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Genetic Analysis of Human Immunodeficiency Virus Type 1 Integrase and the U3 *att* Site: Unusual Phenotype of Mutants in the Zinc Finger-Like Domain

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Retroviral integration is the step which leads to establishment of the provirus. *cis*- and *trans*-acting regions of the human immunodeficiency type 1 (HIV-1) retrovirus genome, including the attachment site (*att*) at the ends of the unintegrated viral DNA and the conserved domains within the integrase (IN) protein, have been identified as being important for integration. We investigated the role of each of these regions in the context of an infectious HIV-1 molecular clone through point mutagenesis of the *att* site and the zinc finger-like and catalytic domains of IN. The effect of each mutation on integration activity was examined by using a single-step infection system with envelope-pseudotype virus. The relative integration efficiency was estimated by monitoring the levels of viral DNA over time in the infected cells. The integration activities of catalytic domain point mutants and *att* site deletion mutants were estimated to be 0.5 and 5% of wild-type activity, respectively. However, in contrast with previous in vitro cell-free integration studies, alteration of the highly conserved CA dinucleotide resulted in a mutant which still retained 40% of wild-type integration activity. The relative levels of expression of each mutant, as measured by a luciferase reporter gene, correlated with levels of integration. This observation is consistent with those of previous studies indicating that integration is an obligatory step for retroviral gene expression. Interestingly, we found that three different HIV-1 constructs bearing point mutations in the zinc finger-like domain synthesized much lower levels of viral DNA after infection, suggesting impairment of these mutants before or at the initiation of reverse transcription. Western blot (immunoblot) analysis demonstrated wild-type levels of reverse transcriptase within the mutant virions. In vitro endogenous reverse transcription assays indicated that all three mutants in the zinc finger-like domain had wild-type levels of reverse transcriptase activity. These data indicate that in addition to integration, IN may have an effect on the proper course of events in the viral life cycle that precede integration.

The retroviral provirus is the species of viral DNA that is stably integrated into host chromosomal DNA. Establishment of the provirus is a critical step for productive and/or latent infection of retroviruses, including human immunodeficiency virus type 1 (HIV-1). The provirus is established through several events of the retrovirus life cycle, including binding of the virus particle to the host cell surface receptor, entry, uncoating, reverse transcription, and integration. Reverse transcription and integration are mediated by the viral gene products reverse transcriptase (RT) and integrase (IN), respectively (61). Both RT and IN are packaged into the viral particle, initially in a *gag/pol* precursor form, and processed to a mature form by viral protease during or after budding from infected cells. Following virion binding and entry of the virus core particle into the host cell, using two copies of the single-stranded viral genomic RNA as the template, RT synthesizes the double-stranded DNA. The newly synthesized proviral DNA intermediate is inserted into the host chromosomal DNA. This last step in establishing the provirus is mediated by IN.

Analysis of integration in vitro and in vivo demonstrates that the reaction proceeds in the following three steps (3, 26, 37, 64): (i) 3′ end processing, i.e., recognition of terminal sequences containing an inverted repeat region at both the 3′ and 5′ viral DNA ends, termed the attachment site (*att*), and removal of two nucleotides from the 3′ terminus of both DNA strands to expose the invariant dinucleotide, CA; (ii) strand transfer reaction, whereby the 3′-OH recessed ends of viral DNA are joined to the protruding 5′-PO_4 termini of the host DNA, which is nicked in staggered fashion by nucleophilic attack; and (iii) gap repair, i.e., removal of the two unpaired dinucleotides at the 5′ end (two bases) and filling in of the gaps between the viral and host DNA, thereby creating a direct repeat sequence that flanks both ends of the proviral DNA at the site of integration.

The steps of the integration process, including 3′ processing and the strand transfer reaction, have been studied through the development of in vitro cell-free assay systems in which bacterially expressed and purified IN and synthetic oligonucleotides mimicking the viral *att* sites are used (5, 23, 31, 56, 63). The process of disintegration (12), or a reversal of the strand transfer reaction, has also been described. Thus, this disintegration activity has been used as an additional index of IN catalytic activity (11). Mutational analyses using this in vitro assay have defined the functional domain of IN, particularly the highly conserved D,D35E motif located in the central region of IN, as the catalytic activity site (6, 19, 21, 38, 40–42, 59).

In the amino-terminal region, IN has a highly conserved HHCC motif that is similar to the zinc finger motif commonly found in the DNA-binding regions of some transcription factors (4, 6, 29, 44). Mutation in this HHCC motif impaired 3′ processing and strand transfer but not the disintegration activity of

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HIV-1 (21, 43, 54, 62), Rous sarcoma virus (7), or Moloney murine leukemia virus (MuLV) (30) IN. These data suggest that the HHCC motif is not directly involved in the catalytic activity of IN but might be important for specific recognition of the *att* site by possibly stabilizing multimerization of IN molecules (21, 30, 62). In addition, no direct evidence of this binding function of the HHCC motif has been demonstrated (7, 33, 54). Thus, the true function of the HHCC motif in the integration process is still unclear, and it is possible that the HHCC motif has another function that cannot be detected by the in vitro assays.

Mutational analyses of the *att* site by using in vivo (13, 14, 45–47, 51) and in vitro (11, 12, 40, 42, 63) assays defined the region of the long terminal repeat *att* sequence required for specific interaction with IN. In the case of HIV-1, at least seven and as many as 13 bp adjacent to the highly conserved CA dinucleotide were shown to be required for efficient interaction of the long terminal repeat with IN (42). Moreover, alteration of the CA dinucleotide significantly impaired IN-mediated 3′ processing and the strand transfer and disintegration reactions (11, 12, 40, 42, 63).

In contrast to these in vitro studies, most of the in vivo studies utilizing infectious retroviruses have addressed IN function(s) by using deletion or frameshift mutants of IN (16, 17, 27, 52, 53, 55, 58). From these studies, it was shown that integration is required for viral gene expression in almost all retroviruses, including MuLV (16, 17, 52, 55) and avian sarcoma virus (27), but may not be required for some retroviruses (25, 47). In the case of HIV-1, both conclusions have been reported (53, 58). These conflicting results might be explained by differences in the assay systems and/or differences in the types of mutations introduced in IN. More recently, the effects of single or double amino acid substitution in the highly conserved regions of HIV-1 IN have been examined in an in vivo infection system (9, 22, 24, 41, 57, 65). These studies showed that some of the highly conserved domains in IN are important for productive infection.

Therefore, although there have been numerous studies investigating biochemical and genetic properties of HIV-1 IN and *att* site requirements, less is known regarding the role of each of these regions in the context of viral replication. We have mutated the known functional regions and assessed the effects of these mutations on viral DNA formation, proviral formation, and viral gene expression. We made several different IN mutants of HIV-1, each with a single or double amino acid substitution in the catalytic D,D35E region or the HHCC zinc finger-like motif, as well as two different *att* site mutants designed to abolish the interaction of IN with viral DNA without mutating IN. Each IN or *att* site mutant virus was tested in a single-step infection system (8, 49) that allowed us to examine the events during a single round of replication of the virus. Our results indicate that IN catalytic site mutants have the expected phenotypes of inability to establish proviruses. However, mutants in the zinc finger-like domain showed greatly decreased levels of viral DNA synthesis, suggesting perturbation of earlier events in the viral life cycle. The *att* site mutants were also decreased in the ability to form stable provirus.

