Human Immunodeficiency Virus Type 1 Integrase Mutants Retain In Vitro Integrase Activity yet Fail To Integrate Viral DNA Efficiently during Infection

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The viral integrase (IN) protein is the only viral protein known to be required for integration of the human immunodeficiency virus type 1 (HIV-1) genome into the host cell DNA, a step in the viral life cycle that is essential for viral replication. To better understand the relationship between *in vitro* IN activity and IN-mediated integration of viral DNA in an infected cell, we characterized the effects of 13 IN mutations on viral replication in cultured cells. Using HIV-1 genomes that express the hygromycin resistance gene and do not express the HIV-1 *env* gene, we generated stocks of pseudotype virus coated with the murine leukemia virus amphotropic envelope glycoprotein, containing either wild-type or mutant HIV-1 IN. All mutants produced normal amounts of physical particles, as measured by reverse transcriptase activity and capsid protein (p24) concentration, but they formed three groups based on infectious titer and synthesis of viral DNA. Changes at the three highly conserved acidic residues in the IN core domain (D-64, D-116, and E-152) impair provirus formation without affecting viral DNA synthesis or the accumulation of viral DNA in the nucleus of the infected cell, a phenotype predicted by each mutant's lack of *in vitro* integrase activity. Mutations at positions N-120, R-199, and W-235 minimally affect *in vitro* integrase activity, but infectious titers are severely reduced, despite normal synthesis of viral DNA, implying a defect during integration *in vivo*. Mutations in the zinc binding region (H12C, H16V, and H16C), S81R, and a deletion of residues 32 through 275 yield noninfectious particles that synthesize little or no viral DNA following infection, despite wild-type levels of reverse transcriptase activity and viral RNA in the particles. The two latter classes of mutants suggest that IN can affect DNA synthesis or integration during infection in ways that are not appreciated from currently used assays *in vitro*.

Retroviral replication requires integration of viral DNA into host cell DNA (40). Attachment (*att*) sites, virus-specific sequences located at each end of viral DNA, and integrase (IN), the protein encoded by the 3' end of the viral *pol* gene, are the only viral factors known to be essential for integration (3, 22). No host cell factors are known to be required for integration, but a human homolog of yeast transcription factor SNF5 has recently been shown to interact with human immunodeficiency virus type 1 (HIV-1) IN (20, 21); its role, if any, in retroviral integration remains unclear.

Reverse transcription is completed after retroviruses enter host cells, resulting in a DNA copy of the viral genome located within a nucleoprotein (preintegration) complex (40). The first step of the integration process, called 3' processing, occurs in the cytoplasm when IN mediates cleavage of the 3' end of each strand of viral DNA immediately beyond a conserved subterminal CA dinucleotide, leaving a hydroxyl group at the 3' end of each strand of viral DNA (3, 22). After the preintegration complex migrates to the nucleus, IN mediates a concerted strand transfer reaction by a nucleophilic attack involving the viral 3' hydroxyl residue and the phosphate backbone of the target DNA (16). The coordinated strand transfer occurs into the major groove of the target DNA (31), and the two viral

ends attack the target DNA in a staggered fashion that determines the length of the virus-specific direct repeats characteristically flanking each provirus.

In vitro assays using wild-type and mutant HIV-1 IN have provided much of the information for the currently accepted mechanism of IN activity (see reference 22 for a recent review). Purified HIV-1 IN performs the 3' processing and strand transfer reactions in the presence of divalent cations and synthetic DNA substrates that mimic the viral *att* sites (5, 25, 27, 33, 43). The initial product of the strand transfer reaction, the gapped intermediate, has the 3' end of the viral DNA attached to the target DNA. It is thought that the 5' ends of the viral DNA are joined subsequently to host cell DNA by cellular repair mechanisms. IN can mediate a reversal of the strand transfer event when supplied with a synthetic gapped intermediate substrate, an activity referred to as disintegration (8).

Amino acid sequence alignment and *in vitro* activity data from wild-type and mutant IN proteins support an IN model with three domains. (i) The amino-terminal domain is characterized by a highly conserved HH--CC sequence resembling a zinc binding domain (4, 6, 19, 23). Mutations involving the conserved histidine and cysteine residues have little if any effect on disintegration while demonstrating variable effects on *in vitro* 3' processing and strand transfer activity (13, 28, 38, 41). Thus, the role of the amino-terminal domain during integration remains poorly understood. (ii) The core region contains a highly conserved D,D-35-E motif found in all retroviral integrase proteins and numerous transposable elements. The D,D-35-E motif refers to three absolutely conserved acidic amino acids (two aspartic acids and one glutamic acid) in the

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order indicated, with a conserved 35-amino-acid spacing between the second and third acidic residues (13, 19, 23). Alteration of any of the three conserved residues leads to loss of all in vitro IN activities, suggesting that this region forms the catalytic core of the protein (13, 28, 38). (iii) The carboxy-terminal domain contains the fewest number of conserved amino acid residues among IN proteins and has a sequence-independent DNA binding capacity (15, 45). In vitro complementation experiments suggest that IN functions as an oligomer (12, 39), and a recently reported crystal structure of the core domain demonstrates a homodimer (11).

