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A Second Origin of DNA Plus-Strand Synthesis Is Required for Optimal Human Immunodeficiency Virus Replication

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We recently reported that human immunodeficiency virus type 1 (HIV-1) unintegrated linear DNA displays a discontinuity in its plus strand, precisely defined by a second copy of the polypurine tract (PPT) located near the middle of the genome (P. Charneau and F. Clavel, J. Virol. 65:2415–2421, 1991). This central PPT appears to determine a second initiation site for retrovirus DNA plus-strand synthesis. We show here that mutations replacing purines by pyrimidines in the HIV-1 central PPT, which do not modify the overlapping amino acid sequence, are able to significantly slow down viral growth as they reduce plus-strand origin at the center of the genome. One of these mutations, introducing four pyrimidines, results in a 2-week delay in viral growth in CEM cells and abolishes plus-strand origin at the central PPT. The introduction in this mutant of a wild-type copy of the PPT at a different site creates a new plus-strand origin at that site. This new origin also determines the end of the upstream plus-strand segment, probably as a consequence of limited strand displacement-synthesis. Our findings further demonstrate the role of PPTs as initiation sites for the synthesis of the retroviral DNA plus strand and demonstrate the importance of a second such origin for efficient HIV replication in vitro.

Replication of retroviruses requires reverse transcription of the viral RNA genome into double-stranded DNA. Synthesis of each strand of retroviral DNA is initiated at a distinct site of its template by a specific primer, which will define each end of the linear, double-stranded retroviral DNA molecule (27). The origin of the minus strand is determined by a tRNA molecule, copackaged with the viral genomic RNA and hybridized to it near its 5′ end, defining the 3′ boundary of the long terminal repeat (LTR). The origin of the plus strand is determined by a polypurine tract (PPT), which defines the 5′ limit of the LTR (14, 16). It is presumed that the PPT can create an RNA primer on the DNA minus strand by virtue of its resistance to the RNase H activity of the reverse transcriptase (18, 20, 23). A specific feature of human immunodeficiency virus (HIV) and other lentiviruses is the presence of a second copy of the PPT located near the center of the genome (24, 29), within the *pol* gene (Fig. 1a). This central PPT appears to be used as a second origin for the plus strand of viral DNA, since it determines a discontinuity (or gap) in that strand, yielding two discrete plus-strand segments on unintegrated linear DNA (2, 4, 8, 10). Similar findings were reported for spumaviruses (12, 26). We recently demonstrated that the downstream segment of the HIV-1 DNA plus strand starts precisely at the last nucleotide of the central PPT (4). From these observations, we proposed that the use of this second origin for the synthesis of HIV-1 plus-strand DNA could allow faster reverse transcription and therefore could mediate faster replication of the virus. We show here that substitution of at least four purines for pyrimidines in the HIV-1 central PPT without a change in the overlapping amino acid sequence eliminates the plus-strand origin at that site and impairs viral growth. Introduction in such a mutant of a wild-type copy of the PPT at a different site creates a new plus-strand origin and appears to improve viral replication. These experiments further demonstrate the role of PPTs in initiation of the synthesis of the retroviral DNA plus strand and reveal the importance of a second plus-strand origin for efficient HIV replication.

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MATERIALS AND METHODS

Mutagenesis. Site-directed mutagenesis was performed as described previously (31) in M13mp18 carrying an *Eco*RI 1.1-kb insert (4684 to 5779) from the HIV-1 infectious molecular clone pBRU2 (4). Following mutagenesis, this *Eco*RI fragment was cloned into pBRU3, a modified pBRU2 missing an *Eco*RI site in the vector sequences. Details of the mutations are shown in Fig. 1b.

To generate 225P and 225X mutant viruses, mutant 225 plasmid DNA was digested with *Sal*I, purified, and ligated to nonphosphorylated double-stranded oligonucleotide 225P or 225X (see Fig. 4a). The resulting clones were screened for the insertion of a *Pvu*I (225P) or *Xba*I (225X) restriction site.

