Genetic Analysis of the Human Immunodeficiency Virus Type 1 Integrase Protein

CHA-GYUN SHIN,[†] BRUNELLA TADDEO,[‡] WILLIAM A. HASELTINE,[§] AND CHRIS M. FARNET*

Division of Human Retrovirology, Dana-Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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Single-amino-acid changes in a highly conserved central region of the human immunodeficiency virus type 1 (HIV-1) integrase protein were analyzed for their effects on viral protein synthesis, virion morphogenesis, and viral replication. Alteration of two amino acids that are invariant among retroviral integrases, D116 and E152 of HIV-1, as well as a mutation of the highly conserved amino acid S147 blocked viral replication in two CD4⁺ human T-cell lines. Mutations of four other highly conserved amino acids in the region had no detectable effect on viral replication, whereas mutations at two positions, N117 and Y143, resulted in viruses with a delayed-replication phenotype. Defects in virion precursor polypeptide processing, virion morphology, or viral DNA synthesis were observed for all of the replication-defective mutants, indicating that changes in integrase can have pleiotropic effects on viral replication.

The integration of a DNA copy of the viral RNA genome into the genome of the infected cell is an essential step in the retroviral replication cycle (57). Genetic studies have shown that two regions of the viral genome are essential for the establishment of an integrated provirus: the ends of the linear viral DNA molecule, including the sequences that are actually joined to the host DNA target (7–9, 18, 42), and the 3' region of the *pol* gene, encoding the integrase protein (6, 14, 15, 43, 46, 48, 50).

Recent *in vitro* studies of the enzymatic activities of purified retroviral integrases have provided the following model of the role of the integrase protein in the retroviral integration reaction (3, 10, 19, 31, 51, 60). The long terminal repeat (LTR) edges at the ends of the linear DNA molecule contain specific *cis*-acting sequences recognized by the integrase protein. The integrase protein catalyzes the endonucleolytic removal of the two 3'-terminal nucleotides from each end of the linear viral DNA molecule in a reaction referred to as the donor cleavage reaction. The newly generated, recessed 3'-hydroxyl termini are then joined to cellular DNA through an integrase-mediated nucleophilic attack on phosphodiester bonds located on the cellular DNA target (the DNA strand joining reaction). Both the donor cleavage reaction and the DNA strand joining reaction are catalyzed by purified retroviral integrases *in vitro* by using short duplex oligonucleotides that match the ends of linear viral DNA as substrates. In addition, purified integrases can catalyze the reversal of the DNA strand joining reaction *in vitro* in a process termed disintegration (5).

Mutational analysis of the *in vitro* enzymatic activities of retroviral integrases has identified three potential functional regions of the protein. The N terminus of the protein contains an array of histidine and cysteine residues that is well conserved among retroviral integrases (16, 29, 32) and is believed to constitute a zinc finger-like domain (2, 4, 29). The N-

terminal domain is required for the donor oligonucleotide cleavage and DNA strand joining reactions *in vitro* and may play a role in the specific recognition of viral DNA sequences present at the ends of the linear viral DNA molecule (19, 29a, 38, 56, 58). The C-terminal region of the protein is the least conserved region among retroviral integrases and most likely forms the major DNA-binding domain of the protein (49, 55, 59, 63, 64). The most highly conserved region of retroviral integrases lies in the central region of the protein. The central region is defined by three acidic amino acid residues in a highly conserved spatial arrangement [the "D, D(35)E" motif] (32, 34). Mutations in any one of the three invariant aspartic or glutamic acid residues coordinately block the donor cleavage, DNA strand joining, and disintegration activities of integrases *in vitro* (17, 20, 34, 36, 38, 56). These studies suggest that a single active site is used to catalyze all of the polynucleotidyl transfer reactions and that the conserved aspartic and glutamic acid residues contribute to the active site of the enzyme (19, 60).

While the enzymatic activities of retroviral integrases are becoming clearly defined through the use of *in vitro* integration assays, other potential roles played by the integrase protein in the establishment of a provirus in an infected cell may be more readily identified by genetic analyses. For example, some features of the integration process, such as the coordinated joining of the two viral DNA ends to precisely spaced phosphodiester bonds in target DNA, the organization of viral DNA and integrase protein into a stable and functional preintegration complex, the entry of the preintegration complex into the nucleus of the cell, and the features of the preintegration complex that serve to prevent the autointegration of viral DNA, may involve functions of the integrase protein that are not assayed by the current oligonucleotide-based integration assays. Dissection of the roles of the integrase protein in viral replication will be facilitated by the identification of single-amino-acid substitutions throughout the integrase protein that result in viruses whose only defect is the conversion of the linear viral DNA molecule into an integrated provirus. As a first step toward this goal, a number of point mutations in the highly conserved central region of the human immunodeficiency virus type 1 (HIV-1) integrase pro-

* Corresponding author. Mailing address: Dana-Farber Cancer Institute, 44 Binney St., JFB824, Boston, MA 02115. Phone: (617) 632-4332. Fax: (617) 632-4338.

