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A Mutation in Integrase Can Compensate for Mutations in the Simian Immunodeficiency Virus *att* Site

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Sequences at the left terminus of U3 in the left long terminal repeat (LTR) and at the right terminus of U5 in the right LTR are important for integration of retroviral DNA. In the infectious pathogenic molecular clone of simian immunodeficiency virus strain mac239 (SIVmac239), 10 of the 12 terminal base pairs form an imperfect inverted repeat structure (5′ TGGAAGGGATTT 3′ [nucleotides 1 to 12] and 3′ ACGATCCCTAAA 5′ [nucleotides 10279 to 10268]). Nineteen different mutant forms of SIVmac239 proviral DNA with changes at one or more of the positions in each of the 12-terminal-base-pair regions were constructed. Viral replication was severely or completely compromised with nine of these mutants. Revertants appeared 40 to 50 days after transfection in two independent experiments with mutant 7, which contained changes of AGG to TAC at positions 5 to 7 in U3 and TCC to GAA at positions 10275 to 10273 in U5. Virus produced at these times from mutant 7 transfection replicated upon reinfection with only a slight delay when compared to the wild type. Sequence analysis of the LTR and integrase regions from infected cultures revealed two predominant changes: G to A at position 10275 in U5 and Glu to Lys at position 136 in integrase. Derivatives of clone 7 in which these changes were introduced individually and together were constructed by site-specific mutagenesis. Each change individually restored replication capacity only partially. However, the combination of both mutations restored replicative capacity to that of the original revertants. These results indicate that changes in integrase can compensate for mutations in the terminal nucleotides of the SIV LTR. The results further indicate that resistance to integrase inhibitors may include both integrase and LTR mutations.

Integration of viral cDNA into the host genome is a crucial step in the life cycle of retroviruses. Integration is catalyzed by a virus-encoded enzyme called integrase. Integrase first cleaves the cDNA near each blunt end, adjacent to CA dinucleotides which are conserved in all retroviruses (3′ processing reaction). Integrase subsequently joins each recessed 3′ end to the 5′ phosphate of a staggered double-stranded cut in host chromosomal DNA (DNA strand transfer reaction). Repair of the recombination intermediate yields the integrated provirus, 5′ TG. . .CA 3′, flanked by the sequence duplication of the double-stranded staggered cut. Purified integrase proteins display 3′ processing and strand transfer activities with recombinant viral DNA substrates (for a recent review of retroviral integration, see reference 15).

Sequences located at each end of linear proviral DNA which are essential for integration define the viral attachment (*att*) site. The conserved terminal two base pairs, 5′ TG. . .CA 3′, are critical for integration. The other nucleotides near the termini, while not conserved in sequence, form an imperfect inverted repeat (IR) in all retroviruses (32).

The human and simian immunodeficiency viruses (HIV and SIV), like other retroviruses, are unable to replicate in the absence of integration. However, little is known about the sequence features within the terminal regions of these viruses that are crucial for integration. We thus set out to investigate which sequence features of the SIV *att* site are important for integration and replication. We constructed 19 mutant forms of SIV strain mac239 (SIVmac239) with changes in the terminal regions and measured the effects on viral replication in a lymphoid cell line. We found that viral replication was severely or completely compromised by many of the mutations. We further show that a second mutation in integrase can compensate for mutations in the SIV *att* site.

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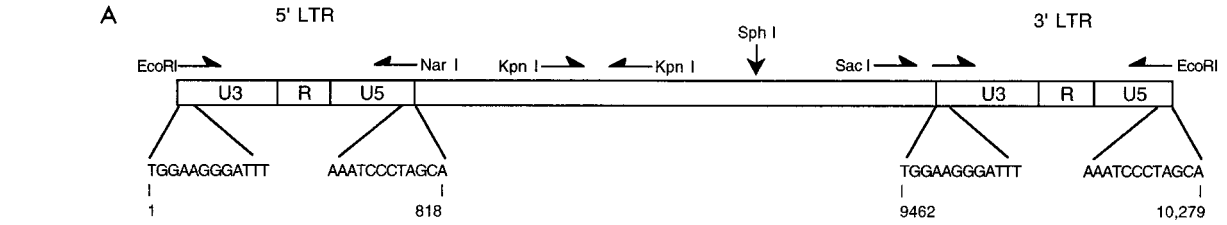
MATERIALS AND METHODS

SIVmac239 plasmids. The SIVmac239 provirus contained in two half-genome plasmids, p239-SpSp-5′ and p239-SpE-3′, has been previously described (16). PCR was used to remove flanking cellular sequences and to introduce *Eco*RI sites immediately flanking the termini of proviral DNA. The resulting plasmid, pSP72-239-5′, contained a 6,450-bp *Eco*RI-*Sph*I fragment, and pSP72-239-3′ contained a 3,829-bp *Sph*I-*Eco*RI fragment. These plasmids were used as the recipient vectors for mutated long terminal repeat (LTR) and integrase fragments.

For expression in *Escherichia coli*, integrase coding regions were amplified by PCR as previously described (2). The N terminus of integrase was chosen as Phe-768 of the 1,060-residue Pol polyprotein (28). Expression plasmids encoded 293-residue integrase proteins fused to a 21-residue N-terminal tag containing six adjacent histidine residues. The His tag facilitates purification by metal affinity chromatography (31).

5′ LTR mutagenesis. The mutagenesis strategy is shown schematically in Fig. 1A. Two mutagenic primers were designed to span the IRs in the 5′ LTR (bases 1 to 30 and 789 to 830). The sense-strand primer contained an *Eco*RI site immediately upstream of the first base of the U3 IR, and the antisense primer spanned the *Nar*I site adjacent to the U5 IR. Primer sequences are listed below. PCR was performed in a 100-μl volume containing 5 U of Vent DNA polymerase (New England Biolabs, Inc., Beverly, Mass.), 1× buffer, 25 μM deoxynucleotide triphosphates, 1 mM $MgSO_4$, 0.8 μM each primer, and 1 to 5 ng of p239-SpSp-5′ template DNA. Reactions were cycled 30 times in a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.), using 50 s at 94°C, 30 s at 55°C, and 1 min at 72°C. At the end of cycling, the reaction was extended for 10 min at 72°C. The reaction products were analyzed by agarose gel electrophoresis, digested with *Eco*RI and *Nar*I, and ligated to *Eco*RI/*Nar*I-digested pSP72-239-5′. Ligation mixtures were transformed into *E. coli* XL1 blue (Stratagene, La Jolla, Calif.) and plated on LB agar containing 100 μg of ampicillin per ml.



