JOURNAL OF VIROLOGY, Sept. 1995, p. 5904–5907 Vol. 69, No. 9
0022-538X/95/$04.00+0

Sequences in the Human Immunodeficiency Virus Type 1 U3 Region Required for In Vivo and In Vitro Integration

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A series of mutants with alterations in the U3 region of the human immunodeficiency virus type 1 long terminal repeat were made, and the effects of these mutations were evaluated both in vitro and in vivo. When the subterminal 6 to 8 nucleotides of the U3 long terminal repeat were mutated, the resulting provirus was unable to efficiently replicate in vivo, and a mutant oligonucleotide which mimicked the mutation could not be efficiently cleaved but could be joined to target DNA by wild-type recombinant integrase protein in vitro. These results suggest that this region is important in the specific recognition of the viral DNA by the integrase protein.

Upon entry of the human immunodeficiency virus (HIV) into a cell, the retroviral RNA genome is reverse transcribed into a linear DNA molecule consisting of a copy of all viral genes flanked by direct sequences termed long terminal repeats (LTRs) (40). This linear double-stranded viral DNA then serves as a substrate for integration into the host genome (2, 15, 30), where it functions as the template for transcription of viral genes and viral RNA (40). The integrase protein of HIV-1 mediates the integration reaction by which a DNA copy of the HIV-1 genome is established as a provirus in the infected cell. (For recent reviews, see references 1, 16, and 17.)

Through the use of in vitro systems, the mechanism by which integration occurs has been elucidated. The integration reaction consists of three major steps. First, the two terminal nucleotides at the 3′ ends of the blunt-ended linear viral DNA are cleaved, resulting in the formation of recessed terminal CA dinucleotides at each end (3, 15, 35). In the next step, the strand transfer step, the 3′-hydroxyl termini are joined to cellular DNA (3, 5, 11, 15, 22) through an integrase-mediated nucleophilic attack on phosphodiester bonds located on the cellular DNA (14). The cleavage and strand transfer reactions are both executed by IN (5, 7, 11, 22, 23, 37). The final step in viral integration is the repair of the mismatched viral 5′ ends and of the single-stranded DNA gaps that flank the host-virus DNA junctions.

Three functional regions of the integrase protein have been identified. In the N terminus of the protein is an array of histidine and cysteine residues (HHCC motif), which reveals strong conservation among retroviral and retrotransposon integrase proteins (20, 24) and potentially defines a zinc finger domain (6, 20). This region has been shown to be necessary for specific endonucleolytic cleavage and strand transfer reactions in vitro. It has been suggested that this region is involved with the specific recognition of the viral ends (6, 18, 21, 28, 29, 39, 41, 42). In the central domain of the protein is another highly conserved region referred to as the D,D(35)E motif. Mutations in this region have been shown to abolish all activities of IN, implying that it is the catalytic site of the protein (12, 13, 25, 28, 29, 39). The C terminus of the protein exhibits nonspecific DNA-binding activity, suggesting that this region plays a role in the binding of IN to its target DNA (38, 42, 44, 45).

The LTR termini are the only viral sequences thought to be required in *cis* for recognition by the integration machinery. In both murine and avian retroviruses, short imperfect inverted repeats are present at the outer edges of the LTRs. These sequences have been shown to be necessary and sufficient for correct proviral integration both in vitro and in vivo (4, 9, 10, 23, 31, 33, 35). It is clear from these studies that the subterminal CA located at retroviral ends is important for these reactions. However, sequences internal to the CA dinucleotide also appear to be required for optimal IN activity.