[†] Present address: Department of Biotechnology, Chung Ang University, Ansong Kyunggi-do, Korea 456-756.

[‡] Permanent address: Istituto Superiore di Sanita, Rome, Italy.

[§] Present address: Human Genome Sciences, Inc., Rockville, Md.

tein were constructed and analyzed for their effects on virus replication in tissue culture cells.

MATERIALS AND METHODS

Construction of mutants. Plasmid targets for in vitro mutagenesis were created by subcloning regions of the *pol* gene of the infectious HXBc2 proviral DNA clone (25). To minimize the likelihood of secondary mutations, small fragments of the *pol* gene encoding portions of the integrase protein were used for site-directed mutagenesis. For mutations in the region of integrase amino acid D116, a 571-bp fragment spanning the *MscI* and *NdeI* sites of HXBc2 (nucleotides 4551 to 5122, according to the numbering scheme of Myers et al. [40]) was subcloned into pGEM11Zf(+) (Promega), and the resulting plasmid was used as a target for site-directed mutagenesis as described before (35). For mutations in the region of integrase amino acid E152, a 474-bp fragment spanning the *EcoRI* and *NdeI* sites of HXBc2 (nucleotides 4648 to 5122) was subcloned into pGEM72f(+), and the resulting plasmid was used as the target for mutagenesis. The mutagenic oligonucleotides used are shown in Fig. 1. The presence of the mutation was confirmed by DNA sequence analysis. To regenerate complete proviral DNA clones, fragments carrying the desired mutation were reinserted into the parental vector pHXB-SV (13), which contains the full-length HXBc2 provirus and provides a simian virus 40 origin of replication for episomal propagation in the COS-7 monkey kidney cell line.

Cell culture, transfection, and infection. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. COS-7 cells (2×10^6) were seeded into 100-mm culture dishes 24 h prior to trans-

fection. The cells were transfected with 10 μ g of proviral DNA by a DEAE-dextran procedure (39). Virus production was evaluated by measurement of reverse transcriptase activity 48 h posttransfection as described previously (12). Alternatively, virion release was measured by using a commercially available HIV-1 p24 enzyme-linked immunosorbent-assay (ELISA) kit (NEN/DuPont, Beverly, Mass.). Human SupT1 and Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. SupT1 or Jurkat cells (10^6) were infected with cell-free virus equivalent to 50,000 cpm of reverse transcriptase activity. Two-thirds of the culture medium was replaced with an equal volume of fresh medium every third day. Virus production was monitored by measuring the culture supernatant reverse transcriptase activity every third day.

Metabolic labeling and immunoprecipitation. COS-7 cell cultures were metabolically labeled for 14 h in medium containing [35 S]cysteine (50 μ Ci/ml) 48 h after transfection. Equivalent numbers of labeled cells were lysed in RIPA buffer (140 mM NaCl, 8 mM Na_2HPO_4 , 2 mM NaH_2PO_4 , 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate [SDS]), viral proteins were immunoprecipitated from the cell lysates with an HIV-1-infected patient serum, and the proteins were analyzed by electrophoresis on a 10 to 15% (wt/vol) polyacrylamide gradient gel and visualized by fluorography.

For pulse-labeling experiments, COS-7 cells were labeled for 20 min before the culture medium was replaced with fresh medium lacking [35 S]cysteine. For the analysis of virion proteins, the labeling medium was collected and cleared of cell debris by centrifugation. Virions were pelleted through a 20% sucrose cushion by centrifugation at $25,000 \times g$ for 1 h and lysed in RIPA buffer, and the virion proteins were immunoprecipitated and analyzed as described above.

Electron microscopy. Forty-eight hours after transfection, COS-7 cells were fixed for 1 h at room temperature and then for 12 h at 4°C in 4% (vol/vol) glutaraldehyde–3% (vol/vol) paraformaldehyde in phosphate-buffered saline. Further processing of the fixed COS-7 cells for thin-section electron microscopy was done as described before (12).