MATERIALS AND METHODS

Construction of mutants. DNA fragments for the mutagenesis of the HIV-1 IN and *att* sites were derived from the infectious clone pNL4-3 (1). For mutagenesis of HIV-1 IN, a 4.3-kb fragment of pNL4-3 spanning the *Spe*I and *Sal*I sites (nucleotides [nt] 1507 to 5785) was subcloned into pBluescript SK− (Stratagene). Following preparation of the single-stranded DNA by using the M13 K07 phage, site-directed mutagenesis was performed according to the protocol for the T7-GEN in vitro mutagenesis kit (United States Biochemical). Mutagenic oligonucleotides introducing single or double amino acid substitutions at each highly conserved amino acid in IN were as follows (altered nucleotides underlined): D64E, 5′-GAATATGGCAGCTCGAGTGATCACATTTAG-3′, corresponding to nt 4405 to 4434; D116G, 5′-ACAGTACATACCGGTAATGGC-3′, corresponding to nt 4563 to 4583; H12L, 5′-GCCCAAGAAGAACTCGAGAAA-3′, corresponding to nt 4251 to 4270; H16Y, 5′-GAGAAATATTACAGTAATTGG-3′, corresponding to nt 4269 to 4286; and C43L, 5′-CAGTGTGATAAGCTTCAGCTA-3′, corresponding to nt 4343 to 4364.

The presence of desired mutations was confirmed by DNA sequencing. We found that the H16Y mutant fragment contained the additional mutation of T to G at nt 4268, resulting in an amino acid change from H to Q at amino acid position 12; therefore, we term this mutant H12Q+H16Y. To exclude the possibility of unwanted mutations in the three zinc finger-like domain (H12L, H12Q+H16Y, and C43L) that may have been inadvertently introduced during the site-directed mutagenesis protocol, the *Asp* 718-*Nsi*I fragment (nt 4154 to 4387) containing the zinc finger-like region was recloned following site-directed mutagenesis into a nonmutagenized *Spe*I-*Sal*I fragment. DNA sequence analysis of the entire *Asp* 718-*Nsi*I region confirmed that each of the three mutants of the zinc finger-like domain had only the expected mutations. The same subcloning procedure was also performed with the *Asp* 718-*Nsi*I fragment of wild-type (WT) IN. Each mutant or WT *Spe*I-*Sal*I fragment was then cloned into the $HIV_{NLluc\text{-}env}$ vector (49), generating mutant or WT proviruses. $HIV_{NLluc\text{-}env}$ is a genetically modified HIV-1 clone that carries a deletion in envelope (nt 7047 to 7609) and in which nt 8786 to 9004, corresponding to the *nef* gene, were substituted by the firefly luciferase gene (15). For the *att* site mutants, the *Xho*I-*Xba*I fragment of $HIV_{NLluc\text{-}env}$ containing the entire 3′ long terminal repeat region was subcloned into the pGEM7Zf(+) vector (Promega). To alter the highly conserved CA dinucleotide sequence to TG (CA→TG) at the U3 *att* site, site-directed mutagenesis was performed with the mutagenic oligonucleotide 5′-AGTGAATTAGCCCTTCTGGTCCCCCCTTTT-3′ (nt 9064 to 9093). To introduce a 10-bp deletion (nt 9076 to 9085) in the U3 *att* site (DEL10), the mutagenic oligonucleotide 5′-GGGGAGTGAATTAGCCCCCCTTTTC-3′ (nt 9063 to 9075 and 9086 to 9096) was used. After the mutations were confirmed by DNA sequencing, the *Mlu*I-*Xba*I fragment containing each U3 *att* site mutation was inserted back into the $HIV_{NLluc\text{-}env}$ vector, replacing the corresponding *Mlu*I-*Xba*I region of the vector.

Cells and viruses. COS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Human rhabdomyosarcoma (RD) cells (ATCC CCL136) and MT-2 cells, a human T-cell line transformed by human T-cell leukemia virus type 1, were cultured in Dulbecco's modified Eagle's medium and RPMI 1640 medium supplemented with 10% fetal calf serum, respectively. Pseudotype viruses were generated by cotransfection of COS cells with the $HIV_{NLluc\text{-}env}$ vector containing either of the mutations in IN or the U3 *att* site and an amphotropic Moloney MuLV envelope ($MuLV_{env}$) expression vector (pJD-1) (18). Pseudotype virus obtained from the $HIV_{NLluc\text{-}env}$ vector was used as a WT control. At 48 h posttransfection, the culture supernatant of the transfected COS cells was harvested and filtered through a 0.45-μm-pore-size Millipore filter. To remove DNA contamination, these virus-containing supernatants were treated with DNase (4 μg/ml; Worthington) in the presence of 10 mM $MgCl_2$ at 37°C for 30 min.

An aliquot of each virus preparation was incubated at 65°C for 30 min and used as a heat-inactivated control. To monitor the amount of virus in each virus preparation, levels of HIV-1 p24 antigen were determined by enzyme-linked immunosorbent assay (ELISA; Coulter Immunology). To monitor viral gene expression from each plasmid vector, cell-associated p24 levels and luciferase activity in transfected COS cells were also determined. At 48 h posttransfection, COS cells were lysed with 2 ml of 1× luciferase lysis buffer (Promega). Aliquots of each cell lysate were subjected to p24 ELISA or the luciferase assay. The levels of p24 and luciferase activity were normalized to total protein, as determined by Bio-Rad protein assay reagents.

Virus infection. RD cells (5×10^4) were incubated in an aliquot of each virus preparation containing 100 ng of p24. For kinetic analyses, multiple infections of each virus were performed in parallel in separate tissue culture wells. Infection was carried out in the presence of Polybrene (10 μg/ml) at 37°C for 6 h. After infection, cells were washed three times with phosphate-buffered saline (PBS) and resuspended with fresh medium to a concentration of 5×10^4 cells per ml. Infected cells were passaged by threefold dilution at 5-day intervals.

Luciferase assay. For luciferase analysis, infected cells were harvested at 6 h and at 1, 2, 3, 5, 8, and 15 days postinfection. The total cell pellet from each well was washed three times with PBS and lysed with 200 μl of 1× luciferase lysis buffer (Promega Corp.). Ten microliters of each lysate was subjected to the luciferase assay (Promega Corp.) with a luminometer Monolight 2010 (Analytical Luminescence Laboratory, San Diego, Calif.). Luciferase activity was normalized to protein content of each lysate. For analyses of viral DNA, total cells were harvested from each well at 6 h and at 1, 2, 5, and 15 days postinfection. After being washed with PBS, nucleic acids were extracted as described elsewhere (66). Briefly, cells were disrupted in urea lysis buffer (4.7 M urea, 1.3% [wt/vol] sodium dodecyl sulfate [SDS], 0.23 M NaCl, 0.67 mM EDTA [pH 8.0], 6.7 mM Tris-HCl [pH 8.0]), and subjected to phenol-chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in 50 μl of water, and a 5-μl aliquot was subjected to quantitative PCR.

