Genetic Analyses of Conserved Residues in the Carboxyl-Terminal Domain of Human Immunodeficiency Virus Type 1 Integrase

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Genetic Analyses of Conserved Residues in the Carboxyl-Terminal Domain of Human Immunodeficiency Virus Type 1 Integrase

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Results of in vitro assays identified residues in the C-terminal domain (CTD) of human immunodeficiency virus type 1 (HIV-1) integrase (IN) important for IN-IN and IN-DNA interactions, but the potential roles of these residues in virus replication were mostly unknown. Sixteen CTD residues were targeted here, generating 24 mutant viruses. Replication-defective mutants were typed as class I (blocked at integration) or class II (additional reverse transcription and/or assembly defects). Most defective viruses (15 of 17) displayed reverse transcription defects. In contrast, replication-defective HIV-1E246K synthesized near-normal cDNA levels but processing of Pr55\textsuperscript{gag} was largely inhibited in virus-producing cells. Because single-round HIV-1E246K,Lac(R-) transduced cells at approximately 8% of the wild-type level, we concluded that the late-stage processing defect contributed significantly to the overall replication defect of HIV-1E246K. Results of complementation assays revealed that the CTD could function in trans to the catalytic core domain (CCD) in in vitro assays, and we since determined that certain class I and class II mutants defined a novel genetic complementation group that functioned in cells independently of IN domain boundaries. Seven of eight novel Vpr-IN mutant proteins efficiently trans-complemented class I active-site mutant virus, demonstrating catalytically active CTD mutant proteins during infection. Because most of these mutants inefficiently complemented a class II CCD mutant virus, the majority of CTD mutants were likely more defective for interactions with cellular and/or viral components that affected reverse transcription and/or preintegration trafficking than the catalytic activity of the IN enzyme.

Retrovirus replication is dependent on the integration of the reverse-transcribed viral genome into a host chromosome. Subsequent to target cell entry, the double-stranded DNA substrate for integration is generated by the viral enzyme reverse transcriptase (RT) upon conversion of the genomic RNA into DNA. Acting on the attachment (att) sites at the cDNA ends, the viral DNA recombinase or integrase (IN) catalyzes two distinct endonucleolytic reactions. For the first reaction, 3'-processing, human immunodeficiency virus type 1 (HIV-1) IN removes the dinucleotide GT from each end. This exposes a 3'-hydroxyl moiety in preparation for the second reaction, strand transfer. Upon recognition and binding to a suitable target site, IN uses the 3'-OHs to cut the chromosome in a staggered fashion, which at the same time joins the viral ends to the 5'-phosphates of the cut. Cellular enzymes are likely involved in the repair of the resultant gapped product, thus fully recombining the viral cDNA with the host (reviewed in references 16 and 38).

IN functions as a multidomain protein consisting of the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD) as defined by limited proteolysis (29), deletion mutagenesis (9, 78), in vitro complementation assays (28, 77), and structural biology (14, 23, 79). The NTD (residues 1 to 49) harbors a conserved HHCC zinc binding motif that contributes to IN multimerization and catalytic function (8, 10, 24, 52, 86). The CCD (residues 50 to 212) contains an invariant triad of acidic residues (Asp-64, Asp-116, and Glu-152 of HIV-1) that form the D,D-35-E active-site motif (21, 29, 49, 51, 76). The CCD has an RNase H-fold, and the catalytic domains of certain bacterial transposases share this fold and also utilize invariant Asp and Glu residues for catalysis (see reference 71 for a review). Various assays have also defined residues within the CCD important for binding to viral and host DNA (20, 32, 36, 37, 41, 44).

Due to the relatively weak degree of sequence homology among retrovirus IN CTDs (a single invariant Trp at HIV-1 position 235), less is known about the precise roles of the CTD (residues 213 to 288) compared to the N-terminal and catalytic core domains in integration and HIV-1 replication. Results of several in vitro assays implicated retrovirus CTDs in DNA binding (31, 62, 78, 81, 82) and multimerization (1, 25, 43, 56). Residues 220 to 270 of HIV-1 IN define a minimal nonspecific DNA binding region (69), and Leu-234 (Val-234 in HIV-1 strain NL4-3, studied here), Arg-262, and Lys-264 contributed to this activity (68, 69). Since DNA protected Gлу-246, Lys-258, and Lys-273 from proteolysis in vitro, these residues were also implicated in DNA binding (20) (Fig. 1A). The HIV-1 CTD also binds the viral att site: through Cys substitution, Gлу-246 was found to cross-link to the adenine located 7 nucleotides from the end of the U5 plus strand (35). A limited number of viral mutants with changes at these amino acid positions, including HIV-1K244A/E246A (80), HIV-1R262A/K264T (11), and HIV-1R262A/R263A/K264H (64), have been analyzed. Although each virus was replication defective, contributions of individual residues to HIV-1 replication were unknown, since...
each mutant carried multiple amino acid substitutions and only a subset of potential DNA binding residues was targeted.

