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The Roles of the Human Immunodeficiency Virus Type 1 Pol Protein and the Primer Binding Site in the Placement of Primer $tRNA_3^{Lys}$ onto Viral Genomic RNA†

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Factors that modulate the placement of primer $tRNA_3^{Lys}$ onto the viral RNA genome in human immunodeficiency virus type 1 (HIV-1) were investigated through analysis of reverse-transcribed products that are extended from the $tRNA_3^{Lys}$ primer. Mutations were introduced into the HIV-1 *pol* gene to result in the appearance of a stop codon in the open reading frame of the reverse transcriptase (RT) gene. These constructs, BH10-RT1 and BH10-RT2, yielded viruses with truncated Pol proteins. Alternatively, we altered the sequences involved in frameshifting by generating the construct BH10-FS. With each of these mutated viruses, we found that the primer $tRNA_3^{Lys}$ that was placed onto viral genomic RNA was present in an unextended state. In contrast, as expected, $tRNA_3^{Lys}$ in the case of wild-type BH10 virus had been extended by 2 bases. Furthermore, the amount of $tRNA_3^{Lys}$ that was placed onto viral RNA in mutated viruses was significantly less than that placed in the wild-type virus. We also generated a mutant within the polymerase-active site of RT (D185H) (Asp→His) that eliminated RT polymerase activity. We found that the placement of primer $tRNA_3^{Lys}$ onto viral genomic RNA was independent of enzyme function; however, the $tRNA_3^{Lys}$ that was placed was present in an unextended state due to the loss of RT activity. In contrast, the elimination of protease activity through a D25A (Asp→Ala) point mutation in the protease-active site (construct BH10-PR) did cause a drop in the efficiency of $tRNA_3^{Lys}$ placement. In this situation, a proportion of the placed $tRNA_3^{Lys}$ was found to be extended by 2 bases, although not to the extent found with wild-type virus (BH10), due to a decrease in RT activity associated with unprocessed Gag-Pol protein that could not be cleaved because of the loss of protease activity. We also investigated the role of the primer binding site (PBS) in the placement of $tRNA_3^{Lys}$ through a series of 2-, 4-, and 8-nucleotide (nt) deletions at the 3′ end of the PBS, i.e., BH10-PBS2, BH10-PBS4, and BH10-PBS8, respectively. In mutated viruses BH10-PBS2 and BH10-PBS4, the 2-base-extended form of $tRNA_3^{Lys}$ was still detected. However, less primer $tRNA_3^{Lys}$ was placed onto viral genomic RNA as more nucleotides were deleted until the percentage of placement seen with wild-type BH10 virus dropped to only 4% in the virus with 8 nt deleted (BH10-PBS8). Consistently, these mutated viruses possessed decreased initial replication capacity compared with that of the wild-type virus, with the extent of incapacity corresponding to the size of the deletion. However, after several days, an increase in replication potential was accompanied by a reversion to a wild-type PBS.

Retroviral reverse transcriptase (RT) is known to catalyze DNA polymerization with RNA as the template (3, 29). This event, as well as that of DNA-dependent DNA polymerization, requires a primer that in the case of human immunodeficiency virus type 1 (HIV-1), has been identified as $tRNA_3^{Lys}$ (20). An 18-nucleotide (nt) segment, located just downstream of the U5 region, in the retroviral RNA genome is complementary in sequence to an 18-nt sequence at the 3′ terminus of the primer tRNA and is termed the primer binding site (PBS). In the virion, primer tRNA is placed onto the PBS to form an 18-bp RNA:RNA hybrid, which serves to initiate reverse transcription once it is recognized by RT, which becomes part of an initiation complex.

The mechanism by which the primer tRNA is placed onto the PBS is still unclear, although the presence of at least part of the PBS is required. Indeed, viruses from which the PBS has been deleted are not viable (15). Furthermore, replacement of the HIV-1 PBS by 18-nt sequences complementary to the 3′ ends of tRNA species other than the cognate tRNA primer led to reduced occupancy of the mutated PBS by these other tRNA species and diminished replicative capacity (7, 15, 32, 34). At the same time, it has previously been reported that deletions or insertions of either 2 or 4 nt at the 5′ end of the PBS did not significantly affect the efficiency of tRNA placement, suggesting that the 5′ terminus of the PBS is not essential for this process (6).