While in vitro assays have been useful in unraveling the mechanism of integration, they fail to fully duplicate the in vivo integration process. Most notably, the coordinated strand transfer required for provirus formation is not required in the commonly used assay, in which synthetic *att* site oligonucleotides are incubated with purified IN protein. Furthermore, altering some highly conserved amino acids has no apparent effect on in vitro IN activities (28), suggesting that these residues may play an important role in some aspect of integration not directly tested by in vitro assays.

To begin to understand the relationship between in vitro IN activity and integration activity in infected cells, we introduced 13 independent IN mutations into the HIV-1 IN coding sequence and determined their effects on viral replication. We have previously characterized the in vitro activities of 10 of these HIV-1 IN mutants (28); the in vitro activities of the other 3 mutants has been characterized by others in the context of HIV-2 IN (38). Our results demonstrate that (i) alteration of any of the three conserved acidic residues in the IN core (D,D-35-E) domain profoundly impairs provirus formation by directly affecting integration; (ii) enzymatic activity in vitro frequently fails to predict provirus formation in culture; (iii) IN mutations sometimes affect intracellular viral DNA synthesis in a manner not appreciated by in vitro reverse transcriptase (RT) assays that use detergent-treated virions and artificial substrates; and (iv) different integration phenotypes can be seen when a single amino acid position in IN is replaced by different amino acids.

MATERIALS AND METHODS

Cell lines. COS-7 cells, HOS (human osteosarcoma) cells (gift from Dan Littman, University of California, San Francisco), and 293T cells (gift from John Young, University of California, San Francisco) were grown at 37°C in 5% CO₂ in high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Gibco), 100 U of penicillin G sodium per ml, and 0.1 mg of streptomycin sulfate per ml.

Virus stocks. All virus stocks consist of pseudotype virus containing an envelope-deleted HIV-1 genome and amphotropic murine leukemia virus (MLV) envelope (26, 30). Stocks were generated by calcium phosphate transfection of 293T cells at 50% confluence, using 10 µg of envelope-expressing plasmid DNA and 10 µg of HIV-1 genome-containing plasmid DNA. Plasmid DNA was removed from the plates after 12 h of incubation, and cells were fed with 20 ml of complete media. Culture supernatant was collected 48 h later and filtered through a 0.45-µm-pore-size surfactant-free cellulose acetate filter (Nalgene) to generate virus stock. Virus was either stored at 4°C for use within 24 h or frozen at -70°C for later use.

Construction of proviral clones with specific IN mutations. The IN coding sequence from amino acid residues 5 through 235 was removed from an HIV-1 IN expression vector and inserted into pBSKS⁻ (Stratagene) by use of *Clal* and *HindIII* digestion sites engineered into the IN nucleotide sequence without affecting the amino acid sequence (2, 27). Site-directed mutagenesis was performed as described previously (24, 28), and all mutations were confirmed by the method of Sanger et al. (32). *Clal* and *HindIII* restriction sites were engineered into the HIV-1_{HXB2} IN coding sequence of pHIV-Hygro, a vector with much of the HIV-1 *env* gene replaced by a sequence encoding the hygromycin-selectable marker (26). The engineered *Clal* and *HindIII* sites leave the IN amino acid sequence unchanged. Each IN mutation was then removed from the pBSKS⁻ plasmid in which it was generated by cleavage with *Clal* and *HindIII* and ligated into the HIV-1 proviral sequence of pHIV-Hygro by use of the engineered *Clal*

and *HindIII* sites, using a two-step construction. Mutagenesis oligonucleotide sequences are available upon request.

Protein assays. The p24 concentration of each virus stock was determined by an enzyme-linked immunosorbent assay, using the protocol recommended by the manufacturer (Coulter Immunology). Standard curves were generated in duplicate, using p24 reagent supplied by the manufacturer, and virus stocks were assayed by using duplicate serial dilutions. Concentrations of p24 were determined by using the linear range of the standard curve. RT assays were performed by a slight modification of a previously described method (17). In brief, 0.5 µl of virus stock was pelleted for 60 min at room temperature in an Eppendorf (model 5257) Microcentrifuge, resuspended in reaction buffer (50 mM Tris (pH 7.8), 10 mM dithiothreitol, 10 mM MgCl₂, 0.05% Nonidet P-40, 34 µg of poly(rA) (Pharmacia) per ml, 10 µg of oligo(dT) (Collaborative Research) per ml, 10 µM dTTP, 40 µCi of [methyl-³H]TTP (Amersham) per ml). The reaction mixtures were incubated at 37°C for 2 h, spotted onto DE-81 paper, washed four times with 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], once with water, and once with 100% ethanol, dried, and counted in a Beckman scintillation counter.