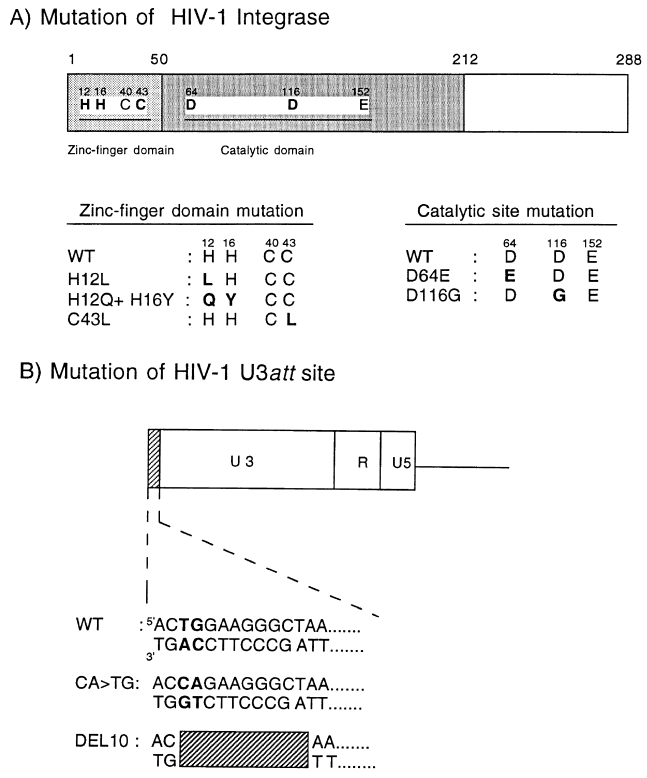

FIG. 1. Mutations of HIV-1 IN and the U3 *att* site. (A) Locations of the highly conserved HHCC (zinc finger-like domain) and D,D35E (catalytic domain) motifs in HIV-1 IN are shown with position numbers in the amino acid sequence. Single or double amino acid substitutions introduced at highly conserved residues in each motif are shown in boldface. WT amino acid sequences are shown for sequence comparison. (B) The boundary of the U3 *att* site at the 5′ terminus of the HIV-1 long terminal repeat/U3 region is indicated with a stippled box. WT DNA sequences of the region are shown, with the highly conserved CA dinucleotides in boldface. The CA→TG mutant has nucleotide substitutions of the CA dinucleotides with TG. DEL10 has a 10-bp deletion in the U3 *att* site. The deleted region is shown by the stippled box in the DEL10 sequences.

Endogenous reverse transcription reaction. The procedure has been described elsewhere (32, 36). DNase-treated virus stocks were prepared as described above, overlaid onto 1-ml cushions containing 20% diatrizoate–80% TEN buffer (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]), and centrifuged in an SW28 rotor at 200,000 × *g* for 30 min. Pelleted virus was resuspended in ice-cold TEN buffer. The amount of pelleted virus was determined by p24 ELISA. Aliquots of virus suspensions containing 60 pg of p24 were incubated in 30 μl of endogenous reverse transcription reaction mixture (0.01% Triton X-100, 50 mM NaCl, 50 mM Tris-HCl [pH 8.0], 10 mM dithiothreitol, 5 mM $MgCl_2$, 100 μM each dATP, dCTP, dGTP, and dTTP). As a negative control, reactions without dTTP were performed in parallel. After 2 h of incubation at 37°C, the reaction was terminated by addition of 270 μl of stop mix (50 μg of proteinase K per ml, 20 μg of tRNA per ml, 1.5 mM EDTA [pH 8.0]). After incubation at 60°C for 1 h, the proteinase K was heat inactivated by boiling for 10 min. Five-microliter aliquots of the reaction mixtures were subjected to PCR analysis.

PCR amplification and quantitation. An aliquot (5 μl) of each sample was subjected to PCR with a ^{32}P-end-labeled primer pair specific for the R/U5 region of HIV-1 (M667/AA55) or the R/*gag* region (M667/M661) as described elsewhere (66). To detect intermediates of the endogenous reverse transcription products, other primer pairs, including M667/BB301, which amplifies the R and primer binding sites (R/PBS), and LA45/LA64, which amplifies the *tat* and *rev* coding region (*tat*/*rev*), were also used. Detection of HIV-1 DNA sequences by each primer pair was performed by 25 cycles of amplification, each consisting of a denaturating step at 94°C for 1 min followed by an annealing-extension step at 65°C for 2 min. For HIV-1 DNA standards, 10 to 25,000 copies of linearized HIV-1_{JR-CSF} DNA (35) were amplified in parallel. Amplified products were resolved on a 6% polyacrylamide gel and visualized by autoradiography. Quantitative analysis of the amplified products was performed with a radioanalytic imager (Ambis, San Diego, Calif.). To normalize the amount of cellular DNA in the samples, a primer pair complementary to the first exon of the human β-globin gene (39, 66) was used. For detection of human β-globin DNA, 20 cycles of amplification were performed under the same conditions as those for HIV-1 DNA amplification. A standard curve for human β-globin DNA was obtained by amplification of known amounts of cellular DNA from RD or MT-2 cells in parallel.

Western blot (immunoblot) analysis. Viruses were concentrated from the DNase-treated virus stock described above for the endogenous reverse transcription assay. Viral proteins containing approximately 10 ng of p24 were subjected to SDS–12.5% polyacrylamide gel electrophoresis (PAGE). Following blotting of proteins to a nitrocellulose membrane (Hybond-ECL; Amersham), the membrane was first incubated with either a human monoclonal antibody to HIV-1 p24 or a mouse monoclonal antibody to HIV-1 RT (NIAID AIDS Research and Reference Reagent Program, ERC Bioservices Corporation) and then incubated with horseradish peroxidase-conjugated sheep anti-human immunoglobulin or anti-mouse immunoglobulin, respectively. HIV-1 Gag-Pol proteins were visualized by the enhanced chemiluminescence detection system (Amersham).

