replication defective due to a negative impact of the Cys-to-Gly substitution compared to the loss of a positive role for Cys at this position. We note that Cys-130 constitutes part of the third alpha helix in the three-dimensional structure of the CCD (19). Since Gly is notorious for destabilizing alpha helices (5, 49), we speculate that the C130G change negatively impacted overall integrase structure and that this in large part contributed to the observed replication defect.

Consistent with this interpretation, Vpr-IN$_{C130G}$ displayed a low level of activity (about 3%) when tested in *trans* to HIV-1$_{D64N/D116N.Luc(R-)}$ (Table 1), the C130G change caused partial mislocalization (Fig. 7) (52) and reduced the stability of Flag-tagged integrase in human cells (52), and the C130S change grossly affected integrase stability in insect cells (4). We note that a recent study reported replication-defective phenotypes for HIV-1 strains carrying the C130S change and that analogous mutant proteins displayed levels of in vitro DNA strand transfer activity that were significantly reduced from that of the wild type (68). In contrast to the differences observed with strains carrying different amino acid substitutions at Cys-130, strains mutated at Cys-56 or Cys-65 with Ala (Fig. 2C) or Ser (68) displayed wild-type replication profiles.

Class II integrase mutant viruses and postnuclear entry interactions. Replication-defective class I integrase mutant viruses are typified by changes in the integrase active site, and thus these viruses encode integrase proteins that lack detectable levels of in vitro activity (20). Paradoxically, class I integrase mutants can support relatively high levels of activity under certain single-round infection conditions such as the multinuclear activation of galactosidase indicator assay, which is likely due to transient expression from unintegrated DNA templates (20, 50, 65).

A variety of integrase changes, including large deletions, cause the class II phenotype (20). In this case the mutant integrase protein will lack detectable levels of catalytic activity. Yet the results presented here extend those of Bouyac-Bertoia et al. (6) and suggest that in contrast to integrases encoded by

replication-defective class I mutant viruses, class II mutants can support substantial levels of integrase activity in vitro (Fig. 8) and in the context of HIV-1 infection (Table 1). Reciprocal complementation assays employing class I and class II mutant viruses and Vpr-integrases defined the class I and class II phenotypes as genetically distinct: each class of Vpr-integrase mutant protein (i) only efficiently complemented the other class of mutant virus and (ii) either failed to complement or only partially complemented its own virus class (Table 1). Since CCD class II Vpr-integrase mutant proteins efficiently complemented CCD class I mutant HIV-1$_{D64N/D116N.Luc(R-)}$ and C-terminal domain class II Vpr-IN$_{K215A/K219A}$ and Vpr-IN$_{Q214L/Q216L}$ proteins did not efficiently complement CCD class II mutant HIV-1$_{V165A.Luc(R-)}$, the pattern of functional class I-class II complementation extends beyond the traditional boundaries of integrase functional domains (Fig. 1A and Table 1). Thus, class I and class II mutant viruses are defective for what would appear to be completely different reasons.

Considering that some class II mutant viruses can encode functional integrase protein, our findings pose the question of the basis of the class II mutant phenotype. Due to the pleiotropic nature of the phenotype, many different factors may contribute. However, in certain cases, for example, HIV-1$_{V165A}$ and HIV-1$_{K186Q}$, viral late events appeared to proceed normally (Fig. 3). In these cases, we therefore propose that the virus mutation perturbs an interaction between integrase and a host cell or viral factor(s) that occurs after virus entry but before locating a chromosomal target for integration and that this interaction(s) is necessary to ensure a productive infectious route. A similar postnuclear entry, preintegration defect was previously proposed for HIV-1$_{V165A}$ (18).

Because the common class II phenotype is reduced levels of cDNA synthesis and because integrase and reverse transcriptase interact with each other in vitro (33, 63, 68), it seems possible that an aberrant integrase-reverse transcriptase interaction could contribute to the class II phenotype. Alternatively, a variety of host cell factors have been shown to interact with integrase, and a subset of these have been proposed to help PICs find their chromosomal targets for integration (reviewed in references 21 and 59). IN$_{L172A/K173A}$ is defective for interaction with uracil DNA glycosidase (UNG2) (55), and IN$_{V165A}$ is defective for interaction with LEDGF/p75 (59). Replication-defective HIV-1$_{L172A/K173A}$ supported wild-type levels of reverse transcription, but the levels of two-LTR circles were not reported (55). Thus, it is currently unclear if HIV-1$_{L172A/K173A}$ is primarily a class I or class II mutant virus. Despite this, it seems possible that the inability to interact with UNG2 and/or LEDGF/p75 might contribute to the class II integrase mutant phenotype. Determining the type(s) of protein-protein interactions that is perturbed during class II integrase mutant viral infection is an active area of research that might lead to novel ways to block HIV-1 integration without necessarily inhibiting the enzymatic activity of integrase protein.

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