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Human Immunodeficiency Virus Type 1 Integrase: Effect on Viral Replication of Mutations at Highly Conserved Residues

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Sequence comparisons of the integrase (IN) proteins from different retroviruses have identified several highly conserved residues. We have introduced mutations at 16 of these sites into the integrase gene of human immunodeficiency virus type 1 and analyzed the phenotypes of the resulting viruses. The viruses were all normal for p24 content and reverse transcriptase activity. In addition, all of the mutants could infect T-cell lines and undergo reverse transcription, as assessed by PCR analysis. Most of the mutant viruses also had normal Western blot (immunoblot) profiles, although three of the mutations resulted in reduced signals for IN relative to the wild type on the immunoblots and mutation of residue W235 completely abolished recognition of the protein by pooled sera from human immunodeficiency virus type 1-positive patients. Mutations that have previously been shown to abolish activity in in vitro studies produced noninfectious viruses. The substitution of W235 was notable in producing a noninfectious virus, despite previous reports of this residue being nonessential for IN activity in vitro (A. D. Leavitt, L. Shiue, and H. E. Varmus, J. Biol. Chem. 268:2113–2119, 1993). In addition, we have identified four highly conserved residues that can be mutated without any affect on viral replication in T-cell lines.

Integration is an essential and characteristic step of the retrovirus life cycle. Following infection of a susceptible cell, the viral RNA genome is copied into a double-stranded DNA molecule, containing terminally repeated long terminal repeat (LTR) sequences, which is then inserted into the host cell DNA. Integration requires the removal of two nucleotides from the 3′ termini of the LTRs (3′ processing), followed by a staggered cleavage of the host cell DNA. This produces 5′ overhangs which are joined to the recessed viral termini (strand transfer). Host enzymes repair and ligate the gapped molecule so created, giving rise to short duplications of the host sequences flanking the integrated provirus (for reviews, see references 17 and 44).

Biochemical studies have demonstrated that only one component, the virally encoded integrase (IN) protein, is required for both the 3′ processing and strand transfer reactions (4, 8, 24, 28). Purified IN catalyzes a nucleophilic attack on a specific phosphodiester bond at the terminus of the viral LTR and then positions the resulting 3′ hydroxyl groups to attack the target DNA. These two cleavage events are coordinated in a one-step reaction, without the involvement of a covalent protein-DNA intermediate (15). Genetic evidence also points to the essential role of IN in this process, as viruses carrying mutations in HIV-1 IN are incapable of integration and correspondingly noninfectious (28, 37).

Sequence comparisons of IN proteins from different retroviruses have identified several residues and motifs that are conserved across species (14, 19, 25, 27). The role of these residues in IN function has been investigated in vitro, using deletion and substitution mutants of IN protein from human immunodeficiency virus type 1 (HIV-1) (11, 14, 30, 41), HIV-2 (39), murine leukemia virus (MLV) (21), and Rous sarcoma virus (RSV) (27). Such studies have identified distinct functional domains within IN, and mutations within individual domains can be complemented in *trans* by wild-type residues within a mixed multimer (5, 13, 40).

The active site of the molecule seems to be contained within the central core and is characterized by three invariant acidic residues with the pattern $D_{51-58}D_{35}E$. This region has homology with similar recombinase proteins from retrotransposons and prokaryotic transposons (25, 27). The amino terminus has a conserved HHCC motif, reminiscent of a zinc finger (19), and the specific interaction between IN and the LTR sequences has been suggested to be a function of this region (5, 21, 41), although no such binding has been demonstrated directly under in vitro conditions (25, 43, 45). IN has a nonspecific affinity for DNA which has been mapped to the C terminus of the protein (43, 45), and this region may be involved in the interaction of IN with the target DNA. There is also evidence that IN functions as an oligomer (20, 22). A possible multimerization region, spanning the central catalytic domain and a region immediately 3′ to this which includes a potential leucine zipper motif (31), has been described for HIV-1 (13).

Although IN protein by itself is sufficient to integrate an LTR oligonucleotide into a target DNA molecule in vitro, it is likely that other viral and cellular components play a role in this process in vivo. Viral DNA rescued from infected cells is contained within a high-molecular-weight complex termed the preintegration complex, which also contains components of the viral core (1, 3). While studies with purified IN have contributed much to our understanding of the mechanism of integration, they will not highlight regions of IN involved in interactions with other components of the preintegration complex. Furthermore, such studies may not be sensitive to mutations that inhibit multimerization of IN, as most in vitro integration events are one-ended reactions that measure the integration of

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a single LTR terminus (4). This contrasts with the concerted integration of two LTRs that occurs when extracts of infected cells are used as the source of IN (16) or that must occur in vivo (32). It is noteworthy that several highly conserved residues can be mutated without any great effect in any in vitro assays (14, 30, 39).