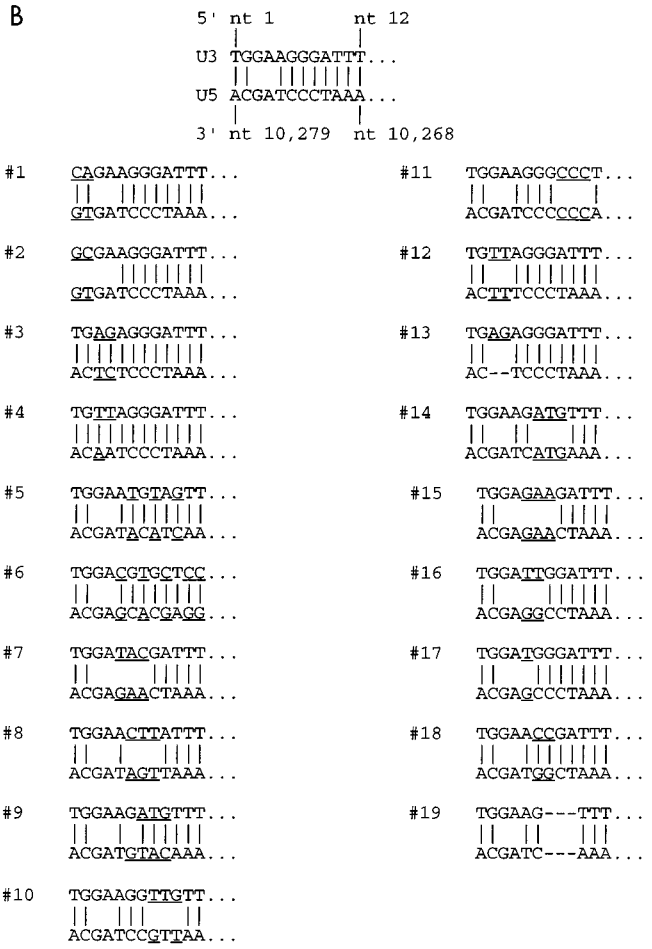


FIG. 1. Mutagenesis strategy. (A) The plus-strand sequences of the LTR regions targeted by site-directed mutagenesis are shown below the genetic map of SIVmac239 (not drawn to scale). The locations and restriction enzyme sites of PCR primers used for mutagenesis and cloning are shown above the map. The position of the unique *Sph*I site is also indicated. (B) The SIV *att* site. Nucleotides (nt) 1, 2, and 5 to 12 of U3 are complementary to nucleotides 10279, 10278, and 10275 to 10268 of U5 (indicated by vertical lines). The bases which were changed in each mutant *att* site are underlined. Bases which were deleted are indicated by dashes. For simplicity, only the sequence of the plus strand is shown.

3′ LTR mutagenesis. Due to the lack of convenient positions of restriction enzyme sites, a three-primer PCR mutagenesis method was used. Two mutagenic primers spanning the IRs were designed. The antisense primer spanned the U5 IR and also contained a flanking *Eco*RI site for cloning. Two sense strand primers were used; the mutagenic primer spanned the U3 IR, and another primer spanned the unique *Sac*I site in envelope (nucleotides 9220 to 9244), which was used for cloning. The three primers were added in one PCR, and cycling was performed as described above, using p239-SpE-3′ as the template. The amplified 3′ LTR was digested with *Sac*I and *Eco*RI and ligated to *Sac*I- and *Eco*RI-digested pSP72-239-3′. The presence of the desired mutations and the absence of off-site mutations in the final clones used in both the 5′ and 3′ LTRs were confirmed by sequencing of the PCR-amplified regions.

Sense primers for 5′ LTR mutagenesis (with the *Eco*RI sites in boldface) were as follows (in all cases, bases which were changed by mutagenesis are underlined and bases deleted by mutagenesis are indicated by dashes):

U3 1 5′GGG**GAATTC**CAGAAGGGATTTATTACAGTGC3′
U3 2 5′GGG**GAATTC**GCGAAGGGATTTATTACAGTGC3′
U3 3 5′GGG**GAATTC**TGAGAGGGATTTATTACAGTGCAA3′
U3 4 5′GGG**GAATTC**TGTTAGGGATTTATTACAGTGCAA3′
U3 5 5′GGG**GAATTC**TGGAATGTAGTTATTACAGTGCAA3′
U3 6 5′GGG**GAATTC**TGGACGTGCTCCATTACAGTGCAAGAAGAC3′
U3 7 5′GGG**GAATTC**TGGATACGATTTATTACAGTGCAA3′
U3 8 5′GGG**GAATTC**TGGAACTTATTTATTACAGTGCAA3′
U3 9 5′GGG**GAATTC**TGGAAGATGTTTATTACAGTGCAA3′
U3 10 5′GGG**GAATTC**TGGAAGGTTGTTATTACAGTGCAA3′
U3 11 5′GGG**GAATTC**TGGAAGGGCCCTATTACAGTGCAA3′
U3 12 5′GGG**GAATTC**TGTTAGGGATTTATTACAGTGCAA3′
U3 13 5′GGG**GAATTC**TGAGAGGGATTTATTACAGTGCAA3′
U3 14 5′GGG**GAATTC**TGGAAGATGTTTATTACAGTGCAA3′
U3 15 5′GGG**GAATTC**TGGAGAAGATTTATTACAGTGCAA3′
U3 16 5′GGG**GAATTC**TGGATTGGATTTATTACAGTGCAA3′
U3 17 5′GGG**GAATTC**TGGATGGGATTTATTACAGTGCAA3′
U3 18 5′GGG**GAATTC**TGGAACCGATTTATTACAGTGCAA3′
U3 19 5′GGG**GAATTC**TGGAAG---TTTATTACAGTGCAA3′

Antisense primers for 5′ LTR mutagenesis (with the *Nar*I sites in boldface) were as follows:

U5 1 5′TCA**GGCGCC**AATCCACTAGGGATTTTCCTG3′
U5 2 5′TCA**GGCGCC**AATCCACTAGGGATTTTCCTG3′
U5 3 5′TCA**GGCGCC**AATCTGAGAGGGATTTTCCTG3′
U5 4 5′TCA**GGCGCC**AATCTGTTAGGGATTTTCCTG3′
U5 5 5′TCA**GGCGCC**AATCTGCTATGTAGTTTCCTG3′
U5 6 5′TCA**GGCGCC**AATCTGCTCGTGCTCCTCCTGCTTCGGTTTCCCA3′
U5 7 5′TCA**GGCGCC**AATCTGCTCTTGATTTTCCTG3′
U5 8 5′TCA**GGCGCC**AATCTGCTATCAATTTTCCTGCTT3′
U5 9 5′TCA**GGCGCC**AATCTGCTACATGTTTTCCTGCTTCG3′
U5 10 5′TCA**GGCGCC**AATCTGCTAGGCAATTTCCTGCTTCG3′
U5 11 5′TCA**GGCGCC**AATCTGCTAGGGGGGTTCCTGCTTCG3′
U5 12 5′TCA**GGCGCC**AATCTGAAAGGGATTTTCCTGCTTC3′
U5 13 5′TCA**GGCGCC**AATCTG--AGGGATTTTCCTGCTTCGGTT3′
U5 14 5′TCA**GGCGCC**AATCTGCTAGTACTTTTCCTGCTTCG3′
U5 15 5′TCA**GGCGCC**AATCTGCTCTTGATTTTCCTGCTTCG3′
U5 16 5′TCA**GGCGCC**AATCTGCTCCGGATTTTCC3′
U5 17 5′TCA**GGCGCC**AATCTGCTCGGGATTTTCCTGCTTCG3′
U5 18 5′TCA**GGCGCC**AATCTGCTACCGATTTTCCTGCTTCG3′
U5 19 5′TCA**GGCGCC**AATCTGCTAG---TTTTCCTGCTTCG3′

Sense primers for 3′ LTR mutagenesis were as follows:

U3 1 5′GGGGGGACCAGAAGGGATTTATTACAG3′
U3 2 5′GGGGGGACGCGAAGGGATTTATTACAG3′
U3 3 5′GGGGGGACTGAGAGGGATTTATTACAGTGC3′
U3 4 5′GGGGGGACTGTTAGGGATTTATTACAGTGC3′
U3 5 5′GGGGGGACTGGAATGTAGTTATTACAGTGCA3′
U3 6 5′GGGGGGACTGGACGTGCTCCATTACAGTGCAAGAAGACA3′
U3 7 5′GGGGGGACTGGATACGATTTATTACAGTGCA3′
U3 8 5′GGGGGGACTGGAACTTATTTATTACAGTGCAA3′
U3 9 5′GGGGGGACTGGAAGATGTTTATTACAGTGCAA3′
U3 10 5′GGGGGGACTGGAAGGTTGTTATTACAGTGCAA3′
U3 11 5′GGGGGGACTGGAAGGGCCCTATTACAGTGCAA3′
U3 12 5′GGGGGGACTGTTAGGGATTTATTACAGTGCAA3′
U3 13 5′GGGGGGACTGAGAGGGATTTATTACAGTGCAA3′
U3 14 5′GGGGGGACTGGAAGATGTTTATTACAGTGCAA3′
U3 15 5′GGGGGGACTGGAGAAGATTTATTACAGTGCAA3′
U3 16 5′GGGGGGACTGGATTGGATTTATTACAGTGCAA3′
U3 17 5′GGGGGGACTGGATGGGATTTATTACAGTGC3′
U3 18 5′GGGGGGACTGGAACCGATTTATTACAGTGCAA3′
U3 19 5′GAAAAGGGGGGACTGGAAG---TTTATTACAGTG3′

