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An Integration-Defective U5 Deletion Mutant of Human Immunodeficiency Virus Type 1 Reverts by Eliminating Additional Long Terminal Repeat Sequences

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Nonoverlapping deletions that eliminated the 5′ (HIV-$1_{U5/603del}$), middle (HIV-$1_{U5/206del}$), and 3′ (HIV-$1_{U5/604del}$) thirds of the U5 region of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) were studied for their effects on virus replication (transient transfection of HeLa cells) and infectivity (T-cell lines and peripheral blood mononuclear cells). All three mutants exhibited a wild-type phenotype in directing the production and release of virus particles from transfected HeLa cells. In infectivity assays, HIV-$1_{U5/206del}$ was usually indistinguishable from wild-type virus whereas HIV-$1_{U5/603del}$ was unable to infect human peripheral blood mononuclear cells or MT4 and CEM cells. Investigations of HIV-$1_{U5/603del}$ particles revealed a packaging defect resulting in a 10-fold reduction of encapsidated genomic RNA. The HIV-$1_{U5/604del}$ mutant either was noninfectious or exhibited delayed infection kinetics, depending on the cell type and multiplicity of infection. Quantitative competitive PCR indicated that HIV-$1_{U5/604del}$ synthesized normal amounts of viral DNA in newly infected cells. During the course of a long-term infectivity assay, a revertant of the HIV-$1_{U5/604del}$ mutant that displayed rapid infection kinetics emerged. Nucleotide sequence analysis indicated that the original 26-nucleotide deletion present in HIV-$1_{U5/604del}$ had been extended an additional 19 nucleotides in the revertant virus. Characterization of the HIV-$1_{U5/604del}$ mutant LTR in in vitro integration reactions revealed defective 3′ processing and strand transfer activities that were partially restored when the revertant LTR substrate was used, suggesting that the reversion corrected a similar defect in the mutant virus.

Retroviral long terminal repeats (LTRs) are generated during the process of reverse transcribing the viral genomic RNA to viral DNA and serve a multitude of functions during the infectious cycle. LTR sequences have historically been subdivided into three discrete regions (U3, R, and U5) on the basis of their locations in the viral genome. By definition, R sequences are situated at the very termini of the full-length retroviral genomic RNA molecules. They participate in the process of reverse transcription by forming intermolecular DNA-RNA hybrid bridges which link short nascent single-stranded DNA molecules and the untranscribed genomic RNA template (15, 17, 30, 52). The U3 and U5 components of LTRs are unique (U) blocks of sequence that map immediately internally to the R regions at the 3′ and 5′ ends of the retroviral RNA, respectively. In the context of the unintegrated linear viral DNA present in the nuclei of recently infected cells, sequences situated near the ends of the LTR mediate the precise and efficient integration into the chromosomal DNA of the host cell (13, 16, 43). Following integration, a major function of the retroviral LTR is to regulate viral mRNA production; the promoter/enhancer elements, needed for efficient transcriptional initiation, are located within the U3 region and function in the context of the 5′ LTR (54). The polyadenylation signal (AATAAA) and addition sites are generally positioned in the R region but are active only as components of the 3′ LTR.

The functional role(s) of U5 sequences during the retrovirus life cycle is less well understood. Deletion mutations introduced into the 5′ portion of U5 markedly impair the packaging of Moloney murine leukemia virus (Mo-MuLV) RNA into progeny particles (41), whereas alterations affecting the 3′ regions of U5 of both avian leukosis viruses and MuLVs severely reduce infectivity by interfering with the initiation of the reverse transcription reaction (4, 12, 14, 41). Mutagenesis of the human immunodeficiency virus type 1 (HIV-1) U5 region has been investigated primarily in the context of LTR-driven reporter gene systems and has focused on the role of clustered G-T residues located near the 5′ terminus of U5 in possibly modulating the polyadenylation of viral mRNAs (10).

In this study, we have examined HIV-1 U5 function by deleting nonoverlapping one-thirds of the U5 region in the context of an infectious molecular clone of the HIV-1 provirus. Deletion of the middle third of the HIV-1 U5 had little effect on HIV-1 infectivity, whereas elimination of the 5′ and 3′ thirds profoundly impaired virus infectivity by interfering with the encapsidation of newly synthesized genomic RNA and viral DNA integration, respectively. During the course of this work, a novel revertant in which the original 26-nucleotide (26-nt) U5 deletion (which had eliminated the 3′ one-third of U5) was extended an additional 19 nt, thereby partially restoring integration function, was identified.

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MATERIALS AND METHODS

Cells and virus infections. Peripheral blood mononuclear cells (PBMCs), purified by Ficoll-Hypaque density gradient centrifugation, were stimulated (10^6 cells per ml) with phytohemagglutinin (PHA-P; Sigma Chemical Corp., St. Louis, Mo.) (5 μg/ml) for 3 days. The phytohemagglutinin-activated PBMCs were washed twice with RPMI 1640 medium containing 10% fetal calf serum and then resuspended in the same volume of RPMI 1640 containing 10% fetal calf serum and 20 U of recombinant interleukin-2 (Boehringer Mannheim) per ml. The PBMCs were cultured in 24-well plates (at a concentration of approximately 5×10^5 cells per ml) and infected with virus produced from transfected HeLa cells. MT4 cells (28) and 12D7 cells (47), the latter subcloned from the CEM-derived A3.01 cell line (24), were both maintained in RPMI 1640 supplemented with 10% FCS. HeLa cells were plated at a concentration of 2×10^5 cells per 25-cm^2 flask in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Virus stocks were prepared by transfecting HeLa cells, 24 h following plating, with cloned HIV-1 proviral DNA (20 μg) in the presence of calcium phosphate by using the Transfinity (Life Technologies, Inc., Gaithersburg, Md.) or Strategene (La Jolla, Calif.) kits. Progeny virus production was monitored by assaying Mg^{2+}-dependent ^{32}P-reverse transcriptase (^{32}P-RT) activity (59) released into the medium 48 h following transfection. When HeLa cells were transfected with the wild-type molecular clone of HIV-1, pNL4-3 (1), approximately 5×10^4 infectious units (determined by end point dilution infectivity assays), equivalent to about 5×10^6 cpm of ^{32}P-RT activity, were present per ml of the culture supernatant. In the comparative cell-free virus infections to be described, equal amounts of wild-type and mutant viruses (based on RT activity) were used as inocula.

Cultures of PBMCs, maintained in 24-well plates (5×10^5 cells per well), were infected with 1.5×10^5 cpm of RT activity of virus produced from transfected HeLa cells. RPMI 1640 medium (containing interleukin-2) changes were made every 2 to 3 days after samples were collected for RT determinations. Kinetic analyses of spreading virus infections were conducted in two different human T-cell systems. 12D7 cells (10^6 cells in 0.1 ml of medium) were incubated with equal amounts of wild-type, mutant, and revertant viruses (approximately 2×10^6 cpm of RT activity) for 1 h, washed, suspended in 10 ml of medium, and plated in T-25-flasks. MT4 cell infections were carried out in 96-well plates containing 2×10^4 cells per 200 μl of medium per well by using approximately 5×10^3 cpm of RT activity of wild-type, mutant, or revertant HIV-1 as inoculum. In both cell systems, two-thirds of the medium was exchanged every 2 to 3 days and an aliquot was saved for RT activity.

In transfection-cocultivation experiments, *Bam*HI-released and -ligated, circularly permuted cloned HIV-1 DNAs from the wild type and mutant derivatives of pILIC constructs were incubated with 12D7 cells (6×10^6 cells) in the presence of DEAE-dextran as previously described (37, 47) and then cocultured with MT4 cells (2×10^6 cells) 48 h later. Virus production was monitored by assaying the medium for RT activity.