To investigate the role of IN in the in vivo integration process, we have created single amino acid changes at 16 highly conserved sites in HIV-1 IN. We have introduced these mutations into an infectious proviral clone and analyzed the resulting virions. We find that mutations that have previously been shown to have a deleterious effect in vitro similarly produce a protein that is unable to function in vivo. Surprisingly, despite their obvious conservation, several residues that were nondeleterious in in vitro assays also appear to be nonessential for integration function in our in vivo system. However, one conserved residue that appears not to be essential in vitro (30) produced noninfectious virus.

MATERIALS AND METHODS

Mutagenesis of HIV-1 integrase and proviral manipulations. The integrase gene from plasmid pBH10 (36) was inserted into M13, and single amino acid substitutions were introduced at highly conserved residues by site-directed mutagenesis. The mutated genes were inserted back into the infectious proviral clone WI3 (26). WI3 is a *nef*$^+$ version of HXB2 and, like pBH10, is derived from HIV_{IIIB}. The sequences of the integrase genes of pBH10 and WI3 are identical (our unpublished data). Plasmid pGEMSSΔE contains the *pol* sequences from WI3 on a 4.3-kb *Spe*I-*Sal*I fragment in a pGEM5Zf backbone (Promega). The integrase gene in this plasmid has been marked by the removal of the central *Eco*RI site following end filling and religation. The mutated integrase genes were first moved from M13 into this construct, using the *Bsp*MI sites at either end of the gene; successful replacement restored the *Eco*RI site. These intermediates were then used to replace the equivalent *Spe*I-*Sal*I fragment of plasmid pSA1, which is a WI3 derivative deleted for the *Eco*RI fragment between nucleotides 4650 and 5745. Inserting the mutant integrase genes into this plasmid restored the *Eco*RI fragment and produced a full-length proviral clone. Mutant viruses were named according to the residue that was changed (e.g., $WI3_{C40A}$ has the cysteine at amino acid 40 in IN changed to an alanine). In addition, the mutant proviral clone WI3ΔIN was derived directly from pGEMSSΔE. It therefore contains a frameshift mutation at residue 142 which adds 14 nonsense codons and a termination codon, producing a protein of 156 amino acids (the wild type contains 288 amino acids). All mutations were confirmed by sequencing both the initial M13 clones and the final reconstituted proviral DNA.

Production of virus containing integrase mutations. Virus was generated by overnight calcium phosphate transfection (18) of COS-7 or 293T (293/tsA1609neo) cells (12) (obtained from D. Baltimore, Rockefeller University). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GIBCO BRL) and transfected at 25% confluence with 20 μg of plasmid DNA per 10-cm-diameter dish. Supernatants were harvested 60 h posttransfection and clarified by centrifugation and filtration through 0.4-μm-pore-size filters. Virus stocks were treated with DNase I (Promega) for 1 h at 37°C in the presence of 6 mM $MgCl_2$ and stored in 1-ml aliquots at −70°C. Virus production was measured by p24 enzyme-linked immunosorbent assay (Coulter) or reverse transcriptase (RT) assay, using both a ^{32}P-based RT assay (33) and a ^3H-based RT scintillation proximity assay kit that is in development from Amersham International. The latter is a modification of a previously reported method (29).

Infection of cells with virus. Virus stocks were assayed for infectivity in the T-cell line C8166. A total of 5×10^5 cells were incubated in 1 ml of virus (titer of approximately 50 ng of p24) for 1 h at 37°C and then washed and plated in 2 ml of fresh medium (RPMI, 10% fetal calf serum). Infectivity was scored 3 to 4 days later by the appearance of syncytia. All of the observations reported here were made with between four and eight independently produced virus stocks for each mutant. Virus stocks were also used to infect the T-cell line H9. A total of 3×10^6 cells were incubated with equal amounts of virus in a total volume of 1 ml for 1 h and then washed and plated in 5 ml of medium. Samples of the culture supernatant were taken every 3 to 4 days and assayed for RT activity to monitor virus production. These infections were performed with at least two independently produced virus stocks for each mutant tested.

Analysis of viral proteins. COS-7 or 293T cells were transfected with proviral DNA as described above. Sixty hours later, virus from the filtered culture supernatant was concentrated by ultracentrifugation at 100,000 rpm for 1 h in a TL100.4 rotor (Beckman Instruments Ltd.), resuspended in 100 μl of loading buffer, and stored at −70°C. Aliquots were heated to 90°C for 5 min before analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. HIV-1 proteins were detected by Western blotting (immunoblotting), using pooled sera from HIV-1-infected donors at a dilution of 1:500. A rabbit antiserum directed against an amino-terminal IN peptide (residues 3 to 18) was made by S. Ranjbar and obtained from the MRC AIDS Directed Program Reagent Project (ADP419). In addition, a rabbit antiserum made against a hybrid Ty-integrase virus-like particle (residues 141 to 288) was supplied by British Biotechnology Ltd. Both of these anti-IN sera were used at a dilution of 1:500. The secondary antibodies used were horseradish peroxidase-conjugated goat anti-human immunoglobulin and goat anti-rabbit immunoglobulin (Vector), used at dilutions of 1:1,000 and 1:4,000, respectively. Specific proteins were visualized by the enhanced chemiluminescence detection system (Amersham).