It has been shown that mutations in the terminal 15 bp of the HIV-1 LTR result in impaired cleavage and strand transfer in vitro (5, 27, 36, 43). However, the specific in vivo requirements for these internal sequences at the HIV-1 LTR end remain unclear. To probe these requirements, a series of mutations were generated in the U3 region of HIV-1 proviral DNA and their effects were analyzed in vivo and in vitro. Regions of U5 have been shown to be important for the encapsidation of RNA and the initiation of reverse transcription (8, 32); therefore, to avoid the generation of mutations that interfered with these processes, the U3 region was chosen for mutagenesis. In HIV-1, unlike murine leukemia virus, the U3 and U5 ends are identical in only 9 of 15 of the terminal base pairs. In some laboratories, but not in others, the cleavage and strand transfer reactions have been shown to be more efficient in vitro with U5 than with U3 termini (4, 27, 43). Mutations which altered residues conserved between the U3 and U5 LTRs were made. The sequences of the mutated U3 regions are shown in Fig. 1.

Mutagenesis of plasmid DNAs was performed by standard oligonucleotide-mediated site-directed techniques with an M13 derivative containing a 1.5-kb *Bam*HI-*Pst*I fragment from the HIV-1 proviral DNA HXBc.2 (26). This fragment contains the 3′-terminal 1,242 bp of the proviral DNA. The resulting M13 clones were sequenced, and the 0.9-kb *Bam*HI-*Bsp*EI fragments containing the U3 mutations were then used to replace the corresponding wild-type fragment of HXBc.2. The HXBc.2 genome lacks a functional *nef* gene, and therefore any potential effects of the mutations on the overlapping *nef* open reading frame were eliminated.

Jurkat cells were transfected with wild-type and mutant proviral DNA by the DEAE-dextran method (34). Viral superna-

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A) HIV-1 U3 LTR Sequence

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5' ACTGGAAGGGCTAATTCA-3'
3' TGACCTTCCCGATTAAGT-5'
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B) SUBSTITUTION MUTATIONS OF THE U3 LTR

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WT  3' TGACCTTCCCGATTAAGT 5'*
XN1 3' ----------CGGG---- 5'*
XN2 3' -----GGG---------- 5'*
BG1 3' -----------G------ 5'*
BG2 3' --------GGT------- 5'*
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FIG. 1. Sequence of wild-type (A) and mutant (B) U3 LTR DNAs. (A) The conserved CA is underlined. The arrow indicates the region of cleavage. Boldface base pairs indicate nucleotides which are conserved between the U3 and U5 ends of HIV-1. (B) The sequence shown is that of the minus strand of the DNA. Differences from the wild-type sequence are indicated for each mutant. Oligonucleotides corresponding to these sequences were used as substrates for the in vitro integration assays. The location of the radiolabel is indicated by an asterisk.

tants were harvested every 2 to 3 days and tested for reverse transcriptase activity. These assays require viral spread throughout the culture to generate sufficient progeny to be detected. Wild-type provirus induced the appearance of reverse transcriptase activity in culture supernatants by day 18 (Fig. 2). Mutants XN1, BG1, and BG2 replicated with wild-type kinetics, whereas mutant XN2 showed a 9-day delay in the production of peak reverse transcriptase activity. To be sure that the delay in growth seen with XN2 was not due to the specific DNA preparation, the experiment was repeated with two different preparations of CsCl-purified DNA; similar results were obtained.

To determine whether the delayed appearance of XN2 viral progeny was due to the emergence of a revertant of the original mutated virus, viral stocks were harvested at the time of peak reverse transcriptase activity and were used to infect fresh Jurkat cells. The stocks were normalized for reverse transcriptase activity and added to the cells in 10-fold dilutions. Mutant XN2 continued to exhibit a delay in the appearance of progeny virus, this time of about 5 days (data not shown), suggesting that reversion to wild type had not occurred.