Cells and viruses. Peripheral blood lymphocytes (PBLs) were obtained from healthy blood donors, stimulated with 1 μg of phytohemagglutinin (Wellcome) per ml, and maintained in the presence of interleukin-2 (10% Lymphocult; Biotest Diagnostics). MT4 cells are human T-cell leukemia virus type I-transformed human CD4+ T cells that allow acute cytopathic HIV-1 infection (7). CEM clone 13 cells were derived from the human lymphoid cell line CEM (ATCC CCL119) and express high levels of CD4 antigen (21). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. For the production of mutant viruses, HeLa cells were transfected by the calcium phosphate method with 10 μg of plasmid DNA from each of the mutants. After incubation for 48 h, culture supernatants were harvested and filtered, and the amount of virus produced was measured by using the DuPont HIV p24 core profile enzyme-linked immunosorbent assay (ELISA). All cells were infected with an equal amount of cell-free virus, corresponding to 10 ng of HIV-1 p24. Infections were carried out in duplicate, using approximately 5×10^5 cells in a final

volume of 1 ml. Virus production in these cultures was monitored with the HIV p24 core profile ELISA.

The H12 indicator cells are HIV-infectable HeLa CD4 cells carrying the bacterial *lacZ* gene under control of the HIV LTR. In this system, infected cells can be detected in an in situ enzymatic assay by the intense blue staining due to the induction by Tat of *lacZ* expression as described previously (22). H12 cells are derived from an HeLa LTR-*lacZ* clone (Z24) obtained by cotransfection of plasmids pMA175 and pSV2neo, followed by G418 selection. pMA175 contains the HIV-1 BRU LTR *Sca*I-to-*Hin*dIII fragment (−139 to +82) driving *lacZ* and simian virus 40 polyadenylation sequences. The Z24 clone was selected for its low basal β-galactosidase activity (undetectable in an in situ assay) and the dramatic induction seen after transfection with *tat* expression vectors or infection with HIV pseudotypes carrying the amphotropic murine leukemia virus envelope. Z24 was further cotransfected with a CD4 expression vector (pMA245) and a plasmid conferring hygromycin resistance (pHygro^R). pMA245 contains the CD4 cDNA as an *Eco*RI-*Bam*HI 1.7-kb fragment from pT4B (13) under control of the simian virus 40 early promoter and inserted in a murine retroviral vector, the 3′ LTR providing the polyadenylation sequence. Hygromycin-resistant clones were selected for surface CD4 expression and strong β-galactosidase induction after HIV infection. A clone fulfilling these properties was further purified by two rounds of single-cell cloning to obtain the H12 subclone.

The H12 cells were used to determine single-round infectious titers of viral suspensions corresponding to the different central PPT mutants. Each mutant was tested at two different dilutions (100 and 10 ng of HIV-1 p24 per ml) in a final volume of 1 ml on monolayers of H12 cells in 6-cm^2 wells. The H12 cultures were then incubated for 24 h, fixed, and stained with 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) for 1 h. The infectious titer of viral suspensions on H12 cells is expressed as the number of blue foci (a focus being a single isolated blue cell or a group of two contiguous blue cells) per nanogram of p24 present in the inoculum. These experiments were repeated four times for each mutant, using supernatants from separate transfections.

Analysis of viral DNA. Viral DNA was analyzed in MT4 cells that were infected by cocultivation with transfected HeLa cells. Cocultivation was carried out 48 h after transfection with the different mutants. On the next day, the MT4 cells were transferred to a new flask, and fresh MT4 cells were added. These cultures were then monitored daily for appearance of HIV cytopathic effect, characterized by dissociation of the MT4 clumps and rounding of the cells. When cytopathic effect was obvious, low-molecular-weight DNA in the corresponding cultures was extracted by the Hirt procedure (9).

Nuclease S1 (Amersham) was used at 1.5 U/μg of DNA after addition of 1:10 volume of 10× S1 buffer (10× S1 buffer is 300 mM sodium acetate [pH 4.6], 500 mM NaCl, and 10 mM $ZnCl_2$) and incubated at 37°C for 30 min. DNA was then subjected to electrophoresis on 0.8% agarose gels that did not contain ethidium bromide and analyzed by Southern blotting (25).