Antisense primers for 3′ LTR mutagenesis (with *Eco*RI sites in boldface) were as follows:

U5 1 5′GTT**GAATTC**CACTAGGGATTTTCCTGCTTCG3′
U5 2 5′GTT**GAATTC**CACTAGGGATTTTCCTGCTTCG3′
U5 3 5′GTT**GAATTC**TGAGAGGGATTTTCCTGCTTCGGTTT3′
U5 4 5′GTT**GAATTC**TGATAGGGATTTTCCTGCTTCGGTTT3′
U5 5 5′GTT**GAATTC**TGCTATGTAGTTTCCTGCTTCGGTTT3′
U5 6 5′GTT**GAATTC**TGCTCGTGCTCCTCCTGCTTCGGTTTCCCAA3′
U5 7 5′GTT**GAATTC**TGCTCTTGATTTTCCTGCTTCGGTT3′
U5 8 5′GTT**GAATTC**TGCTATCAATTTTCCTGCTTCGGTT3′
U5 9 5′GTT**GAATTC**TGCTCATGATTTTCCTGCTTCGGTTT3′
U5 10 5′GTT**GAATTC**TGCTAGGCAATTTCCTGCTTCGGTTT3′
U5 11 5′GTT**GAATTC**TGCTAGGGGGGTTCCTGCTTCGGTTT3′
U5 12 5′GTT**GAATTC**TGAAAGGGATTTTCCTGCTTCGGTTT3′
U5 13 5′GTT**GAATTC**TG--AGGGATTTTCCTGCTTCGGTTT3′
U5 14 5′GTT**GAATTC**TGCTAGTACTTTTCCTGCTTCGGTTT3′
U5 15 5′GTT**GAATTC**TGCTCTTGATTTTCCTGCTTCGGTTT3′
U5 16 5′GTT**GAATTC**TGCTCCGGATTTTCCTGCTTCGGT3′
U5 17 5′GTT**GAATTC**TGCTCGGGATTTTCCTGCTTCGGT3′
U5 18 5′GTT**GAATTC**TGCTACCGATTTTCCTGCTTCGGTT3′
U5 19 5′GTT**GAATTC**TGCTAG---TTTTCCTGCTTCGGTTTC3′

The primer upstream of the 3′ LTR (with *Sac*I site in boldface) was 5′ GGGCT T**GAGCTC**ACTCTCTTGTGAG 3′.

Integrase mutagenesis. Glu-136 in integrase was mutated to Lys by site-directed mutagenesis, creating the E136K mutation. Two primers were used; the mutagenic antisense primer spanned a *Kpn*I site (5′ TAT**GGTACC**CCAAAGG TGTGCTTTATCCCTG 3′, nucleotides 4956 to 4921), and the sense primer spanned another *Kpn*I site (5′ GTAGCATG**GGTACC**AGCACACACAAAGG3′, nucleotides 4445 to 4470 [the base changed by mutagenesis is underlined, and the *Kpn*I sites are in boldface). Both *Kpn*I sites were used for recloning. PCR conditions were the same as described above, using p239-SpSp-5′ as the template.

Preparation of virus stocks. Plasmids containing proviral half genomes with wild-type or mutant LTRs were cut with *Sph*I and ligated with T4 DNA ligase (New England Biolabs). CEMx174 cells were transfected with 3 μg of ligated SIVmac239 or mutant viral DNA by a DEAE-dextran procedure (23). The medium was changed every 3 days, and the supernatants were harvested on day 10. Cells and debris were deposited by centrifugation at 2,000 × *g* for 10 min, and virus contained in the supernatant was stored in aliquots at −80°C. The concentration of p27 antigen was measured by antigen capture assay (Coulter Corporation, Hialeah, Fla.). Viral infections were normalized by using equal amounts of p27 antigen.

Preparation of total cellular DNA. Total cellular DNA was prepared by using an AmpPrep kit (HRI Research, Inc., Concord, Calif.) as recommended by the manufacturer. Briefly, CEMx174 cells (10^5) infected with mutant virus were harvested in microcentrifuge tubes and centrifuged at 3,000 rpm for 1 min. The supernatants were removed, and the cells were resuspended in 50 μl of lysis buffer. Mineral oil was added to each tube, and the tube was vortexed for 5 s. The samples were incubated at 55°C for 5 min and then incubated at 95 to 100°C for 5 min. About 2 μl of total DNA was used for amplification. PCR conditions were essentially as described above.

Viral DNA synthesis. Viral DNA synthesis was measured following infection by using PCR. Virus stocks were generated by transfecting COS-1 cells with 2 μg of ligated SIVmac239 or mutant viral DNA, using Lipofectin (Promega Corp., Madison, Wis.) as recommended by the manufacturer. Culture supernatants were assayed for p27 content, and CEMx174 cells were infected with equal amounts (2 ng of p27) of each virus. Some infections were treated with the reverse transcriptase inhibitors azidothymidine (AZT; 50 μM) and phosphonoformic acid (PFA; 200 μM). Total cellular DNA was isolated 16 h postinfection.

Primers for PCR amplifying β-globin sequences were as described previously (34). SIV U3 (5′ AGAAGGAAACTCGCTGAAACAGCAG 3′, nucleotides 9852 to 9876) and U5 (5′ TTATTGAGTACCGAGTTGACCAGGC 3′, nucleotides 10213 to 10189) primers were designed to detect products of reverse transcription after translocation of minus-strand strong-stop DNA. PCR conditions were essentially as described above. SIV sequences were amplified for 35 cycles; β-globin sequences were amplified for 30 cycles.

Protein expression and purification. Plasmids were transformed into *E. coli* BL21(DE3) (30), and integrase expression was induced as described previously (7). Cells (1.5 liters) were harvested at 4°C, resuspended in 35 ml of 25 mM HEPES (pH 7.6)–0.1 mM EDTA, frozen in liquid N_2, and thawed on ice overnight. Wild-type and E136K integrases were purified at 4°C, using slightly different procedures.

For the wild-type protein, cells were lysed for 30 min in 40 ml of buffer containing 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 2 mM β-mercaptoethanol (β-ME), 5 mM imidazole, and 0.2 mg of lysozyme per ml, the lysate was sonicated as described previously (7) and centrifuged at 40,000 × *g* for 35 min, the supernatant was saved (fraction I), and the pellet was homogenized in 35 ml of 20 mM Tris-HCl (pH 8.0)–1.5 M NaCl–2 mM β-ME–5 mM imidazole. The suspension was stirred for 30 min and centrifuged at 40,000 × *g* for 35 min, the supernatant was saved (fraction II), and the pellet was homogenized in 20 ml of buffer A (20 mM Tris-HCl [pH 8.0], 6 M guanidine-HCl, 2 mM β-ME, 5 mM imidazole). Integrase-containing fractions were identified following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie blue.