PCR analysis. C8166 cells were infected with DNase-treated virus for 1 h as described above. Total DNA was isolated from 10^5 cells at time zero or 48 h following infection by resuspending the cells in 200 μl of lysis buffer containing proteinase K and Triton X-100 (7). Following incubation at 65°C for 2 h and then 95°C for 15 min, PCR was performed on 10 μl of the sample in a 20-μl final volume. In general, the PCR mixtures contained final concentrations of 2.5 mM $MgCl_2$, 4 mM deoxynucleoside triphosphates, 1× *Taq* polymerase reaction buffer (Promega), and oligonucleotide primers at 10 ng of each per ml, with 0.1 U of *Taq* polymerase (Promega) per reaction. The PCR conditions were 35 to 40 cycles at 95°C for 1 min, 61°C for 2 min, and 72°C for 3 min. PCR primers used to detect two-LTR circles were as described previously (38). The 595-bp band produced was detected by ethidium staining, and the identities of the bands were confirmed by Southern blotting using either a ^{32}P-end-labelled oligonucleotide probe specific for the U3 region of the LTR (5′-TGGAAGGGCTAAT TCACTCCCAA-3′) or a riboprobe containing the apical bases of the TAR stem-loop structure (TAR sequence, 5′-CCA GAUCUGAGCCUGGGAGCUCUCUGG-3′).

RESULTS

Generation of virus containing point mutations in the integrase gene. Comparisons of the IN sequences from differ-

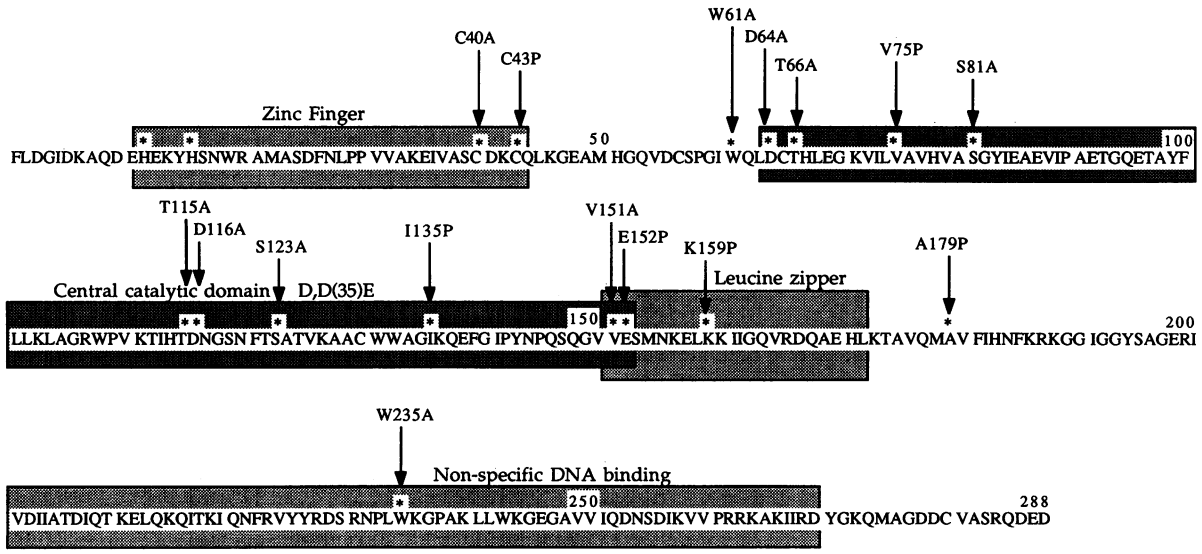

FIG. 1. HIV-1 integrase. The complete amino acid sequence of pBH10 IN is shown. The positions of 18 residues that are completely conserved with RSV, Mo-MLV, HTLV-I, bovine leukemia virus, and mouse mammary tumor virus are identified by asterisks. The 16 single-site mutations are arrowed. Putative IN domains are highlighted. These include an amino-terminal region with conserved HHCC residues (19), a central catalytic domain with invariant D, D, and E residues (27), a potential leucine zipper (31), and a carboxy-terminal region possessing nonspecific DNA binding activity in vitro (42).

ent retroviral species have identified several highly conserved residues. An alignment of HIV-1 IN with those of RSV, bovine leukemia virus, Moloney MLV (Mo-MLV), human T-cell leukemia virus type I (HTLV-I), and mouse mammary tumor virus, for example, produces 18 completely conserved positions (Fig. 1). Substitutions of amino acids were made at 16 of these positions in the HIV-1 IN gene from pBH10 and cloned into the infectious proviral clone WI3. In addition, a control virus (WI3ΔIN) which contains a truncated protein caused by a frameshift mutation at the central *Eco*RI site was made. On the basis of similar constructions described elsewhere (38), this virus will be incapable of integration and therefore noninfectious.

