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Integrase Mutants of Human Immunodeficiency Virus Type 1 with a Specific Defect in Integration

BRUNELLA TADDEO,[1]† WILLIAM A. HASELTINE,[2] AND CHRIS M. FARNET[1]*

Division of Human Retrovirology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115,[1] and Human Genome Sciences, Inc., Rockville, Maryland 20850[2]

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A previous genetic analysis of the human immunodeficiency virus type 1 integrase protein failed to identify single amino acid substitutions that only block the integration of viral DNA (C.-G. Shin, B. Taddeo, W. A. Haseltine, and C. M. Farnet, J. Virol. 68:1633–1642, 1994). Additional substitutions of amino acids that are highly conserved among retroviral integrases were constructed in human immunodeficiency virus type 1 and analyzed for their effects on viral protein synthesis and processing, virion morphology, and viral DNA synthesis and integration in an attempt to identify mutants with a specific defect in integration. Four single amino acid substitutions resulted in replication defective viruses. Conservative, single amino acid substitutions of the two invariant aspartic acid residues found in all retroviral integrases prevented the integration of viral DNA and had no detectable effect on the other stages in the viral replication cycle, indicating that these mutants exhibited a specific defect in integration. Mutations at two positions, S-81 and P-109, blocked the integration of viral DNA but also resulted in the production of viral particles that exhibited reduced reverse transcriptase activity, suggesting additional defects in viral replication. Substitution of the highly conserved amino acid T66 had no effect on viral replication in a CD4$^+$ human T-cell line. This analysis extends the range of possible phenotypes that may be produced by single amino acid substitutions in conserved residues of the integrase protein.

The integration of a DNA copy of the retroviral RNA genome into the host cell genome is essential for viral replication. The viral integrase protein, encoded by the 3′ end of the viral *pol* gene, is required for integration (1, 3, 4, 17, 18, 20). Comparison of the amino acid sequences of a number of retroviral integrases has demonstrated the presence of highly conserved residues and motifs that may be important for integrase function (7, 9). The most highly conserved region of retroviral integrases lies in the central region of the protein and is defined by three acidic amino acid residues in a conserved spatial arrangement [the D,D(35)E motif] (9, 10). Mutations in any one of the three residues result in the complete loss of all integrase enzymatic activities in vitro (5, 6, 10, 12, 13, 24) and can block viral replication in cell cultures (12, 22). Establishing a correlation between specific in vitro enzymatic defects and the resulting virus replication phenotype is complicated by the pleiotropic effects of some integrase point mutants on viral replication. A previous analysis of single amino acid substitutions in the human immunodeficiency virus type 1 (HIV-1) integrase protein failed to identify mutations that only blocked the integration of viral DNA and established that integrase mutations may suffer defects in virion precursor polypeptide processing, virion morphology, or viral DNA synthesis. The integrase protein is initially synthesized as a part of a *gag-pol* precursor polyprotein, possibly accounting for the effect of integrase mutations on the other enzymatic functions encoded by the *pol* gene (19, 21, 23).

In an attempt to identify integrase changes that specifically prevent the integration of viral DNA, additional point mutations affecting highly conserved amino acid residues of the HIV-1 integrase protein were constructed and analyzed for their effects on viral replication in cell culture. Five HIV-1 integrase mutants were constructed by introducing single amino acid substitutions in the highly conserved D,D(35)E region of the protein. The nature and location of each point mutation are indicated in the legend to Fig. 1. The integrase mutants were analyzed for their effects on viral replication by monitoring multiple steps in the viral replication cycle.

Each of the integrase mutations was introduced into an HXBc2 provirus, and wild-type and mutant proviral DNAs were transfected into COS-1 cells. Forty-eight hours following transfection, the cells were metabolically labeled with [^{35}S]cysteine, and viral proteins were immunoprecipitated from cell lysates by using an HIV-1-infected patient serum. As shown in Fig. 1, the pattern of viral proteins produced by the integrase mutants was identical to that produced by wild-type virus, demonstrating that none of the amino acid substitutions had detectable effects on the synthesis or processing of viral proteins.

Virion release from COS-1 cells transfected with wild-type and integrase mutant proviruses was measured 48 h after transfection by assaying culture supernatants for particle-associated viral proteins, viral p24 antigen, and reverse transcriptase (RT) activity. A wild-type pattern of [^{35}S]cysteine-labeled viral proteins was found in the supernatants of cells transfected with all of the integrase mutants (Fig. 1), indicating that the mutations had no detectable effect on the process of virion formation. Consistent with this observation was the demonstration that each of the integrase mutants released wild-type levels of particle-associated p24 antigen into the culture supernatants, as measured by a radioimmunoassay (data not shown). Surprisingly, supernatants recovered from COS-1 cells transfected with mutants S81I and P109S consistently showed a reduced level of particle-associated RT activity (Fig. 2), even though essentially wild-type levels of viral particles were detected in culture supernatants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

* Corresponding author.
† Permanent address: Istituto Superiore di Sanita, Rome, Italy.