Two probes were used in hybridization experiments. The 5′ probe was a 1,932-bp *Bal*I fragment spanning the *pol* region of pBRU3 from positions 2655 to 4587; the 3′ probe was a 2,662-bp *Kpn*I fragment representing the *env* region from positions 6343 to 9005 of HIV-1 molecular clone pNL4-3 (1). The nucleotide position numbers used here start

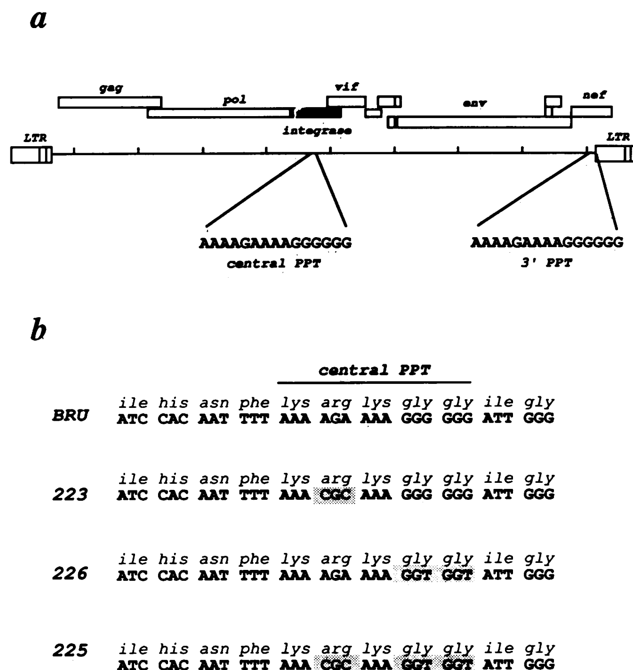

FIG. 1. (a) Positions of the two PPTs on the DNA genome of HIV-1, with respect to the different coding and *cis*-acting sequences. The shaded domain of the *pol* gene encodes the integrase, where the mutations affecting the central PPT were introduced. (b) Details of the different mutations affecting the central PPT.

at the 5′ end of the U3 region in the 5′ LTR. Both probes were labelled by the random hexamer method (5).

RESULTS

Substitution of pyrimidines for purines in the central PPT affects HIV-1 replication. Using site-directed mutagenesis, we replaced several purines (A or G) with pyrimidines (C or T) in the central PPT. Details of these mutations are shown in Fig. 1b. All of the mutations left intact the overlapping *pol* amino acid sequence, corresponding to the integrase (Fig. 1a). HIV-1 proviral molecular clones carrying these mutations were introduced by transfection into HeLa cells. The resulting viruses were analyzed for infectivity in phytohemagglutinin-stimulated PBLs and in CEM cells, which were infected with equal amounts of cell-free virus, as determined from HIV-1 p24 antigen concentration. Central PPT mutations did not affect the amount of virus produced by the transfected cells (data not shown). None of the mutations completely abolished HIV infectivity, but some of the mutants markedly differed in infectivity from wild-type virus BRU, as evidenced by important differences in their kinetics of virus production (Fig. 2a and b) in PBLs and CEM cells. The least affected was mutant 226, in which T replaced G at positions 12 and 15 of the central PPT, and was closest to BRU in its replication kinetics in both cell systems. Mutant 223, replacing A at positions 4 and 6 with C, was comparable to BRU in PBLs, but 223 virus production peaked a week later than BRU production in CEM cells. The most affected of the three mutants was 225, which combined the mutations in 223 and 226, with a total of four pyrimidines replacing purines in its central PPT. The peak of 225 virus production was delayed by 2 weeks in CEM cells and in PBLs. In three