Construction of HIV-1 proviral DNAs containing LTR deletions. Oligonucleotide-directed mutagenesis (34) was used to create nonoverlapping deletions in the 5′ (29-nt), middle (26-nt), and the 3′ (26 nt) thirds (see Fig. 1) of the HIV-1 U5 region present in the circularly permuted HIV-1 clone pILIC (37). These U5 mutations were subsequently transferred to the 5′ LTR of the infectious pNL4-3 proviral clone by (i) digesting wild-type or mutated pILIC DNA with *Bam*HI plus *Sph*I and inserting the 2-kb fragment containing the LTR plus adjacent *nef* and *gag* sequences into similarly cleaved pUC19 DNA and (ii) digesting the pUC19 LTR-containing plasmid with *Bss*HII plus *Aat*II and ligating the 1.3-kb LTR fragment to the purified 13-kb *Bss*HII plus *Aat*II cleavage product of pNL4-3 DNA (which extends from the *gag* leader region through the 3′ LTR into flanking DNA as well as plasmid sequences).

Analysis of particle-associated proteins and RNA. Wild-type and mutant DNAs were transfected into separate cultures of HeLa cells propagated in 25-cm^2 flasks as described above. At 20 to 24 h posttransfection, the cells were scraped, washed in methionine-free RPMI 1640 medium, and resuspended in methionine-free medium. After 10 min at 37°C, the cells were pelleted and resuspended in 1 ml of methionine-free complete medium (9:1, vol/vol) mixture containing 250 μCi of [^{35}S]methionine. An additional 1 ml of 9:1 medium containing 250 μCi of [^{35}S]methionine was added 10 h later, and the cells were incubated for a second 10-h period. Following 20 h of incubation, the medium was harvested, subjected to low-speed centrifugation (1,500 rpm), and filtered through a 0.45-μm-pore-size filter (Millipore) to remove nonadherent cells. Virus particles in the filtrate were centrifuged for 30 min at 35,000 rpm and 20°C in an SW55 rotor (Beckman), and the pellets were resuspended in 400 μl of RPMI 1640 medium containing 10% FCS. Aliquots of the virion suspension (100 to 300 μl) were added to 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, and 0.1% Triton X-100). The resulting lysates were immunoprecipitated by using a mixture of sera from patients with AIDS. Precipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography as previously described (58).

Wild-type and mutant HIV-1 virions, produced by transiently transfecting HeLa cells and pelleted by ultracentrifugation, were suspended in 200 μl of buffer containing 10 mM Tris hydrochloride (pH 7.4), 100 mM NaCl, and 5 mM $MgCl_2$ and digested with 35 U of RNase-free DNase (Boehringer Mannheim) for 2 h at 37°C. Equal amounts of suspended virus particle preparations (determined by RT assay) were disrupted following incubation with 0.5% Nonidet P-40 and then incubated with 5 μg of proteinase K (Boehringer Mannheim) per ml for 20 min at 37°C in the presence of 100 μg of carrier yeast tRNA (Sigma) per ml. The particle-associated RNA was sequentially extracted with phenol, phenol-chloroform (1:1), and chloroform, precipitated with ethanol, and resuspended in 50% formamide–6% formaldehyde–1× standard saline citrate (SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). Viral RNA samples were serially diluted, immobilized on nitrocellulose membranes by using a slot blot manifold (Schleicher and Schuell, Inc.), and hybridized to a ^{32}P-labeled full-length HIV-1 DNA probe consisting of the 9.2-kb *Bam*HI fragment from pILIC (37) DNA in a 5-ml reaction mixture containing 1% SDS, 1 M NaCl, and 10% dextran sulfate. The filters were incubated at 65°C overnight, washed twice with 2× SSC–1% SDS at 65°C for 30 min, dried, and exposed to X-ray film.

Molecular characterization of a U5/LTR HIV-1 revertant. Low-molecular-weight DNA was prepared by differential salt precipitation (29) from separate MT4 cell cultures 4 days following infection with wild-type HIV-1 or a putative HIV-$1_{U5/604del}$ revertant. The U5 regions of both proviral DNAs were amplified with a Perkin-Elmer Cetus thermal cycler using (i) a plus-sense primer with a 5′ terminus located at the beginning of the LTR R region and spanning proviral DNA map positions 455 to 475 (40) (5′-GGTCTCTCTGGTTAGA CCAGA-3′) and (ii) a minus-sense primer situated immedi-

ately downstream from the major splice donor site and spanning positions 736 to 756 (5′-TTGGCGTACTCACCAGTCG CC-3′). PCRs were performed with 100 pmol of each primer in 100-μl reaction mixtures containing 0.2 mM (each) deoxynucleoside triphosphates (dNTPs), 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 2.5 U of *Taq* DNA polymerase (AmpliTaq DNA polymerase; Perkin-Elmer Cetus), and 0.1 μg of unintegrated DNA as the template. The PCR mixtures were overlaid with a drop of mineral oil and subjected to 25 cycles of amplification with denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and polymerization for 1 min at 74°C. The 301-bp PCR product was inserted into PCR II vector DNA (Invitrogen, San Diego, Calif.), the DNA was amplified in *Escherichia coli*, and the HIV-1 LTR regions from seven independent clones were sequenced by the chain termination method (49).

PCR amplification and analysis of newly synthesized viral DNA in cells infected with wild-type, mutant, and revertant viruses. 12D7 or MT4 cells (10^6) were infected with equal amounts (2×10^6 cpm of ^{32}P-RT activity) of wild-type, mutant, and revertant HIV-1 virions prepared from transiently transfected HeLa cells. Each virus inoculum was incubated with 35 U of pancreatic DNase I (Boehringer Mannheim) for 1 h at 37°C prior to use and was adsorbed to cells in 0.1 ml of medium for 1 h. Following a wash, the infected cells were resuspended in 10 ml of fresh medium and harvested at 5, 11, and 24 h postinfection for the preparation of Hirt extracts (29). As a zero-time control, the cells were incubated with similar amounts of all virus preparations for 1 h at 0°C and then processed for unintegrated DNA. The Hirt supernatant DNAs were digested with RNase A (Boehringer Mannheim) (0.02 mg/ml) for 1 h and proteinase K (Boehringer Mannheim) (100 μg/ml) for 1 h at 60°C; extracted with phenol, phenol-chloroform, and chloroform; ethanol precipitated; and suspended in 10 mM Tris HCl–1 mM EDTA, pH 8.0, at a final concentration of 200 ng/μl. The DNA samples were digested with *Dpn*I (Boehringer Mannheim) for 14 h at 37°C to cleave residual contaminating plasmid DNA and then diluted 10-fold prior to PCR.

PCR was performed on 5-μl samples of Hirt DNA from infected cells using primers (5′-CTGAGCCTGGGAGCTCTC TGGC-3′ and 5′-GCCGTGCGCGCTTCAGCAAGC-3′) complementary to sequences in the HIV-1 R region and a region downstream from the primer-binding site. This primer pair amplifies molecules that have completed DNA synthesis. The predicted lengths of the DNA segments amplified from the wild-type, U5/604 mutant, and revertant virus-infected cells are 245, 219, and 200 bp, respectively. In addition to the template (100 ng of Hirt DNA), the reaction mixture included 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus), 500 nM each primer, 0.2 mM each dNTP, 1.75 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.001% (wt/vol) gelatin in a total volume of 0.05 ml. The denaturation, annealing, and polymerization steps were performed at 94°C (1 min), 56°C (1 min), and 72°C (2 min), respectively. The reactions were allowed to proceed for 33 cycles. Before cycling, the mixtures were kept on ice and then held for 5 min at 94°C. For the analysis of PCR products, 5 μl of each sample was loaded on a 4 to 20% polyacrylamide gel (Novex, San Diego, Calif.), run for 2 h, stained with ethidium bromide, and quantitated by using the Image 1 system (Universal Imaging, Inc., West Chester, Pa.).

Care was taken to avoid possible artifacts such as saturation of the PCR and unequal amplification of the different DNA samples. Serial dilutions of plasmid DNAs containing wild-type, mutant, and revertant U5 DNAs or combinations of the three were amplified in separate experiments. All of the reactions were linear during 33 cycles of amplification, and similar amounts of DNA were generated.