Since tRNA molecules possess a highly ordered tertiary structure, other factors are required for placement to occur onto the viral RNA template. Previous cell-free experiments have shown that the viral nucleocapsid protein (NCp) can promote the annealing of the relevant complementary nucleotide sequences and place primer tRNA onto viral genomic RNA (4, 5). In contrast, mutated forms of NCp were not able to efficiently place primer $tRNA^{Trp}$ onto the avian leukosis virus RNA genome (23). The viral RT enzyme may also be involved in this process, since RTs possess higher affinities for primer tRNA species than do other forms of tRNA (1, 2, 17,

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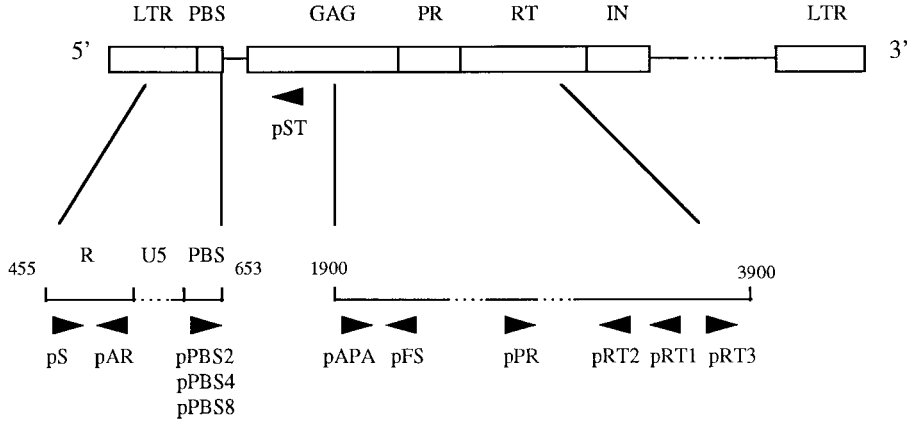
†Dedicated to the memory of Benjamin Freedman, a friend and colleague who devoted his career to easing the suffering of others.

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pS:   5'-AGACCAGATCTGAGCCTGGGAG-3'(468-489)
pAR:  5'-TTATTGAGGCTTAAGCAGTGGG-3'(531-510)
pPBS2:5'-CAGTGGCGCCCGAACAGGGTTGAAAGCGAAAGGG-3'(633-668)
pPBS4:5'-CAGTGGCGCCCGAACAGTTGAAAGCGAAAGGG-3'(633-668)
pPBS8:5'-CAGTGGCGCCCGATTGAAAGCGAAAGGG-3'(633-668)
pST:  5'-CATTCTGCAGCTTCCTCATTGATGG-3'(1421-1397)
pAPA: 5'-TGCAGGGCCCCTAGGAAAAAGGG-3'(1999-2021)
pFS:  5'-GGCCAGATCTTCCCTAGGAAGTTAGCCTGTCTCTCAG-3'(2102-2066)
pPR:  5'-GGAAGCTCTATTAGCAACAGGAGCAGATG-3'(2345-2373)
pRT2: 5'-TCAATGGCCATTACTAAACTTTTGGGCC-3'(2661-2634)
pRT1: 5'-AAAATATGCATCACACATCCAGTACTGTTAC-3'(2930-2898)
pRT3: 5'-CTATCAATACATGGATCACTTGTATGTAGGATCTGAC-3'(3121-3158)
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FIG. 1. Schematic representation of positions and sequences of the primers used in the construction of plasmids and in primer extension assays. LTR, long terminal repeat; IN, integrase; PR, protease.

21, 22, 25, 27, 30, 33, 35, 37, 38) and are responsible for the selective packaging of primer tRNA into the virion (12, 18, 19). However, RT itself cannot efficiently place primer tRNA onto viral RNA and may therefore be only indirectly involved (16).

Two methods have generally been used to verify the placement of tRNA onto the PBS. One is a primer extension assay in which a DNA oligonucleotide is annealed downstream of the PBS (34). The other is based on direct quantitation of extension products from primer tRNA placed onto the viral RNA genome (22). Our group recently showed that primer tRNA placed onto the PBS can exist in both unextended and 2-base-extended forms (9), making it possible to distinguish between them and to study tRNA placement by radiolabelling analysis.

We investigated this subject by studying RT, unprocessed Gag-Pol, and the 3′ terminus of the PBS. Mutations were introduced into viral DNA to generate mutated viruses that contained truncated RT molecules (BH10-RT1 and BH10-RT2) or unprocessed Gag polyprotein (BH10-FS). Mutated viruses that no longer possessed either RT (BH10-RT3) or protease activity (BH10-PR) due to mutagenesis at the active sites of these enzymes were also generated. In addition, a series of deletions at the 3′ end of the PBS, i.e., BH10-PBS2, BH10-PBS4, and BH10-PBS8, that lacked 2, 4, and 8 nt, respectively, were generated. Both unextended primer $tRNA_3^{Lys}$ and the 2-base-extended form of $tRNA_3^{Lys}$ were studied in placement assays with these various recombinant viruses.