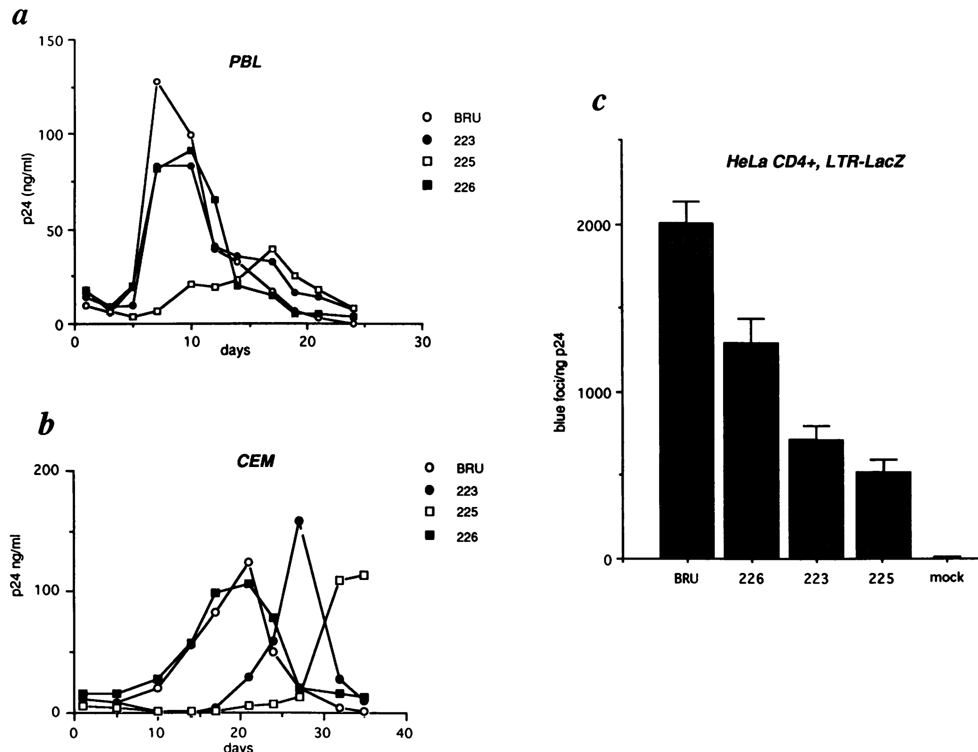

FIG. 2. Phenotypes of the HIV-1 central PPT mutants. (a) Kinetics of replication in phytohemagglutinin-stimulated PBLs. (b) Kinetics of replication in CEM cells. Both PBLs and CEM cells were infected with a volume of viral suspension containing 10 ng of HIV-1 p24, for a total of 5×10^5 cells. (c) Titration of the central PPT mutants on H12 cells (HeLa cells expressing the CD4 receptor and carrying a Tat-inducible *lacZ* gene under the control of the HIV-1 LTR). Monolayers of H12 cells in 6-cm^2 wells were infected with 1 ml of virus suspension containing 100 or 10 ng of HIV-1 p24. H12 monolayers were stained with X-Gal 24 h after infection. The results are presented as the average number of blue foci (single isolated blue cells or groups of two blue cells) per nanogram of HIV-1 p24 contained in the inoculum, obtained from four independent experiments using virus from four different transfections. Error bars represent standard errors of the means.

independent infection experiments using supernatants from three distinct BRU and 225 transfections, adjusted to 10 ng of HIV-1 p24 for each infection, the peak of 225 production in CEM cells was delayed 14, 13, and 16 days compared with the peak of BRU production. Replication of the mutants was also assayed on MT4 cells, which we use mainly for analysis of viral DNA, since they allow very rapid viral propagation and accumulation of high levels of unintegrated viral DNA. In this cell system, only minimal differences between wild-type and mutant viruses could be observed (data not shown).

The onset of viral production in lymphocytic cells, which we monitored as an index of infectivity, proceeds through accumulation of multiple rounds of viral replication. To better understand the nature of the defect introduced in the PPT mutants, we wished to compare the infectivity of these viruses in a system that would allow evaluation of HIV infectivity following a single infectious cycle. Consequently, we constructed a cell line derived from HeLa cells expressing the CD4 receptor and harboring the *lacZ* gene under the control of the HIV-1 LTR. In these cells, which allow entry and replication of HIV-1, the expression of Tat following HIV infection triggers the accumulation of β-galactosidase, easily detected as an intense blue staining in an in situ X-Gal assay. Since the infected H12 cells were stained only 24 h following infection, we believe that the number of blue cells in these cultures reflected the number of cells having harbored one single cycle of HIV replication, up to integration of viral DNA and expression of Tat. The infectious titer of the different viruses was expressed as the number of blue foci per nanogram of p24 measured in the infecting viral suspension. As shown in Fig. 2c, the titer of the mutant viruses in this system was consistently lower than the wild-type BRU titer. As was also observed in PBLs and CEM cells, mutant 225 displayed the most significant reduction, followed by mutants 223 and 226. In four separate experiments using viral suspensions obtained from independent transfections, the average numbers of blue foci per nanogram of HIV p24 were 516 ± 81 (standard error of the mean) for mutant 225 and 2,002 ± 134 for BRU (Fig. 2c).