Transfer of the U5/604del revertant change into a full-length infectious molecular clone of HIV-1. The extended LTR deletion, present in the revertant of the U5 mutant virus HIV-$1_{U5/604del}$, was introduced into the 5′ LTR of pNL4-3 DNA by (i) ligating the LTR-containing, 2-kb *Bam*HI-*Sph*I fragment from pILIC DNA to similarly cleaved pAT153 DNA (Amersham Corp., Arlington Heights, Ill.), generating $pRvt_1$; (ii) *Sac*I and *Bss*HII digestion of the 301-bp PCR product, cloned in PCR II DNA following its amplification from HIV-$1_{U5/604del}$ revertant virus-infected MT4 cells (containing LTR and Gag leader sequences); (iii) inserting the resulting 225-bp cleavage product into the *Sac*I- and *Bss*HII-cleaved $pRvt_1$ DNA, thereby creating $pRvt_2$; and (iv) *Aat*II and *Sph*I digestion of $pRvt_2$ DNA to produce a 2.5-kb LTR-containing fragment (with the extended revertant deletion), which was ligated to the purified 12.3-kb *Aat*II-*Sph*I fragment of pNL4-3 DNA.

In vitro integration assays. The following oligonucleotides were synthesized for use as substrate DNAs in the in vitro integration assays: AE117 (5′-ACTGCTAGAGATTTTCCA CAC-3′), AE118 (5′-GTGTGGAAAATCTCTAGCAGT-3′), oligo1 (5′-AGAGATCCCTCAGACCCCAGT-3′), oligo2 (5′-ACTGGGGTCTGAGGGATCTCT-3′), oligo3 (5′-TTGTGT GACTCTGGTAACAGT-3′), and oligo4 (5′-ACTGTTACCA GAGTCACACAA-3′). AE117 and AE118 correspond to the terminal 21 bp of the U5 end of wild-type NL4-3 DNA, oligo1 and oligo2 correspond to the terminal 21 bp of the U5/604del mutant, and oligo3 and oligo4 correspond to the terminus of the U5/604del-rev4 revertant. Each oligonucleotide was purified following denaturing PAGE prior to use. The 5′ ends of AE118, oligo1, and oligo3 were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as previously described (18). Complementary strands were annealed and unincorporated radionucleotide was removed by spin column chromatography as previously described (22). HIV-1 integrase was purified as previously described (21, 22).

Integration reaction mixtures were incubated at 37°C for 1 h and included 25 mM MOPS (morpholinepropanesulfonic acid) (pH 7.2), 10 mM β-mercaptoethanol, 100 μg of bovine serum albumin per ml, 7.5 mM $MnCl_2$, 10% glycerol (wt/vol), 17 mM NaCl, 0.75 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 25 nM ^{32}P-labeled DNA substrate, and 160 nM integrase in a volume of 16 μl. Reactions were stopped and portions were electrophoresed in 15% polyacrylamide denaturing gels as previously described (22). Quantitation of reaction products was done with a PhosphorImager (Molecular Dynamics).

RESULTS

Properties of HIV-1 U5 deletion mutants in a transfection-cocultivation system. The initial strategy of U5 LTR mutagenesis was to introduce nonoverlapping deletions, each encompassing approximately one-third of the U5 region, into a circularly permuted molecular clone of full-length HIV-1 proviral DNA (designated pILIC [37]). Figure 1 shows that mutant construct p603 is missing 29 nt mapping to the 5′ end of U5 while 26 nt were removed from both the p206 and the p604 proviral constructs, containing deletions in the central and 3′ thirds of the HIV-1 U5 region, respectively. A 4-bp (CAGT) inverted repeat present at both ends of the HIV-1 LTR, located at the 3′ terminus of U5, was not deleted from the p604 mutant (boxed in Fig. 1). Following cleavage with *Bam*HI, the wild-type and the three mutated circularly per-

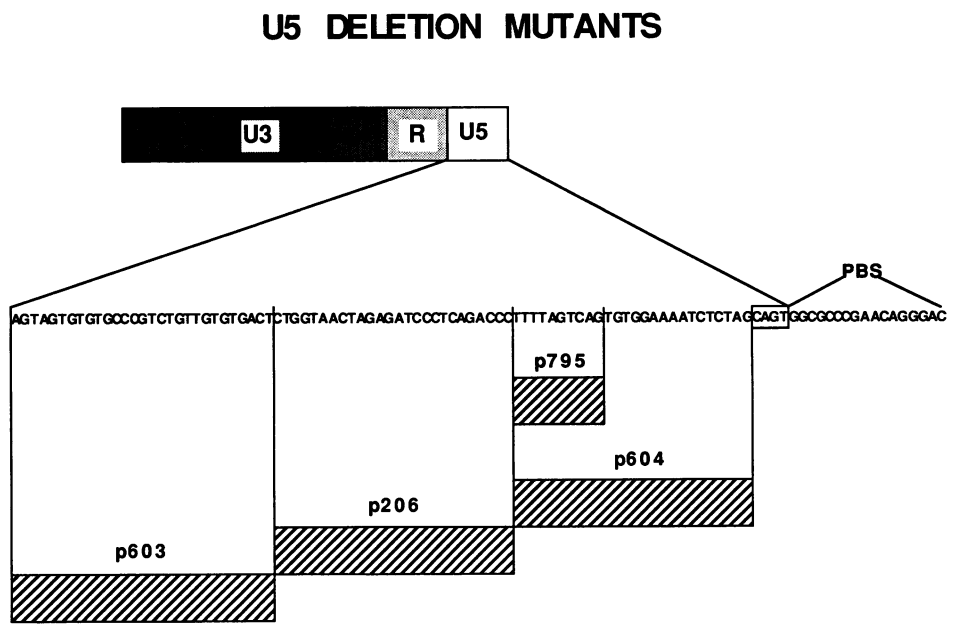


FIG. 1. Locations and sizes of deletion mutations introduced into the U5 region of the HIV-1 LTR. The nucleotide sequence of the HIV_{NL4-3} (1, 40) U5 is shown with the positions of the deletions present in the p603, p206, p795, and p604 proviral constructs indicated. The 4-nt (CAGT) inverted repeat at the 3′ edge of U5 is framed adjacently to the 18-nt primer-binding site (PBS).

muted HIV-1 DNAs were released from their respective pILIC derivatives and separately ligated in vitro to generate viral DNA concatemers, as previously described (37, 47). Head-to-tail oligomers of permuted circular viral DNA that recreated the two-LTR, full-length linear HIV-1 DNA configuration are among the products of the ligation reaction. The biological activities of the ligated wild-type and U5 mutant viral DNAs were then assayed in the following transfection-cocultivation system: 12D7 cells (47), clonal derivatives of CEM cells, were transfected with individual ligation reaction mixtures in the presence of DEAE-dextran and mixed with highly susceptible MT4 (28) cells 48 h posttransfection. Virus production was monitored over the next several weeks by the release of RT activity into the medium of the coculture. As shown in Fig. 2, the deletion of the middle one-third of U5 (p206) had little, if any, effect on HIV-1 replication compared with wild-type virus. In contrast, the elimination of both terminal one-thirds of U5 markedly inhibited HIV-1 infectivity: the 3′ deletion (p604) delayed progeny virion production by more than a week compared with wild-type virus, while the 5′ deletion (p603) rendered the virus completely replication defective in this as well as four other independent experiments, including one lasting 56 days.