Detection of viral DNA by PCR. The synthesis of viral DNA in SupT1 cells infected with the replication-defective integrase mutants and wild-type virus was detected by a PCR technique similar to ones described previously (36, 48). Equivalent doses (100,000 cpm of reverse transcriptase activity) of virus from COS-7 cell supernatants following transfection with wild-type or S147I or E15A mutant proviruses were used to infect one

FIG. 1. Mutation of the HIV-1 integrase protein. (A) Proviral DNA is shown at the top. The *gag*, *pol*, and *env* open reading frames are shown, surrounded by the directly repeated LTRs. The 288-bp integrase protein encoded by the 3' region of the *pol* gene is enlarged below. The central region of the integrase protein targeted for mutagenesis is shown in single-letter code from amino acids T115 to K160. Amino acids that were changed by mutagenesis are underlined, with the amino acid substitution indicated. (B) Mutagenic oligonucleotides used for the construction of the mutations shown in panel A. Nucleotides corresponding to the wild-type HIV-1 integrase sequence are shown in uppercase letters, and the nucleotide changes introduced to create the mutation are shown in lowercase letters.

million fresh SupT1 cells. Because cells transfected with mutant D116A did not release reverse transcriptase activity into cell supernatants, a direct comparison of this mutant with the other replication-defective mutants and wild-type virus could not be made. Twenty-four hours after infection, total DNA was prepared from the infected cells by standard techniques (39). Total DNA was treated with restriction endonuclease *DpnI* prior to PCR to inactivate potentially contaminating plasmid DNA carried over from the COS-7 cell transfections. One microgram of endonuclease-treated total DNA was subjected to 35 rounds of PCR amplification with primers env1 (nucleotides 7950 to 7969 of HXBc2) and env2 (nucleotides 8545 to 8526 of HXBc2) in the envelope region with a commercially available kit and conditions specified by the manufacturer (Perkin-Elmer Cetus). The products of amplification were separated on a 1.5% agarose gel, blotted onto a nylon filter, and hybridized with an envelope-specific oligonucleotide probe (env3; nucleotides 8285 to 8308 of HXBc2) labeled at its 5' end with [γ - 32 P]ATP by standard techniques (39). As a control for plasmid contamination, 10 ng of plasmid pHXB-SV, treated with *DpnI* or not treated, was used as a substrate for amplification under the same conditions. The HXBc2 viral DNA sequence contains five *DpnI* sites located between the primer sequences used for amplification. The plasmid DNA used for transfection of COS-7 cells is digested to completion by *DpnI*, which requires a fully methylated recognition sequence for cleavage; viral DNA synthesized in the cell following infection is fully unmethylated and resistant to cleavage by *DpnI* (data not shown). As a further control to eliminate the possibility of plasmid contamination from the COS-7 cell transfections, PCR experiments were also performed as described above after infection of SupT1 cells with supernatants prepared from stable SupT1 cell lines chronically producing viral mutant S147I or E152A (data not shown). These stable cell lines were constructed by cotransfection of mutant proviral DNA clones and a neomycin resistance plasmid into SupT1 cells, followed by selection of neomycin-resistant, virus-producing cell lines (54a).

RESULTS

Effects of integrase mutations on viral protein synthesis and processing. Single-amino-acid substitutions were introduced into the highly conserved central region of the HIV-1 integrase protein. The nature and location of each point mutation are indicated in Fig. 1. Each of the mutations was introduced into an HXBc2 provirus, and wild-type and mutant proviral DNAs were transfected into COS-7 cells. A full complement of 35 S-labeled viral proteins was found by immunoprecipitation of COS-7 cell lysates with an HIV-1-infected patient serum at 48 h after transfection with wild-type and integrase mutant proviruses (Fig. 2). Similar amounts of the processed capsid proteins p24 and p17 were found after transfection of the wild-type proviruses and all of the integrase mutants except for mutant D116A. Cell lysates from COS-7 cells transfected with mutant D116A consistently showed reduced levels of the p24 and p17 proteins, while the levels of the other viral proteins, including the p55^{gag} precursor, were similar to those in the wild type (Fig. 2, lane 4). Thus, the D116A mutant appeared to exhibit a defect in processing of the p55^{gag} precursor protein, while none of the other single-amino-acid changes examined had major effects on the synthesis or processing of viral proteins.

The nature of the *gag* protein defect observed for mutant D116A was further investigated by pulse-chase labeling analysis of viral proteins produced after transfection of COS-7

FIG. 2. SDS-PAGE analysis of viral proteins synthesized in COS-7 cells after transfection with wild-type and mutant proviral DNAs. COS-7 cells were labeled with [35 S]cysteine at 48 h posttransfection, and the viral proteins were immunoprecipitated from cell supernatants and resolved on a 10 to 15% (wt/vol) gradient polyacrylamide gel. The cells were mock transfected (lane 1) or transfected with wild-type (WT, lane 2), T115A (lane 3), D116A (lane 4), N117K (lane 5), G118A (lane 6), Y143N (lane 7), S147I (lane 8), E152A (lane 9), S153A (lane 10), or K159Q (lane 11) mutant proviral DNAs. The relative positions of the HIV-1 proteins are indicated.