Results of a yeast two-hybrid assay identified Val-260 as important for IN multimerization (45), and 3-dimensional nuclear magnetic resonance (NMR) structures revealed Leu-241 and Leu-242 at or near a CTD dimer interface (25, 56) (Fig. 1B). Whereas HIV-1L260E was replication defective (45), the roles of Leu-241 and Leu-242 in HIV-1 replication have not been investigated. A two-domain CTD-CTD variant solved by X-ray crystallography was also dimeric; however, dimerization was mediated via the CCDs, and the tethered CTDs were separated from each other by 55 Å (14). Nonetheless, novel CTD-CTD interactions were observed between crystallographic dimers, revealing potential roles for Lys-266 and/or Arg-269 in multimerization (14). Whereas a virus mutant altered at Lys-266 has not been described, HIV-1R269A/D270A transduced cells at approximately 15% of the wild-type (WT) level (80).

Replication-defective HIV-1 IN mutant viruses can be grouped into distinct phenotypic classes, for example, those specifically blocked at integration (class I IN mutants) versus those that display additional reverse transcription and/or particle assembly defects (class II mutants). Typified by changes in the D,D-35-E active-site residues, class I mutants support near-normal levels of reverse transcription, and because of this, transient increases in unintegrated viral DNA are observed during acute infection (26). Because the CTD mutant HIV-1W235E was released normally from cells and supported the formation of more 2-long-terminal-repeat (2-LTR) circles than the WT (50), HIV-1W235E was categorized as a class I mutant virus (26). Because HIV-1V260E was released from transfected cells three to fivefold less efficiently than the WT (45), this replication-defective CTD mutant virus is defined here as class II.

In this study 16 CTD residues were targeted by mutagenesis, generating 24 mutant viruses. In addition to their potential roles in multimerization and DNA binding, residues were targeted due to their degree of sequence conservation among a large collection of HIV-1/SIVcpz strains. The majority of replication-defective viruses displayed DNA synthesis and/or virus release defects, characterizing them as class II mutants. Of note, one novel mutant, HIV-1E246K, supported near-WT levels of DNA synthesis and increased levels of 2-LTR circles despite an approximately 20-fold reduction in particle assembly and release. Unexpectedly, the E246K change inhibited proteolytic processing of Pr55gag in virus producer cells. HIV-1E246K displayed about 8% of WT activity in a single-round infection assay, suggesting that perturbations of viral late events contributed significantly to the replication-defective phenotype. Results of Vpr-IN complementation assays revealed that the INs derived from most replication-defective CTD mutant viruses efficiently trans-complemented active-site mutant virus. Thus, despite replication-defective phenotypes, many CTD mutant IN proteins were catalytically active in the backdrop of HIV-1 infection.

MATERIALS AND METHODS

Plasmids. Viral mutations were introduced into pUCWTpol (53) using QuickChange mutagenesis (Stratagene, La Jolla, Calif.), and mutated 1.8-kb AgeI-PflMI fragments were swapped for the corresponding fragments in pNL43/XmaI (6) and envelope (Env)-deleted pNLX.Luc(R) (57) to generate full-length proviral clones and single-round luciferase expression vectors, respectively. Plasmids encoding HIV-1W235E, HIV-1D35E/D116N (63), HIV-1V260E, and HIV-1D64N/D116N (57) have been described previously. Expression vectors for vesicular stomatitis virus G (VSV-G) glycoprotein and HIV-1 NL4-3 Env (pNLXEnv) have been described previously (53). Mutations were introduced into pRL2P-Vpr-IN (84) by QuikChange mutagenesis. Constructs encoding Vpr-INWT, Vpr-INW235E, Vpr-INV260E, and Vpr-IND64N/D116N have been described previously (57). Plasmid regions constructed by PCR were analyzed using DNA sequencing to confirm the desired mutations and the absence of off-site secondary changes.

Cells. 293T and HeLa cells were grown in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin. Jurkat T cells were grown in RPMI 1640 containing 10% fetal calf serum, 100 µg/ml of penicillin, and 100 µg/ml of streptomycin (RPMI). Monocyte-derived macrophages (MDM) were isolated from HIV-1-seronegative donors by plastic absorbance as previously described (63).

Viruses and infections. Transient transfection of 293T or HeLa cells by calcium phosphate generated viral stocks. Cell-free stocks were titered using an exogenous 3P-based RT assay (30, 63) or the Alliance p24 ELISA kit (Perkin-Elmer Life Sciences, Boston, Mass.). Replication assays were performed by infecting 2 × 10⁶ Jurkat T cells with 10⁶ RT cpm of virus (approximate multiplicity of infection, 0.04 [54, 65]) for 17 h at 37°C. Infected cells were washed

FIG. 1. CTD residues targeted in this study. (A) Residues implicated in binding to DNA. Amino acid side chains were highlighted on the dimeric NMR structure (Protein Data Bank entry 1HIV) (56) using the following coloring scheme: Val-234, red; Glu-246, orange; Lys-258, yellow; Arg-262, magenta; Lys-264, pink. The image to the right was rotated 90° along the horizontal axis, revealing the bottom of the structure on the left. Lys-273, also implicated in DNA binding (Table 1), is not shown, because it was not part of the structure. (B) Putative multimerization residues. Side chains were colorized as follows: Leu-241, orange; Leu-242, magenta; Val-260, yellow; Lys-266, red; Arg-269, pink. The image to the right was rotated 90° along the horizontal axis, revealing the bottom of the structure on the left. (C) Residues targeted due to relatively high degrees of sequence conservation. Arg-228, red; Arg-231, yellow; Lys-236, orange; Arg-263, pink; Lys-244, magenta. The image to the right was rotated as in panel A. The images were generated using PyMOL (17).
twice with serum-free RPMI, cultured in 5 ml of RPMI, and split at regular intervals, at which times supernatants were saved for RT assays.