Virus stocks were generated from each of the mutant proviral clones, using transient transfection of the plasmids into COS-7 and 293T cells. This one-step method of generating virus is not dependent on virus-directed integration and therefore will not discriminate against nonintegrative mutants. The virus stocks so generated were measured for p24 concentration and RT activity. Approximately equal amounts of p24 were produced by all of the constructs (typically 1 μg/ml), suggesting efficient transfection and expression of the various plasmid DNAs (data not shown). In addition, all of the virus stocks had reasonable levels of RT activity and a p24/RT ratio that was similar to that of the wild-type WI3 (Table 1).

Analysis of infectivity of viruses containing integrase mutations. Mutant viruses were tested for the ability to establish an infection in T-cell lines. Stocks of virus were standardized for p24 content, and equivalent amounts of virus were used to infect C8166 cells. Three to four days following infection, the C8166 cells were inspected for the appearance of syncytia. The wild-type virus WI3 and virus WI3ΔIN containing the truncated IN were included as positive and negative controls, respectively. Five mutant viruses produced visible syncytia (Table 1). Mutants $WI3_{T66A}$, $WI3_{T115A}$, $WI3_{S123A}$, and $WI3_{V151A}$ produced syncytia at the same rate as WI3, but with mutant $WI3_{K159P}$, the appearance of syncytia was delayed by 24 h. The remaining mutations abolished the ability of the viruses to cause any visible cytopathic effects in C8166 cells. These observations were consistent for several independently produced stocks of each mutant virus. Furthermore, no viral replication could be detected for any of the non-syncytium-

TABLE 1. Properties of HIV-1 IN mutants[a]

Virus	p24/RT ratio[b]	Infectivity[c]	In vitro activity[d]
WI3 (wild type)	1.0	+	
WI3ΔIN	1.2	−	
$WI3_{C40A}$	1.2	−	C40S partial (D only) (14)
$WI3_{C43A}$	0.8	−	C43L partial (D only) (39 [for HIV-2 IN])
$WI3_{W61A}$	1.5	−	ND
$WI3_{D64A}$	0.9	−	D64A inactive (11)
$WI3_{T66A}$	0.8	+	T66A active (14)
$WI3_{V75P}$	1.3	−	ND
$WI3_{S81A}$	0.9	−	S81A active (39 [for HIV-2 IN])
$WI3_{T115A}$	1.0	+	T115A active (14)
$WI3_{D116A}$	1.5	−	D116A inactive (11)
$WI3_{S123A}$	1.1	+	S123A active (14)
$WI3_{I135P}$	1.0	−	ND
$WI3_{V151A}$	1.1	+	ND
$WI3_{E152P}$	1.2	−	E152A inactive (11)
$WI3_{K159P}$	0.9	+	K159V active (39 [for HIV-2 IN])
$WI3_{A179P}$	0.9	−	ND
$WI3_{W235A}$	1.1	−	W235E active (30)

[a] Single amino acid mutations were introduced into infectious clone WI3 as indicated. In addition, WI3ΔIN contains a truncated IN gene caused by a frameshift mutation.

[b] p24 concentration and RT activity were measured for at least two independently produced virus stocks, and the p24/RT ratio was calculated. Values were normalized to 1 for the wild-type virus WI3.

[c] Assessed by the ability to induce syncytia (+) in C8166 cells 3 to 4 days following infection.

[d] Activity of HIV-1 or HIV-2 mutant IN protein in in vitro integration assays. Mutated residues are as indicated. Sources of data are indicated by reference numbers in parentheses. D, disintegration activity; ND, not determined.

producing viruses by supernatant p24 analyses up to 21 days following infection (data not shown). We also tested for the possibility that the nonfunctional mutants were temperature sensitive by carrying out the transfections and subsequent infections with the mutant viruses at 32.5°C. However, no syncytia could be detected up to 8 days postinfection at this lower temperature.

Replication kinetics of viable mutants. Although five mutant viruses produced syncytia in C8166 cells, it remained possible that their ability to integrate had been compromised by these mutations. The kinetics of their infections was therefore compared with that of the wild-type virus in an infection of H9 cells. A total of 5×10^6 H9 cells were infected with 1 ml of virus stock containing equivalent amounts of p24, and the infection was monitored by taking supernatant samples every few days and measuring the RT activity. This analysis demonstrated that viruses $WI3_{T66A}$ and $WI3_{T115A}$ (Fig. 2A) and viruses $WI3_{S123A}$ and $WI3_{V151A}$ (Fig. 2B) replicated with kinetics similar to that of WI3. The mutant virus $WI3_{K159P}$, however, replicated markedly more slowly than the wild-type parent (Fig. 2C). The growth of this virus after 18 days was not due to reversion or suppression of the mutation, as virus taken at this time point showed the same delayed growth kinetics in a new infection (data not shown). The growth curves were repeated at least once for each mutant, using independently produced virus stocks to confirm these observations (data not shown). In addition, the ability of each of these viruses to replicate at a higher temperature (42°C) in C8166 cells was tested in case the mutant IN proteins proved to be less stable. All five of the mutants could still produce syncytia at the higher temperature, although the appearance of syncytia with virus $WI3_{K159P}$ was again delayed by 24 h relative to WI3.