PCR analysis of viral DNA. HOS cells were used in this study because they have been shown to be readily infectable by pseudotype virus containing amphotropic MLV envelope protein and HIV-1 core and enzymatic proteins (26). HOS cells at 50% confluence in 100-mm-diameter plates were infected with virus stock (normalized to 250 ng of p24) in 2 ml of medium containing 8 µg of Polybrene per ml. After 2 h at 37°C, virus was aspirated, cells were washed twice with 5 ml of sterile phosphate-buffered saline (PBS), and 20 ml of fresh medium was added to the cells. The medium was aspirated 18 h after infection, the cells were washed twice with PBS, and DNA was isolated by using a previously described technique (9). DNA isolated from a single 100-mm-diameter plate infection was resuspended in a final volume of 200 µl. PCR was performed with 4 µl of 100×-diluted DNA and previously described primers (35, 37). All infections were performed in duplicate in the presence and absence of 5 µM zidovudine (AZT). The two-long-terminal-repeat (LTR) circle junction PCR product was 595 bp in length (35). Because multiple viral DNA forms are present during the course of infection, we used restriction digest mapping to determine that the observed PCR product migrating near the 600-bp marker was the expected 595-bp product (data not shown). The late-linear PCR product was 199 bp in length (37).

Hygromycin-resistant colony titers. HOS cells were split into six-well plates the day prior to infection to give 50% confluence at the time of infection. Infections were performed in duplicate, using serial dilutions of virus stock in a total of 0.5 ml of medium containing 8 µg of Polybrene per ml. After 2 h at 37°C, 1.5 ml of medium was added, and plates were incubated at 37°C for an additional 20 h. The medium was then aspirated, wells were washed twice with 2 ml of PBS, and 2 ml of complete medium supplemented with 200 µg of hygromycin B (Calbiochem) per ml was placed into each well. The medium was changed every 2 to 3 days, and colonies were counted on day 12 after staining with crystal violet.

Western blots (immunoblots). Virus stock was harvested at 60 h posttransfection, filtered through a 0.45-µm-pore-size filter (Nalgene), and sedimented through 3 ml of 20% sucrose at 100,000 × g for 2 h in a Beckman SW41 Ti rotor. The pelleted virions were lysed in 100 µl of Nonidet P-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris [pH 8.0]), 50 µl of 3× sodium dodecyl sulfate (SDS) sample buffer was added, and the samples were boiled at 100°C for 2 min. The proteins were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad), using an S&K dry blotter. Membranes were blocked in 1% milk at room temperature for 15 min for pooled patient antisera and in 10% milk at room temperature for 1 h for rabbit antiserum. Pooled patient antisera (a gift from K. Steimer, Chiron Corp., Emeryville, Calif.) were used at a dilution of 1:10,000, and rabbit anti-IN (27) was used at a dilution of 1:20,000. Horseradish peroxidase-conjugated secondary antibodies (Boehringer Mannheim Biologicals) were used at a dilution of 1:30,000, and enhanced chemiluminescence Western blot detection was performed as recommended by the manufacturer (Amersham).

RNA slot blot. One milliliter of virus stock was pelleted through 20% sucrose, resuspended in 100 µl of TENS buffer (10 mM Tris [pH 8.0], 10 mM EDTA, 100 mM NaCl, 0.5% SDS), extracted twice with phenol-chloroform, and extracted twice with ether. The aqueous solution was incubated at 65°C for 3 min to remove remaining ether. One volume of 20× SSC was added to make a final concentration of 10× SSC. Slot blots were normalized for p24 and performed in serial dilutions of 1×, 1/5×, and 1/25×, using a nylon membrane (Amersham). Each slot was washed three times with 10× SSC, the RNA was cross-linked with UV light, and the membrane was then washed with water. The probe was generated by the random primer method (1) and a 450-bp fragment from the middle of the hygromycin resistance gene, and hybridizations were performed overnight at 65°C, using Church solutions (1).

RESULTS

Generation of virus stocks containing mutant IN protein. To examine the relationship between IN activity in vitro and IN activity during viral infection, we generated 13 viruses with IN mutations that have been previously analyzed in vitro (28,

TABLE 1. Characterization of virus stocks

Integrase ^a	p24 ^b	RT activity ^c	RT/p24 ratio ^d
Wild type	100	100	100
Δ32-275	74	27	37
HH-CC			
H12C	115	106	92
H16C	68	42	62
H16V	44	25	57
Q53C	154	144	94
Catalytic D,D-35-E			
D64V	163	100	61
S81R	55	27	49
D116I	140	114	81
N120L	199	183	92
N120E	197	165	84
N120G	161	148	92
E152G	166	142	86
DNA binding			
R199C	85	76	89
W235E	105	99	94

^a Each integrase mutant name consists of a number indicating the altered IN residue. The number is preceded by the single-letter code for the amino acid normally found at that location followed by the single-letter code for the amino acid substituted at that location. Δ32-275 is a deletion of IN residues 32 through 275. Wild-type IN is 288 amino acids in length. The location of each mutation relative to the three IN domains is indicated.