cells. As shown in Fig. 3, lanes 2 and 3, similar levels of 35 S-labeled p55^{gag} precursor polyprotein were formed in cells transfected with wild-type and D116A mutant proviruses after a short labeling period with [35 S]cysteine, indicating that viral protein synthesis was not defective in the D116A mutant. The total amount of 35 S-labeled *gag* proteins present in lysates of cells transfected with wild-type provirus decreased during the subsequent chase period (Fig. 3, lanes 5 and 8), presumably reflecting the release of labeled precursor proteins from the cells in the form of virions. However, a considerable fraction of the labeled p55 precursor protein remained cell associated in the form of processed p24 and p17. In contrast, little or no 35 S-labeled *gag* protein was found in lysates of cells transfected with the D116A mutant during the chase period (Fig. 3, lanes 6 and 9). The disappearance of the 35 S-labeled p55^{gag} precursor protein during the chase most likely resulted from the release of the unprocessed precursor from the cell during virion budding (see below). The greatly reduced levels of processed p24 and p17 proteins in the experiment are consistent with the low levels of mature *gag* products seen in the experiment shown in Fig. 2 and suggest that the D116A mutation interferes with the processing of the p55^{gag} precursor polyprotein.

Effects of integrase mutations on virion formation. Virion release from COS-7 cells transfected with wild-type and integrase mutant proviruses was measured 48 h posttransfection by assaying culture supernatants for particle-associated viral proteins and reverse transcriptase activity. A wild-type pattern of viral proteins was found in the supernatants of cells transfected with all of the integrase mutant proviruses except for mutant D116A (Fig. 4, lanes 7, 9, and 10, and data not shown). All of the mutants except D116A also directed the release of wild-type levels of particle-associated supernatant reverse transcriptase activity (data not shown), providing further evidence

FIG. 3. Pulse-chase analysis of viral protein synthesis in COS-7 cells transfected with wild-type and mutant D116A proviruses. COS-7 cells were pulse-labeled with [35 S]-cysteine at 48 h posttransfection with wild-type (WT) or mutant D116A proviral DNAs, as described in Materials and Methods. The cells were lysed immediately after the pulse label (lanes 1 to 3) or after a 3-h (lanes 4 to 6) or 6-h (lanes 7 to 9) chase with unlabeled amino acid, and the viral proteins were immunoprecipitated from the cell lysates and resolved on a 10 to 15% (wt/vol) polyacrylamide gradient gel. Cells were mock transfected (lanes 1, 4, and 7) or transfected with wild-type provirus (lanes 2, 5, and 8) or mutant D116A (lanes 3, 6, and 9) proviral DNAs. The relative positions of HIV-1 proteins are indicated.

that their mutations had no detectable effect on the process of virion formation. In addition, wild-type levels of particle-associated gp120 were found for all of the mutant viruses, indicating that the mutations had no detectable effect on the incorporation of envelope glycoprotein into the budding virions.

The appearance of particle-associated p55^{gag} precursor protein in the supernatant of cells transfected with mutant D116A indicated that virus particles were released from these cells (Fig. 4, lane 8). Greatly reduced amounts of mature p24 and p17 proteins were found in the virions produced by mutant D116A, consistent with the earlier experiments that indicated a defect in p55^{gag} precursor processing for this mutant. Because of the generally high nonspecific background of radiolabeled proteins found in the region of the gel corresponding to the size of p160^{gag-pol}, it was not possible to determine whether unprocessed gag-pol precursor protein was incorporated into virions of mutant D116A (Fig. 4, lane 8). Particle release from cells transfected with the D116A mutant was also confirmed by an HIV-1 p24 ELISA assay (Fig. 5B), which apparently can detect p24 protein sequences in the context of the p55^{gag} precursor. Although viral particles were released from cells transfected with mutant D116A, particle-associated reverse transcriptase activity was not detected in the supernatants of transfected cells (Fig. 5A).

Effects of integrase mutations on virion morphology. Virion

FIG. 4. Viral proteins present in cell lysates and virions after transfection of COS-7 cells with wild-type or replication-defective integrase mutant proviral DNA. COS-7 cells were labeled with [35 S]-cysteine at 48 h after transfection. Viral proteins were immunoprecipitated from cell lysates (lanes 1 to 5) or from virions pelleted through sucrose cushions from culture supernatants (lanes 6 to 10) and resolved on a 10 to 15% (wt/vol) gradient polyacrylamide gel. Cells were mock transfected (lanes 1 and 6) or transfected with wild-type proviral (lanes 2 and 7) or D116A (lanes 3 and 8), S147I (lanes 4 and 9), or E152A (lanes 5 and 10) mutant proviral DNAs. The relative positions of HIV-1 proteins are indicated.

morphology was assessed by thin-section electron microscopy of monolayer preparations of COS-7 cells transfected with wild-type and integrase mutant proviruses. The virions produced by the integrase mutant proviruses fell into three general morphological classes, as shown in Fig. 6. Mutants T115A, N117K, G118A, Y143N, S147I, S153A, and K159Q produced virions with a morphology indistinguishable from that of wild-type virions (Fig. 6A). These virions typically contained an eccentrically placed, cone-shaped, electron-dense core surrounded by a thin envelope layer. In some sections, the core can be seen to include a spherical nucleoid surrounded by the cone-shaped p24 shell. Occasional virion particles having a thick electron-dense shell at the inner circumference of the envelope and an electron-lucent center without a condensed core were produced by these integrase mutants at levels similar to those in wild-type proviruses. These particles most likely represent immature, newly released virions that have not yet undergone precursor processing and core formation (27).