Infections for real-time quantitative PCR (RQ-PCR) assays were performed as previously described (53, 57). Briefly, virus stocks were generated by cotransfecting 293T cells with Env-deleted viral vectors and an Env expression plasmid. Viral stocks were generated by transfecting 293T cells with 2 μg of proviral DNA, respectively. Cells were metabolically labeled with [35S]Cys and [35S]Met (50 μCi/ml of each) from 48 to 60 h posttransfection. Sau3AIV (10 μM final concentration) was added to infected cells where indicated 8 h before radiolabeling. Labeled cells were lysed in a buffer containing 0.3 M NaCl, 50 mM Tris, pH 7.5, 0.5% Triton X-100 with 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and 100 μg/ml leupeptin (Roche Molecular Biochemicals, Indianapolis, Ind.). Cell lysates (1 ml) were spiked with 80 μl of 1% bovine serum albumin and precleared with 50 μg of p24 and Pr55Δp7 of cellular endogenous retrovirus-3 (ERV-3) as described elsewhere (53, 59).

RQ-PCR and single-round infectivity assays. DNAs from infected Jurkat cells and MDM were extracted using the DNeasy tissue kit as recommended by the manufacturer (QIAGEN, Valencia, Calif.). In duplicate 30-μl reaction mixtures, 10 μl of total DNA was analyzed for viral late reverse transcription (LRT) products and 2-LTR circles as previously described (53, 59). Values were normalized to background.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Conservation (%) identity</th>
<th>Criterion for targeting</th>
<th>Enzyme (% activity)</th>
<th>Reference(s)</th>
<th>Mutant(s) analyzed</th>
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<td>K236A, K236E</td>
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</tr>
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<td>E246C</td>
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<td>K258L (5–100)</td>
<td>45, 68</td>
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<td>R262D (10–50)</td>
<td>36, 68, 69, 76</td>
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<td>ii, iii</td>
<td>R263S (50–100)</td>
<td>68, 69, 76</td>
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<td>14, 76, 80</td>
<td>R269A</td>
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<tr>
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<td>i, iii</td>
<td>R269I (50–100)</td>
<td>20</td>
<td>K273A</td>
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<td>i, iii</td>
<td>R271I (50–100)</td>
<td>14, 76, 80</td>
<td>R271A</td>
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</table>

a Relative to a collection of 345 HIV-1 and SIVcpz strains (http://www.hiv.lanl.gov).

b 1, putative role in DNA binding; ii, putative role in IN multimerization; iii, >95% identity as explained in footnote a.

c 3’ processing and/or DNA strand transfer activity.

d IN derived from HIV-2.
dues, Arg-228, Arg-231, Lys-236, Lys-244, and Arg-263 (Fig. 1C), were targeted due to their high degree of sequence conservation among HIV-1/SIVcpz IN proteins (Table 1). Although single missense mutants of most of the residues were previously analyzed for 3′ processing and DNA strand transfer activities in in vitro integration assays (Table 1), only one mutant, HIV-1V260E (45), was previously analyzed for the contribution of an individual residue to virus replication.

Replication profiles of CTD mutant viruses. Virus stocks generated by transient transfection were normalized for RT content, and target cells were challenged with equivalent RT cpm of WT or mutant virus. Jurkat cells infected at an approximate multiplicity of infection of 0.04 supported peak HIV-1NL4-3 replication 4 to 5 days postinfection (Fig. 2). As expected, the negative-control strain, HIV-1Δ–212, which lacked the entire CTD (63), failed to replicate over 2 months of observation (Fig. 2E and data not shown). Also as expected (45), cells infected with HIV-1V260E failed to yield a detectable level of virus growth (Fig. 2B). In contrast, approximately 30% of the mutant viruses replicated with near-WT kinetics (defined by the day at which peak replication was attained) (Table 2). These included HIV-1R231A, HIV-1V234A, HIV-1K236A (Fig. 2A), HIV-1R262A, HIV-1K264A, HIV-1K264E (Fig. 2C), and HIV-1K273A (Fig. 2E). Although the single missense mutants HIV-1R262A, HIV-1K264A, and HIV-1K264E replicated, multiple substitutions within this positively charged region yielded replication-defective mutants HIV-1R262A/R263A, HIV-1R262A/K264A, and HIV-1RRK/DVE (Fig. 2D). HIV-1R228A, HIV-1K236E (Fig. 2A), HIV-1L241A, HIV-1L242A, HIV-1G246K (Fig. 2B), HIV-1K266A, HIV-1K268A (Fig. 2D), HIV-1K244A, and HIV-1K258A (Fig. 2E) were also dead viruses. Three mutants, HIV-1E246A (Fig. 2C), HIV-1K236A/E246A (Fig. 2D), and HIV-1E236A/E246A (Fig. 2E), exhibited significant although reproducible reductions in HIV-1 growth.