Characterization of U5 deletion mutant particles generated by transient transfection of HeLa cells. In contrast to the transfection-cocultivation assays, transient DNA transfections of CD4-negative cells (such as HeLa, SW480, and COS) have proven to be immensely useful for delineating the defect(s) responsible for the loss of or reduction in infectivity attending the mutagenesis of a replication-competent HIV-1 molecular clone (25, 45, 47, 53, 57). Unfortunately, the release and concatemerization of circularly permuted DNA to generate biologically active head-to-tail oligomeric structures yield varying concentrations and heterogeneous populations of ligation products. Consequently, the functional characterization of viral mutants generated in circularly permuted, single-LTR proviral clones cannot be readily characterized by transient transfection of CD4-negative cells. The disabling U5 deletions (p603 and p604), as well as the functionally neutral p206 U5 mutation, were transferred from circularly permuted pILIC clones to the 5′ LTR of the infectious molecular clone pNL4-3 (1), and the biological activities of the resulting two-LTR proviral DNAs were assayed following transient transfection of HeLa cells. As shown in Fig. 3A, wild-type HIV-1 and the

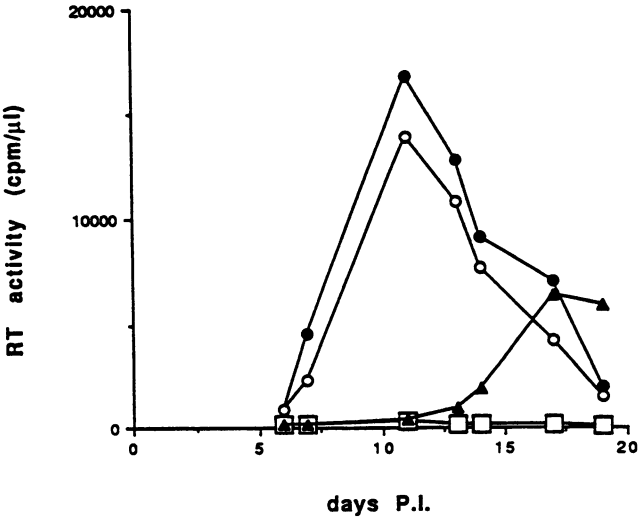
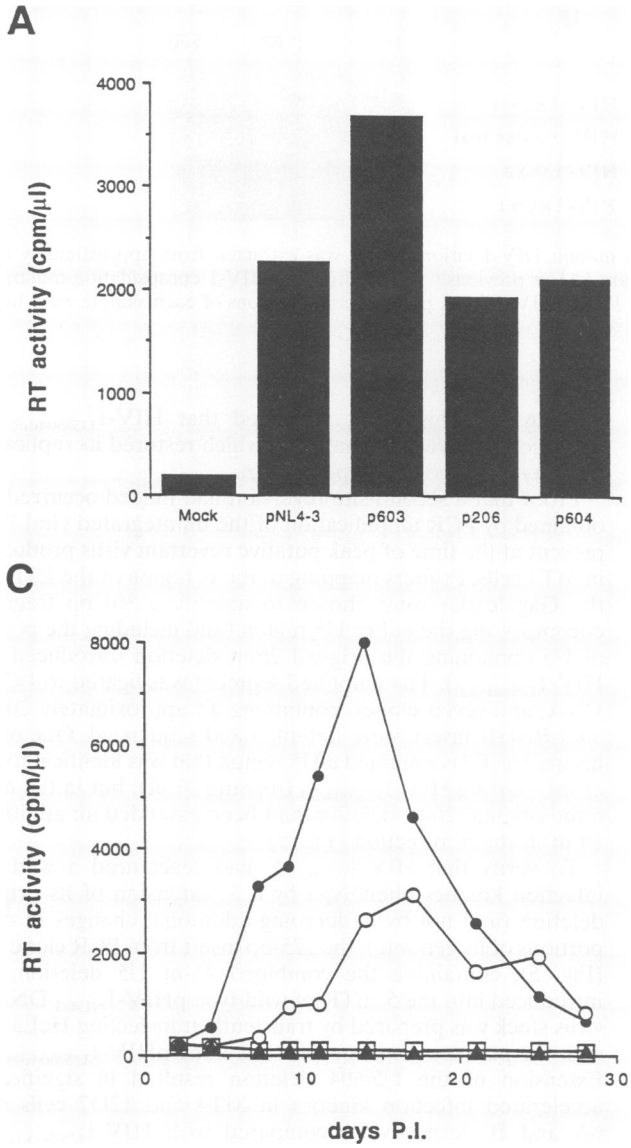


FIG. 2. Transfection-cocultivation of human T-cell lines with HIV-1 wild-type (●), p206 (○), p603 (□), and p604 (▲) proviral constructs. 12D7 CEM cells were transfected in the presence of DEAE-dextran with approximately 5 µg of ligated, circularly permuted cloned HIV-1 DNA, derived from pILIC (37) DNA following cleavage with *Bam*HI. MT4 cells were added to the transfected cultures 48 h posttransfection, and virus production was monitored by RT assay of the medium at the indicated times.



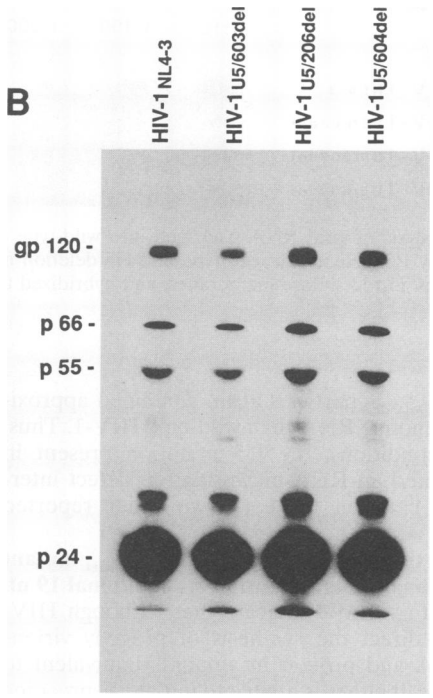


FIG. 3. Properties of HIV-1 U5 deletion mutant virions released from transfected HeLa cells. (A) HeLa cells were transfected with 20 μg of wild-type or the indicated U5 deletion mutant HIV-1 DNA in the presence of calcium phosphate, and progeny particle production was monitored by the RT activity released into the medium. (B) [^{35}S]methionine-labeled virions, released from HeLa cells transfected with cloned wild-type or the indicated U5 deletion mutant HIV-1 DNA, were pelleted in an ultracentrifuge, lysed in the presence of Triton X-100, immunoprecipitated by using a pool of sera from patients with AIDS, and analyzed by SDS-PAGE. The gp120 envelope, p66 RT, and p55 and p24 Gag proteins are indicated. (C) The infectivities of wild-type HIV-1_{NL4-3} (●) and the U5 deletion mutants HIV-$1_{U5/206del}$ (○), HIV-$1_{U5/603del}$ (□), and HIV-$1_{U5/604del}$ (▲) were assayed in activated human PBMCs.

three large U5 deletion mutants released comparable amounts of progeny virions (RT activity) into the medium 48 h post-transfection, indicating that none of the U5 mutations affected viral RNA or protein synthesis. To analyze the protein composition of the progeny virions released from the transfected HeLa cells, labeled virus particles present in the supernatant medium were pelleted by ultracentrifugation, resuspended in medium, lysed in the presence of Triton X-100, immunoprecipitated with a mixture of sera from patients with AIDS, and electrophoresed in 10% polyacrylamide gels. Figure 3B demonstrates that wild-type HIV-1 and the three U5 deletion mutant virion preparations contain similar amounts and types of viral proteins. However, both the HIV-$1_{U5/603del}$ and the HIV-$1_{U5/604del}$ mutants failed to infect activated PBMCs when wild-type HIV-1 and the HIV-$1_{U5/206del}$ mutant exhibited similar infection kinetics, although the latter replicated to lower levels (Fig. 3C).

The 5′ one-third of the U5 region contains sequences required for the efficient packaging of viral RNA into particles. Although the protein composition of HIV-$1_{U5/603del}$ particles and that of HIV-$1_{U5/604del}$ particles were indistinguishable from that of wild-type virus, their impaired-infectivity phenotype could still reflect a defect in virion structure such as inefficient encapsidation of genomic RNA. This was investigated by purifying RNA from equivalent amounts (determined by particle-associated RT activity) of pelleted wild-type and U5 mutant virus particles released from HeLa cells, immobilizing the RNA on nitrocellulose membranes, and hybridization to a ^{32}P-labeled full-length HIV-1 proviral DNA probe. As shown in Fig. 4A, HIV-$1_{U5/603del}$ particles contained substantially less viral RNA compared with wild-type, HIV-$1_{U5/206del}$, and HIV-$1_{U5/604del}$ particles. The average RNA content of HIV-$1_{U5/603del}$, determined in six independent RNA slot blot hybridization experiments, was approximately 10-fold lower than that of the wild type (HIV-1_{NL4-3}). In these analyses, HIV-$1_{U5/206del}$ and HIV-$1_{U5/604del}$ encapsidated genomic RNA in amounts similar to those for wild-type virus.