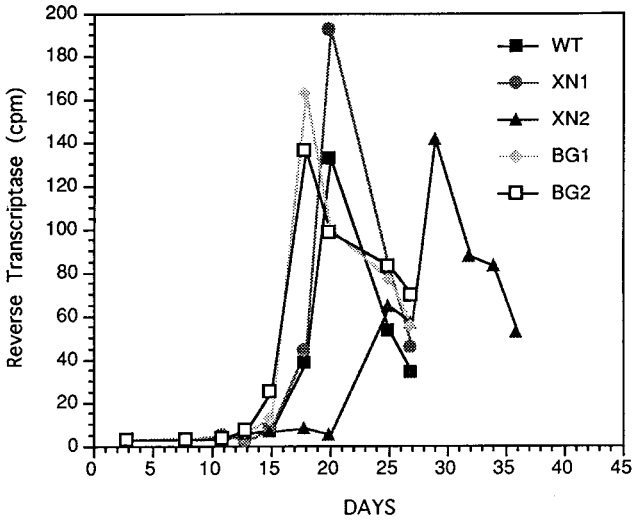


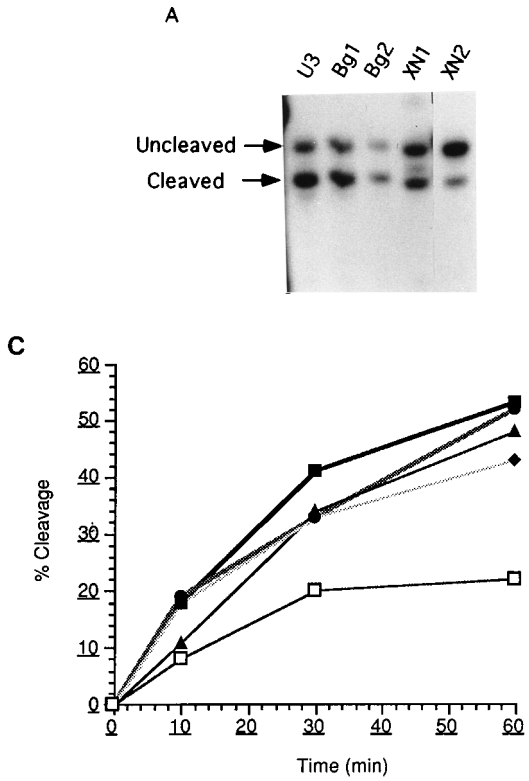
FIG. 2. Growth kinetics of the U3 mutants in Jurkat cells. The cells were transfected with HXBc.2 (WT) or mutant proviral DNAs. The infectivity was monitored as reverse transcriptase activity in the culture supernatant over time. Each reverse transcriptase value represents the amount of activity in 1.5 μl of culture supernatant.

Unintegrated proviral DNA was harvested from mutant XN2 by the method of Hirt (19) at the time of peak virus production. DNA was subjected to 25 rounds of PCR amplification with primers mapping to the *nef* coding region (nucleotides 8865 to 8885 of HXBc.2) and to the R region of the LTR (nucleotides 9628 to 9647 of HXBc.2) under standard conditions (Perkin-Elmer Cetus). The resulting 782-bp product was cleaved with the restriction enzymes *Kpn*I and *Hin*dIII and ligated to corresponding sites in pBluescript KS (Stratagene); four clones containing the insert were obtained and sequenced. All four clones retained the original mutation and showed no linked second-site mutations. These results suggest that the virus replicating after transfection with mutant XN2 was still mutant and had not reverted.