Analysis of viral proteins. The absolute levels of p24 and RT activity in the supernatants of the transfected 293T cells were similar for all of the panel of mutant viruses, and the ratios of p24/RT were also normal, suggesting that the mutations that we had created had no gross effect on virus particle structure or production. To confirm this, viral particles were concentrated from the supernatants of transfected cells by ultracentrifugation and analyzed by Western blotting. HIV-1 proteins were detected with pooled human patient sera, and the identities of individual bands were confirmed by using a panel of more specific antibodies (data not shown). The truncated IN mutant, WI3ΔIN, as expected did not produce a protein corresponding to normal full-length IN (Fig. 3, lane 2). (This mutation is predicted to code for a protein of approximately half the normal size, which may or may not be stable.) The protein profiles produced by the majority of the mutants were identical to the wild-type profile (Fig. 3 and data not shown). However, four viruses had abnormal protein profiles; the mutations V75P, W61A, and I135P always produced viruses with a relatively low amounts of IN protein compared with the other viral proteins (lanes 3, 4, and 7), and no IN protein could be detected for virus $WI3_{W235A}$ (lane 9). Analysis of $WI3_{W235A}$ particles grown at 32.5°C also failed to show any IN protein, and no IN could be seen in extracts of transfected cells (data not shown). Because the profile of this virus on Western blots mirrored that of the truncated IN construct WI3ΔIN, we were concerned that a second-site mutation could have arisen during our DNA manipulations. We therefore reconstructed the virus from the original M13 clone and sequenced across the entire IN coding region. The phenotype of this second $WI3_{W235A}$ mutant virus was the same as the original (data not shown).

Residue W235 is part of an immunodominant epitope of IN. The apparent absence of IN in the Western blots of the