A 2-ml Chelating Sepharose Fast Flow (Pharmacia Biotech, Piscataway, N.J.) column was precharged as described previously (7) and then equilibrated with buffer B (20 mM Tris-HCl [pH 8.0], 2 M NaCl, 2 mM β-ME, 5 mM imidazole). Fractions I and II were combined and loaded onto the column, and the column was washed with about 50 ml of buffer B and then with about 50 ml of buffer B containing 60 mM imidazole. Integrase was eluted by using a linear gradient of imidazole (60 mM to 1 M) in buffer B. Integrase-containing fractions were identified by SDS-PAGE and pooled, and EDTA was added to a final concentration of 5 mM. The protein was dialyzed against buffer C (20 mM HEPES [pH 7.6], 2 M NaCl, 5 mM EDTA, 2 mM β-ME, 10% [wt/vol] glycerol), then dialyzed against buffer C containing 0.3 M imidazole, and finally dialyzed against buffer D (20 mM HEPES [pH 7.6], 0.5 M NaCl, 1 mM EDTA, 2 mM β-ME, 0.3 M imidazole, 10% glycerol). The His tag was removed by thrombin cleavage essentially as previously described (7), and the thrombin was removed by passage over 0.4 ml of Benzamidine Sepharose 6B (Pharmacia) equilibrated with buffer D. The flowthrough was dialyzed against buffer E (20 mM HEPES [pH 7.6], 1 mM EDTA, 0.5 M NaCl, 10% glycerol, 1 mM dithiothreitol [DTT]) and then centrifuged at 19,000 × *g* for 10 min. The supernatant, which contained integrase at the concentration of 0.75 mg/ml, was frozen in liquid N_2 and stored at −80°C.

For the E136K mutant, the cells were lysed in 40 ml of buffer F (20 mM Tris-HCl [pH 8.0], 1 M NaCl, 2 mM β-ME, 5 mM imidazole) containing 0.2 mg of lysozyme per ml. After 30 min, the lysate was sonicated (7) and centrifuged at 40,000 × *g* for 35 min, the supernatant was saved (fraction IA), and the pellet was homogenized in 20 ml of buffer A. After 30 min, the suspension was centrifuged at 40,000 × *g* for 35 min, the supernatant was saved, and appropriate fractions were identified by SDS-PAGE.

Fraction IA was loaded onto a 2-ml Chelating Sepharose Fast Flow column equilibrated with buffer F. The column was washed with about 50 ml of buffer F, then with about 50 ml of buffer B containing 20 mM imidazole, and then with about 50 ml of buffer F containing 60 mM imidazole. The protein was eluted with a linear gradient of 60 mM imidazole to 1 M imidazole–10% glycerol in buffer F. EDTA was added to 5 mM to pooled column fractions, the protein was dialyzed against buffer D, and the His tag was removed as described above. Thrombin was removed as described above; the protein was dialyzed against buffer E and then centrifuged at 19,000 × *g* for 10 min. E136K integrase was recovered at the concentration of 1.2 mg/ml. The protein was frozen in liquid N_2 and stored at −80°C.

In vitro integration substrates. Blunt-end U5 and U3 substrates were 23 and 22 bp, respectively (see Fig. 6A). The U5 substrate was prepared by end labeling AE388 (5′-AAGCAGGAAAATCCCTAGCAGAT-3′) and annealing the complement strand, AE389, as previously described (7). For the U3 substrate, AE407 (5′ ACTGGAAGGGATTTATTACAGT 3′) was labeled and the complement strand was annealed. For the precleaved U5 substrate (see Fig. 6B), AE421 (5′ AAGCAGGAAAATCCCTAGCA 3′) was labeled and AE389 was annealed.

Tethered U3-U5 substrates contain three strands, two of which are labeled (see Fig. 6C) (17). AE424 (175 ng) (5′ CGAAGCAGGAAAATCCCTAGCA GATACTGGAAGGGATTTATTAC 3′) and AE425 (75 ng) (5′ GTAATAAAT CCCTTCCAGT 3′) were labeled in separate tubes, the kinase was heat inactivated (7), and the two strands were mixed with 100 ng of AE426 (5′ TCTGCT AGGGATTTTCCTGCTTCG3′). This yielded an approximate equimolar mixture of the three strands. NaCl was added to 0.1 M; the DNA was heated to 85°C for 3 min and then cooled to room temperature over about 30 min. Unincorporated nuclide was removed as previously described (10). Native PAGE revealed that ≥95% of the DNA annealed under these conditions.

Mutant substrates were prepared after incorporating base changes into separate DNA strands.

In vitro integration reactions. Mutant blunt-ended DNA was assayed under conditions which yielded about one-third of the maximum activity with the wild-type substrates (see Results). These reaction mixtures (16 μl) contained 25 mM morpholinepropanesulfonic acid (MOPS; pH 7.2), 0.1 mg of bovine serum albumin (BSA) per ml, 10 mM β-ME, 10% glycerol, 7.5 mM $MnCl_2$, 100 nM DNA, 50 mM NaCl, and 0.4 μM integrase. Reactions were terminated after 60 min at 37°C and analyzed by denaturing PAGE as described previously (7).

Similar to observations for HIV (10), DTT, dimethyl sulfoxide, and polyethylene glycol 8000 were found to stimulate Mg^{2+}-dependent SIV integrase activity but suppress Mn^{2+}-dependent activity (19). Mn^{2+}- and Mg^{2+}-dependent reaction kinetics were therefore assayed by using two different buffer conditions. Conditions which yielded about one-half of the maximum activity after 1 h at 37°C with the wild-type U5 blunt end were chosen for this analysis. Mn^{2+}-dependent conditions were 25 mM MOPS (pH 7.2), 0.1 mg of BSA per ml, 10 mM β-ME, 5 nM DNA, 5 mM $MnCl_2$, 60 mM NaCl, 10% glycerol, and 0.28 μM integrase. Mg^{2+}-dependent reaction mixtures contained 25 mM MOPS (pH 7.2), 20 μg of BSA per ml, 10 mM DTT, 5 nM DNA, 5 mM $MgCl_2$, 25 mM NaCl, 15% glycerol, 15% dimethyl sulfoxide, 5% polyethylene glycol 8000, and 0.28 μM integrase. Reactions (16 μl) were terminated after 1, 2, 4, 8, 16, 32, and 64 min at 37°C and analyzed following denaturing PAGE.

Precleaved U5 ends were assayed with pUC19 target DNA. Reaction mixtures (16 μl) contained 25 mM MOPS (pH 7.2), 0.1 mg of BSA per ml, 10 mM β-ME, 10% glycerol, 7.5 mM $MnCl_2$, 75 mM NaCl, 25 nM SIV DNA, 2.5 nM pUC19, and 0.5 μM integrase. U5 and integrase were mixed on ice for 5 min, pUC19 was

TABLE 1. Replication of SIV with mutated U3-U5 following transfection of CEMx174 cells

Virus	p27 production[a]		
	1st transfection	2nd transfection	3rd transfection
SIVmac239	WT	WT	WT
Mutant			
1	↓ ↓ ↓	↓ ↓ ↓	ND
2	↓ ↓ ↓	ND	ND
3	↓	ND	ND
4	WT	WT	ND
5	↓	↓	↓
6	↓ ↓ ↓	↓ ↓ ↓	↓ ↓ ↓
7	↓ ↓	↓ ↓	↓ ↓
8	↓ ↓	↓ ↓	↓ ↓
9	↓ ↓	ND	ND
10	WT	WT	ND
11	↓	WT	ND
12	↓	WT	ND
13	↓ ↓ ↓	ND	ND
14	WT	WT	ND
15	↓ ↓	ND	ND
16	ND	↓ ↓	↓ ↓
17	↓	↓	↓
18	ND	ND	↓
19	ND	↓	↓

[a] CEMx174 cells were transfected with 3 μg of cloned plasmids; WT, peak p27 antigen production 11 days posttransfection; ↓, peak 27 production delayed 3 to 10 days; ⇓, p27 production delayed 20 to 60 days; ↓ ↓ ↓, p27 production delayed more than 60 days; ND, not done.

added, and incubation continued for 5 min at room temperature and then at 37°C for 60 min. Reactions were terminated by adding 4 μl of 2.5% (wt/vol) SDS–25% (wt/vol) Ficoll 400–50 mM EDTA–0.025 (wt/vol) bromophenol blue and then analyzed by agarose gel electrophoresis as described previously (10).