Table 2 summarizes the phenotypes of the different mutant viruses.

DNA synthesis profiles of WT and IN mutant viruses. The preceding section revealed that a majority of CTD mutant viruses were blocked in their ability to replicate in Jurkat T cells, and the nature of the replication defects was investigated next. Replication-defective IN mutant viruses can be categorized into two distinct phenotypic groups (26). Class I IN mutants are specifically blocked at the integration step. Because reverse transcription proceeds normally, transient increases in unintegrated DNA such as 2-LTR circles are observed at a time when integration normally occurs (26). In contrast, class II mutants display pleiotropic defects that, in addition to integration, can affect late events such as particle assembly/release and/or the preintegration step of reverse transcription (26). Since class I IN mutant viruses yield increased levels of 2-LTR circles and class II mutants often display overall reductions in cDNA synthesis, RQ-PCR analysis of cDNA metabolism in acutely infected cells was utilized as an initial approach toward phenotypic classification. Taqman primers and probes were selected to detect products after the second template switch of reverse transcription as well as at the unique 2-LTR circle junction. Because reverse transcription peaks approximately 7 hpi and the peak of 2-LTR circles occurs about 24 hpi (12, 13, 46, 59), viral DNAs were analyzed at 7 and 24 hpi. To permit analyses in the absence of virus spread, mutations were introduced into the single-round, Env-deleted HIV-1NLX.Luc(R−) strain, which carried firefly luciferase in the viral nef position (57). Controls included the previously described CTD class I mutant HIV-1W235E.Luc(R−) (50) and the CCD class II mutant HIV-1V165A.Luc(R−) (53, 57).

HIV-1W235E.Luc(R−) supported near-WT levels of DNA synthesis (differing by twofold at most) at 7 and 24 hpi (Fig. 3A, C,
TABLE 2. HIV-1 IN CTD mutant phenotypes

| Mutant     | Replication
gaina | DNA synthesisb | 2-LTR circlesb | Infectivityc | Pheno-
typed |
<table>
<thead>
<tr>
<th></th>
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<th></th>
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<tr>
<td>R228A</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>II</td>
</tr>
<tr>
<td>R231A</td>
<td>+</td>
<td>NA</td>
<td>N</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>V234A</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>K256A</td>
<td>+</td>
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<td>NA</td>
<td>NA</td>
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<tr>
<td>K236E</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>4.0 (0.3)</td>
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<tr>
<td>L241A</td>
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<td>+</td>
<td>+</td>
<td>0.00 (0.00)</td>
<td>II</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>0.13 (0.19)</td>
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<td>+</td>
<td>0.21 (0.13)</td>
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<td>E246K</td>
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<td>+</td>
<td>+</td>
<td>26.3 (3.2)</td>
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<td>0.09 (0.01)</td>
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<td>NA</td>
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<td>−/+</td>
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<tr>
<td>R262A/K264A</td>
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<tr>
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*a As determined in Fig. 2, + replication peak detected up to 4 days delayed relative to the WT; +/− peak detected up to 12 days delayed relative to the WT concurrent with an 8- to 20-fold reduction in RT activity; −, replication not detected after 2 months of observation. Values represent results of duplicate infections.

*b Late reverse transcription products at 7 h postinfection (Fig. 3). +/+ >50% of WT; +, 20 to 50% of WT; +/−, 10 to 20% of WT; −, <10% of WT.

*c 2-LTR circles at 24 h postinfection (Fig. 3). +/+ >50% of WT; +, 20 to 50% of WT; +/−, 10 to 20% of WT; −, <10% of WT.

*d Average values for duplicate luciferase assays and a minimum of two independent infections, with standard deviations given in parentheses.

*e Based on results in Fig. 2 to 6. Whereas class I mutants are specifically blocked at integration, class II mutants display additional reverse transcription and/or release defects.

*f NA, not applicable; the virus was classified as WT.

*g Although the class II phenotype is clearly not a simple loss of one or more viral functions, here it indicates whether the primary replication block (Fig. 2) was at integration (class I) or also encompassed reverse transcription and virus release (class II).

and E). As predicted for the class I mutant phenotype, HIV-
11W235E.Luc(R−) yielded up to 12-fold more 2-LTR circles than the WT at 24 hpi (Fig. 3B, D, and F). In contrast, the class II control HIV-11V165A.Luc(R−) supported significantly less (approximately 10 to 20-fold) reverse transcription than the WT (Fig. 3A, C, and E), and as previously described (57), mutant 2-LTR circle levels mirrored these overall reductions in DNA synthesis (Fig. 3B, D, and F). In other words, the percentages of total viral cDNA converted to 2-LTR circles were similar for HIV-11V165A.Luc(R−) and HIV-11W235E.Luc(R−).