The amount of gapped viral DNA in central PPT mutants is directly proportional to their replicative capacity. In an attempt to determine whether the mutations affecting HIV replication also altered the structure of the reverse transcription product, unintegrated viral DNA from MT4 cells infected with the different mutants was tested for the presence of the central plus-strand gap by nuclease S1 cleavage (Fig. 3). In untreated preparations, there appeared to be no significant difference between BRU and the mutants in the total amount of viral DNA, nor was there a difference in the relative amounts of linear and circular molecules. However, the mutations clearly affected the proportion of gapped linear molecules, as evidenced by the release, after nuclease S1 treatment, of a 5-kb fragment reactive with an HIV-1 *env* probe. This DNA fragment represents the portion of HIV linear DNA 3′ to the gap (4). The amount of this 5-kb fragment therefore reflects the amount of gapped viral DNA

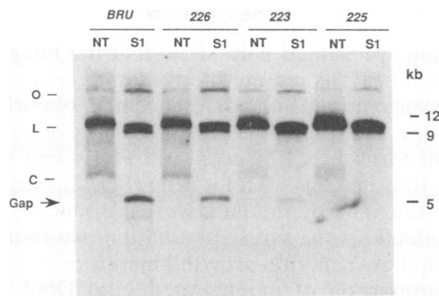

FIG. 3. Southern blot of Hirt supernatants from MT4 cells infected with the indicated central PPT mutants, before (NT) and after (S1) treatment with nuclease S1, and hybridized with a probe specific of the *env* region of the HIV-1 genome. The three forms of unintegrated viral DNA observed in untreated DNA preparations are linear molecules (L), closed circles (C), and open or relaxed circles (O). Following nuclease S1 treatment, the closed circles disappear, and cleavage of linear molecules at the central gap releases a 5-kb fragment (4), reactive with the *env* probe (nucleotides 6343 to 9005) used in this experiment. The intensity of this 5-kb band is an indication of the proportion of linear molecules carrying a central gap. In this experiment, the cleavage of viral DNAs by nuclease S1 was not complete. Therefore, substantial amounts of uncleaved linear molecules remained after nuclease S1 treatment. Consequently, the double band around 9 kb in S1-treated preparations corresponds to uncleaved linear molecules (upper band) and to linearized one-LTR circles (lower band), which were previously shown to lack a central gap (4).

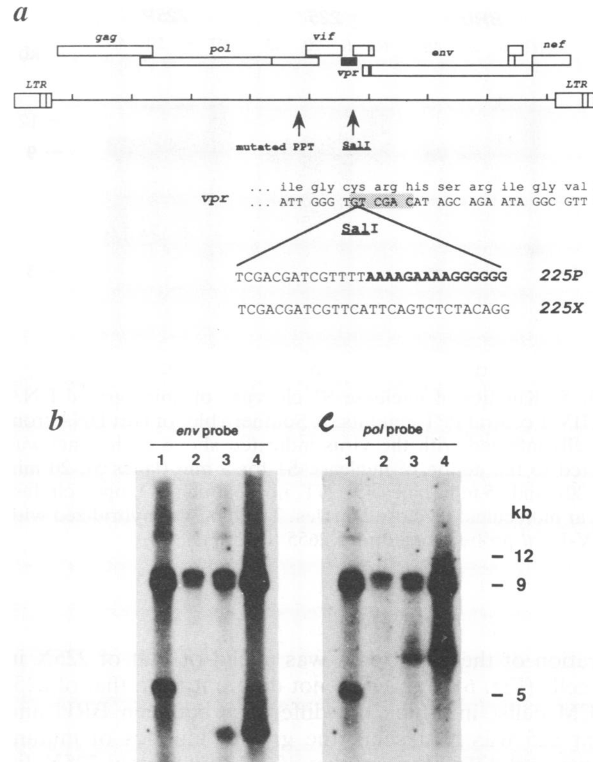

FIG. 4. Insertion of a new PPT downstream of the original central PPT site in mutant 225. (a) Oligonucleotide linkers used to insert a PPT in the unique *Sal*I site at position 5821 of mutant 225 (PPT mutant substituting four pyrimidines for purines). Linker 225P contains a PPT identical to the central and 3′ PPTs of HIV-1. Linker 225X is a control with no PPT sequence. (b and c) Southern blots of Hirt supernatants from MT4 cells infected with different viruses after treatment with nuclease S1. Lanes: 1, BRU Hirt DNA digested with nuclease S1; 2, 225 DNA digested with nuclease S1; 3, 225P DNA digested with nuclease S1; 4, unintegrated BRU DNA digested with *Sal*I. The same Southern blot was first hybridized with the *env* probe (nucleotides 6343 to 9005), dehybridized, and rehybridized with a *pol* probe (nucleotides 2655 to 4587).