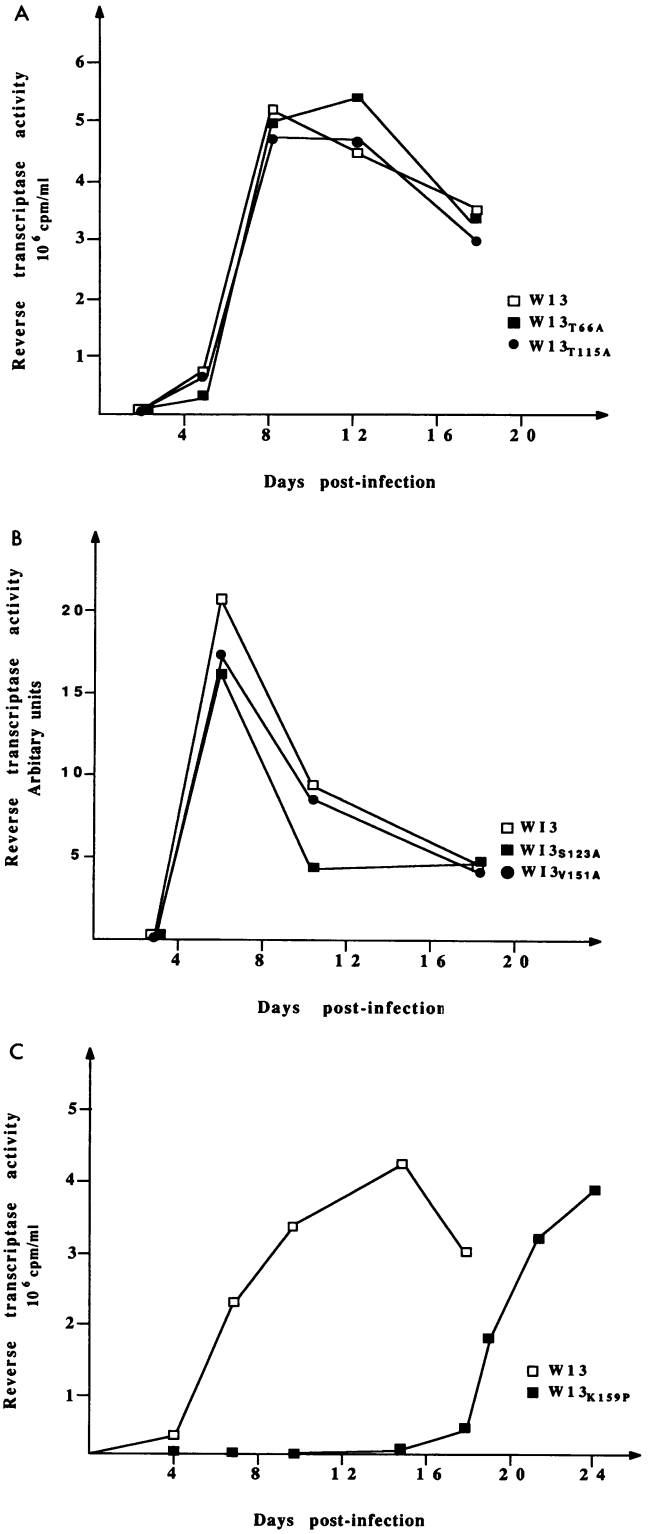

FIG. 2. Replication kinetics of viable mutants. Equivalent amounts of wild-type (WI3) and mutant viruses were used to infect H9 cells. Virus production was monitored for 18 days by analyzing RT activity in the culture supernatants by a ^3H-based scintillation proximity assay (A and C) or a ^{32}P-based assay (B).

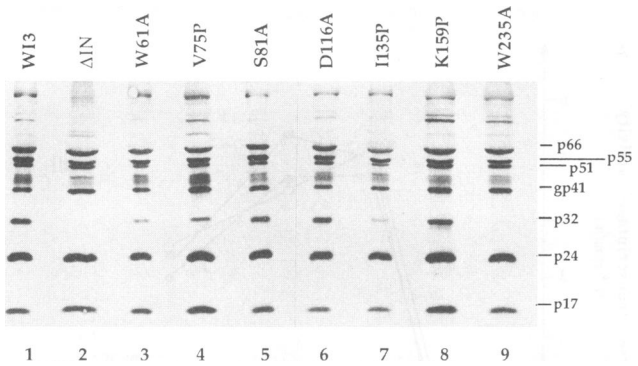

FIG. 3. Immunoblot analysis of viral proteins. Virions were concentrated from the supernatant of transfected 293T cells by ultracentrifugation and separated on an SDS–12.5% polyacrylamide gel. HIV-1 proteins were detected with pooled human patient serum. The positions of the major viral proteins are shown.

$WI3_{W235A}$ viral particles was unexpected. It was possible that mutation of W235 had produced a protein that was very unstable or could no longer be incorporated into the virion. As IN is presumed to be targeted to the virion as part of the larger *gag-pol* precursor, it was unlikely that the latter explanation was correct. Furthermore, there was no apparent effect on the relative amounts of the other viral proteins, RT activity was normal, and the ratio of p24 levels to RT activity was very similar to that of the wild-type virus. Similarly, if this mutation had caused any structural changes in IN that prevented correct processing by the viral protease at the RT/IN junction, this would be reflected in reduced levels of the processed RT proteins on the Western blots. We therefore considered the possibility that the mutant protein was no longer recognized by the patient sera that we were using as the source of anti-IN antibodies, even though this serum was pooled from three different individuals. Accordingly, we tested both the wild-type and the W235A mutant viruses with two additional anti-IN sera, one derived from inoculation of rabbits with an amino-terminal peptide (residues 3 to 18) and one made against a Ty-virus-like particle containing residues 141 to 288. As expected, the wild-type IN could be detected by both of these antisera (Fig. 4). The W235A protein, as before, failed to be detected by the patient sera and was also not detected by the polyclonal serum made against residues 141 to 288. However,

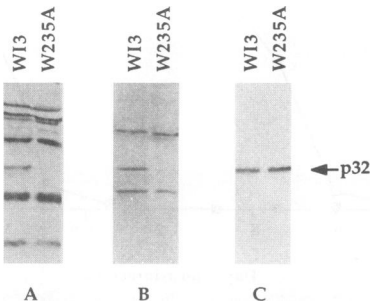

FIG. 4. Mutation W235A prevents recognition by a major epitope in human HIV-1-positive serum. WI3 and $WI3_{W235A}$ virions were subjected to immunoblot analysis using pooled human HIV-1-positive sera (A), a rabbit antiserum directed against residues 141 to 288 (B), and a rabbit antiserum directed against amino-terminal residues 3 to 18 (C). The position of p32 IN is arrowed.

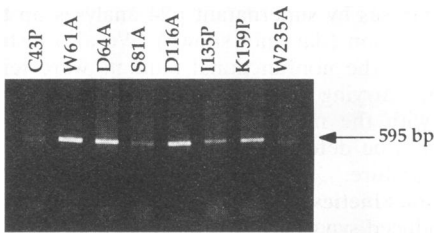

FIG. 5. PCR of two-LTR circles. Extracts of C8166 cells infected 3 days previously with virus stocks were subject to 40 cycles of PCR amplification using primers specific for the two-LTR circle junction. Amplification of such a structure results in a 595-bp band.

the antiserum raised against residues 3 to 18 reacted with both the W235A protein and the wild type, revealing approximately equal levels of each in the virions. The substitution of W235 had therefore prevented recognition of IN by the pooled patient sera, suggesting that W235 is essential for an immunodominant epitope in IN.