The extent of the HIV-$1_{U5/603del}$ packaging defect was also compared with those exhibited by two previously described HIV-1 encapsidation mutants (5, 11). In each case, pelleted virion RNAs, isolated from HeLa cells transiently transfected with individual packaging mutants, were analyzed by slot blot hybridization (Fig. 4B). Compared with wild-type HIV-1_{NL4-3}, the packaging mutants HIV-1_{NLW1} and HIV-1_{3HXB} incorporated 5- and 100-fold less viral RNA, respectively; in the same

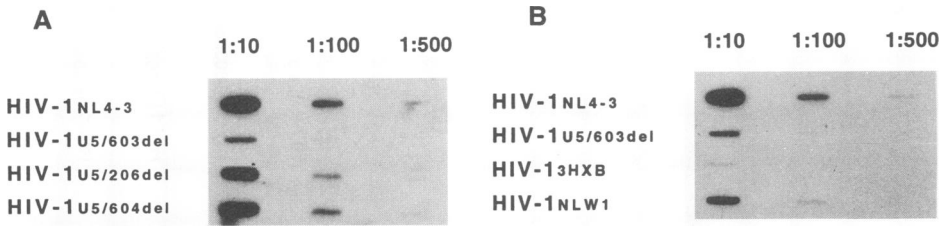


FIG. 4. Slot blot analysis of viral RNA packaged into wild-type and mutant HIV-1 virions. RNA was extracted from approximately equal amounts (determined by RT activity) of wild-type and U5 deletion mutant (A) or previously reported (5, 11) HIV-1 encapsidation mutant (B) particles, immobilized on nitrocellulose membranes, and hybridized to a 9.2-kb HIV-1 DNA probe. Serial dilutions of each sample were loaded in the indicated lanes.

experiment, HIV-$1_{U5/603del}$ particles again contained approximately 10-fold less genomic RNA than wild-type HIV-1. Thus, under these assay conditions, the U5 mutation present in HIV-$1_{U5/603del}$ exhibited an RNA encapsidation defect intermediate in severity between those of two other reported packaging mutants.

During passage in tissue culture, the HIV-$1_{U5/604del}$ mutant reverts by extending its original mutation an additional 19 nt. The experiments in Fig. 2 to 4 indicate that, although HIV-$1_{U5/604del}$ is able to direct the synthesis of progeny virions containing viral RNA and protein in amounts equivalent to those for the wild type, it either is unable to initiate a spreading infection or exhibits delayed infection kinetics. This is to be contrasted with the results obtained with a closely related HIV-1 U5 mutant, derived from proviral clone p795 (Fig. 1), which lacked a subset of the 26 nt missing from HIV-$1_{U5/604del}$. HIV-$1_{U5/795del}$ exhibited infection kinetics in T-cell lines that were equivalent to the infection kinetics of the wild type (data not shown).

During a long-term passage of HIV-$1_{U5/604del}$ in 12D7 cells, progeny virus particles were detected in one of four replicate cultures 4 to 5 weeks following infection. In our experience, the delayed appearance of virions following infection with HIV-1 mutants is frequently associated with the emergence of a second-site revertant of an original mutated virus (25, 26, 47, 57, 59). When the virus released on day 30 from the single RT-positive HIV-$1_{U5/604del}$-infected culture was used as inoculum, it exhibited infection kinetics only slightly delayed compared with those of wild-type HIV-1 (data not shown, but see Fig. 6). This result suggested that HIV-$1_{U5/604del}$ had undergone a revertant change(s) which restored its replication phenotype to the wild-type pattern.

Proof that a second-site reversion had indeed occurred was obtained by PCR amplification of the unintegrated viral DNA present at the time of peak putative revertant virus production in MT4 cells. Primers mapping to the R region of the LTR and the Gag leader were chosen to amplify a 301-bp fragment encompassing the entire U5 region (and including the portion of U5 containing the original 26-nt deletion introduced into HIV-$1_{U5/604del}$). The amplified segment was ligated to PCR II DNA, and seven clones, containing an approximately 200-bp *Sac*I-*Bss*HII insert, were obtained and sequenced. One of the inserts (revE2) contained a U5 region that was identical to that of the starting HIV-$1_{U5/604del}$ (missing 26 nt), but in the other 6 the original 26-nt deletion had been extended an additional 19 nt in the 5′ direction (Fig. 5).

To verify that HIV-$1_{U5/604del}$ had reacquired a wild-type infection kinetics phenotype by a 5′ extension of its original deletion (and not by undergoing additional changes in other portions of its genome), the 225-bp insert from PCR clone rev4 (Fig. 5), containing the combined 45-nt U5 deletion, was introduced into the 5′ LTR of wild-type pHIV-1_{NL4-3} DNA. A virus stock was prepared by transiently transfecting HeLa cells with the putative revertant proviral clone pHIV-$1_{U5/604del-rev4}$. Extension of the U5/604 deletion resulted in significantly accelerated infection kinetics in MT4 and 12D7 cells (Fig. 6A and B, respectively) compared with HIV-$1_{U5/604del}$. In both T-cell lines, peak RT production in the revertant HIV-

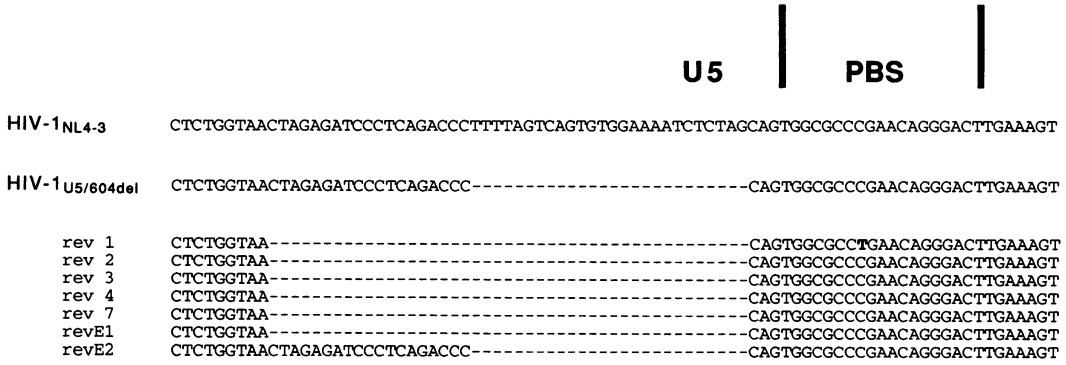


FIG. 5. Nucleotide sequence of seven PCR clones from cells infected with a putative HIV-$1_{U5/604del}$ revertant. Hirt supernatant DNA was prepared from cells infected with putative revertant virus harvested on day 30 from the single positive culture of HIV-$1_{U5/604del}$-infected 12D7 CEM cells. The amplified PCR products were inserted into PCR II, and the inserts from seven independent clones were sequenced and aligned with the U5 regions of wild-type HIV-1_{NL4-3} and HIV-$1_{U5/604del}$. Dashes, deleted nucleotides. A nucleotide substitution is boldfaced. PBS, primer-binding site.