MATERIALS AND METHODS

Plasmid construction. A proviral DNA clone of infectious HIV-1, SVC21-BH10, was employed to generate all mutated constructs by PCR (26). To generate the mutated constructs pBH10-RT1, pBH10-RT2, and pBH10-FS, a series of antisense primers containing the expected mutations, pRT1, pRT2, and pFS, respectively, and the sense primer pAPA were employed (Fig. 1). The following convenient restriction sites were included in the primers to facilitate cloning: ATGCAT (*Nsi*I) in pRT1, TGGCCA (*Bal*I) in pRT2, AGATCT (*Bgl*II) in pFS, and GGGCCC (*Apa*I) in pAPA. The construction of pBH10-RT3 and pBH10-PR was carried out with primers pRT3 and pPR (Fig. 1) as previously described with a Chameleon double-stranded site-directed mutagenesis kit (Stratagene Inc., La Jolla, Calif.). To generate nested deletions at the 3′ end of the PBS, sense primers pBS2, pBS4, and pBS8 and antisense primer pST were used (Fig. 1). Restriction sites were also included in primers as follows: GGCGCC (*Nar*I) in pPBS2, pPBS4, and pPBS8 and CTGCAG (*Pst*I) in pST. The presence of the mutations generated was confirmed by sequencing.

Cells, transfections, and virus infections. MT-2 and COS-7 cells were maintained in RPMI 1640 medium and Dulbecco's modified Eagle's medium, respectively, each supplemented with 10% fetal calf serum.

COS-7 cells were transfected with wild-type (wt) HIV-1 (pBH10) or mutated (pBH10-RT1, pBH10-RT2, pBH10-RT3, pBH10-FS, pBH10-PR, pBH10-PBS2, pBH10-PBS4, or pBH10-PBS8) DNA in the presence of calcium phosphate (26). The production of progeny virus was assessed by measuring p24 (capsid [CA]) antigen levels by enzyme-linked immunosorption assay (Abbott Laboratories, Abbott Park, Ill.) or RT activity (15) in culture fluids at 48 h after transfection. To compare the infectivities of the wt virus (pBH10) and mutated viruses (pBH10-PBS2, pBH10-PBS4, and pBH10-PBS8), similar amounts of virus, based on p24 levels or RT activities (approximately 4×10^5 cpm per 10^6 cells), were treated with RNase-free DNase I (Canadian Life Technologies, Inc., Toronto, Ontario, Canada) in the presence of 10 mM $MgCl_2$ to digest any plasmid DNA that might potentially have been transferred from the transfection experiments and then were used to infect MT-2 cells (approximate multiplicity of infection, 1:10) (15, 31, 32). After 2 h, cells were washed twice with serum-free RPMI 1640 medium and maintained in serum-supplemented medium. Culture fluids were collected at various times, depending on the appearance of cytopathology, for determinations of RT activity (see Results).

Western blot. The pelleted virus particles from 10 ml of culture fluid were suspended in 50 μl of lysis buffer. Samples (10 μl) were fractionated on sodium dodecyl sulfate–12% polyacrylamide gels and transferred to nitrocellulose filters. After being blocked with 5% skim milk–0.05% Tween 20–phosphate buffer at 37°C for 2 h, the filters were incubated with anti-HIV p24 and anti-HIV RT immunoglobulin G1 (IgG1) monoclonal antibodies (MAbs) (ID Labs Inc., London, Ontario, Canada) at 37°C for 1 h for the visualization of p24 and RT, respectively. After extensive washing with 0.05% Tween 20–phosphate buffer, secondary sheep anti-mouse IgG-horseradish peroxidase antibody (Amersham Life Science, Oakville, Ontario, Canada) was added for 1 h at 37°C. After

thorough washing, proteins were visualized with an ECL chemiluminescence detection kit (Amersham Life Science, Amersham Place, England).

Viral RNA extraction. Culture fluids (30 ml) of transfected COS-7 cells were clarified at 3,000 rpm for 30 min at 4°C, after which viruses were pelleted through a 20% sucrose cushion at 40,000 rpm for 1 h at 4°C with an SW41 rotor in a Beckman L8-M ultracentrifuge. Virus pellets were suspended in TN buffer (50 mM Tris HCl [pH 7.5], 10 mM NaCl). Viral RNA was extracted by using the ultraspecTM-II RNA isolation system (Biotecs, Houston, Tex.). Relative amounts of viral RNA were analyzed in primer extension experiments, in which 5′-end-labelled antisense primer pAR (Fig. 1) was annealed with viral genomic RNA and extended with avian myeloblastosis virus RT in the presence of deoxynucleoside triphosphates (dNTPs).