molecules in the acutely infected cells. As we anticipated, the amount of gapped viral DNA in the various mutants directly correlated with the replicative capacity of the corresponding viruses (Fig. 3). Mutant 226 differed little from BRU, but in mutant 223, the proportion of gapped linear molecules was markedly reduced. In mutant 225, which had the slowest replication profile in lymphocytes and the lowest titer on CD4+ HeLa cells, the amount of 5-kb fragment was always extremely low and usually undetectable. The relative amount of gapped DNA should reflect the capacity of the central PPT to promote priming of HIV plus-strand synthesis at the center of the genome. Taken together, our observations on infectivity and DNA structure of the central PPT mutants suggest that reduction of plus-strand initiation at that site directly affects replication of HIV.

Introduction of a new PPT at a different location creates a new gap at that site and can affect viral growth. We next attempted to create a new plus-strand priming site in mutant 225, since the utilization of the mutated PPT in this virus was nearly abolished. A linker containing an exact replica of the HIV-1 PPT (Fig. 4a) was therefore introduced in the *Sal*I site at position 5821, within the *vpr* open reading frame (mutant 225P). In this mutant genome, the introduced PPT was located approximately 1,000 bp downstream of the site where the PPT would be found in wild-type HIV-1. The presence and position of a plus-strand gap in the 225P reverse transcription product were evaluated by nuclease S1 cleavage of viral unintegrated linear DNA from infected MT4 cells (Fig. 4b and c). Introduction of a PPT at the *Sal*I site indeed created a new priming site, as evidenced by the presence of a new S1-sensitive site in 225P DNA at this position. The 225P DNA fragment released by nuclease S1 and reactive with the *env* probe was ≈4 kb in size (instead of 5 kb in wild-type BRU) and comigrated with the fragment released by *Sal*I cleavage on wild-type viral DNA (Fig. 4b, lane 3). Interestingly, when we used a *pol*-specific probe, we found that the 5′ fragment released by S1 cleavage was larger (≈6 kb) than the 5-kb fragment released from wild-type virus (Fig. 4c, lane 3), indicating that the stop in elongation of the upstream plus-strand segment is determined by the PPT itself. This observation seems to imply that the origin of the 3′ segment at the PPT cannot be displaced by reverse transcriptase-driven DNA synthesis. A kinetic analysis of S1 cleavage (Fig. 5) revealed that the relative amounts of DNA fragment specifically released with time by S1 cleavage at their respective gaps were comparable for BRU and 225P (Fig. 5a and c), whereas very little, if any, such fragment was released from 225 DNA (Fig. 5b). The comparable sensitivities of BRU and 225P DNAs to nuclease S1 cleavage indicate that the new plus-strand discontinuity on 225P DNA has a general structure similar to that of wild-type DNA and is found in a similar proportion of DNA molecules.

The introduction of the linker sequence created a frameshift in the *vpr* gene of the 225P mutant. Therefore, the replication of 225P had to be compared with that of a control in which a linker containing a random sequence was introduced at the same site (mutant 225X), also creating a frameshift at the same position in *vpr* (Fig. 4a). Clearly,

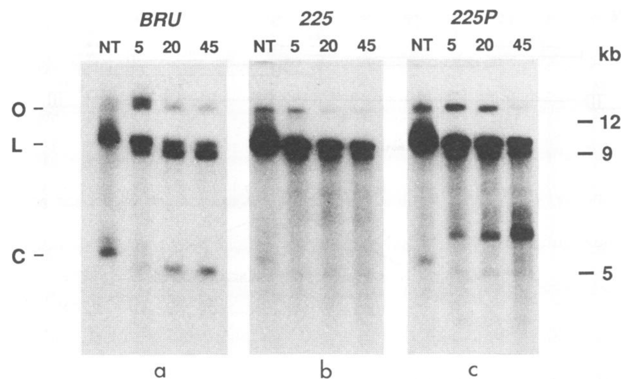

FIG. 5. Kinetics of nuclease S1 cleavage of unintegrated DNA from HIV-1 central PPT mutants. A Southern blot of Hirt DNA from MT4 cells infected with the virus indicated above each panel was subjected to the action of nuclease S1 for 5 min (lanes 5), 20 min (lanes 20), and 45 min (lanes 45). NT, no treatment. O, open circles; L, linear molecules; C, closed circles. The blot was hybridized with an HIV-1 *pol* probe (nucleotides 2655 to 4587).