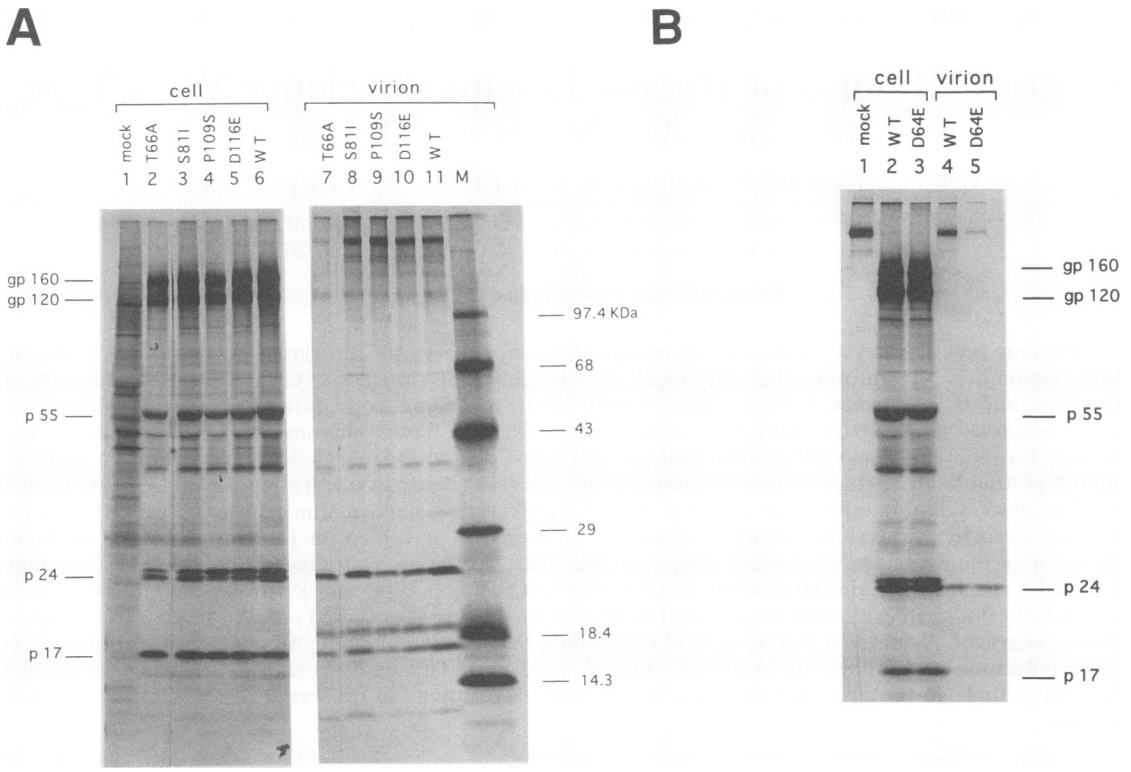

FIG. 1. SDS-PAGE analysis of viral proteins present in cell lysates and virions after transfection of COS-1 cells with wild-type or integrase mutant proviral DNA. The integrase mutants were constructed as described by Shin et al. (22). Briefly, a 1,671-bp fragment spanning the *Eco*RV and *Eco*RI sites of HXBc2 (nucleotides 2977 to 5122, according to the numbering scheme of Myers et al. [16]) that encodes a portion of integrase protein was subcloned into pSL1180 (Pharmacia), and the resulting plasmid was used as a target for site-directed mutagenesis using procedures already described (11). The mutagenic oligonucleotides used are D64E (5′GGAATATGGCAACTAGAgTGTACACATTTA3′), T66A (5′TG GCAACTAGATTGTTgCACATTTAGAAGGA3′), S81I (5′GCAGTTCATGTAGCCAtTGGATATATAGAA3′), P109S (5′TTAGCAGGAA GATGGtCAGTAAAAACAATA3′), and D116E (5′AAAACAATACATACTGAaAATGGCAGCAAT3′) (nucleotide changes introduced to create the mutation are indicated by lowercase letters). COS-1 cells were transfected with 10 μg of proviral DNA by a DEAE-dextran procedure (15). The procedures for metabolic labeling of COS-1 and viral protein immunoprecipitation were performed as previously described (22). COS-1 were labeled with [^{35}S]cysteine 48 h after transfection, and the viral proteins were immunoprecipitated from cell lysates (A, lanes 1 to 6; B, lanes 2 and 3) or from virion pelleted through a sucrose cushion from culture supernatants (A, lanes 7 to 11; B, lanes 4 and 5) and resolved on a 10 to 15% (wt/vol) polyacrylamide gradient gel. (A) The cells were mock transfected (lane 1) or transfected with T66A (lanes 2 and 7), S81I (lanes 3 and 8), P109S (lanes 4 and 9), or D116E (lanes 5 and 10) mutant proviral DNA or wild-type proviral DNA (WT; lanes 6 and 11). The relative positions of HIV-1 proteins and protein markers (lane M; GIBCO) are indicated, on the left and right, respectively. (B) Cells were mock transfected (lane 1) or transfected with wild-type proviral (WT; lanes 2 and 4) or D64E mutant proviral (lanes 3 and 5) DNA. The positions of HIV-1 proteins are also shown.