In vitro reverse transcription. In vitro reverse transcription reactions were performed with genomic viral RNA that had been extracted from virions and onto which $tRNA_3^{Lys}$ had been placed in vivo. Recombinant HIV-1 RT (p66/51) was prepared as described previously (35). Reactions were performed in a volume of 20 μl containing 50 ng of RT, 50 mM Tris HCl (pH 7.5), 75 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, and 0.1 mM (each) dNTPs and were incubated at 37°C for up to 30 min. To specifically label both unextended and 2-base-extended forms of $tRNA_3^{Lys}$ placed onto viral genomic RNA, only [α-^{32}P]dCTP and [α-^{32}P]dGTP were employed as previously described (9). For the full-length negative-strand strong-stop DNA (ssDNA) product, generated from $tRNA_3^{Lys}$ placed onto viral genomic RNA, all four dNTPs (0.1 mM [each]) were used. The product was detected in a primer extension assay with a 5′-end-labelled sense primer, pS (Fig. 1), that had been annealed with negative-strand ssDNA at 94°C for 3 min and at 55°C for 5 min and extended by avian myeloblastosis virus RT at 37°C for 30 min. Products were fractionated on 5% denaturing acrylamide gels. Experiments dealing with $tRNA_3^{Lys}$ extension were repeated on at least three occasions, with similar results obtained each time. All results were quantified by molecular imaging on a Bio-Rad (Mississauga, Ontario, Canada) molecular imager.

DNA sequencing of the PBS and its flanking region after infection of MT-2 cells by mutated viruses. MT-2 cells infected by mutated viruses (BH10-PBS2, BH10-PBS4, and BH10-PBS8), which had been treated with RNase-free DNase I, were maintained in supplemented RPMI 1640 medium. When about 30% of cells presented with cytopathology, infected MT-2 cells were collected and suspended in lysis buffer containing 0.5% sodium dodecyl sulfate and 1 mg of protease K per ml. After 6 h at 37°C, lysed suspensions were extracted twice with phenol-chloroform (1:1) and precipitated with 2.5 volumes of 95% ethanol. PCR was used to amplify the region containing the PBS with primers pS and pST (Fig. 1). PCR products were digested with *Bgl*II and *Pst*I and cloned into pSP72 vector (Promega, Madison, Wis.). Sequencing reactions were performed with a double-stranded DNA cycle sequencing system (GIBCO BRL, Montreal, Quebec, Canada).

RESULTS

Impairment of placement of $tRNA_3^{Lys}$ onto genomic RNA in the absence of complete Pol protein. To study the role of Pol in the placement of $tRNA_3^{Lys}$, mutations that generated truncated forms of Pol protein were introduced into the *pol* gene (Fig. 2A). BH10-RT1 contained a deletion of only 2 nt (2916-GG-2917) that resulted in a UGA stop codon at positions 2918 to 2920; this yielded a truncated RT protein containing only amino acids 1 to 111 (Fig. 2A). BH10-RT2 was constructed by introducing two adjacent stop codons 2646-UAG-2648 and 2649-UAA-2651 to yield an RT protein containing only 21 amino acids (Fig. 2A). BH10-FS contained mutations at the frameshift site, i.e., from 2082-TTTTTT-2087 to 2082-CTT CCT-2087; this prevented frameshifting during the translation of Gag protein to generate viruses that contained only *gag*, not *gag-pol* (8, 11, 36). These nucleotide substitutions did not alter amino acid sequences (Fig. 2A).

The mutated viral DNAs studied, BH10-RT1, BH10-RT2, and BH10-FS, and wt BH10 were transfected into COS-7 cells. Virus particles were collected from culture fluids after 48 h, and Western blotting was performed. The results in Fig. 2B show that viral pr55, p40, and p24 were present in mutated viruses BH10-RT1 and BH10-RT2 and in wt BH10. Consistent with previous observations (24), more pr55 and p40, relative to the amount of p24, were observed in mutated viruses, showing that they possessed protease activity but their processing of Gag proteins was less efficient than that in wt BH10. No band corresponding to CA p24 was observed with BH10-FS, showing that the frameshift mutation resulted in a relative absence of protease. The presence of relevant mutations was also confirmed on Western blots with anti-RT MAb (Fig. 2C) by the absence of RT proteins in mutated viruses (BH10-RT1, BH10-RT2, and BH10-FS). To further verify the presence of these mutations, culture fluids of transfected COS-7 cells were tested for RT activity. The results in Fig. 2D show that no RT activity was associated with mutated viruses BH10-RT1, BH10-RT2, and BH10-FS.

To analyze the amount of $tRNA_3^{Lys}$ placed onto viral RNA, cell-free RT reactions were performed with recombinant HIV-1 RT enzyme and viral RNA that had been extracted from virus particles produced by transfected COS-7 cells. The amount of cDNA product was analyzed by primer extension and directly quantified to reveal the relative extent of placement of $tRNA_3^{Lys}$ onto viral RNA. The quantity of viral RNA used in reverse transcription assays was determined by primer extension assay (Fig. 2E); no significant differences were observed between mutated and wt viruses in regard to the amount of viral RNA. However, an analysis of cDNA extended from primer $tRNA_3^{Lys}$ that had been placed onto either mutated or wt viral RNA revealed much less product for mutated viruses BH10-RT1, BH10-RT2, and BH10-FS than for wt BH10 (Fig. 2F). This suggests that less $tRNA_3^{Lys}$ was annealed onto the PBS in mutated viruses.