RESULTS

Site-directed mutagenesis of HIV-1_{NL4-3} IN and the *att* site. We generated mutations within the catalytic domain, zinc finger-like domain, and *att* site through site-directed mutagenesis of subgenomic fragments (Fig. 1), followed by reconstruction of the mutations into a derivative of the infectious HIV-1_{NL4-3} clone (1). To allow efficient monitoring of HIV-1 expression, we used an HIV-1_{NL4-3} clone that we modified by replacement of the *nef* gene with the firefly luciferase gene (8, 10, 48, 49). This virus was also defective for envelope (48, 49), and therefore infectious virus was recovered by cotransfection with an expression construct encoding murine amphotropic envelope to allow formation of HIV-1 (ampho) pseudotypes. These pseudotypes were previously shown to be effective in infecting a variety of different human cell types and expression of HIV-1 protein, but no further rounds of HIV-1 replication occur as a result of the *env*-defective nature of these vectors (8, 48, 49). Mutations involved amino acid residues previously shown to be important for HIV-1 integration functions in vitro. The mutations analyzed are summarized in Table 1. All of the mutants were shown to have comparable levels of p24 and luciferase activity within cell lysates of transfected COS cells and comparable levels of p24 in culture supernatants harvested from

TABLE 1. Gene expression of each mutant proviral DNA after transfection of COS cells[a]

Virus	Site of mutation	Cell lysate[b]		p24[c] in culture supernatant (ng/ml)
		Luc activity (10^6 U/μg)	p24 (ng/μg)	
WT	None	4.2	12.1	101.2
CA→TG	U3 *att*	8.1	27.2	113.3
DEL10	U3 *att*	7.3	10.0	76.5
D64E	IN catalytic site	3.7	9.3	95.3
D116G	IN catalytic site	3.8	17.7	143.3
H12L	IN zinc finger	3.8	18.7	67.7
H12Q+H16Y	IN zinc finger	2.7	23.3	74.1
C43L	IN zinc finger	2.1	17.5	77.8

[a] This experiment was repeated three times; results of a representative experiment are shown.

[b] Luciferase (Luc) activity and p24 levels in cotransfected COS cells, with each viral DNA and pJD-1 (amphotropic envelope), measured at 2 days posttransfection. COS cells (10^7) were transfected and harvested 2 days posttransfection by being washed twice with PBS and resuspended with 5 ml of cell lysis buffer (Promega Corp.). A 1-μl aliquot was subjected to luciferase assay or p24 ELISA. Luciferase activity was determined after background levels were subtracted in mock-transfected cells and normalized to cellular protein amounts. The background level in 1 μl of mock-transfected COS extract was less than 200 U. Levels of p24 are normalized to 1 μg of cellular protein.

[c] Measured in each culture supernatant harvested at 2 days posttransfection.

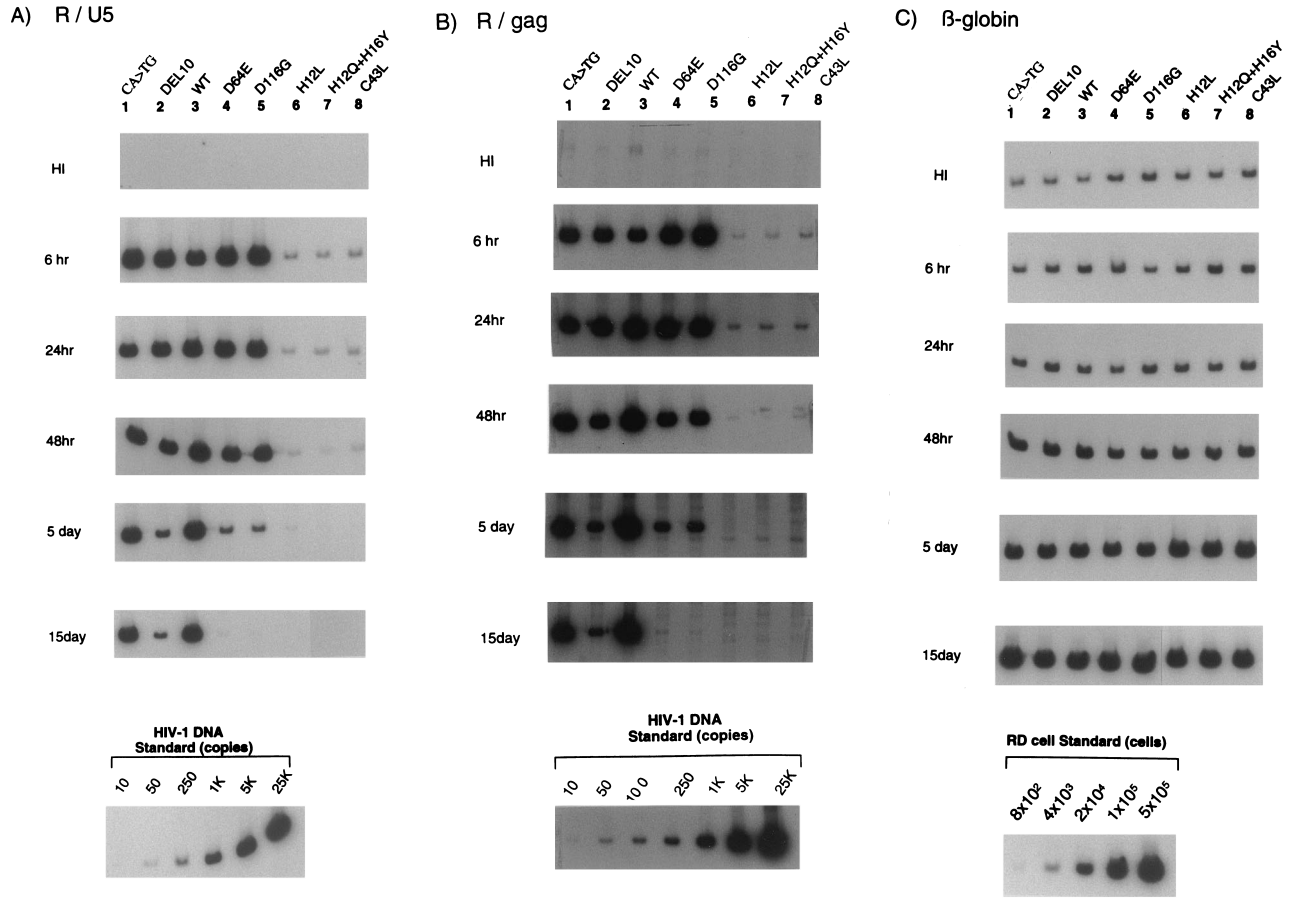

FIG. 2. Quantitative analysis of de novo-synthesized viral DNA after infection. For each virus, the same number of RD cells (5×10^4) were infected at each time point by inoculating an aliquot (1 ml) of DNase-treated COS cell virus-containing supernatant. The amount of p24 in each 1-ml aliquot was 67 to 143.3 ng (see Table 1). At each time point (6 h, 24 h, 48 h, 5 days, or 15 days), as indicated at the left, the entire culture was harvested. Total DNA isolated from each culture was resuspended with 50 μl of H_2O. For each PCR reaction, a 5-μl aliquot of the DNA sample was used. Quantitative PCR amplification with ^{32}P-end-labeled primers was performed as previously described (66). Fragments amplified with either M667/AA55 (R/U5) (A) or M667/M661 (R/*gag*) (B) were resolved on a 6% polyacrylamide gel and visualized by autoradiography. HI indicates DNA from cells infected with heat-inactivated virus and extracted at 6 h postinfection. Cloned HIV-1 DNA standards (linearized HIV-$1_{JR\text{-}CSF}$ [35]) from 10 to 25,000 copies containing tRNA (20 μg/ml) as the carrier were amplified in parallel. (C) As an internal control for cellular DNA, PCR with human β-globin primers (39, 66) was also performed. Total DNA from RD cells corresponding to 8×10^2 to 5×10^5 cells was amplified in parallel as human DNA standards. Note the increasing β-globin signal with time as a result of cell growth. This experiment was repeated three times with independent virus preparations. A representative experiment using virus preparations shown in Table 1 is presented.

the transfected cells. As expected, the mutations had no effect on transfected proviral gene expression, for which retroviral integration is not required. Other experiments described below, using Western blot analysis of viral proteins and endogenous reverse transcription reactions (see Fig. 5), were also consistent with this conclusion.