Replication-defective CTD mutants displayed a wide range of DNA synthesis profiles that varied from WT levels (for example, HIV-11R228A.Luc(R−) at 24 hpi [Fig. 3A]) to approximately 2% of WT (HIV-11R228A.Luc(R−) [Fig. 3A]). Mutants with >10-fold DNA synthesis defects at 7 hpi included HIV-11R228A.Luc(R−), HIV-11L241A.Luc(R−), HIV-11K258A.Luc(R−), and HIV-11V260E.Luc(R−) (Fig. 3C), and HIV-11K266A.Luc(R−) (Fig. 3E) (Table 2). Somewhat less severe 5- to 10-fold defects were detected for HIV-11R262A.R263A.Luc(R−), HIV-11R262A.K264A.Luc(R−), HIV-11K268E.Luc(R−) (Fig. 3C), and HIV-11R269A.Luc(R−) (Fig. 3E).

Mutant virus release from cells. In addition to reverse transcription, some class II mutants are defective for virus assembly and release (26). To assess release, levels of cell supernatant RT activity were quantified following transient transfection of CD4+ HeLa cells. The CD4-negative phenotype permitted comparison of replication-defective and replication-compotent strains. Previous analyses revealed that class
II mutant strains expressed HIV-1 proteins at WT levels (reference 57 and references therein).

The class I mutant control strain HIV-1 D64N/D116N was released from HeLa cells at the WT level (Fig. 5A). In contrast, the CTD deletion mutant HIV-1Δ1–212 was about fourfold defective for release (Fig. 5A). Most of the replication-defective CTD mutants either were released at the WT level or showed marginal (approximately 1.5- to 3-fold) release defects. Two notable exceptions were HIV-1RRK/DVE and HIV-1 E246K: whereas HIV-1 RRK/DVE behaved similarly to the HIV-1Δ1–212 control (approximate fourfold defect), HIV-1 E246K was released at only about 5% of the WT level (Fig. 5A). Because RT interacts with IN through the CTD (40, 87), CTD mutations could in theory alter RT packaging and/or virion-associated activity. To address this potential caveat, a subset of the mutant viruses was analyzed for p24 levels, and these values were compared to corresponding RT activities in HeLa cell supernatants. The results of this analysis revealed that p24 and RT levels closely mirrored each other in the majority of cases, although the RT activity of HIV-1RRK/DVE was approximately twofold lower than the corresponding p24 value (Fig. 5B). These results are in line with previous observations that mutations only minimally altered the p24-to-RT ratio of CTD mutant viruses (11).

Based on WT and mutant cDNA synthesis (Fig. 3 and 4) and virus release (Fig. 5) profiles, we concluded that the majority of the replication-defective CTD mutants were phenotypically class II (Table 2). Since HIV-1R269A was released at the WT level (Fig. 5), supported near-WT levels of DNA synthesis, and converted more of its cDNA to LTR circles than the WT (Fig. 3), it was typed as a class I mutant (Table 2).

Processing defect of HIV-1E246K. The release defect associated with IN deletion mutant viruses can be overcome by inhibiting the activity of the viral protease through mutation or treatment with antiviral compounds (7). To further probe the dramatic reduction in HIV-1E246K release, transfected HeLa cells metabolically labeled with [35S]Met and [35S]Cys were either left untreated or treated with the protease inhibitor saquinavir, and cell- and virion-associated HIV-1 proteins were visualized following SDS-PAGE and fluorography. To aid detection of cell-associated proteins, lysates were immunoprecipitated with AIDS patients’ sera prior to electrophoresis. In contrast, virion proteins were recovered following direct pelleting of cell supernatants through sucrose cushions.
Processed p24 and precursor Pr55\textsuperscript{gag} proteins were identified in WT HIV-1\textsubscript{NL4-3}-expressing cells (Fig. 6A, lane 3). Saquinavir effectively inhibited WT polyprotein processing: the level of cellular Pr55\textsuperscript{gag} increased at the expense of p24 production (Fig. 6A, lane 4), and Pr55\textsuperscript{gag} instead of p24 was released from cells (Fig. 6B, compare lane 4 to lane 3). As previously observed for IN deletion mutants (7, 58, 70), intracellular p41\textsuperscript{gag} and p25 processing intermediates were detected in HIV-1\textsubscript{1–212}-expressing cells (Fig. 6A, lane 5), and in agreement with Fig. 5A, extracellular HIV-1\textsubscript{1–212} p24 levels were reduced about fourfold from the WT level (Fig. 6B, compare lane 4 to lane 3). As previously observed for IN deletion mutants (7, 58, 70), intracellular p41\textsuperscript{gag} and p25 processing intermediates were detected in HIV-1\textsubscript{1–212}-expressing cells (Fig. 6A, lane 5), and in agreement with Fig. 5A, extracellular HIV-1\textsubscript{1–212} p24 levels were reduced about fourfold from the WT level (Fig. 6B, compare lane 4 to lane 3). Also as previously established (7), saquinavir effectively counteracted the HIV-1\textsubscript{1–212} release defect: substantial levels of pelletable Pr55\textsuperscript{gag} were recovered after drug treatment (Fig. 6B, lane 6). In contrast to both the WT and the HIV-1\textsubscript{1–212} deletion mutant, HIV-1\textsubscript{E246K} was poorly processed in cells; only a minimal level of p24 was detected (Fig. 6A, lane 7). Whereas saquinavir effectively blocked HIV-1\textsubscript{E246K} processing (Fig. 6A, lane 8), it did not enhance the release of polyprotein precursors (Fig. 6B, lanes 7 and 8). Based on this, we concluded that the E246K mutation impacted HIV-1 late events differently than previously characterized class II IN deletion mutations.