It has previously been shown that the $tRNA_3^{Lys}$ placed onto viral genomic RNA in HIV-1 virions is present in two predominant forms, unextended and 2-base extended (9). To further quantify the placement of $tRNA_3^{Lys}$ onto viral RNA, the two major forms of $tRNA_3^{Lys}$ were labelled with [α-^{32}P]dCTP and [α-^{32}P]dGTP in cell-free reverse transcription reactions as previously described (9). The results in Fig. 2G show that in wt BH10, two major bands, corresponding to the unextended form that has a single C added during the labelling reaction (tRNA-C) and the 2-base-extended form that has an additional G added (tRNA-CTG), were present. In contrast, only one weak band, corresponding to unextended $tRNA_3^{Lys}$, was seen with mutated viruses BH10-RT1, BH10-RT2, and BH10-FS. This result was probably due to the loss of RT activity in these mutated viruses and further supports the notion that less $tRNA_3^{Lys}$ was placed onto viral RNA in mutated viruses.

Nonessentiality of RT polymerase activity for placement of $tRNA_3^{Lys}$. As shown above, mutated viruses BH10-RT1, BH10-RT2, and BH10-FS did not possess RT activity and contained only unextended $tRNA_3^{Lys}$ placed onto viral RNA. We wished to examine whether complexes between primer $tRNA_3^{Lys}$ and viral genomic RNA are less stable in the absence of the 2-base extension and therefore decrease levels of $tRNA_3^{Lys}$ placement. To pursue this possibility, we generated a construct, BH10-RT3, that contained only one amino acid substitution D185H (3135-GAT-3137→3135-CAC-3137), at the active site of RT to eliminate polymerase activity (13). In this scenario, the $tRNA_3^{Lys}$ placed onto viral genomic RNA could not be extended. Western blotting with anti-p24 MAb and purified viruses confirmed that mutated virus BH10-RT3 contained the same protein bands (pr 55, p40, and p24) as did wt virus (Fig. 3A). Furthermore, Western blotting with anti-HIV RT MAb showed that RT protein was present in mutated virus BH10-RT3 (Fig. 3B). However, no RT polymerase activity was present in the BH10-RT3 sample, i.e., the RT generated by the mutant virus was inactive (Fig. 3C). The results in Fig. 3D show that $tRNA_3^{Lys}$ that was placed onto viral genomic RNA in mutated BH10-RT3 existed only in unextended form due to an absence of RT polymerase activity. wt BH10 virus and mutated virus BH10-RT3 contained similar levels of viral RNA, as analyzed by primer extension assays (Fig. 3E). Moreover, cell-free reverse transcription reactions with isolated viral RNA