^b Wild-type virus p24 of 526 ng/ml, an average of five independent transfections, is assigned a value of 100, and mutant stock p24 concentrations are standardized to that of wild-type p24.

^c Wild-type virus stock RT activity of 3.4×10^5 cpm/ml, an average of five independent transfections, is assigned a value of 100, and mutant stock RT activities are standardized to that of wild-type virus.

^d An indication of RT activity relative to p24 content, an attempt to correct the RT activity for the number of virions present in the stock.

38). Each mutant virus was tested for its ability to mediate provirus formation during a single round of viral replication. Virus containing the wild-type IN coding sequence served as the positive control for our experiments; we also prepared virus with a deletion that removed amino acids 32 through 275 from IN, which is normally 288 amino acids long. Virus stocks were generated by transfecting 293T cells with proviral clones expressing either wild-type or mutant IN. A hygromycin resistance gene replaced much of the HIV-1 *env* sequence in each viral genome; thus, a functional *env* gene was supplied in *trans* by cotransfection with a vector expressing the amphotropic MLV envelope glycoprotein. The resulting pseudotype virions have amphotropic MLV envelope glycoprotein surrounding an HIV-1 core, with HIV-1 proteins and genomic RNA. The hygromycin resistance gene in the packaged genome allows for clonal outgrowth of infected cells in the presence of the antibiotic if viral DNA is made, integrated, and expressed. This provides a means of quantifying the number of integration events per unit of virus stock (26, 30).

The virus stocks were initially characterized for p24 concentration and in vitro RT activity. The p24 concentration for wild-type virus (526 ± 112 ng/ml; average $\pm$ standard deviation of five independent transfections) was assigned a value of 100; p24 concentrations of mutant virus stocks were recorded relative to that of the wild type and ranged from approximately one-half to twice the p24 concentration of wild-type virus (Table 1). The RT activity was determined by using detergent-lysed virions and the artificial template poly(rA). A value of 100 was assigned for wild-type RT activity [$339 \pm 82 \times 10^3$ cpm/ml; average $\pm$ standard deviation of five independent transfections]; RT activity of mutant virus stocks was again recorded relative to that of wild-type virus and ranged from

approximately one-quarter to twice that of wild-type virus (Table 1). When the RT activity of each stock is corrected for the p24 concentration, indicated in Table 1 as the RT/p24 ratio, the virus stocks are remarkably similar, with the RT/p24 ratios of most mutants ranging from 0.8 to 1.2, within 20% of the normalized ratio for wild-type virus. The few exceptions show mild reductions in relative amounts of RT activity, with RT/p24 ratios of 0.37 for Δ32-275, 0.57 and 0.62 for the two H16 mutants, 0.61 for D64V, and 0.49 for S81R. Thus, the RT activities per virion are quite similar among the mutant viruses, allowing us to compare provirus formation by normalizing infections for p24.

IN mutations do not alter the processing and incorporation of viral proteins into viral particles. To determine if the IN mutations affect the protein content of virus particles, virions were sedimented through 20% sucrose and then subjected to Western blot analysis, using pooled antisera from HIV-infected individuals. Similar amounts of capsid (CA) protein (p24), its Gag precursor proteins (p41 and p55), and RT proteins (p66 and p51) were detected for wild-type and each mutant virus (Fig. 1A). IN is readily visualized in all lanes

FIG. 1. Western blot analysis of viral proteins. (A) Virus was pelleted through 20% sucrose and lysed with radioimmunoprecipitation assay buffer, and the proteins were electrophoresed through an SDS-10% polyacrylamide gel. Proteins were transferred to nitrocellulose, incubated with pooled antisera from HIV-1-infected patients, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. Signal was generated by using enhanced chemiluminescence. Positions (in kilodaltons) of molecular weight markers are indicated on the left, known viral proteins are identified on the right, lanes are numbered at the bottom, and virus stocks (nomenclature as defined in Table 1, footnote a) are indicated at the top. WT, wild type. (B) Western blot of wild-type, Δ32-275, and W235E viral proteins and antisera used for panel A, subjected to a longer exposure demonstrating IN (p32) in lane 1 (wild type) and lane 3 (W235E) but not in lane 2 (Δ32-275). (C) Western blot of the same viral proteins as in panel B, using polyclonal rabbit anti-IN antisera, demonstrating similar amounts of IN from wild-type and W235E viral lysates and no IN from the lysate of Δ32-275.