**Single-round infectivities of CTD mutant viruses.** The experiment for which results are shown in Fig. 2 measured infectivity as a function of virus spread. Since some CTD mutants were released from cells less efficiently than the WT (Fig. 5 and 6), it was of interest to also determine infectivity in the absence of virus spread. In other words, by quantifying luciferase activity in cell extracts following single-round infections, infectivity would be determined independently of potential viral late event (release/processing) defects. A subset of the cells that
were infected for RQ-PCR measurements was lysed at 48 hpi, and luciferase activity was normalized to total cell protein concentration.

Whereas the infectivity of the class I mutant control strain HIV-1W235E.Luc(R-) was 0.39% of the WT, the class II HIV-1V165A.Luc(R-) mutant supported 0.01% ± 0.02% of WT activity. Each replication-defective CTD mutant fell within this low to background range of luciferase activity with the exception of HIV-1E246K.Luc(R-), which displayed about 8% of the WT titer (Table 2). HIV-1K236A/E246A, HIV-1E246A, and HIV-1R269A, each of which supported low but reproducible levels of virus spread (Fig. 1), also supported luciferase activities that were significantly above background (Table 2).

Vpr-IN complementation. Results of in vitro complementation assays established that IN comprised three distinct functional domains, the NTD, CCD, and CTD. Whereas two proteins containing mutations in different domains could functionally complement each other, proteins with mutations in the same domain failed to restore IN function (28, 77). Complementation-dependent rescue of IN function can also occur during HIV-1 infection. The infectivity defect of IN mutant viruses can be rescued by trans-incorporating INWT as a Vpr-IN fusion protein during HIV-1 assembly (33, 84). In addition, Vpr-IN proteins derived from certain replication-defective IN mutant viruses restored infectivity to class I active-site CCD mutant viruses (5, 33, 57, 58, 67). Notably, since a subset of these (class II) IN mutations also resided in the CCD, complementation in vivo can extend beyond the traditional definition of IN domain boundaries (57, 58). Class II CCD mutant INs efficiently trans-complemented class I CCD mutant viruses because the class II mutant proteins retained catalytic activity (57).

![FIG. 5. Virus release from HeLa cells.](http://jvi.asm.org/article-pdf/79/17/10363/10363_1-2.pdf)

FIG. 5. Virus release from HeLa cells. (A) Results of duplicate RT assays following a minimum of three independent transfections, expressed as percentages of WT activity. (B) Results of duplicate RT and p24 assays following two independent transfections, expressed as percentages of WT values.


FIG. 6. Defective HIV-1E246K processing. (A) Cell-associated proteins. Cells were treated with saquinavir as indicated prior to radiolabeling, and cell lysates were immunoprecipitated prior to SDS-PAGE. (B) Virion proteins. Cell supernatants pelleted through sucrose were lysed prior to electrophoresis. Migration positions of molecular mass standards are indicated on the left, and Pr55Gag and p24 are indicated on the right. Similar results were observed in three independent experiments.
The results presented in Fig. 2 to 6 led to phenotypic classification of the majority of CTD mutant viruses as class II (Table 2). To investigate the catalytic potential of CTD mutant proteins as well as the interplay between the CCD and CTD during integration, a subset of mutations that addressed the multiple roles of the CTD, including R228A, L242A, E246K, K258A, V260E, K264E, and K266A (see Table 1), was introduced into a Vpr-IN expression vector. The following control changes were also studied: W235E (a CTD class I mutation), Q62K and D116A (CCD class I mutations [57]), and V165A (a CCD class II mutation [57]). Vpr-IN proteins were tested for their ability to trans-complement four different single-round mutant viruses: HIV-1 D64N/D116N.Luc(R-), HIV-1 V165A.Luc(R-), HIV-1 W235E.Luc(R-), and HIV-1 1–212.Luc(R-). Levels of Vpr-IN mutant complementation activity were quantified as percentages of Vpr-INWT activity (Table 3). We note that the baseline infectivity of 0.39% reported for HIV-1 W235E.Luc(R-) in the preceding section is greater than the Table 3 value of 0.02% due to different multiplicities of infections that were utilized in the different experiments.