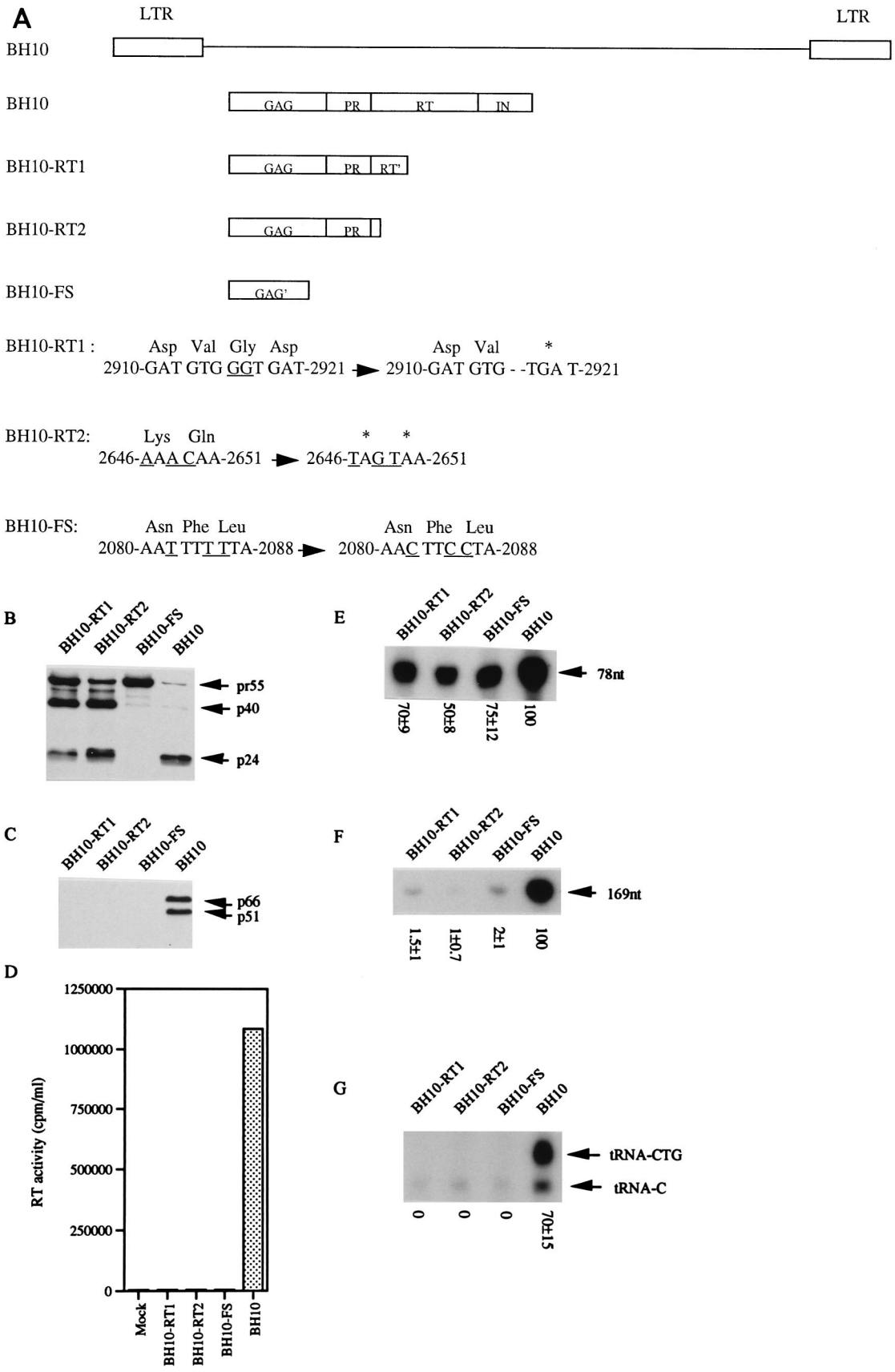

A
LTR
LTR
BH10
BH10
GAG
PR
RT
IN
BH10-RT1
GAG
PR
RT'
BH10-RT2
GAG
PR
BH10-FS
GAG'
BH10-RT1 : Asp Val Gly Asp Asp Val *
2910-GAT GTG GGT GAT-2921 → 2910-GAT GTG - -TGA T-2921
BH10-RT2: Lys Gln * *
2646-AAA CAA-2651 → 2646-TAG TAA-2651
BH10-FS: Asn Phe Leu Asn Phe Leu
2080-AAT TTT TTA-2088 → 2080-AAC TTC CTA-2088
B
BH10-RT1
BH10-RT2
BH10-FS
BH10
pr55
p40
p24
C
BH10-RT1
BH10-RT2
BH10-FS
BH10
p66
p51
D
RT activity (cpm/ml)
1250000
1000000
750000
500000
250000
0
Mock
BH10-RT1
BH10-RT2
BH10-FS
BH10
E
BH10-RT1
BH10-RT2
BH10-FS
BH10
78nt
70±9
50±8
75±12
100
F
BH10-RT1
BH10-RT2
BH10-FS
BH10
169nt
1.5±1
1±0.7
2±1
100
G
BH10-RT1
BH10-RT2
BH10-FS
BH10
tRNA-CTG
tRNA-C
0
0
0
70±15

FIG. 2. Truncated Gag-Pol proteins result in decreased placement of primer $tRNA_3^{Lys}$ onto HIV-1 genomic RNA. (A) Schematic representation of mutations to generate truncated Gag-Pol proteins. BH10-RT1 represents a deletion of nucleotides 2916-GG-2917 to yield a stop codon at positions 2918 to 2920; BH10-RT2 contains substitutional mutations to yield two adjacent stop codons at positions 2646 to 2651; BH10-FS contains mutations that prevent frameshifting. Mutated nucleotides are underlined. Stop codons are represented by asterisks. LTR, long terminal repeat; IN, integrase; PR, protease. (B) Viral protein analysis by Western blotting. Virus particles harvested from culture fluids of COS-7 cells at 48 h after transfection were lysed and subjected to Western blotting with anti-HIV p24 CA IgG1 monoclonal antibody. The sizes (in kilodaltons) of HIV proteins are indicated on the right. (C) Western blot analysis as described for panel B with anti-HIV RT IgG1 MAb. The positions of the two RT peptides, p66 and p51, are indicated on the right. (D) RT assay of mutated and wt viruses. Samples were collected from culture fluids of transfected COS-7 cells. (E) Quantitation of viral RNA template by primer extension assay. Viral RNA was extracted from purified viral particles, and primer pAR was employed in primer extension reactions. The size of the product (78 nt) is shown on the right. The relative amounts of products were quantitated by molecular imaging with a Bio-Rad molecular imager and are shown below each lane, with the wt level set at 100. Each experiment was performed three times, and the standard deviations (SD) obtained are noted below each gel in panels E through G. (F) Negative-strand ssDNA synthesis from primer $tRNA_3^{Lys}$ that was placed onto the viral RNA template. Negative-strand ssDNA was analyzed by primer extension experiments and quantified by molecular imaging as described for panel E. The relative amounts of products are shown below each lane, with the wt level set at 100. (G) In vitro labelling of the unextended and 2-base-extended forms of primer $tRNA_3^{Lys}$ placed onto viral RNA templates. This experiment was performed three times, and the mean ± SD of extended $tRNA_3^{Lys}$ for wt BH10 was 70% ± 15%. For each of the mutated viruses, no extension from $tRNA_3^{Lys}$ was observed.

generated similar levels of cDNA products when RNAs from wt virus and mutated virus BH10-RT3 were employed as templates (Fig. 3F). Therefore, the decreased placement of $tRNA_3^{Lys}$ onto the PBS in these studies with mutated viruses, BH10-RT1, BH10-RT2, and BH10-FS, is not attributable to either the presence or absence of RT polymerase activity in viral p66/51 protein.