Tethered U3-U5 substrates were assayed under conditions which optimized for coupled cleavage of U3 and U5. Reaction mixtures (16 μl) contained 25 mM MOPS (pH 7.2), 0.1 mg of BSA per ml, 10 mM β-ME, 10% glycerol, 7.5 mM $MnCl_2$, 5 nM DNA, 20 mM NaCl, and 0.5 μM integrase. Reactions were terminated after 60 min at 37°C and analyzed by denaturing PAGE.

Results were visualized by autoradiography and quantitated by using either densitometry (IS 1000 digital imaging system; Alpha Innotech Corp., San Leandro, Calif.) or a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

RESULTS

Mutant construction and viral replication. In this study, we constructed 19 mutant forms of SIVmac239 proviral DNA with changes introduced into the inverted terminal repeats by site-directed mutagenesis (Fig. 1A). Ten of the 12 bp that comprise the SIV *att* site display sequence complementarity (Fig. 1B). The roles of certain base pairs in SIV replication were studied by introducing paired, nonpaired, and deletion mutations into both LTRs. Whereas some of the mutations were designed to maintain the wild-type pattern of complementarity (for example, mutants 1, 6, and 12), others were chosen to either disrupt the complementarity (for example, mutants 8, 10, and 11) or enhance it (mutants 3 and 4). To help ensure that the mutations were preserved in the process of replication, each U3 and U5 mutation was introduced into both the 5′ and 3′ LTRs. Thus, for mutant 2 (Fig. 1B), TG/AC at bp 1 and 2 in U3 of the 5′ LTR, and 9462 and 9463 in U3 of the 3′ LTR, were changed to GC/CG; CA/GT at bp 10278 and 10279 in U5 of the 3′ LTR, and 817 and 818 in U5 of the 5′ LTR, were changed to TG/AC.

Viral replication was measured after transfection of cloned DNA into CEMx174 cells. Mutant clones 4, 10, 11, 12, and 14 each replicated as well as wild-type SIVmac239. Clones 3, 5, 17, 18, and 19 had replication delayed from 3 to 10 days. Clones 7, 8, 9, 15, and 16 had replication delayed from 20 to 60 days. Clones 1, 2, 6, and 13 showed no signs of replication after more than 60 days (Table 1).

Compensatory mutation in U5 and integrase of clone 7. Clone 7 showed severely delayed replication. Once it began to replicate 35 days after transfection, however, virus production increased at a rate similar to that for wild-type SIVmac239 (Fig. 2). This finding suggested that a reversion or compensatory mutation may have occurred. Virus obtained from cells transfected with clone 7 at 45 days posttransfection replicated with only a 3-day delay compared to the wild type upon reinfection of CEMx174 cells (Fig. 3). These results further supported the suspicion that reversion or compensatory mutations were present in this recovered virus.

To determine whether mutations had indeed occurred in clone 7, total DNA was prepared from transfected cultures after emergence of the revertant virus, as well as from cells freshly infected with this virus. The U3 and U5 regions of the LTRs and the 3′ end of the *pol* gene, encompassing the entire integrase coding region, were amplified by PCR. Sequence analysis showed that the original mutations in U3 had not reverted in 26 clones from three independent DNA preparations (Table 2). However, the U5 region had several changes. The predominant change was G to A at nucleotide 822 (position 5 from the right terminus of proviral DNA). Some of the clones showed reversion from G to T at this position (Table 2).

Interestingly, the 3′ end of the *pol* gene exhibited sequence changes as well. The predominant change was G to A at nucleotide 4934 (numbering based on reference 28), resulting in the substitution of lysine (K) for glutamic acid (E) at amino acid position 136 in integrase (Table 2). This change was found in DNA prepared from two independent cultures. Further experiments were thus planned to investigate the possible contribution of sequence changes in U5 and in integrase to viral replication.

Contribution of the U5 and integrase mutations to restored replication of clone 7. Since the G-to-A mutation in U5 and the E-to-K substitution in integrase were the predominant changes recovered from clone 7-infected cultures, we tested whether these mutations could compensate for the original clone 7 mutations. The following site-specific mutants were constructed: SIVmac239 with only the E-to-K change at amino acid 136 in integrase (SIV239INE136K); mutant 7 with the E-to-K change in integrase (SIV239 7INE136K); mutant 7 with the G-to-A change in U5 (U3-U5 7-1); and mutant 7 with both the E-to-K change in integrase and the G-to-A change in U5 (U3-U5 7-1INE136K) (Fig. 4). These clones were transfected into CEMx174 cells and tested for viral replication.

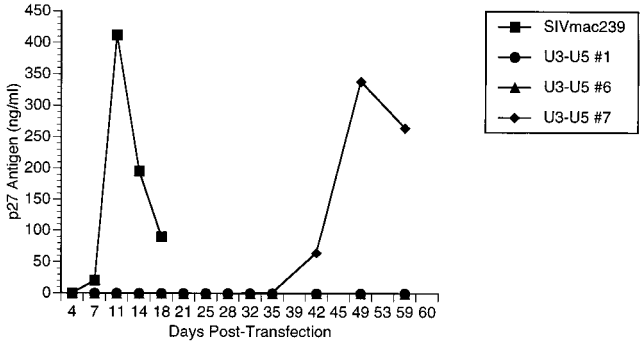


FIG. 2. Replication kinetics of wild-type and mutant SIV. CEMx174 cells were transfected with ligated plasmid DNA containing the wild-type SIVmac239 (SIV239) provirus or the indicated mutant. The cells were split every 3 days, and aliquots were tested for p27 content by using an antigen capture assay.

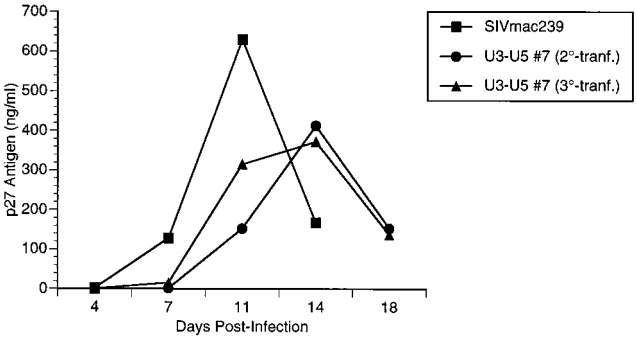


FIG. 3. Replication kinetics of virus recovered 45 days posttransfection with clone 7 DNA. CEMx174 cells (2×10^6) were infected with wild-type SIVmac239 (2 ng of p27 antigen) or the supernatants derived from two independent transfections with clone 7 DNA. The cultures were treated as described for Fig. 2.

Parental SIVmac239 and clone SIV239INE136K exhibited the same replication kinetics in CEMx174 cells (Fig. 5). The original clone 7 consistently exhibited grossly delayed replication. Clones U3-U5 7-1 and SIV239 7INE136K replicated with 10- and 14-day delays compared to the wild type, indicating that either mutation alone partially compensated for the original clone 7 mutations. Replication of U3-U5 7-1INE136K was delayed only 3 days compared to the wild type, similar to the replication profile of the virus recovered 45 days posttransfection with original clone 7 DNA (Fig. 3). These results demonstrate that an amino acid change in integrase is able to compensate functionally for mutations in the terminal nucleotides of the LTR.

Effect of the integrase E136K mutation on replication of clone 6. The results described above indicated that the E-to-K change alone in integrase could partially compensate for the mutations in clone 7. We next examined whether it could also compensate for the mutations in clone 6, which did not detectably replicate even after 60 days from the time of transfection (Fig. 2). The E-to-K change in integrase was introduced into clone 6 (U3-U5 6E136K) (Fig. 4). Replication of clone U3-U5 6E136K was detected 50 days after transfection (Fig. 5), while replication of the original clone 6 was not detected even 120 days after transfection (data not shown). The delayed replication of clone U3-U5 6E136K suggests that the mutation in integrase partially compensated for the LTR mutations in clone 6 but could not fully restore replication.