replication of the 225P virus was ahead of that of 225X in MT4 cells (Fig. 6), but it was not different from that of 225. In CEM cells, in which the difference between BRU and mutant 225 was best seen, the growth kinetics of mutant 225P was not different from that of 225, but mutant 225X did not give rise to a productive infection during the 40-day observation period (Fig. 6). Therefore, it appeared that the introduction of a new PPT, even not exactly where the wild-type PPT is normally found, could promote more efficient viral replication in lymphocytic cells. However, the clear and reproducible reduction in infectivity characteristic of the 225X mutant in MT4 and CEM cells (Fig. 6) did not correlate with its one-round infectious titer on H12 cells, which was repeatedly found to be comparable to those of mutants 225 and 225P (data not shown). This discrepancy could be a consequence of the interruption of the *vpr* gene, whose function is poorly understood. It may also reflect a qualitative difference between the assay on H12 cells and the replication kinetics on lymphocytic cells.

DISCUSSION

The results presented here show that the integrity of the HIV-1 central PPT is required for optimal replication of this virus in tissue culture. Indeed, replacement of purines within this structure by pyrimidines resulted in delayed growth of the mutant virus in CEM cells and PBLs and in a lower infectious titer on CD4+ HeLa cells (Fig. 2). Strikingly, we found that the more pyrimidines were introduced, the slower and less infectious the virus. In addition, we found a direct relationship between viral growth kinetics or infectious titer and the proportion of unintegrated viral DNA molecules carrying a central single-stranded gap, as measured by the amount of specific DNA fragments released following nuclease S1 treatment (Fig. 3). Taken together, these observations strengthen the evidence that the central PPT determines an additional site for initiation of HIV-1 plus-strand synthesis and establish that this additional origin site allows more efficient replication of the virus.

One possible explanation for this phenomenon is that this second origin determines faster synthesis of full-length viral DNA and, from there, faster overall replication of HIV-1. Another explanation could be that this additional plus-strand DNA origin not only affects the speed of viral DNA synthesis but also increases the overall efficiency of the reverse transcription process. This could be made possible, for example, by allowing synthesis of more stable reverse transcription intermediates. This hypothesis is supported by the difference in titer that we observed between the different mutants in cells that harbored a single round of HIV replication. We also found that this difference was not affected by a longer incubation period between infection and staining (data not shown). We cannot exclude the possibility that the presence of a gap in linear viral DNA is required for further steps of the retroviral life cycle involving processing of the viral DNA genome (i.e., transport to the nucleus, integration, etc.). This is unlikely, however, since none of the mutants has its replication completely impaired. In particular, mutant 225, in which the proportion of linear molecules with a central gap was dramatically reduced, exhibited a delayed peak of virus production but was still infectious, and its infectious titer on CD4+ HeLa cells was never reduced more than sixfold compared with the wild-type virus titer. In addition, the replication of this mutant in MT4 cells was

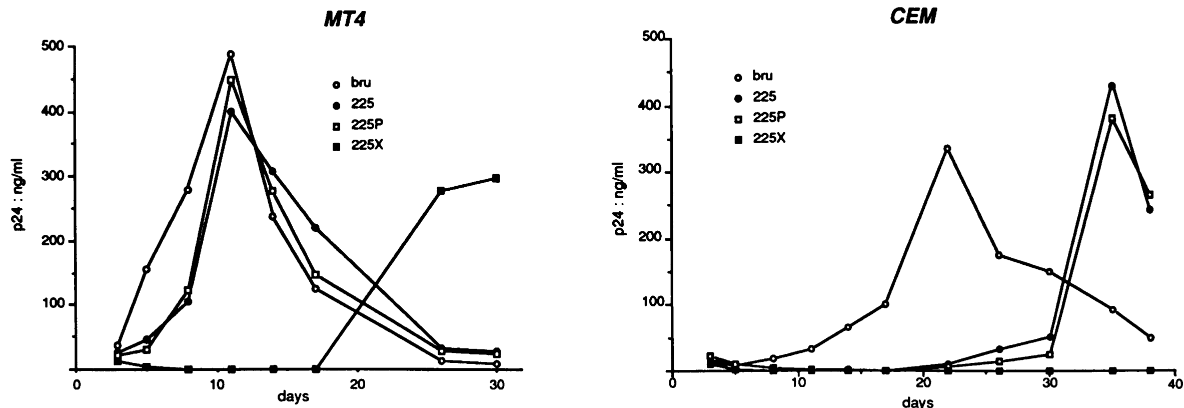

FIG. 6. Replication kinetics of 225P and 225X mutants, compared with kinetics of wild-type BRU and PPT mutant 225, in CEM and in MT4 cells infected with a volume of viral suspension from transfected HeLa cells containing 10 ng of HIV-1 p24. For each infection, a total of 5 $\times 10^5$ cells was used in a final volume of 1 ml.