Importance of protease in extension and placement of $tRNA_3^{Lys}$. HIV undergoes a remarkable rearrangement during maturation, at which time the Gag and Gag-Pol polyproteins are processed by the viral protease. To evaluate the extent to which the placement of primer $tRNA_3^{Lys}$ is dependent on such maturation, viral protease activity was abolished by the introduction of a D25A point mutation, [2358-GAT(25Asp)-2360→2358-GCA(25Ala)-2360] at the protease-active site to generate construct BH10-PR (14). Western blot analysis of viruses purified from culture fluids of transfected COS-7 cells showed that BH10-PR contained only pr55 and pr160, indicating that the Gag and the Gag-Pol polyproteins had not been processed (Fig. 4A). The results in Fig. 4B show that mutated virus

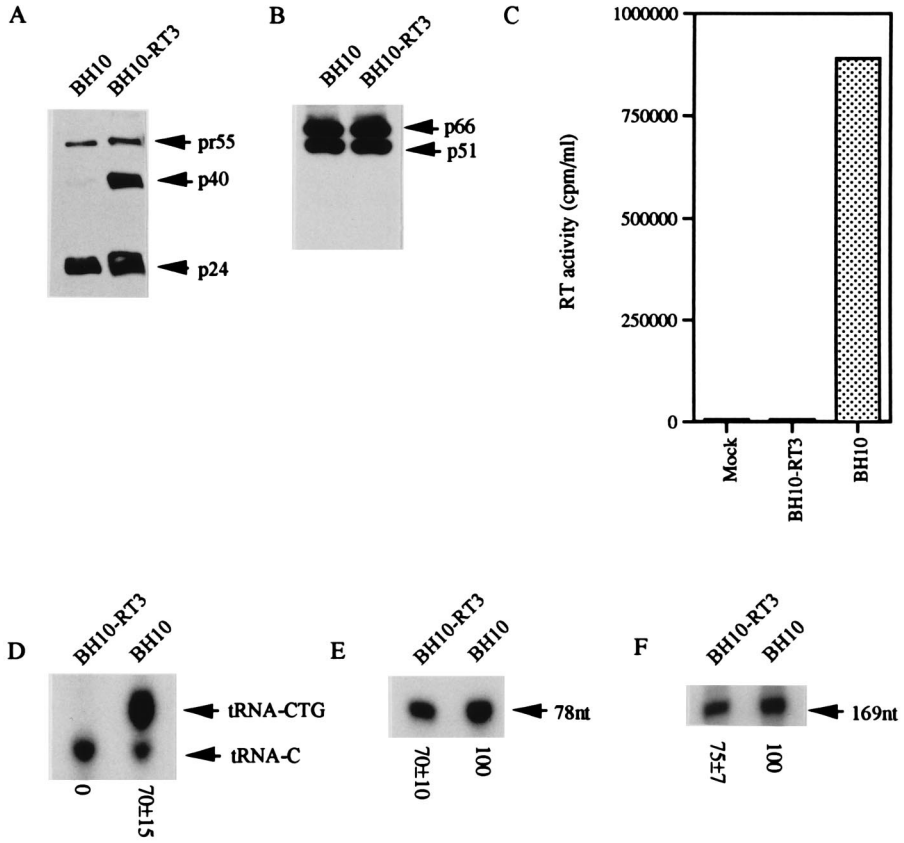


FIG. 3. Loss of RT polymerase activity does not affect the placement of primer $tRNA_3^{Lys}$. (A) Viral protein analysis of mutated and wt viruses by Western blotting with MAbs against HIV CA p24. (B) Viral protein analysis of mutated and wt viruses with MAbs to HIV RT. (C) RT assays of mutated (BH10-RT3) and wt (BH10) viruses. Samples were collected from culture fluids of transfected COS-7 cells. (D) In vitro labelling of the unextended (tRNA-C) and 2-base-extended forms (tRNA-CTG) of primer $tRNA_3^{Lys}$ placed onto viral templates. Each experiment was performed three times, and the SD are noted below each gel in panels D through F. The mean ± SD of extended $tRNA_3^{Lys}$ for wt BH10 was 70% ± 15%. No extension was observed for the mutated virus. (E) Detection of viral RNA template by primer extension assay. The relative amounts of products are indicated below each lane, with the wt level set at 100. (F) Analysis of negative-strand ssDNA products synthesized from primer $tRNA_3^{Lys}$ placed onto viral RNA templates. The relative amounts of products are shown below each lane, with the wt level set at 100.