To further investigate the nature of the defect that affected the replication rate of mutant XN2, a series of in vitro integration assays were performed. Recombinant HIV-1 IN protein was isolated from bacterial cultures carrying plasmid pINC6H, expressing an HIV-1 IN tagged with six histidine residues at the C terminus (generously provided by Stuart LeGrice). Bacterial cultures were grown and lysed, and the IN protein was purified by affinity chromatography on Ni-nitrilotriacetic acid agarose essentially as described previously (12). Integrase activity assays were performed with a substrate consisting of an 18-bp double-stranded DNA oligonucleotide corresponding to either the wild-type or mutant U3 terminus with the 5′ end of the processed strand labelled with ^{32}P (Fig. 1). A 0.5-μg portion of 5′-end-labelled oligonucleotide representing the terminal 18 bp of the U3 substrate minus strand was annealed with 2 μg of its complement by heating the reaction mixture at 85°C for 5 min and cooling it to room temperature. To test for cleavage, approximately 0.5 ng of the duplex substrate was incubated with 75 ng of IN protein for 1 h at 37°C in a reaction volume of 30 μl of buffer R (7.5 mM $MnCl_2$, 1.4 mM β-mercaptoethanol, 5% glycerol, 1 mg of bovine serum albumin per ml, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.0]). The reactions were stopped by the addition of EDTA to a final concentration of 50 mM, and the products were analyzed by electrophoresis on a 20% polyacrylamide gel containing urea as described previously (14). Oligonucleotides XN1, BG1, and BG2 were cleaved by IN 65 to 100% as efficiently as the wild-type oligonucleotide was, while oligonucleotide XN2 was cleaved by IN only 30% as efficiently as the wild-type oligonucleotide (Fig. 3). Similar results were consistently found with independent preparations of gel-purified, quantitated oligonucleotides, confirming that the effects seen for the mutants could not be attributed to a particular preparation.

To assess the kinetics of the cleavage reaction for the various mutant oligonucleotides, similar reactions were set up and aliquots were removed at various times and analyzed by electrophoresis as above (Fig. 3C). The initial rate and extent of cleavage at the end of the reaction were significantly reduced for the XN2 mutant. There was a slight reduction in the extent of cleavage observed for the XN1 oligonucleotide.

To test the mutants as substrates for the strand transfer reaction, oligonucleotides which resembled precleaved substrate (lacking the 5′-GT-3′ dinucleotide pair at the 3′ end of the labelled strand) were used. A 0.5-μg portion of 5′-end-labelled oligonucleotide representing the terminal 16 bp of the U3 "precleaved" substrate minus strand was annealed with 2 μg of its 18-bp "uncleaved" complement by heating the reaction mixture at 85°C for 5 min and cooling it to room temperature. A 2-ng portion of the radiolabelled substrate was incu-



B

	Cleavage Efficiency	% Wild-type
U3	67%	100
BG1	62%	92
BG2	64%	95
XN1	45%	67
XN2	20%	30

FIG. 3. Cleavage reactions with the HIV-1 wild-type and mutant U3 LTR substrates. Sequences of the substrates are shown in Fig. 1. (A) A 0.5-ng portion of the radiolabelled duplex substrate was incubated for 1 h at 37°C with 75 ng of purified IN. Products were analyzed by electrophoresis on 20% sequencing gels. The positions of the substrate (18 nucleotides) and the primary cleavage product (16 nucleotides) are indicated. (B) Radiographs of the cleavage reactions were analyzed by densitometry. The cleavage efficiency was determined as 100 × cleaved product/(cleaved product plus uncleaved substrate). The cleavage efficiency of each of the mutant substrates is reported as a percentage of wild-type activity. (C) A 1.5-ng portion of the radiolabelled duplex substrate was incubated with 225 ng of purified IN at 37°C in a reaction volume of 90 μl. Aliquots (30 μl) were removed after 10, 20, and 60 min, and the reaction was stopped by the addition of EDTA. Products were analyzed by electrophoresis, and radiographs of the cleavage reactions were analyzed by densitometry. The percent cleavage was determined as 100 × cleaved product/(cleaved product plus uncleaved substrate). Symbols: ■, U3; ●, BG1; ▲, BG2; ◆, XN1; □, XN2.

bated for 1 h at 37°C with 75 ng of purified IN and 10 ng of the target substrate pBluescript in a reaction volume of 30 μl of R buffer. The reactions were allowed to proceed for 1 h at 37°C, and the products were treated with proteinase K and sodium dodecyl sulfate, extracted with phenol and chloroform, and analyzed by electrophoresis on a 1% agarose gel. The gel was dried and exposed to autoradiography to monitor transfer of the oligonucleotide to the relaxed circular target DNA. All of the oligonucleotides could be joined to the target DNA as efficiently as the WT oligonucleotide (Fig. 4), implying that the XN2 mutation interfered preferentially with cleavage but not with the joining of the mutant U3 LTR. We note that the four mutants were also indistinguishable from each other, further indicating that none of the mutations had a measurable impact on the strand transfer.