Formation of two-LTR circles by nonreplicative mutants. We used PCR analysis to confirm that the noninfectious mutants were not blocked in a step of the life cycle prior to integration. Two-LTR circles are a form of retroviral DNA present in the nucleus. They are thought to be produced by host enzymes as an alternative to correct integration by IN and are therefore formed in the absence of a functional IN (2). Two-LTR circle junctions are not present in the input plasmid DNA used in the initial transfection (which can be carried over into the infections and contaminate subsequent PCRs). The PCR amplification of such a structure is therefore confirmation of entry, uncoating, and reverse transcription by the mutant viruses. Using the primers described in Materials and Methods, we could amplify a 595-bp fragment corresponding to the two-LTR circle junction for all of the noninfectious mutants (Fig. 5 and data not shown). This finding indicates that the block to productive infection occurs at a step in the life cycle after reverse transcription and is presumably at integration.

DISCUSSION

We have mutated 16 residues in the integrase gene of HIV-1 at positions that are highly conserved among retroviruses and introduced them into infectious proviral clones. The mutations had no gross effect on viral particle structure or levels of virus production, as assessed by Western blotting and p24 measurement. Furthermore, all of the mutants displayed wild-type levels of RT activity in in vitro assays and were competent for all stages of the HIV-1 life cycle up to and including reverse transcription, as demonstrated by the production of two-LTR circles. The inability of several of the mutant viruses to establish an infection in T-cell lines is therefore highly likely to be the result of a defect in integration.

Zinc finger mutants (C40A and C43P). The amino termini of all retroviral IN proteins contain two conserved histidines and two conserved cysteines, separated by the spacing H_3 $H_{22-32}C_2C$ (19). This structure is similar to the zinc finger motif found in some transcription factors, and the region has been demonstrated to bind zinc (5). Because of the known DNA binding properties of zinc fingers, it has been proposed that this structure may be involved in the interaction of IN with DNA. In vitro binding assays have not detected any LTR-specific or nonspecific affinity for DNA in this region, and indeed the nonspecific DNA binding ability of whole IN has

been mapped to its carboxy terminus (42, 45). However, other analyses suggest an involvement of this region in LTR recognition (21, 41).

Substitution of any one of the conserved histidine or cysteine residues in this motif would be expected to disrupt the putative zinc finger, but mutation of IN at these residues does not completely abolish catalytic activity in vitro. Disintegration activity remains unaffected (14), and two groups report nearly wild-type levels of strand transfer activity for a C43S substitution of HIV-1 (11) and a C40S mutation in HIV-2 (39). However, mutation of the conserved cysteines in the context of the provirus completely abolished infectivity, suggesting a more rigid requirement for this region in vivo. These data are in agreement with previous studies of the effects of mutations in the analogous region of MLV IN (9, 35).

Central catalytic domain. Amino acids D64, D116, and E152 are the key invariant residues in the central catalytic domain of IN. This motif is also conserved across retrotransposons and prokaryotic transposases, and the carboxylic acid side chains of these acidic residues have been proposed to be involved in the coordination of the divalent metal cations needed for IN function, such as Mg^{2+} or Mn^{2+} (27). Mutation of these residues has been shown to have the most drastic effect on all in vitro IN activities (11, 14, 27, 28, 30, 39) and not unexpectedly produces completely noninfectious viruses. Other groups have also reported noninfectious viruses for the mutations D116A and E152A (37) and also for the V151D E152Q double mutation (28).

None of the $D,D_{35}E$ substitutions had any effect on the profile of the viruses on Western blots, and all three had wild-type levels of RT activity. This finding contrasts with the observation by Shin et al. (37) that a D116A mutation in an HXB2 provirus has a gross effect on Gag protein processing and abolishes in vitro RT activity. In our hands, the identical substitution in a similar proviral backbone (WI3 is a *nef*$^+$ version of HXB2) has no such effects. At present, we cannot account for these discrepancies.

Other conserved residues in this region, including V75, S81, and I135, abolished infectivity when they were mutated. Viruses containing mutations V75P and I135P, together with W61A, appeared to have abnormally low amounts of IN protein in the viral particles relative to the wild type. As all three of these mutant particles had wild-type levels of RT activity, normal RT/p24 ratios, and expected levels of RT p66 on Western blots, reduced recognition by the human antisera used for Western blotting is the most likely explanation for this observation.

Mutation of S81 to alanine also produced a noninfectious virus, but the data from in vitro studies for this residue are conflicting. Several different substitutions at this residue in both HIV-1 and RSV IN have been reported to severely affect 3′ processing and strand transfer (23), and other reports state that mutations at this site produce a protein of low solubility that is difficult to purify (30). Despite its lower solubility, one group has been able to purify enough of an S81A mutant of HIV-2 to test and reported wild-type activity in all of their assays (39). This finding suggests that substitutions at this residue may affect protein folding rather than catalytic activity directly and that such mutations are better tolerated in some expression configurations than others.