All of the virions produced by cells transfected with mutant D116A had the appearance of immature virions (Fig. 6B). All of the particles produced by mutant D116A contained thick electron-dense outer shells and electron-lucent centers, with no evidence of a condensed core, indicating that this mutation resulted in a defect in virion core formation. Virions produced by mutant E152A showed two general morphological types (Fig. 6C). Some particles contained an eccentrically located, electron-dense spherical core, characteristic of the spherical

FIG. 5. Viral particle release from COS-7 cells transfected with wild-type or mutant D116A proviruses. Forty-eight hours after transfection, culture supernatants were assayed for viral particles by measuring viral reverse transcriptase (RT) activity (A) or p24 antigen (B) as described in Materials and Methods. O.D., optical density.

nucleoid found in wild-type viral particles. Other particles contained, in addition to the spherical nucleoid structure, an adjacent, irregularly electron-dense structure, giving the core of the mutant virion a distended, bilobed appearance.

Replication of integrase mutants in tissue culture. The effects of the integrase point mutations on viral replication were assessed by infecting cells of the human SupT1 T cell line with supernatants prepared from COS-7 cells 48 h after transfection with mutant or wild-type proviruses. Parallel cultures of SupT1 cells were infected with equivalent doses (reverse transcriptase units) of wild-type or mutant virus. The course of virus replication was monitored by supernatant reverse transcriptase level every 3 days.

Three replication phenotypes were observed for the integrase mutants examined after infection of SupT1 cells. Similar results were obtained after infection of Jurkat cells (data not shown), demonstrating that the observed phenotypes were not strictly cell type dependent. Mutants T115A, G118A, S153A, and K159Q had replication profiles indistinguishable from that of the wild-type virus (Fig. 7A). Two of the mutants, N117K and Y143N, exhibited delayed-replication phenotypes (Fig. 7C). For both of these mutants, peak virion production occurred 20 to 30 days postinfection, compared with 10 to 15 days postinfection for wild-type virus. Virus harvested from the supernatants of cells infected with the N117K and Y143N mutant viruses during the peak of replication (day 21 in Fig. 7C) showed similar delays in replication when used to infect fresh SupT1 cells (Fig. 7D), demonstrating that the delayed appearance of peak reverse transcriptase levels displayed by these two mutants was the result of a delayed course of replication and was not due to the appearance of revertant viruses. Two mutants, S147I and E152A, failed to replicate to detectable levels in SupT1 cells, with no detectable virus production for up to 30 days postinfection (Fig. 7B). Both of these mutants also failed to replicate after direct transfection into SupT1 or Jurkat cells (data not shown).

Mutant D116A did not release reverse transcriptase activity after transfection into COS-7 cells, precluding a direct com-

parison of its replication phenotype with those of the other mutants. However, this mutant was shown to be completely replication defective by the following criteria. No virus replication was detected in SupT1 or Jurkat cells infected with supernatants prepared from COS-7 cells 48 h after transfection with D116A mutant proviruses or by cocultivation of the transfected COS-7 cells with fresh SupT1 or Jurkat cells (data not shown). In addition, no virus replication was detected after direct transfection of SupT1 or Jurkat cells with D116A mutant proviruses, even though viral protein production was readily detected by metabolic labeling of the transfected cells with ^{35}S -labeled amino acids (data not shown).

Detection of viral DNA in cells infected with the replication-defective integrase mutants. Mutant viruses having a specific defect in the formation of integrated proviruses are expected to be able to support the synthesis of viral DNA upon infection of a susceptible target cell. Each of the replication-defective HIV-1 integrase mutants was tested for the ability to synthesize viral DNA after infection of SupT1 cells. Viral DNA was readily detected by PCR 24 h after infection of SupT1 cells with wild-type virus or mutant E152A (Fig. 8, lanes 5 and 7). No viral DNA was found in cells infected with mutant S147I (Fig. 8, lane 6). Similar results were found whether virus was prepared from the supernatants of transfected COS-7 cells or from the supernatants of cells chronically producing wild-type or mutant viruses (Fig. 8, lanes 8 and 9, and data not shown). Because cells transfected with mutant D116A did not release reverse transcriptase activity into the supernatant, a direct comparison of this mutant with the others or with wild-type virus was not possible. However, a dose of D116A mutant virions could be estimated by electrophoretic analysis of virion pellets and normalized to wild-type virus by the intensity of the gp120 envelope glycoprotein (for example, see Fig. 4, lanes 7 and 8). By this standard, D116A mutant virions were unable to direct the synthesis of viral DNA at levels detectable by the PCR assay, while equivalent doses of wild-type virus synthesized readily detectable levels of viral DNA (data not shown).