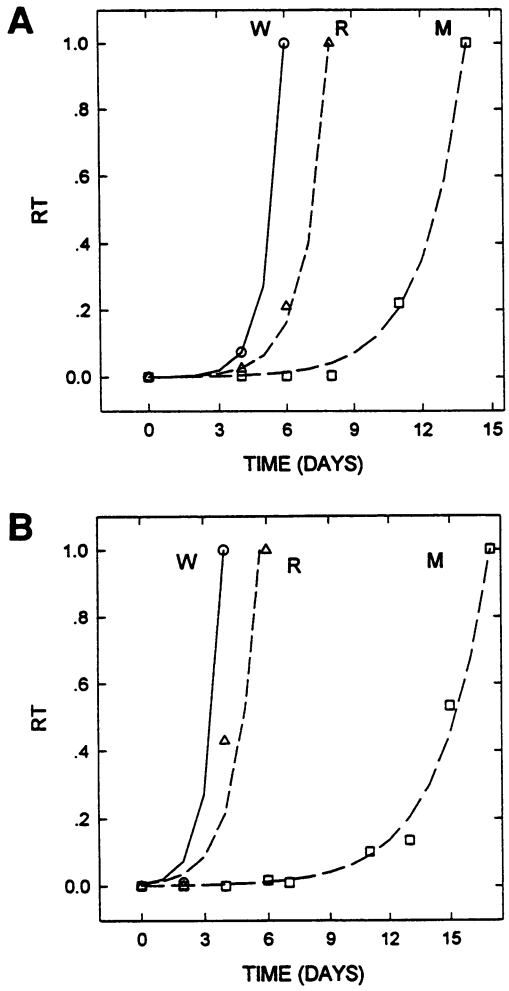


FIG. 6. Infection kinetics of wild-type, HIV-$1_{U5/604del}$ mutant, and HIV-$1_{U5/604del\text{-}rev4}$ revertant viruses in human T-cell lines. 12D7 CEM (A) and MT4 (B) cells (10^6) were infected with equal amounts (approximately 2×10^6 cpm of RT activity) of wild-type (W), HIV-$1_{U5/604del}$ mutant (M), and HIV-$1_{U5/604del\text{-}rev4}$ revertant (R) viruses in 0.1 ml of medium for 1 h, washed, and then cultured in 10 ml of medium. The cells were split threefold every 2 or 3 days. Samples from the culture supernatants were collected at the indicated times, and their RT activities were measured. As previously reported (19), the curves are the best fits of the experimental data with exponential functions: $RT = e^{k(t - t_p)}$, where RT is normalized to its maximal value (15,536, 16,453, and 17,349 cpm/μl for the wild-type, mutant, and revertant viruses, respectively).

$1_{U5/604del\text{-}rev4}$ infections was delayed only 2 to 3 days compared with wild-type virus. In contrast, progeny virus production was delayed for nearly 2 weeks in HIV-$1_{U5/604del}$-infected cells. Taken together, these data indicate that the extension of the original 26-nt deletion to encompass 45 nt of the U5 sequence is solely responsible for the revertant change in HIV-$1_{U5/604del}$.

We have previously described a method (19) for analyzing HIV-1 infection kinetics which allowed us to determine the infection rate constant, k, by fitting experimental data with the exponential function $RT = e^{k(t - t_p)}$ using SigmaPlot 4.0. In this equation, RT is the amount of virion-associated RT activity in the culture supernatant, t_p is the time required to attain peak virus production, and t is the time of RT measurement. The infection rate constant, k, depends on the number, n, of infectious particles produced during a single cycle of infection and the time, t_i, required to complete such a cycle: $k = \ln n/t_i$. Assuming that t_i is approximately the same for infections by the wild-type, HIV-$1_{U5/604del}$, and revertant HIV-$1_{U5/604del\text{-}rev4}$ viruses (previously shown to be approximately 3 to 4 days for several wild-type and mutant virus infections [19]), one can calculate the number (n) of infectious virions produced, which would also be equal to the number of newly infected cells in each cycle of infection. These equations have been used to calculate k, t_p, and n from the three infections shown in Fig. 6. The resulting values are presented in Table 1 and indicate that the original HIV-$1_{U5/604del}$ mutant and HIV-$1_{U5/604del\text{-}rev4}$ revertant are approximately 10-to-15- and 3-fold less infectious than wild-type virus, respectively.

TABLE 1. Decrease in the number of cells productively infected by HIV-1 U5 deletion mutants compared with the wild-type virus

Virus	k (day^{-1})		t_p (days)		Fold decrease	
	12D7	MT4	12D7	MT4	12D7	MT4
Wild type	1.30	1.3	6	4		
Mutant	0.50	0.40	14	17	11	15
Revertant	0.91	0.96	8	5.7	3.3	2.8

The mutation in HIV-$1_{U5/604del}$ does not impair the synthesis of reverse transcripts in acutely infected cells. Mutations analogous to U5/604, which affect the 3′ half of U5, have been reported to block the initiation of the reverse transcription reaction in both avian and murine retrovirus systems (4, 12, 14, 41). To further investigate the nature of the defect(s) that severely compromised the infectivity of HIV-$1_{U5/604del}$, the possible inhibition (by the U5/604 mutant) and subsequent restoration (by the revertant) of viral DNA synthesis during productive infection were examined. 12D7 CEM cells were infected with equal amounts (RT activity) of wild-type, HIV-$1_{U5/604del}$, and revertant HIV-$1_{U5/604del\text{-}rev4}$ viruses, and Hirt supernatant DNAs, harvested at 0, 5, 11, and 24 h postinfection from approximately 10^6 cells, were subjected to PCR using a primer pair that would selectively amplify 245-, 219-, and 200-bp segments, whose sizes are equivalent to the sizes of the U5 regions plus adjacent sequences present in the wild-type, mutant, and revertant viruses, respectively. As shown in Fig. 7A, approximately equal amounts of the three HIV-1 DNAs were first detected 5 h postinfection and peaked around 11 h; by 24 h, the amount of the three viral DNAs present in the Hirt supernatant had fallen. In an attempt to quantitatively compare the amounts of newly synthesized viral DNA present in the three infected cultures, equal (approximately 33-ng) amounts of Hirt supernatant DNA were added to a single reaction mixture and allowed to compete with one another in a PCR. A 100-ng sample of wild-type DNA was similarly analyzed. As shown in Fig. 7B, the amounts of HIV-1 DNA reverse transcribed from the wild-type, HIV-$1_{U5/604del}$, and revertant HIV-$1_{U5/604del\text{-}rev4}$ viruses were not significantly different. A similar result was obtained following infection of MT4 cells with the three viruses (data not shown).

The mutation in HIV-$1_{U5/604del}$ severely impairs 3′ processing and strand transfer in in vitro integration assays, and the activities are partially restored in the revertant LTR substrate. Sequences located at the 3′ end of U5 are also located at the 3′ terminus of the full-length linear form of HIV-1 viral DNA and participate in the integration of the reverse transcript into the host cell chromosome. Precise and efficient integration requires not only the integrity of the edges of both U5 and U3 in both avian and murine retroviral systems but the presence of

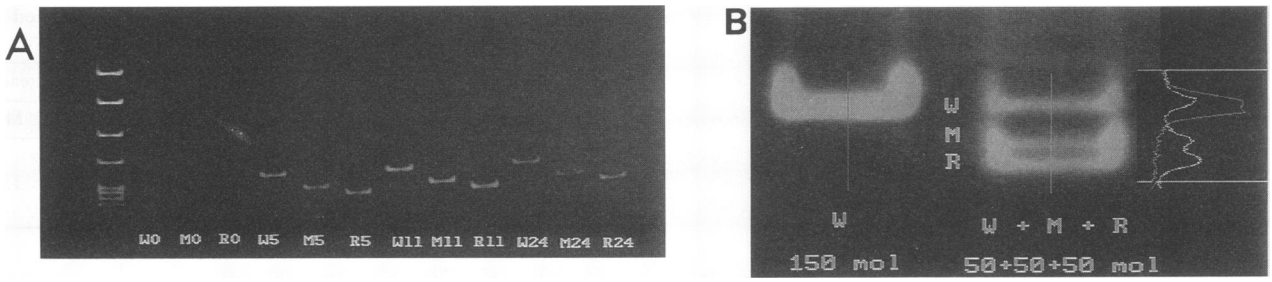


FIG. 7. HIV-$1_{U5/604del}$ is not defective in viral DNA synthesis. (A) 12D7 CEM cells (10^6) were infected with equal amounts of wild-type, HIV-$1_{U5/604del}$ mutant, and HIV-$1_{U5/604del\text{-}rev4}$ revertant viruses as described for Fig. 6 and cultured at 10^6 cells/ml for 5, 11, or 24 h at 37°C. As a control (time zero), 12D7 cells were also incubated with the three virus inocula for 1 h on ice. DNA was extracted by the Hirt procedure (29), and PCR was performed using primers spanning the U5 region as described in Materials and Methods. PCR products (5 μl) were analyzed on 4 to 20% polyacrylamide gels and quantitated by using the Image 1 system. (B) A competitive PCR was carried out in a single reaction mixture containing approximately equal amounts of Hirt supernatant DNAs from 12D7 cells infected with wild-type (W), mutant (M), and revertant (R) viruses. For comparison, the PCR product of wild-type virus DNA is shown on the left. The amount of the wild-type PCR product is approximately equal to the total amount of DNA from the mixture of the wild-type, mutant, and revertant DNAs shown on the right. The graph in the inset depicts the relative intensity of gel staining as quantitated by a video imaging system.