Analysis of HIV-1 DNA synthesis and proviral formation. We assessed the ability of each of the mutants to form viral DNA following infection of susceptible RD cells. PCR was used to monitor the formation of various species of the viral DNA, with primers M667/AA55 for the early species of viral DNA formed (R/U5) and M667/M661 for formation of complete or nearly complete viral DNA (R/*gag*) (66). Since integration results in the stable maintenance of the provirus in a population of cells, we used the persistence of viral DNA in infected cells relative to the WT as an estimate of the extent of integration. We assayed by PCR the amount of the viral DNA initially synthesized, representing unintegrated viral DNA, and that which was subsequently maintained, representing integrated viral DNA. We recently demonstrated by gel fractionation that the stably maintained viral DNA is the form of viral DNA associated with high-molecular-weight cellular DNA (49), substantiating this assay as a measure of integration. It is important to note that the HIV-1 *vpr* gene product impairs growth of infected cells, leading to a decreasing proportion of cells in the culture harboring HIV-1 over time (28, 49, 50). Our recently published studies also confirm that catalytic domain mutants synthesize DNA, but the DNA does not become stably associated with high-molecular-weight cellular DNA. Therefore, quantitative assessment of HIV-1 DNA levels relative to cellular β-globin levels shows decreases over time. The levels of viral DNA shown in Fig. 2 represent equivalent fractions of all cultures rather than equal amounts of cellular DNA (note the increasing β-globin signal over time in Fig. 2).

Consistent with previous studies of HIV-1 DNA formation (34, 67), the earliest species of WT viral DNA were detected within 6 h of infection and persisted throughout the 15-day observation period (Fig. 2A and B, lanes 3). In contrast to WT infection, infection with catalytic domain mutants D64E and D116G resulted in equal levels of viral DNA synthesized initially, but the amount of viral DNA detected decreased over time. Since these infections represent single-cycle infections

without reinfection, the failure of viral DNA to persist is a result of loss or dilution of unintegrated viral DNA from the culture. These results with the catalytic domain mutants are consistent with those for similar mutations reported previously (9, 41).

Three mutations in the zinc finger-like domain were examined: H12L, H12Q+H16Y, and C43L. Infection with these mutants showed an unexpected phenotype. Initially, very low levels of viral DNA were synthesized, less than 4% of those for the WT construct or catalytic domain mutants, as evidenced by PCR detection of early reverse transcription steps with M667/AA55 (Fig. 2A, lanes 6 to 8). We examined the mutants in the zinc finger-like domain further by analysis of the extent of reverse transcription. PCR primers designed to detect complete or nearly completely synthesized viral DNA showed a pattern similar to that of the primers that detect early reverse transcription products, indicating that once initiated, completion of reverse transcription appeared to be normal. This decreased level of viral DNA did not appear to be due to less virus in the inoculum, since these mutants produced similar levels of luciferase expression and p24 in the lysates and supernatant of transfected cells (Table 1) and virions had comparable levels of virion RT and Gag proteins (see Fig. 5 and 6). We considered the possibility that the site-directed mutagenesis protocol had inadvertently introduced mutations elsewhere, which could account for the unexpected phenotype. Therefore, as described in Materials and Methods, a restriction fragment including each mutation and the WT as a control was isolated and confirmed for genotype by complete nucleotide sequence analysis. The respective proviruses were then reconstructed by restriction endonuclease digestion and ligation steps only without further site-directed or PCR intervening steps. Therefore, the mutants in the zinc finger-like domain block the HIV-1 life cycle at a step prior to or at initiation of reverse transcription.

The low level of viral DNA that was synthesized also failed to persist over time, indicating that the mutants in the zinc finger-like domain were also impaired in integration; however, the low levels of viral DNA did not allow a quantitative assessment of the extent of integration (Fig. 2). Thus, mutants in the zinc finger-like domain appear to have a dual phenotype: (i) failure to efficiently synthesize viral DNA and (ii) failure of the DNA that was synthesized to stably integrate.

We also tested the *att* site mutants, the CA→TG point mutation, and the DEL10 deletion within the U3 *att* site. As with the WT construct and catalytic domain mutants, comparable levels of viral DNA were formed efficiently by 6 h postinfection. Over time, approximately 95% of the viral DNA synthesized by the deletion mutant of the U3 *att* site, DEL10, disappeared (Fig. 2A and B, lanes 2); 5% of the viral DNA appeared to be stable. Thus, the DEL10 *att* site mutant was diminished in its ability to integrate stably but was not as severely affected as the D64E and D116G catalytic site mutants. The U3 *att* site CA→TG mutant was even less affected in its ability to stably integrate. This mutant maintained 40% of WT levels of integration (Fig. 2A and B, lanes 1). Therefore, it is noteworthy that the *att* site mutants affected integration less severely than might have been expected from the various in vitro cell-free integration reactions, in which *att* site mutants showed considerably less integration (11, 12, 40, 42, 63).

Expression of HIV-1 integration mutants following infection. We constructed the mutations within an HIV-1 provirus bearing the luciferase gene marker in place of the *nef* gene to allow monitoring of viral expression following infection. We showed, as described above (Table 1), that the mutants have comparable levels of luciferase activity following transfection of COS cells. We also found that the mutants, with the exception of the mutants in the zinc finger-like domain, yielded comparable levels of viral DNA initially following infection of cells (Fig. 2). Thus, we can directly compare luciferase activity following infection relative to viral DNA levels. Infection with the WT construct yielded 2.3×10^5 U of luciferase activity per μg of cellular protein 3 to 5 days following infection. Although comparable amounts of DNA were synthesized at early time points by the catalytic site mutants (Fig. 2), considerably less (<1%) luciferase activity was detected (Fig. 3). This result indicates that unintegrated viral DNA of the mutants is not expressed at detectable levels and that expression requires integration. Each of the three mutants in the zinc finger-like domain had undetectable levels of luciferase activity, consistent with the low levels of viral DNA synthesized and even lower levels maintained.

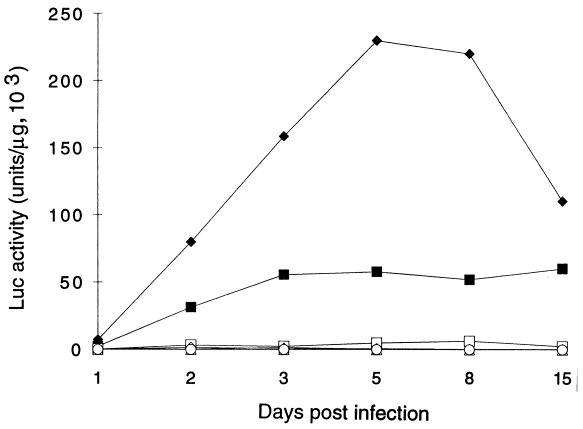

FIG. 3. Kinetics of virus gene expression after infection. Infection of RD cells with virus was performed as described for Fig. 2. At each time point, the entire culture was harvested and washed twice with PBS. The cell pellet was resuspended with 200 μl of cell lysate buffer (Promega Corp.). Ten microliters of each cell lysate was subjected to luciferase assay as described in Materials and Methods. Luciferase (Luc) activity was determined after subtraction of background obtained from each heat-inactivated control infection and normalization to 1 μg of cellular protein. Background levels ranged from 100 to 200 U/10 μl of cell lysates. This experiment was repeated three times, and a representative experiment using the same HIV-1 infection as that in Fig. 2 is shown. Symbols: ■, CA→TG; □, DEL10; ◆, WT; ◇, D64E; ▲, D116G; △, H12L; ●, H12Q+H16Y; ○, C43L.