In addition to the PCR assay described above, a Southern blotting and hybridization procedure was used to detect viral DNA synthesized in SupT1 cells after cocultivation with cell lines chronically producing the replication-defective viruses (data not shown). These experiments confirmed that mutant E152A synthesized nearly wild-type levels of full-length linear viral DNA, while viral DNA synthesis by mutants D116A and S147I was not detectable (54a).

DISCUSSION

The genetic analysis presented here demonstrates that a variety of viral replication phenotypes can result from single-amino-acid substitutions in the integrase protein. Three replication-defective viruses were generated by mutagenesis of the highly conserved central region of the HIV-1 integrase protein, each apparently blocked at a different stage of the viral replication cycle. Previous genetic studies have established that the integrase protein is required for only one stage of the retroviral replication cycle, the formation of an integrated provirus from the unintegrated linear viral DNA intermediate (14, 15, 43, 46, 50). However, this work, together with previous work that demonstrated that some integrase mutants can have deleterious effects on viral protein processing and virion release (47, 50, 53), demonstrates that mutant integrase proteins can interfere with a variety of steps in the early and late stages of the viral replication cycle. This observation is not surprising, since the integrase protein is synthesized as part of

FIG. 6. Electron microscopic analysis of virions produced by wild-type and integrase mutant proviruses. COS-7 cell monolayers were fixed at 48 h posttransfection and analyzed by thin-section electron microscopy. Representative virions produced after transfection with wild-type provirus (A) or the D116A (B) or E152A (C) mutant provirus are shown. Bars, 100 nm.

a polyprotein precursor that must fold and be correctly processed to produce all of the *gag-pol* products.

Only one of the replication-defective mutants, mutant E152A, was able to direct the synthesis of full-length viral DNA after infection of SupT1 cells, a phenotype expected for a mutant with a specific defect in integration. However, electron microscopic analysis indicated that this mutation resulted in an aberrant virion core structure. Viral protein synthesis and virion assembly and release were apparently normal, but virion core morphology suggested a defect in viral precursor polyprotein processing. Wild-type virions contain a spherical nucleoid surrounded by a bullet-shaped p24 core shell (27). The p24 shell appeared to be situated adjacent to the nucleoid of mutant E152A virions, giving the virion core a bilobed appearance. Previous morphological analysis of virions produced by a p17-p24^{gag} protease cleavage site mutant demonstrated that condensation of the C-terminal *gag* nucleocapsid proteins and viral genomic RNA to form a spherical nucleoid can occur independently of the formation of the surrounding p24 core shell (28). The aberrant core structures

of mutant E152A may result from the uncoordinated condensation of the nucleoid and the p24 core shell. The observed effects of mutation E152A on the virion core make it impossible to assign the phenotype of this mutant to defects in integrase function, as the apparent integration defect may be secondary to defects in virion core structure.

An HIV-1 mutant carrying a double amino acid substitution that included E152 (V151D and E152Q) of the integrase protein was previously shown to be unable to replicate in a human T-cell line (36). The late stages in the mutant replication cycle appeared to be normal, as transfected cells released particles that contained a wild-type pattern of viral proteins. Viral DNA synthesis was detected after infection of T-cell targets, leading to the conclusion that the mutant virus suffered a specific defect in integrase function that was responsible for the replication defect. However, electron microscopic analysis of mutant virions was not performed, so the effects of the double substitution mutation on virion core structure are not known. Electron microscopic analysis of virion morphology provides an additional means to assay the effects of mutations

FIG. 7. Replication of wild-type and integrase mutant viruses in SupT1 cells. (A to C) Cells were infected with equivalent amounts (reverse transcriptase [RT] units) of virus harvested from supernatants of COS-7 cell transfections. The culture medium was changed and assayed for reverse transcriptase activity every 3 days. (A) Wild-type and T115A, G118A, S153A, and K159Q mutant viruses. (B) Wild-type and S147I and E152A mutant viruses. (C) Wild-type and N117K and Y143N mutant proviruses. (D) Virus collected from the supernatants of SupT1 cells at 21 days postinfection with mutant N117K or Y143N (as shown in panel C) was normalized for reverse transcriptase activity and used to infect fresh SupT1 cells. The replication of wild-type virus was also measured for comparison.

on viral protein processing. As shown here in the analysis of mutant E152A, aberrant virion morphology can result even though the pattern of viral particle-associated proteins appears to be normal. While the effects of virion core structure on reverse transcription and integration are not known, even

minor alterations in the architecture of the virion core may be sufficient to have deleterious effects on one or both of these processes.