TABLE 2. Infectious titer of HIV-1 with wild-type or mutant integrase^a

Integrase ^b	No. of hygromycin-resistant colonies/ μ g of p24 ^c	In vitro activity ^d	DNA synthesis in culture ^e	Packaged viral RNA ^f
Wild type	8.5×10^5	100	+++	++
Δ 32-275	<1	ND	—	ND
HH-CC				
H12C	20	ac	+	++
H16C	<1	75–100	—	++
H16V	<1	75–100	—	++
Q53C	8.2×10^4	100	++++	++
Catalytic D,D-35-E				
D64V	90	<1	++++	ND
S81R	<1	ac	—	++
D116I	260	<1	+++	ND
N120L	630	10–50	+++	ND
N120E	370	10–50	++	ND
N120G	2.8×10^4	10–50	++	ND
E152G	60	<1	+++	ND
DNA binding				
R199C	10	100	++	ND
W235E	70	100	+++	ND

^a Titers were generated on HOS cells, using HIV-1 pseudotyped with amphotropic MLV envelope as described in Materials and Methods.

^b See Table 1, footnote a.

^c <1 indicates that no colonies were obtained.

^d Taken from previous reports (28, 38). Values are relative to that for wild-type IN. ND, not done; ac, activity present but difficult to assess because of purification problems.

^e A semiquantitative indication of the intracellular DNA synthesis based on visual inspection of the late-linear PCR data in Fig. 2.

^f Taken from Fig. 3. ND, not done; ++, present. See Fig. 3 for graphic demonstration of the data.

except those for the IN deletion mutant Δ 32-275 and the IN point mutant W235E. A longer radiographic exposure of a gel containing protein from wild-type, Δ 32-275, and W235E virus stocks detects W235E IN, using the same pooled antisera from HIV-1-infected patients, but the band is much less intense than that seen for wild-type virus (Fig. 1B). We reprobbed the blot in Fig. 1B with a polyclonal rabbit antiserum raised against denatured HIV-1 IN and detected similar amounts of IN (p32) from wild-type and W235E viruses (Fig. 1C). Our data suggest that W235E is altered in a manner that changes the primary antigenic epitope recognized in HIV-1-infected patients without reducing the amount of IN contained within viral particles.

Integration activity of the virus stocks. Having a hygromycin-selectable marker in place of the viral *env* gene imparts two important characteristics to the virus particles: (i) the absence of a functional *env* gene prevents the production of infectious

particles from infected cells, thereby preventing virus spread and the emergence of secondary mutations, and (ii) selection for growth in hygromycin allows us to quantify the infectious titer of each virus stock by counting hygromycin-resistant colonies; each hygromycin-resistant colony was assumed to represent the formation of a single provirus. The titer of wild-type virus was roughly 3.8×10^5 colonies per ml, or 8.5×10^5 colonies per μ g of p24 (Table 2).

The mutants can be placed into three groups based on their infectious titers, and none has an infectious titer as high as that of wild-type virus (Table 2). Mutants Q53C and N120G are the least impaired, having infectious titers within 1 log unit of that of wild-type virus. The second group includes mutants with infectious titers 3 (D116I, N120L, and N120E) or 4 (H12C, D64V, E152G, R199C, and W235E) log units less than that of wild-type virus. Mutants in the third group, H16C, H16V, S81R, and Δ 32-275, have no detectable infectious titer, as they consistently failed to demonstrate hygromycin-resistant colonies (Table 2). Notably, mutations at each of the two histidines that comprise the HH-CC domain, H12 and H16, produce different phenotypes in this assay; H12C consistently yielded low numbers of hygromycin-resistant colonies, while H16C and H16V failed to yield any colonies.

IN mutations influence the production of viral DNA. To determine whether factors other than impaired integration activity are responsible for decreased infectious titers of any of the IN mutants, we harvested DNA 18 h after infection of HOS cells and looked for evidence of complete or nearly complete linear products of reverse transcription (late-linear DNA), using previously described PCR primers (35, 37). Because viral DNA synthesis can begin in virus particles prior to infection of the target cell (29, 37), HOS cell infections were performed in the presence or absence of 5 μ M AZT to determine what portion of the late-linear DNA signal came from DNA synthesized after cell entry (Fig. 2). The DNA detected after infections in the presence of AZT was indistinguishable from that detected when PCR was performed directly on virus stock, evidence that 5 μ M AZT effectively blocked reverse transcription within the HOS cells (data not shown).