The U3 *att* site mutants had higher levels of luciferase activity, in accordance with the relatively higher levels of viral DNA persisting over time (Fig. 2). The CA→TG mutant showed levels of luciferase activity ranging from 20 to 50% of WT levels, consistent with the levels of viral DNA persisting (Fig. 2). The same phenotype for each virus was obtained when an HIV-1 envelope pseudotype virus and MT-2 cells were used (data not shown). Since in all cases the relative levels of expression correlated with the relative abilities to integrate but did not correlate with the relative levels of DNA synthesized initially, we conclude that stable maintenance of proviral DNA, presumably through integration, is essential for HIV-1 gene expression.

It has previously been reported that in cell-free assays, complementation between the zinc finger-like and catalytic domain mutants can be observed (20, 60). Since retroviruses package many IN molecules per virion, coexpression of different IN mutants within a cell would lead to the production of virus particles containing phenotypic mixtures of IN proteins. We used this approach to test complementation in vivo, allowing copackaging of zinc finger-like and catalytic domain mu-

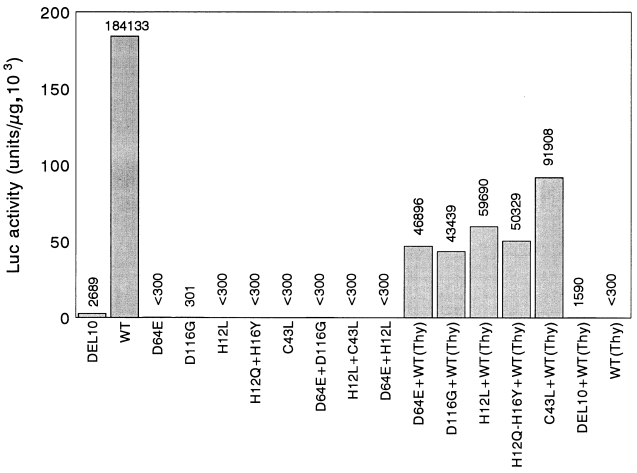

FIG. 4. Coexpression and copackaging of mutant IN. Pseudotype viruses were obtained by cotransfection of COS cells with 20 μg of pJD-1 ($MuLV_{env}$) and 20 μg of IN WT or mutant $HIV_{NLluc\text{-}env}$ vector. In rescue experiments, pseudotype viruses were prepared by cotransfection with the pJD-1 vector and 10-μg pairs of each of two different mutant $HIV_{NLluc\text{-}env}$ vectors that are indicated below the columns. For pairs of mutant and WT, $HIV_{NLthy\text{-}env}$ vector [WT(Thy)], which contains the WT IN and which replaces the mouse *thy-1.2* gene for the *luc* gene, was used. Infection was performed as described for Fig. 2. A 1-ml aliquot of each virus containing 100 ng of p24 was inoculated into 5×10^4 RD cells. Three days postinfection, the entire culture was harvested and subjected to luciferase assay as described for Fig. 3. Luciferase (Luc) activity was determined after subtraction of background level and normalization to 1 μg of cellular protein. This experiment was performed three times with independently prepared virus. A representative experiment is shown.

tants via cotransfection. We observed that luciferase activity following infection of rescued virus was not greater than that of the individual parental viruses, indicating that complementation did not occur under these experimental conditions (Fig. 4). To demonstrate that in fact copackaging of different IN proteins did occur under these experimental conditions, we cotransfected the mutants with a WT provirus that did not bear the luciferase gene (carrying an irrelevant marker, Thy-1) (48). Luciferase production was observed after infection, showing that the WT IN protein could apparently be packaged with the mutant genome. The *att* site mutant, DEL10, could not be complemented by the WT protein, as expected for a *cis*-acting mutation. This latter result also demonstrated that the higher levels of luciferase activity seen with the WT IN-mutant IN complementations were not likely to result from genetic recombination between cotransfected genomes, leading to generation of IN-expressing WT provirus bearing the luciferase gene.

RT activities of mutants in the zinc finger-like domain. The decreased early DNA levels observed for the three mutants in the zinc finger-like domain indicated that steps in the HIV-1 life cycle at or prior to reverse transcription were affected. Since RT and IN result from processing of a common precursor, we tested whether synthesis of RT itself might have been altered by the mutants in the zinc finger-like domain. Western blots were prepared with lysates from virions isolated by ultracentrifugation of zinc finger-like domain mutants and the WT construct, with the U3 *att* site DEL10 mutant and catalytic domain mutant D116G serving as controls. Probing with monoclonal antibodies to RT showed the presence of both p66 and p51 RT proteins at levels comparable to those of the WT construct and other mutants (Fig. 5A). Similarly, p24 Gag protein levels were also unaffected (Fig. 5B). Thus, the IN mutants in the zinc finger-like domain appear to have no effect on the levels of RT in virions or on Gag-Pol polyprotein processing.

Although RT levels were normal, it was possible that the RTs may have altered ability to reverse transcribe genomic RNA within the virion. We directly tested the RT activity of virion preparations from each of the mutants by using a quantitative virion-disrupted endogenous reverse transcription assay (2, 32, 36). DNase-treated virions were purified by ultracentrifugation. Virus was subjected to an endogenous reverse transcription assay without exogenous templates or primer. HIV-1-specific cDNA was detected by PCR. For detection of HIV-1 DNA products in the reaction, we used PCR primers, as in Fig. 2, to detect early (R/U5) and late (R/*gag*) cDNA products. We also used PCR primers for intermediate reverse transcription products (R/PBS and *tat*/*rev*) (66). Our results show that all of the mutants, including the mutants in the zinc finger-like domain, had levels of early and late DNA comparable to levels of the WT synthesized in the endogenous reactions (Fig. 6). The only difference that we noted was a consistent two- to threefold reduction in R/*gag* or full-length viral DNA synthesis in the case of the three mutants in the zinc finger-like domain. Various other detergents, including Tween 20, octylglucoside, various ionic conditions, and permeabilizing agents such as melittin (2) were tested to determine whether conditions that would show a greater difference between the mutants in the zinc finger-like domain and the WT could be found, but no significant differences were observed. Thus, we conclude that the intrinsic RT activity measured in an endogenous reaction of the mutants in the zinc finger-like domain is essentially normal, and the slight differences observed cannot account for the more severe effects on initial levels of HIV-1 DNA seen following infection of cells.