Mutation D116A had a dramatic effect on the late stages of the viral replication cycle. Virions produced by this mutant contained unprocessed p55^{gag} precursor polypeptide and undetectable levels of reverse transcriptase activity and failed to direct the synthesis of viral DNA after infection of CD4-positive target cells. The release of unprocessed p55^{gag} precursor polypeptide into the culture supernatant suggests that the D116A mutation interferes with viral precursor polypeptide interactions during virion assembly. Many of the essential reactions of virion morphogenesis require specific protein-protein interactions. For example, intermolecular interactions between p55^{gag} precursors are believed to provide the primary driving force for viral particle formation (reviewed in reference 62), while specific interactions between the *gag* precursor and the p160^{gag-pol} precursor may be required for incorporation of the latter polypeptide into budding virions (24, 44, 52, 61). An alteration in the conformation of the *gag-pol* precursor by the D116A mutation in the integrase region may reduce its stability or interfere with its incorporation into budding virions, resulting in particles that lack the *pol* gene products. Alternatively, the D116A mutation may interfere with the dimerization of the *gag-pol* polypeptide, a process that is required for activation of the viral protease (37, 41, 54). In either case, mutant virions would lack active protease and would be unable to process the p55^{gag} precursor into mature *gag* proteins, resulting in particles with an immature morphology.

FIG. 8. PCR analysis of viral DNA synthesized after infection of SupT1 cells with replication-defective HIV-1 integrase mutants. Viral DNA sequences were amplified by PCR, resolved by agarose gel electrophoresis, and detected by Southern hybridization analysis, as described in Materials and Methods. Lanes 1 and 2, control amplification of 10 ng of wild-type proviral plasmid pXHB-SV DNA either with (lane 1) or without (lane 2) prior restriction with *Dpn*I. Lanes 3 to 7, amplification of 1 μ g of total SupT1 DNA after infection with equivalent doses of virus produced by COS-7 cell transfection. SupT1 DNA was prepared after mock infection (lane 3) or 30 s postinfection with wild-type virus (lane 4), 24 h postinfection with wild-type virus (lane 5), 24 h postinfection with mutant S147I (lane 6), or 24 h postinfection with mutant E152A (lane 7). Lanes 8 and 9, amplification of 1 μ g of total SupT1 DNA after infection with equivalent doses of virus prepared from stable virus-producing cell lines. SupT1 DNA was prepared 24 h postinfection with mutant E152A (lane 8) or wild-type virus (lane 9).

The virions produced by mutant D116A are similar in morphology to protease-defective retrovirus mutants (11, 28, 30, 45, 54). Unlike mutant D116A virions, however, viral particles produced by protease-defective HIV-1 (28), murine leukemia virus (11), and avian leukosis virus (54) contain reverse transcriptase activity, demonstrating that the reverse transcriptase can function, at least in the *in vitro* assays, when contained within the *gag-pol* precursor. Similar arguments can be made for the absence of reverse transcriptase activity in mutant D116A virions as were made for the failure of the mutant particles to process the *gag* precursor. Failure to incorporate the *gag-pol* precursor would result in particles that lack reverse transcriptase. Alternatively, activation of the reverse transcriptase, like the activation of protease, may require dimerization of the *gag-pol* polyprotein, and the primary defect of mutant D116A may be in precursor dimerization. Some mutations in the reverse transcriptase region of the *pol* gene have also been shown to cause the production of immature viral particles (1, 26). An immature virion morphology may be a common feature of *pol* mutations that alter the conformation of the *gag-pol* precursor polyprotein and interfere with precursor interactions.

All of the late events in the replication of mutant S147I appeared to be similar to those in the wild-type virus, yet this mutant was unable to synthesize detectable viral DNA after infection of SupT1 cells. Mutant S147I may suffer defects in virion assembly or stability that were not detectable by the assays used in this study or may be defective in the early stages of the replication cycle, such as entry, uncoating, or reverse transcription. The analysis of this mutant highlights the importance of using multiple assays to assess the replication competence of integrase mutants. Additional assays for specific steps in the replication cycle, such as the incorporation of viral genomic RNA into virions or the synthesis of strong-stop viral DNA in cells after infection, may be useful in characterizing mutants like S147I. Additionally, some integrase mutations may directly or indirectly affect the stability of newly generated reverse transcripts, so that the absence of detectable viral DNA after infection may not necessarily indicate a defect in the initiation or completion of reverse transcription. Effects such as this may be detected by time course analysis of mutant viral DNA synthesis (21, 33).