To examine if other changes had occurred in clone U3-U5 6E136K, total DNA was prepared from three independent cultures after growth of this mutant. The U3 and U5 regions, as well as the 3′ end of the *pol* gene, were amplified by PCR. The results of sequencing 14 clones from three independent cultures again showed that no changes occurred in U3 (Table 3). However, nucleotide 822 of U5 had mutated from G to T in 30 of 33 clones (Table 3). T is present at this position in parental wild-type SIVmac239. Sequencing of the *pol* gene revealed only valine (V) changed to isoleucine (I) at position 141 of integrase from one culture, whereas 10 clones from two other cultures showed no additional changes in integrase (Table 3). These results further demonstrate the importance of position 5 from the terminus of U5 for virus replication and the ability of an E-to-K change at position 136 in integrase to compensate for terminal nucleotide mutations.

In vitro integration. A subset of the *att* site mutants were analyzed for effects on the in vitro integration activities of recombinant SIV integrase. Blunt-end U5 DNA substrates were tested initially (Fig. 6A). Conditions which yielded about one-third (20% of the wild-type substrate processed; 2% integrated) maximum activities were chosen for mutant analysis.

Substrate 1, which contained the substitution of TG/AC for the invariant CA/GT, supported 2 to 5% of wild-type 3′ processing activity (Table 4). This low level of in vitro activity is consistent with the replication-defective phenotype of SIV carrying this change in both U5 and U3 (Table 1). In vitro activities of most of the other mutant substrates also correlated roughly with the corresponding viral replication phenotypes (Tables 1 and 4). The notable exceptions were mutants 6 and 7. Each of these mutations severely impaired the ability of virus to replicate but had little effect on integrase activity with blunt-end U5 integration substrates. We thus analyzed integrase activity by using blunt-end U3 and precleaved U5 substrates.

The U3 substrates were also analyzed under conditions which supported one-third (10 to 15% of the wild-type substrate processed) maximum activity. As observed for the U5 substrates, mutants 6 and 7 supported the wild-type level of activity (Table 4). Precleaved U5 substrates were reacted with pUC19 target DNA. Mutant 1 displayed approximately 5% of wild-type strand transfer activity under these conditions. Mutant 7 displayed the wild-type level of activity, while the activity of mutant 6 was approximately 50% of that of the wild type (Table 4).

Tethered U3-U5 DNA substrates. We found that SIV integrase readily cut and joined mutant 6 and 7 single-LTR-end substrates, even though the replication of these viruses was severely impaired. One possible explanation is that in vitro integration assays are limited in their ability to recapitulate

TABLE 2. Analysis of IR and integrase gene sequences in cells infected with mutant 7[a]

Viral DNA	U3 sequence (nt 9462–9473)	U5 sequence (nt 818–807)	Integrase gene
SIVmac239	TGGAAGGGATTT	ACGATCCCTAAA	WT
U3-U5 mutation 7	TGGATACGATTT	ACGAGAACTAAA	WT
DNA from transfected cells	TGGATACGATTT (10[b])	ACGAGAACTAAA (7)	ND
		ACGAAAACTAAA (1)	
DNA from infected cells			
Expt 1	TGGATACGATTT (7)	ACGAGAACTAAA (5)	E136K (2)
		ACGAAAACTAAA (13)	E136K and D163H (1)
		ACGATAACTAAA (1)	
Expt 2	TGGATACGATTT (9)	ACGAGAACTAAA (1)	WT (3)
		ACGAAAACTAAA (1)	E136K (1)
		ACGATACCTAAA (8)	A54V and E136K (1)
		ACGAAGAACTAAA (7)	E10K, G27E, and E276K (1)

[a] nt, nucleotides; WT, wild type; ND, not done. Underlined bases differ from the wild-type sequence.
[b] Number of clones with indicated sequence.

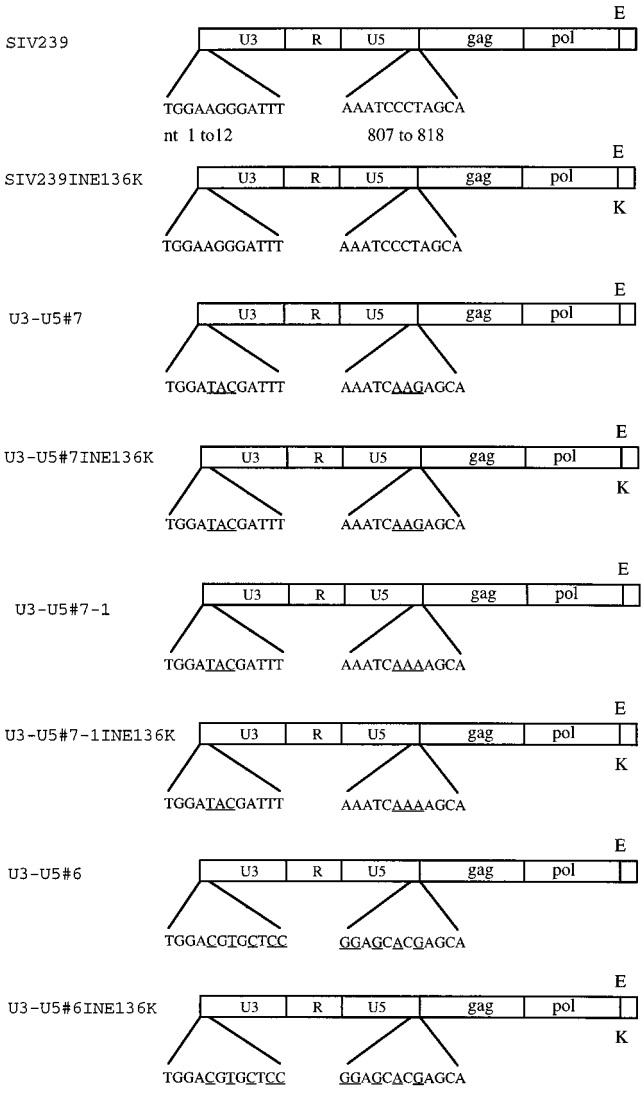


FIG. 4. Structures of site-directed revertant mutant viruses. The sequences of the plus strands of the U3 and U5 IRs are indicated below a genetic map of the left half of SIV (not drawn to scale). The identity of either E or K at position 136 in integrase is indicated on the right. Base changes are underlined. nt, nucleotides.

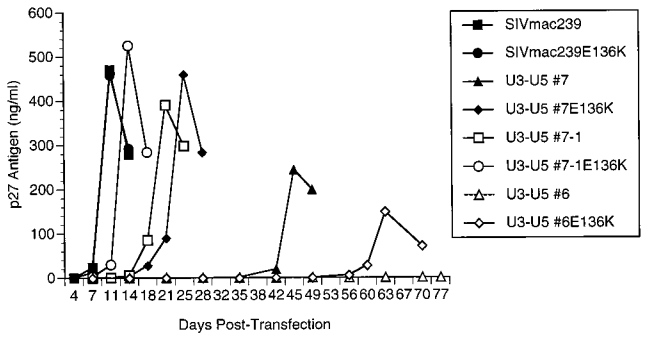


FIG. 5. Replication kinetics of reconstructed revertant viruses. CEMx174 cells were transfected with either ligated wild-type SIVmac239 or the indicated mutant plasmid DNA. Cultures were treated as described for Fig. 2.

certain viral mutant phenotypes. Another concern is that the viruses contained mutations in both U3 and U5. We therefore tested mutant 6 and 7 tethered U3-U5 DNA substrates (17). These 3′ processing substrates contain both viral termini tethered by a single-nucleotide gap (Fig. 6C). A tethered substrate containing the G-to-A change (substrate 7-1) identified in the U5 end of the 7 revertant virus was also analyzed.