The IN mutants fall into three groups when DNA synthesis is compared with infectious titer (Table 2). Mutants Δ 32-275, H16C, H16V, and S81R, which failed to produce any hygromycin-resistant colonies, and mutant H12C, which produced very few hygromycin-resistant colonies, appear to have a profound defect in DNA synthesis (Fig. 2). The failure to synthesize intracellular viral DNA makes it impossible to determine the intracellular integration activity of these mutants. Although these five mutants synthesize negligible amounts of late-linear viral DNA following HOS cell infection (Fig. 2), RT

FIG. 2. Late-linear PCR performed on DNA harvested 18 h after virus infection as described in Materials and Methods. One-quarter of each late-linear PCR mixture was electrophoresed through 6% polyacrylamide in 1 $\times$ Tris-borate-EDTA. Lanes are numbered on the bottom, virus stock (nomenclature as defined in Table 1, footnote a) is indicated at the top, and the desired band is indicated by the arrow on the right. Each experiment was performed in the presence (+) and absence (–) of 5 μ M AZT as indicated. Base pair markers are indicated on the left. WT, wild type.

FIG. 3. RNA slot blot of encapsulated viral RNA. Virions were pelleted through 20% sucrose, and RNA was prepared as described in Materials and Methods. For each virus, 100 ng of p24 was applied in duplicate to a nylon membrane (row A). Row B is a 5 $\times$ dilution of row A; row C is a 5 $\times$ dilution of row B. The 32 P-radiolabeled probe was directed against a 450-bp fragment of the hygromycin resistance gene in each viral genome. WT, wild type.

activities of the mutant virus stocks are very similar to that of wild-type virus when assayed *in vitro* with a poly(rA) template (Table 1). The *in vitro* RT activity is therefore a very poor predictor of intracellular viral DNA synthesis for these mutants. To determine if a failure to package viral RNA is the cause of this discrepancy, we performed RNA-DNA slot blot hybridizations with viral particles pelleted through 20% sucrose, normalizing for p24 and using a radiolabeled probe to the hygromycin resistance gene. H12C, H16C, H16V, and S81R, mutants with significantly impaired intracellular reverse transcription, package at least as much viral RNA as do wild-type virus and mutant Q53C, which has an infectious titer close to that of wild-type virus (Fig. 3). Interestingly, S81R synthesizes negligible amounts of viral DNA following infection (Fig. 2, lanes 15 and 16), yet it packages wild-type levels of viral RNA (Fig. 3) and contains an unusually large amount of intravirion viral DNA (Fig. 2, lane 16).

Mutants D64V, D116I, N120L, N120E, E152G, and W235E each have a 3- or 4-log-unit decrease in infectious titer in culture, yet they each generate complete or nearly complete viral DNA at levels similar to that of wild-type virus (Fig. 2). Consequently, these mutants appear to have a primary integration defect. Three of these mutants, D64V, D116I, and E152G, have alterations at the highly conserved acidic residues of the IN catalytic core (D,D-35-E) and demonstrate very little or no 3' processing, strand transfer, or disintegration activity *in vitro* (13, 28, 38); their reduced infectious titers are therefore most likely due to a lack of IN enzymatic activity. In contrast,

all three *in vitro* IN activities of W35E, N120L, and N120E are equal to those of wild-type IN with the exception that N120L and N120E perform *in vitro* strand transfer at roughly 10 to 50% of the wild-type IN level (28, 38). The modest decrement in viral DNA detected in cells infected with R199C, compared with that in cells infected with virus containing wild-type IN (Fig. 2), and the fact that R199C retains wild-type levels for all three *in vitro* IN activities place R199C in a group similar to that comprising W235E, N120L, and N120E: mutants with an apparent integration defect that cannot be explained simply by a lack of IN enzymatic activity or a failure to synthesize viral DNA. Mutants in the third group, Q53C and N120G, have near-wild-type levels of colony (and hence provirus) formation (Table 2) and wild-type levels of intracellular viral DNA synthesis (Fig. 2). These mutants demonstrate that not all IN mutations produce a profound defect in viral replication.

Nuclear localization of viral DNA is not altered by the IN mutations. Viral DNA is synthesized in the cytoplasm of infected cells and then migrates to the nucleus as part of a preintegration complex. To determine whether failure of the viral DNA to migrate to the nucleus contributes to the low infectious titers seen in any of the mutants, we looked for evidence of viral DNA in the nuclei of infected cells by exploiting the fact that the two-LTR circle form of viral DNA is present in the nucleus and not in the cytoplasm of infected cells. DNA was harvested after wild-type and mutant virus infection of HOS cells, and two-LTR circle junctions were identified by PCR amplification using previously described primers (35). The close relationship between the presence of complete or nearly complete forms of viral DNA (Fig. 2) and the presence of DNA in the nucleus (Fig. 4) do not suggest that any of the IN mutants has a nuclear migration defect. The small amount of two-LTR circle DNA following H12C infection likely reflects a low level of intracellular viral DNA synthesis not achieved by mutants Δ 32-275, H16C, H16V, and S81R.