adjacent subterminal sequences as well (13, 16, 36, 42, 43). For this reason, care was taken to preserve the 4 nt positioned at the very 3′ end of U5 during construction of the HIV-1 U5/604 deletion mutant. However, because the creation of the U5/604 deletion completely altered the subterminal region of U5 in HIV-$1_{U5/604del}$, in vitro integration reactions measuring 3′ processing and DNA strand transfer were carried out using oligomers modelling the 21 bp located at the U5 ends of wild-type, HIV-$1_{U5/604del}$, and revertant HIV-$1_{U5/604del\text{-}rev4}$ viral DNAs. The autoradiogram shown in Fig. 8A indicates that 3′ processing of the U5/604 mutant terminus by HIV-1 integrase is markedly reduced compared with the wild-type U5; this processing impairment was partially restored when the revertant U5 substrate was employed. Similarly, strand transfer of the U5/604 mutant U5 end was barely measurable in the assay presented in Fig. 8B, whereas the revertant U5 terminus generated readily detectable products of the strand transfer reaction. Phosphorimage analyses of two independent in vitro integration assays are summarized in Table 2 and reveal that 3′ processing and strand transfer of the U5/604 mutant U5 terminus were reduced approximately 10- and 23-fold, respectively, compared with the wild-type U5 oligomer. When the revertant U5 substrate was used, the 3′ processing and strand transfer activities were restored to 25 and 22% of the wild-type levels, respectively.

DISCUSSION

The U5 region of the HIV-1 LTR is 85 nt in length, highly conserved among different HIV-1 isolates (40), and like analogs present in other retroviral genomes, is not known to encode a protein product. To a certain extent, the functional consequences of disrupting the U5 region of the HIV-1 LTR mirror those described for members of other retrovirus subfamilies. Similarly to our results with the HIV-$1_{U5/603del}$ mutant, deletions introduced into the 5′ half of the Mo-MuLV U5 region caused a defect in the packaging of genomic RNA into progeny virions (41). The deletion mutation situated near the 3′ edge of the HIV-1 U5 in HIV-$1_{U5/604del}$ severely impaired integration of viral DNA and compromised infectivity, a finding similar to that reported for both murine and avian retroviruses (13, 16, 43). In contrast to studies of Rous sarcoma virus and Mo-MuLV (4, 12, 14, 41), no defect in reverse transcription could be demonstrated by deletion of the 3′ portion of the U5 region of HIV-1. Finally, our failure to observe a significant loss of virus infectivity subsequent to the removal of the central one-third of U5 in HIV-$1_{U5/206del}$ is similar to the result for a previously reported deletion mutant of Mo-MuLV missing 17 nt from the central region of its U5 region (41). Extension of the 26-bp HIV-$1_{U5/206del}$ deletion by 15 nt in either the 5′ or the 3′ direction, however, resulted in complete loss of infectivity (data not shown).

The packaging of retroviral genomes involves the specific interactions of an 8- to 9-kb viral RNA molecule with defined regions of both the Gag and the Gag-Pol precursor proteins (generally the Cys-His boxes of the NC domains). Since the sequences located between the major splice donor and the initiating codon of the *gag* gene are invariably present in full-length retroviral genomes and absent from spliced viral mRNAs, this region has received the greatest attention as the primary determinant for encapsidation. In the case of Mo-MuLV and spleen necrosis virus, the principal encapsidation signal (Ψ) is, in fact, situated downstream from the major 5′ splice donor (2, 38, 56); Rous sarcoma virus has important packaging elements located upstream of the major splice donor as well as near the 3′ end of the genomic RNA, flanking the v-*src* gene (7, 31, 51). During the development of murine retroviral vectors engineered for the efficient delivery of RNA into mammalian cells, it was realized that other regions of the viral genome, such as portions of the *gag* leader, could augment the incorporation of heterologous RNA into virus particles (6, 9). Subsequent studies demonstrated that deletion of Mo-MuLV sequences located in the 5′ half of U5 severely impaired the packaging of genomic RNA (41) but did not significantly increase RNA encapsidation when present as a component of murine retroviral vectors, possibly because of unique requirements for incorporating full-length viral RNA into progeny particles (2). A similarly located U5 deletion in HIV-$1_{U5/603del}$ caused a significant reduction of packaged genomic RNA that was coupled to a loss of virus infectivity. The analogous Mo-MuLV U5 mutation resulted in a less severe encapsidation defect than a Ψ-region mutation (100- to 200-fold versus 300- to 1,000-fold [38, 41]), a result similar to that observed for comparable HIV-1 mutants HIV-$1_{U5/603del}$ and HIV-1_{3HXB}, respectively, both of which exhibited packaging deficiencies that differed from one another by about 10-fold (Fig. 4B).

In contrast to results reported for both avian and murine retroviruses (4, 12, 14, 41), the 26-bp deletion adjacent to the