In vitro reaction conditions were optimized for coupled cleavage of U5 and U3 (60 to 70% of U5 processed; about 20% of U3) (Fig. 7, lane 2). Substrates 7 (Fig. 7, lane 8) and 7-1 (Table 4) each supported close to this level of activity (about 40% of U5 processed; 20% of U3). In contrast, mutant 6 supported only about 10% processing of U5 and about 10% processing of U3 (Fig. 7, lane 5, and Table 4).

In vitro activities of E136K integrase. In the course of this study, we identified a mutation at position 136 in integrase which in part compensated for the mutations in viral clone 7 (Fig. 5). Based on this result, we had hypothesized that recombinant E136K integrase might show preferential in vitro activity for mutant 7 substrates. However, during the study we found that four different types of mutant 7 substrates, blunt-end U5 and U3, precleaved U5, and tethered U3-U5, supported nearly wild-type levels of 3′ processing and DNA strand transfer activities (Table 4). This observation indicated that differences between wild-type and E136K integrase activities with wild-type and mutant 7 substrates would be subtle. Reaction components were systematically varied in an attempt to detect altered specificity of purified E136K integrase for mutant 7 DNA substrates.

Wild-type and E136K integrase displayed similar 3′ processing activities with wild-type and mutant 7 blunt-end U5 DNA over a range (25 to 200 mM) of NaCl concentrations (data not shown). 3′ processing reaction kinetics were analyzed under Mn^{2+}- and Mg^{2+}-dependent conditions. In general, activities were unaffected by the identity of the integrase, DNA substrate, or divalent metal ion (data not shown). Blunt-end U3, precleaved U5, and tethered U3-U5 DNA substrates were also analyzed. Convincing evidence of E136K integrase specificity

TABLE 3. Analysis of IR and integrase gene sequence in cells infected with mutant 6INE136K[a]

Viral DNA	U3 sequence (nt 9462–9473)	U5 sequence (nt 818–807)	Integrase gene
SIVmac239	TGGAAGGGATTT	ACGATCCCTAAA	WT
U3-U5 mutation 6INE136K	TGGACGTGCTCC	ACGAGCACGAGG	E136K
DNA from transfected cells			
Expt 1	TGGACGTGCTCC(2[b])	ACGATCACGAGG(6)	E136K & V141I (6)
Expt 2	TGGACGTGCTCC(2)	ACGATCACGAGG(10)	E136K (1)
Expt 3	TGGACGTGCTCC(10)	ACGATCACGAGG(14)	E136K (9)
		ACGAGCACGAGG(3)	

[a] nt, nucleotides; WT, wild type. Underlined bases differ from the wild-type sequence.
[b] Number of clones with indicated sequence.

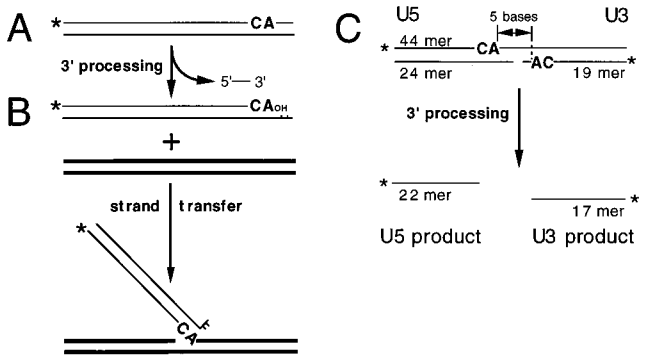


FIG. 6. In vitro integration substrates. (A) Single-end 3′ processing substrate. The DNA is labeled (marked ∗) at the 5′ end of the strand that is cleaved by integrase. Previous sequence analysis of the two-LTR circle formed in SIV-infected cells indicated that a GAT trinucleotide is cleaved from the U5 end in the 3′ processing reaction (26). The GAT trinucleotide was incorporated into the blunt-end U5 substrate 3′ of the conserved CA. The blunt-end U3 substrate contained the predicted GT dinucleotide at the 3′ end (26). 3′ processing yields a labeled product which is two (U3) or three (U5) bases shorter than the starting substrate. (B) DNA strand transfer of the processed viral end (thin lines) into a target DNA (bold lines) yields a labeled product which is longer than the starting substrate. (C) Tethered U3-U5 substrate. The phosphodiester bonds in U3 and U5 cleaved in the 3′ processing reaction are separated by 5 bp, mimicking the 5-bp target sequence duplication produced upon SIV integration in vivo (28). The substrate is labeled (∗) at the 5′ ends of the strands that are cleaved by integrase. Processing yields two labeled shorter strands, a 22-base U5 product and a 17-base U3 product.

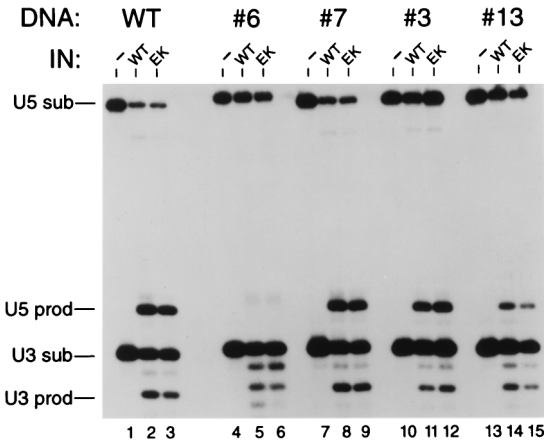


FIG. 7. Activities of tethered U3-U5 substrates. The type of DNA substrate (wild type [WT] or mutant) is indicated above the gel. The reactions in lanes marked WT and EK contained wild-type and E136K integrase proteins, respectively. Integrase (IN) was omitted from the reactions marked −. The migration positions of the 44-base labeled U5 ends and 19-base labeled U3 ends are marked U5 sub and U3 sub, respectively. The migration positions of the 22-base U5 products and 17-base U3 products are marked U5 prod and U3 prod, respectively.

for mutant 7 DNA substrates was not observed (Fig. 7, lanes 8 and 9, and data not shown).

DISCUSSION

Effective retroviral integration requires *cis*-acting sequences located at each end of linear cDNA and the *trans*-acting integrase protein. Results of mutagenesis experiments which targeted the integrase established that integration is required for retroviral replication (8, 11, 12, 18, 25, 29). Imperfect IRs at the U3 and U5 termini of linear DNA comprise the *cis*-acting *att* site (32). Sequence features of the *att* site important for lentiviral integration and replication are not well defined. In this study, we analyzed mutations in both termini of SIVmac239 DNA for effects on viral replication in tissue culture.

TABLE 4. In vitro activities of wild-type and mutant oligonucleotide substrates

Virus	Activity[a]			
	Blunt U5[b]	Blunt U3[b]	Precleaved U5	Tethered U3-U5
Wild type	++++	++++	++++	++++
Mutant				
1	+	ND	+	ND
3	++	ND	ND	++++
4	++++	ND	ND	ND
6	++++	++++	+++	++
7	++++	++++	++++	++++
8	++	ND	ND	ND
12	+++	NA	ND	ND
13	++	NA	ND	+++
7-1	ND	NA	ND	++++

[a] ++++, 50 to 100% of the activity of the wild-type substrate; +++, 20 to 50% of wild-type activity; ++, 10 to 20% of wild-type activity; +, 1 to 10% of wild-type activity. Values are averages of a minimum of two experiments. ND, not done; NA, not applicable. The U3 changes in mutant 12 were the same as in mutant 4 (similarly for mutants 13 and 3 and for mutants 7-1 and 7).

[b] Activities relative to wild-type activity were similar at 4 and 25 nM substrate.