PAGE) analysis (Fig. 1A, lanes 8 and 9) and p24 radioimmunoassay (data not shown), and levels of p160$^{gag\text{-}pol}$ precursor were similar in wild-type and S81 and P109 mutant viruses (Fig. 1A, lanes 8, 9, and 11).

Virion morphology was assessed by thin-section electron microscopy of monolayer preparations of COS-1 cells transfected with wild-type and integrase mutant proviruses. All of the mutants released virions with a morphology indistinguishable from that of wild-type virions (data not shown), indicating that the mutations did not have major effects on the late stages of viral replication, including virion assembly and maturation.

The effects of the integrase point mutations on viral replication were assessed by infecting cells of the CD4$^+$ human SupT1 T-cell line with supernatants prepared from COS-1 cells 48 h after transfection with mutant or wild-type provirus. Parallel cultures of SupT1 cells were infected with equivalent doses (RT units) of wild-type or mutant virus. The course of virus replication was monitored by measuring supernatant RT levels every 3 days. Only integrase mutant T66A showed a

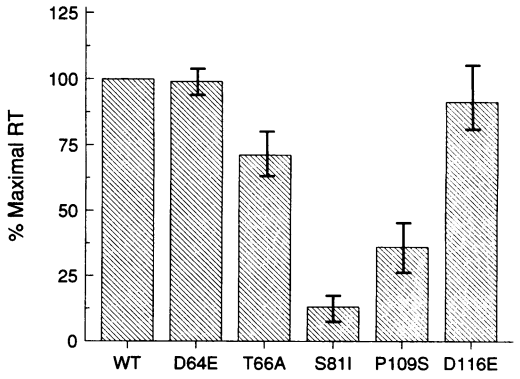

FIG. 2. Viral particle release from COS-1 cells transfected with wild-type (WT) or integrase mutant proviral DNA. Virus production was monitored 48 h after transfection by determination of RT activity on the supernatants of transfected COS-1, using procedures already described (2). Bars indicate the range of values obtained from four experiments.