DISCUSSION

To better understand the effect of IN mutations on the HIV-1 life cycle and to determine the relationship between IN activity *in vitro* and IN-mediated provirus formation in infected cells, we generated a series of viruses, each with a point mutation in the IN coding sequence. Virus stocks were initially characterized for p24 concentration, RT activity, and virion protein content (Table 1 and Fig. 1). The *in vitro* enzymatic

FIG. 4. Two-LTR circle junction PCR. PCR for two-LTR circle junctions was performed on DNA harvested 18 h after virus infection. One-quarter of each PCR mixture was electrophoresed through 6% polyacrylamide in 1 $\times$ Tris-borate-EDTA. Lanes are numbered on the bottom, virus stock (nomenclature as defined in Table 1, footnote a) is indicated at the top, and the desired band is indicated by the arrow on the right. Because multiple viral DNA forms are present during the course of infection, we used restriction digest mapping to determine that the observed PCR product migrating near the 600-bp marker was the expected 595-bp product (data not shown). Each experiment was performed in duplicate in the presence (+) and absence (-) of 5 μ M AZT as indicated. Base pair markers are indicated on the left. WT, wild type.

activity of each purified IN mutant was compared with its ability to mediate provirus formation and expression during infection of cells in culture (Table 2). All viruses were pseudotypes of HIV-1 with amphotropic MLV envelope surrounding HIV-1 cores, and each viral genome contained a hygromycin-selectable marker in place of the HIV-1 *env* gene. This design prevents the production of infectious particles from integrated proviruses and thereby prevents the rescue of IN mutations by secondary mutations. Furthermore, infection followed by selection in hygromycin allowed us to quantify the infectious titer, and indirectly the integration activity, of each mutant, because colony outgrowth in hygromycin requires the direct and sustained expression of the hygromycin resistance gene.

Characterization of viral particles containing mutant IN protein. We found that the ratio of in vitro RT activity to p24 concentration, a measure of virion RT content, was highly consistent among the virus stocks (Table 1). This allowed us to compare infectious titer, defined as the number of hygromycin-resistant colonies per unit of virus stock, by normalizing infections for the amount of p24 used in each infection. There were no significant differences in the protein content of any of the mutants compared with wild-type virus (Fig. 1A), consistent with recent reports by others (7, 44).

Western blotting with antisera from HIV-1-infected patients produces a markedly reduced signal with W235E compared with IN from wild-type virus or from virus with other IN point mutations (Fig. 1A and B). Yet similar amounts of wild-type and W235E IN protein are detected when Western blotting is performed with an anti-IN antibody generated by means other than a natural infection (Fig. 1C). Others have reported that IN from HIV-1 mutant W235A is not detectable by Western blot analysis using pooled sera from HIV-1-infected people but that it is detectable with an antibody directed against the amino-terminal 18 amino acids of IN (7) and that anti-IN antibodies from HIV-1-infected people map predominantly to the carboxy terminus of IN (42). Taken together, these findings suggest that the tryptophan at position 235 contributes significantly to the primary antigenic epitope recognized by the human antisera.

The D,D-35-E residues of the IN core domain are critical for provirus formation in culture. Each of the three highly conserved acidic residues of the IN core domain (D,D-35E) is independently critical for 3' processing, strand transfer, and disintegration reactions in vitro (13, 28, 38). Consistent with the in vitro IN activity and with recent reports by others who have tested for the effects of mutations at these three residues on virus replication (7, 14, 34, 44), we find that mutants D64V, D116I, and E152G each have impaired provirus formation (Table 2). Mutations at these three critical acidic residues have no apparent defect in steps of the virus life cycle that precede integration, as demonstrated by the normal or enhanced levels of late-linear viral DNA and of two-LTR circle forms of viral DNA (Fig. 2 and 4). This finding strongly suggests that D64V, D116I, and E152G have a specific defect in proviral integration as the cause of the marked reduction in hygromycin-resistant colony formation. One report (36) of a D116A mutation resulting in no detectable RT activity cannot be reconciled with the bulk of the data now available.

Mutations at residues D-64, D-116, and E-152 consistently produce the most profound defects in 3' processing, strand transfer, and disintegration in vitro (13, 28, 38), yet mutants D64V, D116I, and E152G have a higher infectious titers than do mutants H12C, H16C, H16V, and S81R, which retain at least some in vitro IN activity (Table 2) (28). The likely explanation for this difference is that the amount of viral DNA

synthesized and transported to the nucleus is much greater following infections with D64V, D116I, and E152G than following infections with H12C, H16C, H16V, S81R, and Δ 32-275 (Fig. 4). The finite number of hygromycin-resistant colonies generated by infections with mutants D64V, D116I, and E152G could be the result of integration mechanisms unrelated to retroviral IN activity. Prior studies of the host-virus DNA junctions following infections with MLV IN mutants demonstrated that the MLV DNA was frequently integrated by mechanisms other than those mediated by MLV IN protein (10, 18). We are analyzing the virus-host junctions from hygromycin-resistant clones derived from mutants D64V, D116I, and E152G to look for any evidence of retained IN activity.