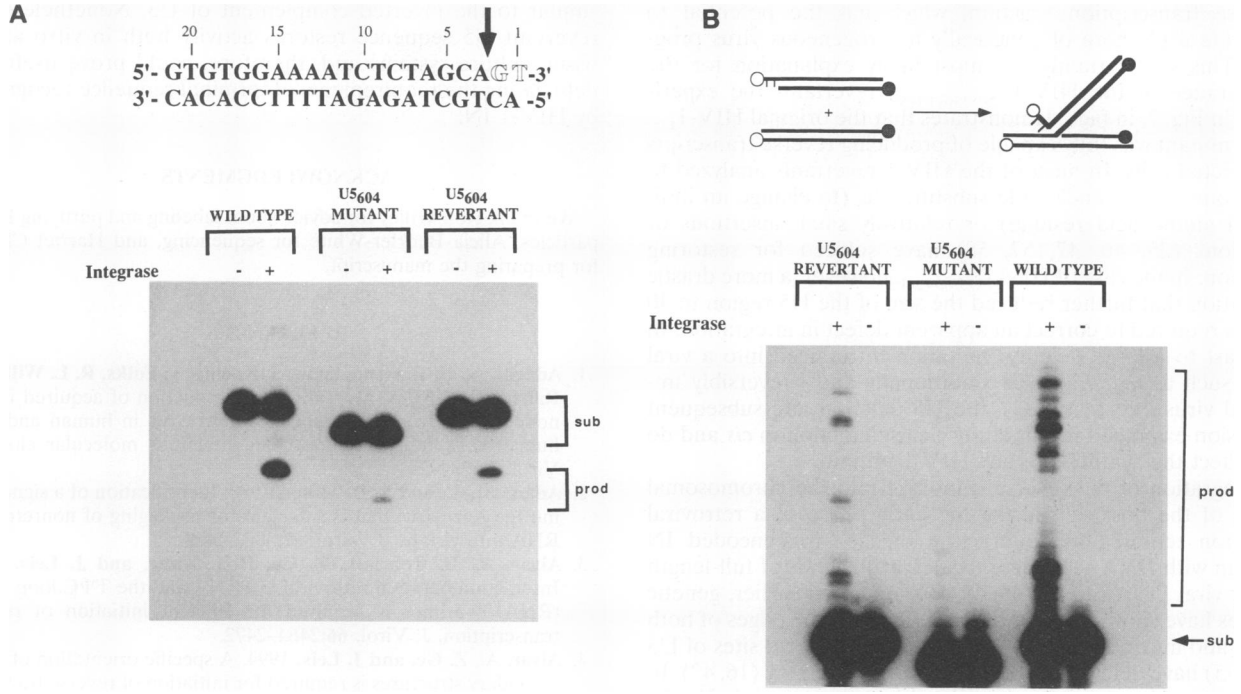


FIG. 8. 3′ processing and DNA strand transfer reactions with wild-type, U5/604del mutant, and U5/604del-rev4 revertant U5 DNA substrates. (A) The sequence of the 21-bp wild-type U5 DNA substrate is shown. The phosphodiester bond that is cleaved in the 3′ processing reaction is indicated (arrow); cleavage removes the outlined GT dinucleotide, resulting in a 19-base product. The substrate is labeled at the 5′ end of the cleaved strand. Integrase was included in the lanes marked + and was omitted from the lanes marked −. The migration positions of the labeled 21-base substrate strands (sub) and the 19-base cleaved products (prod) are shown; the different substrate and product strands display slightly different electrophoretic mobilities because of their different base compositions. (B) Schematic of the DNA strand transfer reaction. The recessed 3′-OH generated in the 3′ processing reaction is the nucleophile that attacks the target DNA, concomitantly cleaving the target strand and joining the viral DNA end to a 5′-phosphate in the target (23). The substrate is labeled at the 5′ end of the strand that becomes joined to the target DNA; a separate substrate molecule serves as the target in this reaction. Integrase was included in the lanes marked + and was omitted from the lanes marked −. DNA strand transfer results in a ladder of joined products (prod) that are larger than the substrates (sub), as strand transfer can occur into essentially any position in the target DNA backbone. Circles, 5′ ends of the oligonucleotide substrates (filled circles, 5′ end of the strand cleaved at its 3′ terminus by integrase). The autoradiogram is a longer exposure of the gel in panel A.

3′ end of the U5 region of HIV-1 in HIV-$1_{U5/604del}$ had no detectable effect on reverse transcription. In the avian system, the defect in viral DNA synthesis is thought to affect initiation of reverse transcription and depend on the formation of two RNA stem structures, the U5 inverted repeat and the U5 leader stem (3, 12, 14). An analogous RNA structure proposed for HIV-1 (14) would include sequences deleted from HIV-$1_{U5/604del}$, HIV-$1_{U5/206del}$, and HIV-$1_{U5/795del}$. Although one could argue that the loss of infectivity seen with HIV-$1_{U5/604del}$ reflects the inability to form these RNA structures, the wild-type replication phenotype observed with both HIV-$1_{U5/206del}$ and HIV-$1_{U5/795del}$, neither of which is capable of forming a U5 leader stem, suggests that initiation of the HIV-1 reverse transcription reaction may be dependent on other RNA structures. Furthermore, the competitive PCR analysis presented in Fig. 7B indicates that HIV-$1_{U5/604del}$, which cannot form either RNA stem structure, suffers no impairment in viral DNA synthesis. A different U5 RNA structure involving the interaction of an A-rich U5 loop, located upstream of the primer-binding site, with the anticodon loop of $tRNA^{Lys}$ has recently been proposed for HIV-1 RNA (29a). The biological significance of this structure is unclear, however, since no impairment in reverse transcription was observed following infections with either the original HIV-$1_{U5/604del}$ mutant or the revertant virus, neither of which contains a U5 region with this motif (Fig. 7).

TABLE 2. In vitro integration assays

U5 substrate	3′ processing[a]	DNA strand transfer[a]
Wild type	1.0 ± 0.3	1.0 ± 0.02
HIV-$1_{U5/604del}$	0.1 ± 0.03	0.04 ± 0.03
HIV-$1_{U5/604del-rev4}$	0.25 ± 0.07	0.22 ± 0.07

[a] Relative to the activity of the wild type. The data are averages for two experiments.

A most unexpected finding of this study was the correction of a severe infectivity defect, secondary to a preexisting 26-nt U5 deletion, by eliminating an additional 19 nt from the HIV-$1_{U5/604del}$ mutant during passage in tissue culture. Previously reported HIV-1 revertants have been primarily *env* mutants in which secondary changes restored alterations in tropism, fusion, virus neutralization properties, gp120-CD4 binding, and gp120-gp41 association (8, 26, 32, 39, 57, 58, 60). Second-site revertants of mutations affecting HIV-1 *cis*-acting elements, mapping to the TATA box within U3 and the primer-binding site, have also been described (46, 47). In tissue culture systems, the underlying mechanism responsible for restoration of biological activity to replication-incompetent viral mutants is invariably the error-prone RNA-dependent

reverse transcription reaction, which has the potential to generate a plethora of genetically heterogeneous virus progeny. This was certainly the most likely explanation for the appearance of the HIV-$1_{U5/604del-rev4}$ revertant: the experiment in Fig. 7, in fact, demonstrates that the original HIV-$1_{U5/604del}$ mutant was fully capable of producing reverse transcripts in infected cells. In most of the HIV-1 revertants analyzed to date, one or two nucleotide substitutions (to change an individual amino acid residue) or relatively small insertions or deletions (25, 46, 47, 57, 58) have sufficed for restoring function. In the case of HIV-$1_{U5/604del}$, however, a more drastic alteration that further reduced the size of the U5 region to 40 nt was required to correct an apparent defect in integration. In contrast to a large deletion mutation introduced into a viral gene such as *nef*, which unconditionally and irreversibly impaired virus infectivity (33), the U5 deletion and subsequent reversion examined in this study clearly function in *cis* and do not affect the synthesis of any HIV-1 protein.

Integration of the reverse transcript into the chromosomal DNA of the host cell during the early phase of a retroviral infection requires the interaction of the virus-encoded IN protein with DNA sequences located at the ends of full-length linear viral DNA (20, 27, 44, 48, 50). As noted earlier, genetic studies have shown that mutations located at the edges of both avian and murine retroviral DNAs (altering the *att* sites of U3 and U5) have deleterious effects on virus infectivity (16, 43). In particular, changes affecting the highly conserved 3′ CA dinucleotides near the ends of U5 and U3 markedly reduce virus infectivity as well as the 3′ processing and strand transfer reactions assayed in vitro (35, 36, 55). For this very reason, the terminal 4 nt at the 3′ terminus of U5, including the CA dinucleotide, were not mutagenized during construction of HIV-$1_{U5/604del}$ (Fig. 1). Although the conserved CA is required for efficient integration in in vitro integration assays, its mere presence is not sufficient either for integration in vitro (36) or, as was observed for the HIV-$1_{U5/604del}$ mutant, for virus production during tissue culture infections. The similar properties of the U5/604 deletion mutant and its revertant in both in vitro integration and virus infectivity assays are quite striking in this regard (Tables 1 and 2).

An early study of the role of subterminal LTR sequences during integration focused on the 15-bp inverted repeat present at the termini of the Mo-MuLV LTR (42). When a 7-bp deletion was introduced near the edge of U3 (removing nt 5 to 11 of the inverted repeat), 3′ processing of both LTRs was blocked and the resultant MuLV was rendered replication defective. This result suggested that an oligomerized form of IN recognized both ends of the linear viral DNA substrate prior to cleaving the 3′ dinucleotide from either end. An analysis of the HIV-1 U3 and U5 terminal sequences present in multiple HIV-1 isolates has failed to reveal an inverted repeat longer than the terminal 4-bp motif, 5′-CAGT-3′; furthermore, the subterminal positions of both U3 and U5 are also highly conserved in different isolates (40). Of relevance to this work is a recent report showing that certain alterations of blocks of nucleotides, subterminal to the CA dinucleotide, resulted in impairment of both 3′ processing and strand transfer in vitro (36). Our results suggest that U5 sequences critical for integration during productive HIV-1 infection are situated within the terminal 16 nt of U5. This conclusion is based on the wild-type infection kinetics observed for HIV-$1_{U5/795del}$ and the loss of infectivity or markedly delayed replication properties exhibited by the HIV-$1_{U5/604del}$ mutant (see Fig. 1 for the structures of these two HIV-1 mutants). The sequence of the HIV-$1_{U5/604del-rev4}$ revertant LTR revealed no obvious homology to the wild-type U5 subterminus, nor was it similar to the inverted complement of U3. Nonetheless, the revertant U5 sequence restores activity both in vitro and in tissue culture systems and, therefore, could prove useful for delineating the requirements of terminal sequence recognition by HIV-1 IN.

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