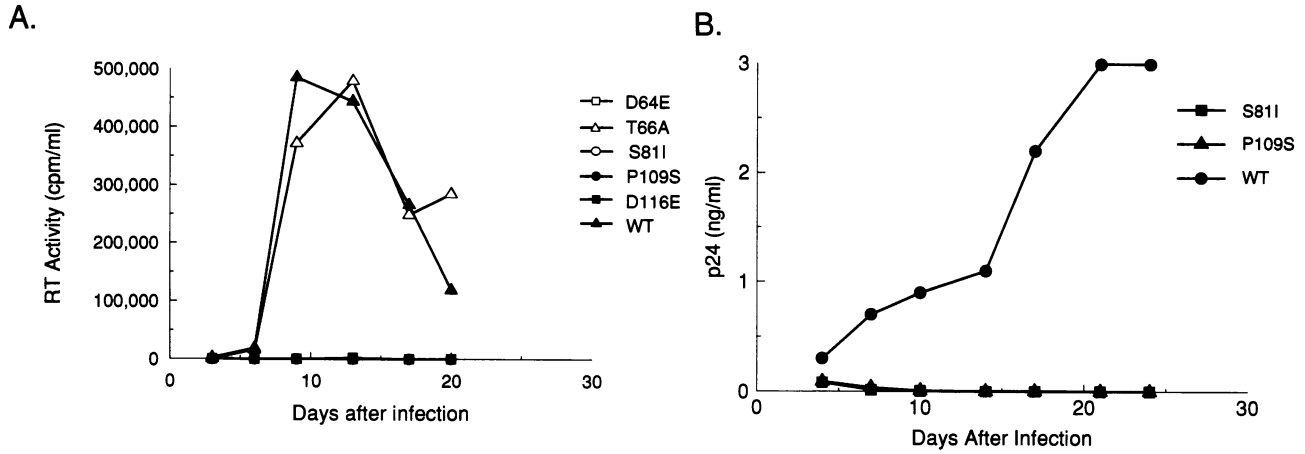

FIG. 3. Replication of wild-type (WT) and integrase mutant viruses in human SupT1 cells. Human SupT1 cells (10^6) were infected with an aliquot of filtered supernatant recovered from transfected COS-1 equivalent to 10^5 cpm of RT activity. The infected cells were fed with fresh medium every third day, and the supernatants were assayed for RT activity (A) and p24 antigen production (B) by using a commercially available HIV-1 p24 enzyme-linked immunosorbent assay kit (NEN/DuPont, Boston, Mass.). The p24 antigen measured at day 4 includes antigen contributed by residual input virus.

replication profile similar to that of wild-type virus (Fig. 3A). The other integrase mutants were unable to establish a productive infection of SupT1 cells, showing no production of RT activity for up to 25 days postinfection (Fig. 3A). Because viral particles produced by mutants S81I and P109S showed reduced levels of RT activity, replication of these two mutants was also monitored by measuring supernatant p24 antigen levels. No viral p24 antigen was produced by cells infected with the S81I or P109S integrase mutant (Fig. 3B).

Each of the replication-defective integrase mutants was tested for the ability to synthesize viral DNA following infection of SupT1 cells and to integrate the viral DNA into the cellular genome. Total DNA was extracted from cells 24 h after infection with wild-type or integrase mutant virus. Viral DNA was readily detected by PCR amplification following infection with wild-type or integrase mutant virus (Fig. 4A), indicating that viral DNA synthesis was not detectably affected by the integrase mutations. The same DNA samples were subsequently analyzed by using a method for the detection of viral DNA integrated into the cellular genome (14). None of the replication-defective integrase mutants generated detectable levels of integrated viral DNA (Fig. 4B, lanes 3 to 6), in contrast to wild-type virus (Fig. 4B, lane 2), demonstrating that these mutants suffer a defect in viral DNA integration.

The replication phenotypes of the mutant viruses described here are consistent with previous biochemical analyses of recombinant HIV-1 integrase proteins carrying similar amino acid substitutions. The T66A mutation had no detectable effect on the in vitro enzymatic activities of recombinant HIV-1 integrase (6, 8) or on viral replication in cell culture (Fig. 3A). The replication defect displayed by mutants D64E, S81I, P109S, and D116E may result from the loss of integrase enzymatic activity. Conservative substitutions of D-64 and D-116 resulted in the loss of the oligonucleotide cleavage, DNA strand transfer, and disintegration activities of HIV-1 integrase in vitro (6). Similarly, the P109S mutation reduced the DNA binding and oligonucleotide cleavage activities of purified HIV-1 integrase (5, 6, 10). Mutation of S81 has been reported to completely (8) or partially (24) reduce the in vitro enzymatic activities of HIV-1 integrase, but these effects may result from the reduced solubility of the mutant protein (24).

The integrase protein is synthesized in infected cells as part of a *gag-pol* polyprotein precursor that is subsequently processed to form the structural and enzymatic proteins of the mature virion. Previous analyses of the replication of integrase mutant viruses demonstrated that single amino acid substitutions in the integrase protein can produce defects in virion precursor polypeptide processing, virion morphology, and viral DNA synthesis (22), most likely by interfering with the proper folding and proteolytic processing of the viral precursor polyprotein. In contrast, two of the replication-defective integrase mutants described here, D64E and D116E, specifically blocked the integration of viral DNA and had no detectable effect on other steps in the viral replication cycle, providing strong support for the idea that integrase function is absolutely required for retroviral replication in cell lines. The nature of the amino acid substitution appeared to be an important determinant of the observed viral replication phenotype. For example, the nonconservative substitution of alanine for amino acid D-116 had dramatic effects on the late events of the viral replication cycle (22), resulting in immature virion particles that contained no detectable RT activity, while the conservative D116E mutation described here had no detectable effect on the replication cycle outside of a block to integration.