As expected (5), Vpr-IN V165A efficiently complemented HIV-1 D64N/D116N.Luc(R-) under conditions where it failed to complement HIV-1 V165A.Luc(R-) (57) (Table 3). Conversely, neither Vpr-IN Q62K nor Vpr-IN D116A complemented HIV-1 D64N/D116N.Luc(R-) but each partially restored HIV-1 V165A.Luc(R-) infectivity (57) (Table 3). In contrast to the differential behavior of CCD class I versus class II Vpr-IN mutant proteins with class I and class II CCD mutant viruses, the CCD mutant proteins behaved similarly with class I (HIV-1 W235E.Luc(R-)) and class II (HIV-1 1–212.Luc(R-)) CTD mutant viruses. Whereas each functionally complemented HIV-1 W235E.Luc(R-), none functioned with HIV-1 1–212.Luc(R-) (Table 3). The inability to complement HIV-1 1–212.Luc(R-) was somewhat surprising, since each CCD mutant protein contained an intact CTD.

Vpr-IN W235E rescued the infectivity of each virus tested, including HIV-1 W235E.Luc(R-) (Table 3). Complementation of defective IN mutant virus by Vpr-mediated trans-incorporation of the same mutant protein has been observed previously (57, 67) and likely depends on the inherent activity of the IN mutant protein (INW235E displayed WT function in an in vitro integration assay [51]) and the incorporation of greater than normal levels of IN protein during virus assembly (57, 58, 67).

With the exception of Vpr-IN V260E, each novel CTD mutant fusion protein efficiently complemented HIV-1 D64N/D116N.Luc(R-) (~18% to 320% of Vpr-INWT activity [Table 3]). Because of this, we concluded that each of these IN enzymes could support substantial levels of integration during infection and, by extension, that each Vpr-IN protein was efficiently incorporated into particles during cotransfection. Since Vpr-IN R228A...

### Table 3: Complementation of IN mutant viruses with Vpr-IN

| IN mutant       | Without | After complementation with Vpr-IN  
<table>
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<tr>
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<tr>
<td></td>
<td></td>
<td>Activity (%)</td>
</tr>
<tr>
<td>HIV-1 W235E.Luc(R-)</td>
<td>0.02 (0.01)</td>
<td>27.2 (2.5)</td>
</tr>
<tr>
<td>HIV-1 V165A.Luc(R-)</td>
<td>0.00 (0.00)</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>HIV-1 D64N/D116N.Luc(R-)</td>
<td>0.00 (0.00)</td>
<td>0.8 (0.2)</td>
</tr>
<tr>
<td>HIV-1 1–212.Luc(R-)</td>
<td>0.00 (0.00)</td>
<td>0.2 (0.0)</td>
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Values represent an average of at least two infections.

Values expressed as a percentage (with the standard deviation in parentheses).
Vpr-IN_{242A} and Vpr-IN_{258A} failed to appreciably complement HIV-1/V165A.Luc(R-) and Vpr-IN_{235E}, unlike the WT, yet HIV-1 K264E, HIV-1 R262A/R263A, and HIV-1V234A replicated like the WT. Since Lys-234 is invariant among HIV-1 and related chimpanzee strains (47), it seemed that Glu-246 within the CTD can interact with the viral site during integration (31, 78, 82). A variety of residues, including Val-234, Glu-246, Lys-258, Arg-262, Lys-264, and Lys-273, were implicated in nonspecific DNA binding activity (20, 68, 69).

Recently, it has become clear that the IN CTD contains important nonspecific DNA binding determinants (3, 39, 72) and that Glu-246 within the CTD can interact with the viral site during integration (35). Whereas HIV-1K244A/E246A (80), HIV-1R262A/K264T (11), and HIV-1R262A/R263A/K264H (64) were noninfectious, the contribution of individual DNA binding residues to HIV-1 replication was unknown. Since HIV-1V234A (Fig. 2A) and HIV-1K273A (Fig. 2E) grew like WT HIV-1 NL4-3, our results failed to support an important role for either Val-234 or Lys-273 in HIV-1 replication. Although the majority of HIV-1/SIV gp120 strains harbor Ile, Ser and His are occasionally found at position 234 (47). In this light, it was not overly surprising that HIV-1V234A replicated like the WT. Since Lys-273 is invariant among HIV-1 and related chimpanzee strains (47), a potential role for this residue in HIV-1 replication could lie outside the realm of spreading infection in Jurkat T cells.

HIV-1R262A, HIV-1R263A, and HIV-1K264A grew similarly to the WT, yet HIV-1K244E/HIV-1R262A/R263A, and HIV-1R262A/K264A were replication defective (Fig. 2 and Table 2). This suggests that the HIV-1K244E replication defect was due primarily to the negative impact of the nonconservative Glu substitution rather than to a necessity for Lys at this position. Since HIV-1K244E/HIV-1R262A/R263A and HIV-1R262A/K264A were 5- to 10-fold defective for cDNA synthesis and 1.4- to 3-fold reduced for viral release, they were typed as class II IN mutant viruses (Fig. 3 and 5; Table 2).