Some IN mutants impair intracellular viral DNA synthesis without affecting in vitro RT activity or RNA packaging. Mutants Δ 32-275, H12C, H16C, H16V, and S81R each have a profound impact on intracellular viral DNA synthesis (Fig. 4), a defect not appreciated when virus stocks were assayed for RT activity in vitro with detergent-lysed virions and poly(rA) substrate (Table 1). The failure of these IN mutants to synthesize viral DNA following infection could be due to altered protein-protein or protein-RNA interactions at the time of virus assembly, or after proteolytic cleavage of the Gag-Pol polyproteins, resulting in a failure of RT to become properly positioned within the preintegration complex. It is clearly not due to a failure to package viral RNA (Fig. 3). The discrepancy between RT activity in vitro and the synthesis of viral DNA in an infected cell prevents us from determining the integration activities of these mutants in culture. Previous reports that mutants H12N (14), H12A (44), H16A (44), and S81A (7) fail to direct integration of viral DNA in culture did not distinguish between a primary integration defect and a defect earlier in the viral life cycle. The abnormal ultrastructure seen on thin-section electron microscopy of H12N (14) could be related to a defect in the virus life cycle other than integration.

Reduced provirus formation by IN mutants cannot be explained by impaired IN activity or viral DNA synthesis. Mutants R199C and W235E have in vitro IN activities near or at the activity of wild-type IN (11, 28). Our analysis of these mutants in the context of an infecting virus demonstrates efficient synthesis of linear viral DNA (Fig. 2) and transport of the DNA to the nucleus (Fig. 4) but a profoundly impaired ability to integrate viral DNA (Table 2). The most plausible interpretation of these data is that mutants R199C and W235E have a nonenzymatic integration defect. Mutations R199C and W235E may alter the architecture of the preintegration complex so that a fully active IN protein may not be in the proper location to act on the viral ends or to interact with the target DNA. Alternatively, these mutant IN proteins may be able to act on a single viral end, as assayed in vitro, but be unable to coordinate integration of the two viral ends at a single point on the target DNA, a requirement for proper provirus formation. IN mutant W235F is replication competent (14), suggesting that the substitution of a phenylalanine for the highly conserved tryptophan at position 235 reflects the need for an aromatic side chain at that residue. If so, our data suggest that the aromatic ring structure is important for a nonenzymatic function of the IN protein during provirus formation.

The three mutations at N-120 demonstrate the importance of the replacement amino acid in determining the integration phenotype of IN point mutations. The asparagine at position 120 is conserved in many retroviral INs, and HIV-2 IN mutants N120L, N120G, and N120E retain 10 to 50% of wild-type IN activity in vitro (38). Consistent with its in vitro activity, our HIV-1 N120G mutant has only a slight reduction in infectious titer compared with wild-type virus, but HIV-1 IN mutants

N120E and N120L have markedly impaired infectious titers (Table 2). Since purified N120E and N120L proteins are at least as active *in vitro* as purified N120G (38), and since viral DNA synthesis following infection with N120E and N120L is similar to that seen following infection with wild-type virus (Fig. 2), N120L and N120E are similar to R199C and W235E: they appear to have a primary integration defect that cannot be explained by a loss of IN activity as measured *in vitro* or by a defect in intracellular viral DNA synthesis.

In summary, we have found that none of the 13 IN mutations studied affect the viral protein content of the mutant virions, but some of the IN mutations have unexpected effects on the virus life cycle. Changes at any of the three acidic residues defining the D,D-35-E motif of the catalytic core result in virus with a primary integration defect, a phenotype predicted by *in vitro* IN activity assays. Mutations at positions H-12, H-16, and S-81, all located within the amino-terminal one-third of the IN protein, produced an unexpected and profound reduction in the intracellular synthesis of viral DNA, yet the mutant virions package normal amounts of viral RNA and have wild-type levels of RT activity when assayed on an artificial substrate. The impaired intracellular viral DNA synthesis prevented us from determining the intracellular integration activities of the mutants with changes in the highly conserved, amino-terminal HH-CC domain. Furthermore, mutations at IN residues N-120, R-199, and W-235 result in integration-defective virions that synthesize wild-type levels of intracellular viral DNA and contain enzymatically active IN as measured *in vitro*. The latter two groups of mutants raise interesting questions about the possible role of IN in intracellular viral DNA synthesis and in the assembly and function of integration-competent, viral nucleoprotein complexes.

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