The analysis of the S81I and P109S mutants extends the range of possible effects of integrase mutations on the viral replication cycle. Both of these mutants were similar to wild-type virus in the synthesis and processing of viral proteins, in the assembly and maturation of virions, and in the synthesis of viral DNA following infection of SupT1 cells, as measured by the assays described here. However, viral particles produced in both of these mutants contained lower levels of RT activity than wild-type virions, making it impossible to assign the replication defect of these mutants to a sole defect in integration. The RT activity expressed by these mutants was apparently sufficient to drive the synthesis of viral DNA in infected cells (Fig. 4A). However, the reduced enzyme activity in the in vitro RT assay indicates that these mutations have pleiotropic effects on *pol* gene functions. The S81I mutation has been reported to reduce the solubility and stability of the HIV-1 (8) and HIV-2 (24) integrase proteins in vitro, presumably by interfering with the proper folding of the polypeptide. Im-

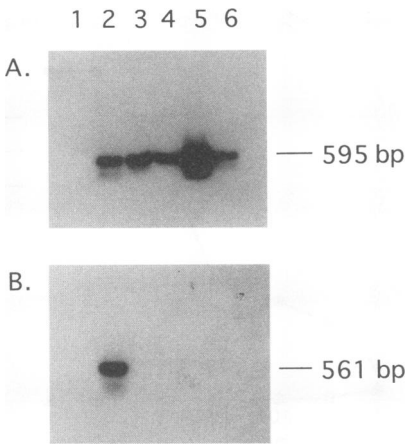

FIG. 4. PCR analysis of synthesis and integration of viral DNA in SupT1 cells infected with wild-type or replication-defective integrase mutant virus. (A) The analysis of viral DNA synthesis in infected SupT1 cells was carried out as described by Shin et al. (22). Briefly, total DNA was extracted from infected cells 24 h after infection, using standard techniques (15). DNA samples were pretreated with restriction endonuclease *Dpn*I (New England Biolabs, Inc., Beverly, Mass.) to remove plasmid DNAs potentially contaminating COS-1 cell supernatants. DNAs (1 μg of each) were subject to 35 cycles of PCR amplification with a pair of primers, *env*-1 (nucleotides 7950 to 7969 of HXBc2) and *env*-2 (nucleotides 8545 to 8526 of HXBc2), which are able to amplify a 595-bp fragment in the *env* gene of HIV-1. Each cycle consisted of 1 min 15 s of denaturation (94°C), 1.5 min of annealing (57°C), and 2.5 min of extension (72°C). Amplified products resulting from PCR were analyzed by electrophoresis on 1.5% agarose gel, transferred to a nylon filter (Hybond-N; Amersham), and hybridized at 42°C with a ^{32}P-labeled specific oligonucleotide, *env*-3 (nucleotides 8285 to 8308 of HXBc2), that is complementary to an internal region of amplified fragment. (B) The analysis of integrated viral DNA in the infected SupT1 cells was performed as described by Li et al. (14). Briefly, each high-molecular-weight DNA sample was subjected to a double digestion with restriction endonucleases *Sac*I and *Xba*I overnight at 37°C. Digested DNAs were separated by electrophoresis on 1% low-melting-point agarose, and sections of the gel containing DNA fragments between 1 and 5 kb in size were excised. DNAs were extracted by using the Rapid Gene Clean kit (Bio 101, Inc., La Jolla, Calif.) and were subjected to ligation in order to reconstruct a full-size long terminal repeat (LTR). The ligation mixtures were used directly for PCR amplification. Primers used in this PCR assay were LTR 1 (nucleotides 75 to 98 of HXBc2) and AA55 (25), able to amplify a 561-bp fragment between the U3 and R regions of the HIV-1 LTR. The amplified products were analyzed as described above, using the oligonucleotide M669 (25) as a probe. The critical step of this procedure is the size exclusion of DNA fragments smaller than 1 kbp: molecules of this size are expected to contain fragments of the HIV-1 LTR derived from *Sac*I digestion of linear and circular viral DNAs (note that *Xba*I does not cut HIV-1 DNA). LTR-containing DNA fragments longer than 1 kbp will be derived from viral LTRs that have joined to genomic DNA as a result of integration. SupT1 DNA was prepared after mock infection (lane 1), 24 h after infection with wild-type virus (lane 2), 24 h after infection with mutant D64E (lane 3), 24 h after infection with mutant S81I (lane 4), 24 h after infection with mutant P109S (lane 5), or 24 h after infection with mutant D116E (lane 6). The sizes of amplified products are also indicated.

proper folding of the integrase polypeptide in the *gag-pol* precursor may produce subtle defects in precursor processing or incorporation into virions, resulting in reduced particle-associated RT activity.

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