DISCUSSION

We used a PCR-based assay system to assess the levels of stably maintained viral DNA in a single cycle of infection by

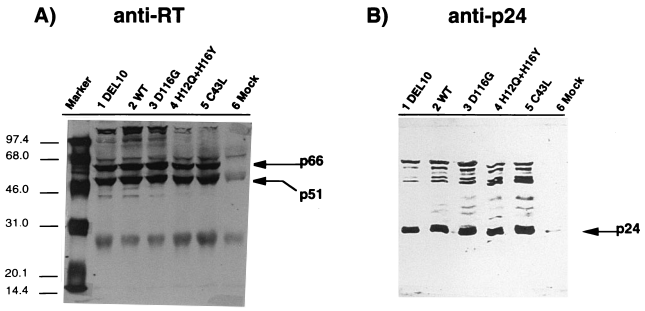

FIG. 5. Western blot analysis of RT and p24 in virions. Viruses ($MuLV_{env}$) were concentrated from the DNase-treated virus stock by ultracentrifugation (200,000 × *g* for 30 min) through 20% diatrizoate–80% TEN buffer. The virus pellet was resuspended with TEN buffer. Viral proteins containing 10 ng of p24, as determined by ELISA, were subjected to SDS-PAGE. As a negative control, culture supernatants of mock-transfected COS cells were precipitated and subjected to SDS-PAGE in parallel (lane 6, Mock). After blotting of proteins to nitrocellulose membrane (Hybond-ECL; Amersham), the membrane was reacted with either a mouse monoclonal antibody to HIV-1 RT (obtained from the NIAID AIDS Research and Reference Reagent Program, ERC Bioservices Corporation) (A) or a human monoclonal antibody to HIV-1 p24 (obtained from the NIAID AIDS Research and Reference Reagent Program, ERC Bioservices Corporation) (B) and then incubated with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin or anti-human immunoglobulin, respectively. HIV-1 Gag and Pol proteins were visualized by the enhanced chemiluminescence detection system (Amersham). Major immunoreactive proteins are indicated by their sizes (in kilodaltons) relative to those of molecular weight markers (lane labeled Marker).

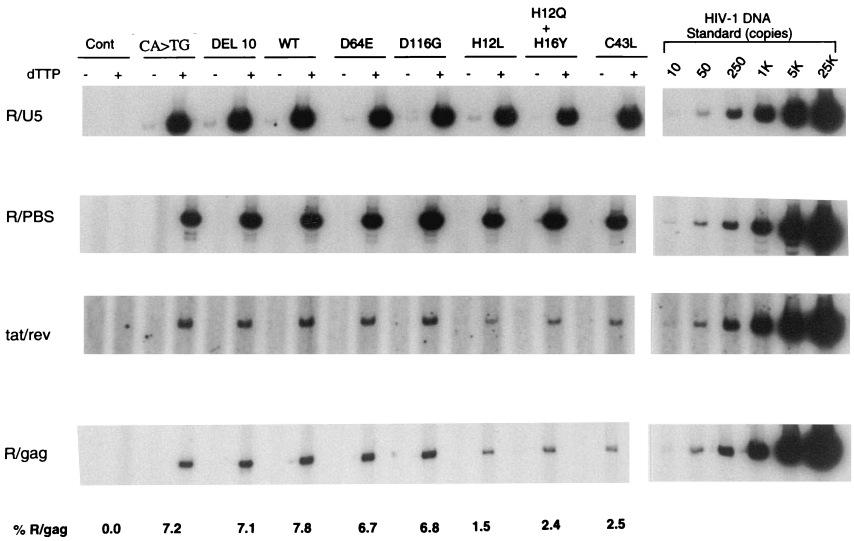

FIG. 6. Endogenous RT activity of each virus. DNase-treated envelope-pseudotype virus ($MuLV_{env}$) was obtained through cotransfection of COS cells as described in Materials and Methods. Virus in the transfected COS culture supernatant (10 ml) was concentrated through a 20% diatrizoate–80% TEN buffer cushion by ultracentrifugation (200,000 × *g* for 30 min). Virus pellets were resuspended with 100 μl of TEN buffer, and the aliquots containing 60 pg of p24 were subjected to the endogenous reverse transcription assay. Reactions were performed with 100 μM each deoxynucleoside triphosphate with (+) or without (−) dTTP. As a buffer control (Cont), TEN buffer alone was reacted in parallel. After the reaction was stopped with proteinase K treatment, the endogenous reverse transcription products were analyzed by quantitative PCR (see Materials and Methods) with several primer pairs, including M667/AA55 (R/U5), M667/BB301 (R/PBS), LA45/LA64 (*tat*/*rev*), and M667/M661 (R/*gag*). At the bottom of each + lane, the percent extension of the RT products for each virus through the LTR/*gag* region (% R/*gag*) was calculated by the ratio of copy number detected by the M661/M667 pair (R/*gag*) to that detected by the M667/AA55 pair (R/U5). This experiment was performed three times with independent preparations of virus, and a representative experiment is shown.

HIV-1 vectors (8, 48, 49) as a measure of the degree of integration. Throughout this study, we make the assumption that maintenance of HIV-1 DNA indicates integration. The unintegrated viral DNA has not been shown to replicate or persist in cells, whereas the definition of the provirus is the form of retroviral DNA that persists as an integrated form. Furthermore, in recently published studies using the same vectors as those described here (49), we provide support for the use of this assay by showing through agarose gel fractionation that unlike the WT construct, a catalytic domain mutant synthesizes DNA but does not associate with high-molecular-weight cellular DNA. Using the luciferase gene as a reporter gene in the *nef* region of the viral DNA (8, 10, 49), we correlated levels of HIV-1 expression, as monitored by luciferase expression, with the presence of a stable, presumably integrated form of viral DNA. With the exception of the mutants in the zinc finger-like domain, our results are generally in agreement with those of other investigators conducting similar experiments with viral infection (9, 22, 41, 53, 57, 65) and with studies in in vitro cell-free integration assays (6, 19, 21, 40–42, 59). Expression of the viral DNA correlates with the ability or inability of viral DNA to integrate, as the levels of expression measured by

TABLE 2. Characterization of each mutant

Virus	Site of mutation	Relative integration efficiency[a]	Relative efficiency of Luc activity[b]	RT activity[c]			
				In vivo		In vitro	
				R/U5	R/*gag*	R/U5	R/*gag*
WT	None	100	100	100	97.8	100	7.2
CA→TG	U3 *att*	40.5	26.2	168	76.9	90.7	7.1
DEL10	U3 *att*	5.1	2.8	145	59.3	117	7.8
D64E	IN catalytic site	<0.5	0.6	181	48.6	118	6.7
D116G	IN catalytic site	<0.5	0.8	126	47.0	122	6.8
H12L	IN zinc finger	ND[d]	<0.1	2.1	57.9	146	1.5
H12Q+H16Y	IN zinc finger	ND	<0.1	1.3	60.0	109	2.4
C43L	IN zinc finger	ND	<0.1	3.5	86.7	85	2.5

[a] Each value was determined from PCR experimental data shown in Fig. 2. Relative integration efficiency was calculated as the ratio of the R/U5 levels detected by the M667/AA55 primer pair at 15 days postinfection to the level at 6 h postinfection in comparison with WT IN and is expressed as a percentage of the WT level.