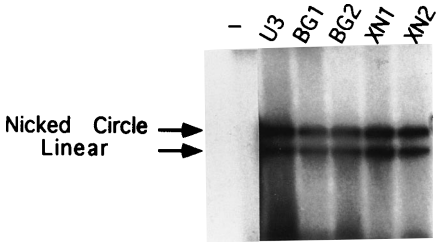


FIG. 4. Strand transfer reactions with the HIV-1 wild-type and mutant U3 LTR substrates. The reactions, performed as described in the text, were stopped by the addition of EDTA to a final concentration of 50 mM. The products of integration were analyzed on an agarose gel and were subjected to autoradiography. − represents the strand transfer reaction with the wild-type U3 substrate without purified IN. The positions of nicked circles and linear plasmid DNA are indicated. Nicked circles are the result of the integration of a single oligonucleotide into the circular plasmid. Linear plasmid DNAs arise by nonspecific nicking of the circular DNAs.

The results of the in vitro data appear to correlate with the in vivo data. XN2, the mutant with the most severe defect in the in vitro cleavage reaction, also had a significant defect in viral replication in Jurkat cells. However, mutant XN1, which was cleaved only 65% as efficiently as the wild-type oligonucleotide, had wild-type kinetics in vivo, while mutant XN2, which was cleaved only twofold less efficiently than XN1, was severely affected in vivo. These findings highlight the fact that it is hard to predict which defects in the in vitro integration assay will be tolerated in vivo, and they emphasize the need to check the phenotype of mutants in vivo.

The results reported here and by others suggest that the in vitro activity of IN does not show profound sequence requirements apart from the subterminal CA dinucleotide. However, nucleotides internal to the conserved CA do influence IN activity, since HIV-1 IN will not cleave or join oligonucleotides which correspond to the tips of murine leukemia virus despite the presence of the subterminal CA (27, 36). We observed that alteration of the subterminal 6 to 8 nucleotides significantly reduced the ability of IN to cleave the terminal GT dinucleotide; however, oligonucleotides with the same mutation which were "precleaved" could be efficiently strand transferred by IN. This is the first report of a mutant oligonucleotide which affected the ability of IN to cleave the oligonucleotide while retaining its ability to strand transfer the oligonucleotide. There has, however, been a report of a mutant oligonucleotide which affected the ability of IN to strand transfer the oligonucleotide without affecting its ability to cleave the oligonucleotide (36). Oligonucleotides with mutations in the subterminal 5 to 8 bp of U5 have previously been reported to result in a moderate reduction in the in vitro cleavage assay (27, 43). In these studies, the strand transfer reaction was not done or was done with oligonucleotides which were not precleaved; therefore, joining could not be separately assessed.

It is interesting that for both HIV-1 and HIV-2, there have been reports of mutations in the HHCC region of IN which resulted in a mutant protein which was unable to efficiently cleave oligonucleotide substrates in vitro but was able to efficiently mediate strand transfer of such oligonucleotides if they were precleaved (28, 42). In addition, IN mutations have been reported to abolish the specific cleavage of viral substrates but have no effect on the nonspecific endonucleolytic cleavage (28). It has been hypothesized that the HHCC motif is involved in the specific recognition of the viral ends and that the region of recognition involves the nucleotides beyond the subterminal CA (41). The data reported here lend credence to this hypothesis and may imply that the region of IN which includes the Cys-His motif was no longer able to efficiently recognize the U3 region of mutant XN2.

This work was supported by grant AI 24845 from the National Institute of Allergy and Infectious Diseases to S.P.G. A.S.R. is an Aaron Diamond Foundation Fellow and a Pfizer Postdoctoral Fellow. S.P.G. is an investigator of the Howard Hughes Medical Institute. G.K. is a fellow of the Helen Hay Whitney Foundation.

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