Leucine zipper (V151A, K159P, and A179P). These residues lie within a putative helical region of the protein containing a possible leucine zipper (31). Residues V151, L158, V165, and L172 could contribute a hydrophobic face to a helix which could function as a dimerization domain for IN. The introduction of helix-breaking residues (proline) at A179 and in the center of the region at the highly conserved K159 would be expected to disrupt such a structure, and the resulting viruses were either completely ($WI3_{I179P}$) or partially ($WI3_{K159P}$) defective in their replicative ability.

$WI3_{K159P}$ replicated at a reduced level compared with the wild type, suggesting that this mutation had compromised integration. The ability of this mutant to replicate at all was especially surprising since K159 is one of the most highly conserved of all the residues (14). The alignment of the potential members of the leucine zipper also abuts residues E152, K159, and R166 (31), with the first two residues being invariant and R166 also highly conserved. E152 is a member of the proposed active site of IN, and the side chains of these adjacent amino acids may be essential for the correct environment for this residue. However, the tolerance of K159 to substitution has also been demonstrated by the wild-type replication kinetics seen in SupT1 cells for the somewhat less disruptive replacement by a glutamine (37).

The location of V151 suggests that as well as being important for leucine zipper formation, it may also influence the adjacent catalytic domain residue, E152. Accordingly, the mutation that we chose to make at this site was fairly conservative and resulted in no effect on viral replication. It is possible that a different mutation would have had a greater effect.

Nonspecific DNA binding (W235A). The carboxy terminus of IN is the area of least sequence homology among different retroviruses. In HIV-1, it has been shown to account for the nonspecific DNA affinity of the protein which may be involved in target DNA interactions (42, 45). Mutation of the highly conserved tryptophan in this region to a glutamate has previously been shown to have no effect in any in vitro integration assay (30), and the authors did not report any difficulty purifying and assaying the mutant protein. However, our W235A mutation totally abolished the ability of the provirus to replicate, so it is clearly an important residue for in vivo function of IN. We speculate that this region may therefore be involved in the correct positioning of the processed retroviral LTRs to interact with the target host cell DNA, either by virtue of an inherent affinity for DNA or by interactions with protein molecules associated with the chromatin.

W235 is probably also a key component of a major immunodominant region of IN. The observation that reactivity with both the pooled human sera and the rabbit serum is lost in the W235A mutant supports this notion and suggests that W235 is essential for the integrity of some local structure that is preserved in Western blotting. It is interesting that a large insertion is present just upstream of the equivalent tryptophan residue in the Mo-MLV IN relative to other retroviruses and, in addition, that Mo-MLV IN can tolerate an insertion of a further 36 amino acids into this adjacent region and still retain function (34). This finding is consistent with a role for this residue in key structural interactions within the core of the protein, possibly adjacent to a looped-out region of somewhat variable length.

Viable mutants (T66A, T115A, S123A, and V151A). It was surprising that four highly conserved residues in the central region (T66 and T115, together with S123 and V151) could be mutated without affecting the replicative ability of the viruses in T-cell lines. To guard against the possibility of revertant wild-type viruses in these cultures, we repeated these infections in H9 and C8166 cells several times with independently produced virus stocks. In every experiment, these mutants showed the same phenotype as the wild-type virus. Previously, mutation T66A has been shown to be defective in 3′ processing in vitro (22% of wild-type activity) and to be partially defective

for integration (42% of wild-type activity) (14), although others have reported wild-type activity for mutations of the equivalent residue in RSV IN (23, 27). Mutation of residue T115 has also produced variable results in in vitro analyses. Several substitutions at this residue have been reported to retain wild-type activity in all in vitro assays by some groups (14, 27, 39), but a T115A substitution is reported to be selectively defective in strand transfer activity by another (11). Furthermore, the same T115A substitution that we introduced has also been reported to have no effect on HIV-1 infectivity in a T-cell line (37).

Varying in vitro observations have been made also for proteins mutated at S123. The same serine-to-alanine replacement that we made resulted in an insoluble protein for HIV-2 IN (39) but produced a protein with almost wild-type in vitro activity for HIV-1 (14). No studies have been reported for single mutations at V151, although a double mutant, V151D/E152Q, is defective both in vitro and in the context of the provirus (28). However, it is not possible to deduce anything about the role of V151 from this double mutant, as the substitution of E152 alone is known to be deleterious.

Overall, our analysis of the effect of mutations at conserved sites in the integrase gene on viral replication fits with much of the current data on IN. The drastic effect of mutations at key residues in the $D,D_{35}E$ region was as expected, and the absolute requirement for C40 and C43 in vivo confirms an important role for the HHCC region in IN function. Residue W235 has the interesting phenotype of being completely nonessential to IN activity in vitro while abolishing integration in vivo. It will be interesting to analyze at which stage in the integration process this block occurs. The finding that four highly conserved residues can be mutated without deleterious effect on viral replication is also intriguing. It raises the possibility that these mutations have a phenotype that is not apparent in T-cell lines, and we are currently analyzing the role of these residues in integrase function in primary cells.

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