[b] The peak level of luciferase (Luc) activity in RD cells after infection (3 or 5 days) for each virus was obtained from Fig. 3. Each value is presented as a percentage of the WT level.

[c] RT activity in vivo was determined from Fig. 2 by quantitating the level of the viral DNA (R/U5) (initial cDNA) copies detected by the M667/AA55 primer pair at 6 h postinfection of RD cells. Values are presented as a percentage of the WT level. R/*gag* is the ratio of viral DNA (R/*gag*) copies (nearly full length) detected by the M667/M661 primer pair at peak levels (24 or 48 h postinfection) to the viral DNA (R/U5) (initial cDNA) detected by M667/AA55 at the same time points. RT activity in vitro was determined from endogenous RT values in Fig. 6. The viral DNA (R/U5) copies are presented as a percentage of the WT level. R/*gag* is the ratio of viral DNA (R/*gag*) (nearly full length) detected by the M667/M661 primer pair to the viral DNA (R/U5) (initial cDNA) detected by M667/AA55.

[d] ND, not determined since no viral DNA (R/U5 or R/*gag*) was detected at 15 days postinfection.

luciferase activity correspond to the relative levels of stable DNA maintained. Mutants such as those of the U3 *att* site that have an intermediate level of stable viral DNA show an intermediate level of expression. Our conclusions agree with those of some groups (9, 22, 24, 41, 53, 57, 65), which showed that integration is essential for HIV-1 expression, but they differ from the studies of Stevenson et al. (58), who showed HIV-1 p24 expression in the absence of integration. Thus, our results support the hypothesis that integration of HIV-1 DNA is a required step for its life cycle, similar to that of other retroviruses (16, 17, 27, 55).

IN mutants. Two catalytic site mutants, D64E and D116G, were designed on the basis of previous in vitro studies of HIV-1 IN (6, 21, 41). Consistent with the previous in vitro studies, these mutants synthesized WT levels of viral DNA but were defective in integration. The relative integration efficiencies of these mutant viruses, as judged from DNA stability, were estimated to be less than 0.5% of the WT levels (Table 2). Similarly, expression was equally low. It is interesting that there did appear to be a low level of stable luciferase gene expression from these mutant viruses, which correlates with the similarly low levels of DNA. If one assumes that this is due to integrated viruses, these results suggest that there is a low level of integration for these catalytic site mutants.

In contrast to the catalytic site mutants, the mutants in the zinc finger-like domain showed severely impaired synthesis of viral DNA, and the DNA that was synthesized did not appear to be efficiently integrated, as evidenced by loss of detectable DNA over time. The mutants in the zinc finger-like domain are discussed further below.

***att* site mutants.** An integration-deficient phenotype was also obtained by deletion of the 10 terminal nucleotides of the U3 *att* site (DEL10). This mutation reduced the relative integration efficiency, estimated by proviral stability to be approximately 5% of WT levels (Table 2). In contrast to the deletion mutant, mutation of the highly conserved CA nucleotides, in mutant CA→TG, showed much less severity in its effects. Integration was at a level of approximately 40% of the WT level. A greater tolerance to alterations of CA sequences was also reported for MuLV following infection (13, 14, 45); however, those results and those of our study contrast sharply with those of in vitro studies, in which CA→TG *att* site mutants much more severely affect in vitro 3′ processing, strand transfer, and integration activities of IN (11, 12, 40, 42, 63). The differences observed between in vivo and in vitro studies may reflect a less stringent requirement for terminal nucleotide sequences in vivo than in cell-free reactions as a result of other cellular and/or viral factors that may participate in integration.

IN zinc finger-like domain. Other studies have reported effects of mutation in the zinc finger-like domain upon retroviral replication (9, 16, 65); however, the step of the viral life cycle that was affected was not characterized. The most striking effects of mutations studied here were those at three different positions within the HHCC zinc finger-like domain of IN. We observed greatly reduced levels of viral DNA in infected cells. In addition, although precise quantitation was not possible, because of the low levels of viral DNA, the low levels of viral DNA synthesized shortly after infection did not appear to be integrated, since this DNA was lost over time. Thus, there appear to be two phenotypic effects of the mutants in the zinc finger-like domain: impairment of viral infectivity at or prior to reverse transcription and an impairment in integration.

The zinc finger-like region of IN was defined as evolutionarily conserved pairs of histidines and cysteines (the HHCC motif) located at the amino terminus of retroviral INs (21, 29). The HHCC motif has structural similarity to prototypic zinc finger proteins (29). Structural analysis of the amino-terminal 55 amino acids of HIV-1 (4) as well as direct evidence of zinc-binding abilities of this domain (6, 44) showed that the zinc finger-like region is a discrete domain that can be stable in the presence of zinc (4). The role of the zinc finger-like domain has been extensively addressed through cell-free integration assays (21, 30, 33, 43, 54, 62). These studies show preferential impairment of 3′ processing and strand transfer activities, but not disintegration activity, following mutation of the HHCC conserved residues. Taken together with the structural analysis, these in vitro data suggest a possible function of the zinc finger-like domain in DNA recognition by IN (21, 30, 62). However, no direct evidence for binding to DNA has been reported (33, 54). Recently, Bushman and Wang (7) showed that HHCC mutants defective for in vitro Rous sarcoma virus RSV functions could be restored by fusion of short peptides, suggesting that the zinc finger-like region is not strictly required for substrate binding, formation of oligomers, or catalysis of IN. Thus, the true function of the zinc finger-like region remains to be determined.

Our results indicate that one major effect of mutations in the zinc finger-like domain is at or prior to initiation of reverse transcription. The levels of RT enzyme are normal in mutants in the zinc finger-like domain, indicating that the levels and/or processing of reverse transcription are not altered. Furthermore, use of the endogenous reverse transcription reaction indicates that virions derived from the mutants in the zinc finger-like domain are apparently competent for RT activity, including minus- and plus-strand synthesis and template switching (as evidenced by detection of cDNA products [R/*gag*] with M667/M661 PCR primers). We did note a reproducible two- to threefold decrease in synthesis of full-length viral DNA in the endogenous reverse transcription reaction. This result was in contrast to the more severe impairment of initiation of reverse transcription observed following infection of cells. We were unable to find endogenous reaction conditions that mimicked those of the in vivo situation. Thus, it appears that the major effect of the mutants in the zinc finger-like domain is not on RT or enzymatic processes of the reverse transcription process itself but may be on events proximal to reverse transcription that determine proper and efficient initiation of reverse transcription. We find that the amounts of RT and p24 that are packaged appear normal, but IN or RT p61 may interact directly with these or other components of the virion through the zinc finger-like domains to facilitate as yet unknown events in uncoating that culminate in the initiation of reverse transcription. It is also possible that the mutants in the zinc finger-like domain interfere with proper assembly of virions. A recent study indicated that a point mutation (H12N) in the zinc finger-like domain was defective and showed abnormal virion morphology (22).

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