We recently determined via Vpr-IN complementation assays that certain CCD class I and class II mutants functioned as separate complementation groups, which revealed that this type of class/III complementation can extend beyond the traditional boundaries of IN domain structure (57). Because the class II CCD mutant proteins retained catalytic function, we and others proposed that the mutations were likely to affect higher-order interactions between IN and other proteins specific to the infected cell such as RT and/or host cell factors (22, 57). By extending these analyses to include a variety of CTD mutant proteins and viruses, we determined additional examples of class I/II complementation as well as examples of traditional domain boundary complementation. Because Vpr-IN_{K264E} efficiently complemented HIV-1K273A.Luc(R-) and HIV-1W235E.Luc(R-) under conditions where HIV-1L242A.Luc(R-) and HIV-1W235E.Luc(R-) complementation was more marginal (Table 3), we conclude that the Lys-to-Glu change disrupted a CTD function that was readily complemented by CTD mutant viruses regardless of their class I or class II phenotype. Since IN_{K264E} was defective for DNA binding (69), we speculate that this CTD function can be provided in trans by different classes of CTD mutant viruses during HIV-1 infection. Because of this, we concluded that class I and class II need not always define separate complementation groups in vivo. On the other hand, Vpr-IN_{R262A} and Vpr-IN_{R263A} were replication defective (Fig. 2 and Table 3). Since Lys-258 was also implicated in DNA binding (20), our results suggest that the K258A mutation affected a function(s) that is similarly perturbed by other class II mutations and thus might affect protein-protein interactions (57) in addition to potential IN-DNA interactions.

**CTD multimerization and HIV-1 replication.** Previous in vitro work highlighted the importance of CTD-dependent multimerization in IN function (1, 43), and the virus-based results reported here support this contention. Viruses mutated at previously implicated multimerization determinants were either dead (HIV-1L242A, HIV-1L242A, HIV-1V260E, HIV-1K266A, HIV-1K266E) or severely impaired (HIV-1K266A) in their ability to replicate (Table 1 and Fig. 2). Since HIV-1K266A and HIV-1K266E were both replication defective, our results indicate that it is particularly important for HIV-1 to harbor a positively charged side chain at this position. Since Lys-266 is solvent inaccessible and diametrically opposed to the dimer interface in the crystallographic CTD-CTD contact mediated via Lys-266 (14) may be of importance during HIV-1 infection. HIV-1K266A and HIV-1K266E, as well as HIV-1L242A, HIV-1L242A, and HIV-1V260E, were typed as class II mutant viruses (Fig. 3 to 5; Table 2). Like Val-260, Leu-242 is for the most part buried within the core of the CTD structure (Fig. 1B). Because of this, it was not overly surprising that HIV-1L242A displayed pleiotropic replication defects.

Vpr-IN_{L242A} complemented class I mutant viral strains under conditions where class II mutant complementation was not observed (Table 3). Thus, Vpr-IN_{L242A} functioned as a class II mutant compared to a traditional CTD mutant under these assay conditions. The function of Vpr-IN_{K266A} was somewhat less clear, although due to the strikingly different results obtained with HIV-1K266E.Luc(R-) and HIV-1V165A.Luc(R-), it too seemed to demonstrate mainly class II behavior during...
complementation (Table 3). The finding that changes in putative multimerization determinants imparted class I/II behavior in Vpr-IN complementation assays is consistent with the model that higher-order interactions are likely to be impaired by this type of (class II) IN mutation (22, 57, 58).

**Other conserved CTD residues and HIV-1 replication.** Although not previously ascribed specific tasks in CTD function, Arg-228, Arg-231, Lys-236, Lys-244, and Arg-263 were targeted due to their relatively high degrees of sequence conservation among HIV-1 strains (Table 1). Since HIV-1R228A and HIV-1R231A grew similarly to the WT (Fig. 2A), our assays failed to define an essential role for either Arg-231 or Lys-236 ascribed IN deletion mutants. The first was an overall reduction in levels of Pr55<sup>pp65</sup> processing (Fig. 6A, lanes 3, 5, and 7). The second was the failure of the protease inhibitor saquinavir to rescue release from HIV-1<sub>E246K</sub>-expressing cells (Fig. 6B, lane 8). Despite the inability to support spread in infected Jurkat cell cultures (Fig. 2B), HIV-1<sub>E246K Luc(R-)</sub> supported approximately 8% of the level of WT HIV-1<sub>NLX Luc(R-)</sub> transduction activity (Table 2). HIV-1<sub>K236A,E246A Luc(R-)</sub> (Fig. 2D) and HIV-1<sub>R269A</sub> (Fig. 2E) supported weak but detectable levels of virus spread, and HIV-1<sub>K236A,E246A Luc(R-)</sub> and HIV-1<sub>R269A Luc(R-)</sub> transduced cells at approximately 4.0% and 11.5% of the WT level, respectively (Table 2). Based on this, we concluded that E246K was the first example of an IN mutation where a late-stage processing defect contributed significantly to the inability of the mutant virus to support multiple rounds of replication. This observation is in line with the hypothesis that antiviral drugs targeted against IN might inhibit HIV-1 replication without necessarily inhibiting the catalytic function of the DNA recombinase (57, 85).

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