close to the wild-type level despite the near absence of a central gap. It should be noted that we did not detect the reappearance of a gap in 225 viral DNA upon serial passage on MT4 cells (data not shown), indicating that the minimal differences found between wild-type and mutants in this cell system were not due to a correction of the PPT mutation. These observations suggest that the presence of the central PPT confers a relative replication advantage but does not constitute an absolute requirement for viral replication.

The three mutations analyzed here clearly differed one from another in their effects on viral growth and use of the central PPT as an origin site, as reflected by the proportion of gapped DNA molecules in acutely infected cells. The least affected, 226, had two pyrimidines replacing purines, and the longest stretch of purines in the mutated 226 PPT was 11, compared with 15 in wild-type virus BRU. Comparatively, mutant 223, which appeared to be more affected than 226 in the ability of its PPT to generate a plus-strand origin site, also had two replacements, but its longest purine stretch consisted of only nine purines. Finally, mutant 225, in which only a very small proportion of DNA molecules showed use of the mutated central PPT as a plus-strand origin, had four replacements but also had only five purines in a row at the PPT site. These observations could suggest that the efficiency of the PPT is determined by the overall length of the longest stretch of purines. It is also possible that the priming efficiency of a PPT is determined by an overall sequence configuration, which could have been most disrupted by the 225 mutation. In this respect, it has been shown that single base changes in the PPTs of murine retroviruses, including those that are the most conserved among retroviral PPTs, have little effect on priming efficiency in vitro (19).

Interestingly, in all of our mutants, the only discrete S1-sensitive site observed in the genome appeared to be still determined by the modified central PPT. We cannot explain why other possible plus-strand origin sites on the HIV genome are not detected by nuclease S1 cleavage. In particular, other stretches of purines present elsewhere in the genome either may not be used as origins for plus-strand synthesis or may be unable to define a discrete and stable strand discontinuity, as the central PPT is. This could be due to the particular sequence configuration of the PPT or to the participation of yet unidentified neighboring sequences.

We show here that the introduction of a new PPT in mutant 225 is able to recreate an origin site precisely where the new PPT is inserted, as evidenced by the presence of a new nuclease S1-sensitive gap at that site (Fig. 4b and c). This finding further demonstrates the role of the PPT in the generation of an origin for retroviral plus-strand synthesis. In addition, we found that the new gap in mutant 225P remained narrow and discrete, since the 3′ end of the upstream plus-strand segment appeared to coincide with the origin of the new downstream segment, at the site of the inserted PPT. This finding indicates that the stop in progression of synthesis of the upstream segment is determined by the PPT itself or, more likely, by the origin of the downstream segment at that site. Although we do not provide here data on what the precise 3′ end of the upstream segment is, we hypothesize that the termination of its synthesis is a consequence of an inability or limited ability to carry strand displacement-synthesis at the PPT. This mechanism is also thought to generate the strand discontinuities observed in caulimoviruses (17, 28). It has been proposed that in avian retroviruses, strand displacement-synthesis by reverse transcriptase is able to remove short segments of plus strand initiated at nonspecific sites by the elongating main plus strand initiated at the 3′ PPT (3). The resulting lagging strands were also speculated to play a role in retroviral genetic recombination (11). It has also been proposed that strand displacement-synthesis is necessary to generate the ends of the full-length, two-LTR retroviral DNA molecule (6, 18). More detailed in vitro studies on strand displacement-synthesis by reverse transcriptase are required to ascertain whether the stop that we observe is a specific feature of PPTs or a general property of reverse transcription. Whatever its exact mechanism, the inability to displace the plus-strand origin at the central PPT could be a prerequisite for the optimal use of this additional priming site, leading to faster or more efficient synthesis of the whole retroviral plus strand.

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ADDENDUM

Shortly after this work was performed, a new designation was proposed for the HIV-1 isolate used in this study (15, 30). Therefore, the virus and the corresponding molecular clones designated here HIV-1 BRU should be now referred to as HIV-1 LAI.

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