Features of the SIV *att* site important for viral replication. The results with mutant clones 1 and 2 demonstrate the importance of the invariant terminal two base pairs, 5′ TG. . .CA 3′, in SIV replication. These results are consistent with a number of previous studies. Deletions which removed the terminal CA/GT base pairs from the U5 end of Moloney murine leukemia virus inhibited viral integration and replication (5, 6). Spleen necrosis virus integration was inhibited by deleting the invariant base pairs from either LTR terminus (24). In contrast, substituting AC/TG for TG/AC at the 5′ end of HIV reduced integration only about threefold (20). Unlike the study reported here, none of these previous studies simultaneously targeted both IRs.

Clone 6, which has five mutations, at positions 5, 7, 9, 11, and 12, and clone 13, which carries substitutions and deletions at positions 3 and 4 (Fig. 1B), also did not replicate detectably in these assays. Thus, in addition to the conserved terminal two base pairs, subterminal positions in the SIV IRs are also important for replication. Previous studies have shown that mutations in the subterminal regions of Moloney murine leukemia virus (5, 6, 22), spleen necrosis virus (24), Rous sarcoma virus (3, 4), and HIV (20, 27, 33) can affect integration.

SIV replication seemed to be somewhat tolerant to point mutations at positions 3 and 4. These 2 positions are the only ones in the terminal 12 of the SIV *att* site which are not complementary (Fig. 1B). Mutants 4 and 12, which targeted positions 3 and 4, replicated as well as wild-type upon DNA transfection and virus infection. Mutant 4 was designed to create the potential for base pairing at positions 3 and 4. Thus, the absence of the potential for base pairing at these positions is not an essential element of the SIV *att* site. There does, however, appear to be some contribution from these positions, since mutant 3 showed a slight (3- to 10-day) delay in virus replication.

Mutants 7, 15, and 16 replicated with a severe (20- to 60-day) delay in replication. Thus, positions 5 to 7 from the termini appear to be very important for SIV replication. Mutants 17 and 18, which affect position 5 and positions 6 and 7 from the

termini, respectively, had smaller delays (3 to 10 days) (Fig. 1B and Table 1). Mutant 8, with changes at positions 6 to 8, and mutant 9, with changes at positions 6 to 9, replicated with severe (20 to 60-day) delays. Mutant 14, with changes at positions 7 to 9, however, replicated with wild-type kinetics. Since the U3 ends of mutants 9 and 14 were identical, clone 9 highlights contributions of U5 subterminal positions 6 to 9 to SIV replication. Likewise, mutant 13 was completely defective yet differed from mutant 3 only at U5 positions 5 and 7 to 9.

Mutant 19, with deletions of positions 7 to 9 from both termini, replicated with only a slight (3- to 10-day) delay. This deletion maintained complementarity at positions 7 to 9 and effectively changed U3 positions 7 to 10 and U5 positions 7 to 9, 11, and 12. Point mutations at positions 8 to 10 from the termini (mutant 10) and at positions 9 to 11 (mutant 11) did not have a noticeable effect on virus replication (Fig. 1B and Table 1).

In summary, the nucleotides at positions 1, 2, and 5 to 7 from the termini of proviral DNA appear to be the most critical for SIV replication. We also conclude that the potential for base pairing in the subterminal region of the *att* site plays little if any role in replication.

In vitro activities of mutant *att* sites. Eight of the mutant *att* sites were tested for in vitro integration activities by using recombinant SIV integrase. When assayed as single U5 ends, six of the mutants displayed activities which were roughly consistent with the replication profiles of the corresponding mutant viruses. Two of the mutants, 6 and 7, however, did not show this correlation. Although these viruses were severely impaired for replication, their U3 and U5 ends supported wild-type levels of in vitro integration activities.

We therefore considered the possibility that mutants 6 and 7 affect a step in the viral life cycle other than integration. In the viral genome, the U5 terminal region forms RNA secondary structures which are important for the initiation of reverse transcription (1, 13, 14). To detect early products of reverse transcription in mutant 6- and 7-infected cultures, total cellular DNA was isolated 16 h postinfection and analyzed by PCR using U3- and U5-specific primers (Fig. 8). In two independent amplifications, mutant 6 yielded similar levels of DNA as did wild-type-infected cultures (Fig. 8A; compare lane 9 to lane 7 and lane 13 to lane 12). Cells infected with mutant 7 yielded less PCR product; however, this level was consistently above that observed for cells infected with wild-type SIV in the presence of reverse transcriptase inhibitors (Fig. 8A; compare lane 10 to lane 8 and lane 14 to lane 11). We conclude that the replication-defective phenotype of mutant 6 is due to a block in integration, while the defective phenotype of mutant 7 may in part be due to lowered reverse transcription. However, since E136K integrase partially restored replication to each of these viruses, we speculate that mutant 7 is primarily integration defective in infected cells.

Interestingly, the mutations in clone 6 affected the in vitro activity of the tethered U3-U5 integration substrate. This finding implies that these mutations exert their damaging effect through a synapsed protein-DNA complex involving both LTR termini. This interpretation is consistent with previous models of retroviral integrase function (17, 21). More sophisticated in vitro assays may be needed to understand the nature of the integration defect with mutant 7.

A mutation in integrase partially overcomes SIV *att* site mutations. Mutant 7 replicated after a prolonged (20- to 60-day) delay. Virus which grew after this delay replicated similarly to wild-type SIVmac239 upon reinfection. Analysis of this revertant virus revealed a complex population (Table 2). Two changes, however, were detected in independent experiments:

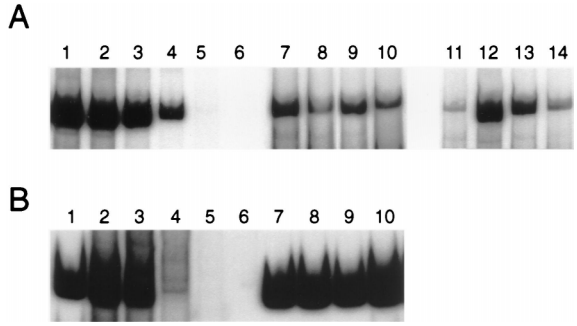


FIG. 8. Viral DNA synthesis in cells infected with wild-type and mutant SIV. (A) PCR amplification with SIV-specific primers. Lane 1, 10^6 copies of SIV plasmid DNA; lane 2, 10^5 copies; lane 3, 10^4; lane 4, 10^3; lane 5, 10^2 copies. Plasmid DNA was omitted from the PCR in lane 6. Lanes 7 and 12, DNA isolated from cells infected with wild-type SIV; lanes 8 and 11, DNA from cells infected with wild-type in the presence of AZT and PFA; lanes 9 and 13, DNA from cells infected with mutant 6; and lanes 10 and 14, DNA from cells infected with mutant 7. (B) Amplification using β-globin-specific primers. Lanes 1 to 4, 5, 2, 0.4, and 0.08 μg of uninfected CEMx174 cellular DNA, respectively. Cellular DNA was omitted from the reaction in lane 5. Lane 6, empty; lanes 7 to 10, DNAs isolated from cells infected wild-type SIV, wild-type SIV plus AZT-PFA, mutant 6, and mutant 7, respectively.

G to A at position 10275 in U5 and E to K at position 136 in integrase. Reconstruction experiments showed that each of these changes partially restored replication to the original clone 7 and that combining both changes yielded a replication profile identical to that of the original revertant population. These results highlight the importance of position 5 in U5 for SIV replication and show that a mutation in integrase can compensate for changes in the terminal nucleotides of the LTRs. Replication of mutant 6 was also partially restored by changes at position 5 in U5 and 136 in integrase (Table 3). This is the first example of a retroviral integrase mutation which compensates for mutations in the terminal regions of the LTRs. Lys-136, the residue in HIV analogous to Glu-136 in SIV, is solvent accessible in the crystal structure of the integrase catalytic domain (9). It is possible that Glu-136 interacts directly with SIV DNA in the context of the viral preintegration complex. Our results also support the contention that resistance to integrase inhibitors may include LTR mutations in addition to changes in integrase.

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