Two amino acid substitutions in the integrase protein resulted in viruses with a delayed-replication phenotype. Neither the N117K nor the Y143N mutation had any detectable effect on viral protein expression or virion assembly or release from transfected cells, and virion morphologies were indistinguishable from that of the wild type, indicating that the late events of viral replication were unaffected by these mutations. The delays in replication seen for these mutants may result from defects in the early stages of the replication cycle. Tyrosine 143 is well conserved among mammalian retroviral integrases, although nonconservative amino acid substitutions occur naturally at the analogous positions of some integrases (20, 29, 32). A tyrosine at this position is not absolutely required for enzyme activity *in vitro*, as a nonconservative amino acid substitution at this position of the HIV-2 integrase (Y143L) had no effect on the oligonucleotide cleavage, strand joining, or disintegration reactions (57). The nature of the Y143N mutation may be at least partly responsible for the delayed replication of this mutant. Conservative substitutions at this position should be analyzed before any conclusions can be made about the potential contributions of amino acid Y143 to integrase function. Asparagine 117 is also highly conserved among retroviral integrases. All known retroviral integrases contain an asparagine or, rarely, the closely related glutamine

at this position (20). Even the conservative amino acid substitution N117Q of HIV-1 integrase was shown to reduce oligonucleotide cleavage and strand joining *in vitro* (20). The delayed replication of mutant N117K may result from a defect in the known enzymatic activities of the integrase, although a true test of this hypothesis awaits the analysis of conservative amino acid substitutions at this position.

Four amino acid substitutions had no effect on viral replication in tissue culture cells. Threonine 115 of HIV-1 integrase represents a well conserved amino acid, as all retroviral integrases contain a threonine or a structurally related serine residue at the analogous position (20, 29, 32). However, the occurrence of a hydroxylated amino acid at this position is apparently not important for integrase enzyme activity *in vitro* or for viral replication in cell cultures, as nonconservative substitutions for T115 in HIV-1 and HIV-2, or the analogous threonine of Rous sarcoma virus integrase (T120) had no effect on the enzymatic activities of mutant proteins *in vitro* (17, 20, 34, 56), and the replication of mutant T115A was similar to that of wild-type virus in cell cultures (Fig. 7A). The conservative substitution of alanine for glycine at position 118 of integrase also had no effect on HIV-1 replication. Glycine 118 is highly conserved among retroviral integrases, but an alanine does occur naturally in this position in the mouse mammary tumor virus integrase (20, 29, 32). Among the known retroviral integrases, only HIV-1 and the African green monkey strain of simian immunodeficiency virus (SIV_{agm}) contain a serine at position 153; the HIV-2, SIV_{mac}, and SIV_{sm} integrases contain alanines at the analogous position, while all other integrases contain an arginine at this position (20). In light of this, the wild-type replication of mutant S153A is not surprising. Mutational analysis of HIV-1 integrase implicated S153 in enzyme function, as an S153R mutant was reduced in all of the *in vitro* enzymatic activities tested (20). The particular amino acid substitution tested *in vitro* may have indirectly affected enzyme activity by altering protein conformation, pointing to potential limitations of the *in vitro* enzymatic assays in identifying catalytically important amino acid residues. Finally, mutant K159Q replicated like wild-type virus. Lysine 159 is invariant among retroviral integrases (20, 29, 32). However, a lysine at this position is evidently required neither for viral replication in cell culture nor for enzyme activity *in vitro*, as nonconservative substitutions for K159 of HIV-2 integrase had no effect on the oligonucleotide cleavage or disintegration reactions and only minor effects on DNA strand joining (57).

All of the replication-defective mutants analyzed here carried nonconservative substitutions for highly conserved amino acid residues of the integrase protein. The nature of the amino acid change may influence the observed replication phenotype, so that conservative substitutions may be less likely to have pleiotropic effects on the viral replication cycle. Consistent with this possibility is the recent demonstration that a conservative substitution of integrase amino acid D116 (D116E) results in a replication-defective virus that appears to be similar to the wild type in the late stages of the replication cycle (54a). It may be necessary to analyze a number of conservative substitutions at individual amino acid positions before mutations that specifically affect integrase function are identified.

Three of the integrase mutants altered in viral replication, N117K, Y143N, and E152A, catalyzed the synthesis of detectable viral DNA after infection of SupT1 cells. These mutants can be further analyzed for their effects on the rate of viral DNA synthesis, viral DNA end processing, nuclear entry, formation of circular viral DNA, and the joining of viral DNA to cellular DNA in order to pinpoint the basis of the altered replication phenotype. The stability and function of the viral

preintegration complexes formed by these mutants can also be assessed *in vitro* (21, 22). Similar mutations in other regions of the integrase protein may be identified by the type of analysis described here, permitting an analysis of the role(s) of the integrase protein in the structure and function of the retroviral preintegration complex.

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