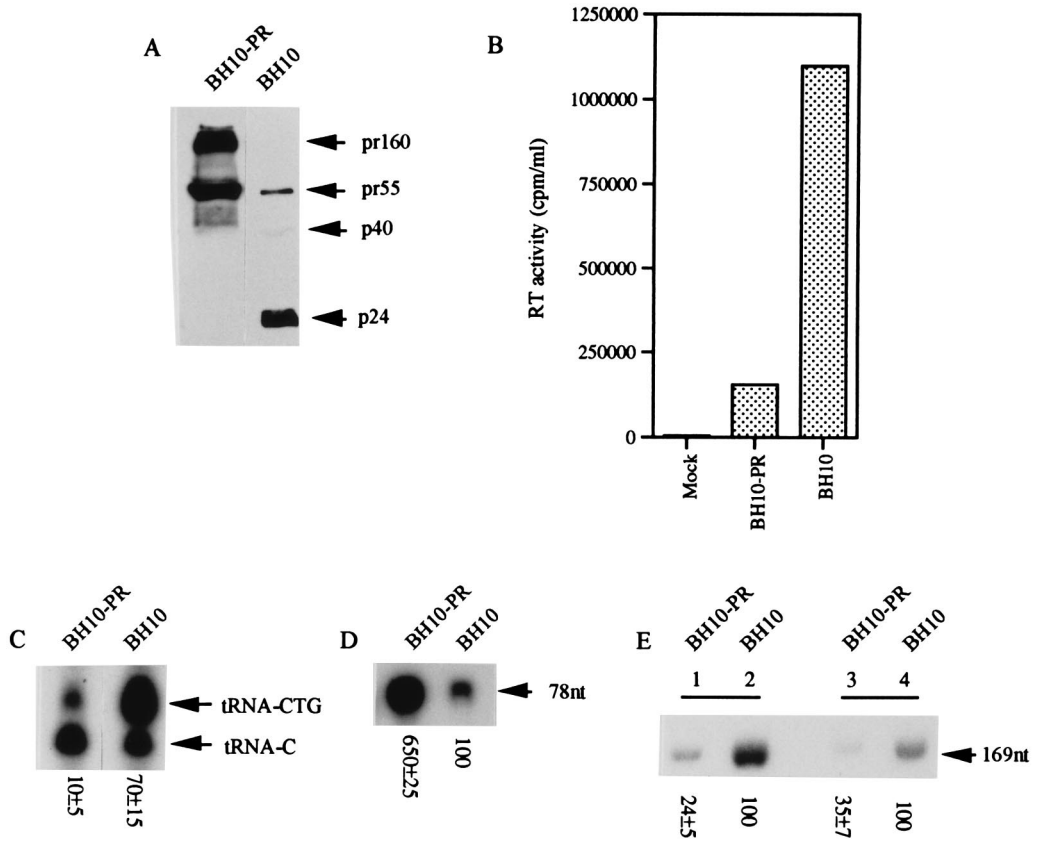


FIG. 4. Effects of a point mutation at the protease-active site on the placement of primer $tRNA_3^{Lys}$. (A) Western blot of viral proteins in purified viral particles with MAbs against HIV CA p24. (B) RT activities of mutated (BH10-PR) and wild-type (BH10) viruses. Samples were collected from culture fluids of transfected COS-7 cells. (C) In vitro labelling of the unextended and 2-base-extended forms of primer $tRNA_3^{Lys}$ placed onto viral RNA templates. Each experiment was performed three times, and the SD are noted below each gel in panels C through E. The means ± SD of extended $tRNA_3^{Lys}$ were 10% ± 5% and 70% ± 15% for mutated virus BH10-PR and wt virus (BH10), respectively. (D) Analysis of viral RNA templates extracted from purified viral particles by primer extension experiments. (E) Analysis of the negative-strand ssDNA products synthesized from primer $tRNA_3^{Lys}$ with the same amounts of viral RNA template as targets of $tRNA_3^{Lys}$ placement. The reaction mixtures in lanes 1 and 2 included viral RNA corresponding to viruses containing 300 ng of p24, whereas the reaction mixtures in lanes 3 and 4 included RNA from viruses containing 100 ng of p24. The relative amounts of products are shown below each lane, with the wt level set at 100.

BH10-PR contained only 15% of the level of RT activity possessed by wt BH10 virus.

We next isolated viral RNA from purified virus particles obtained from transfection of COS-7 cells and labelled both the unextended and 2-base-extended forms of $tRNA_3^{Lys}$ that had been placed onto the viral RNA template in cell-free reverse transcription reactions. The results in Fig. 4C show that both BH10-PR and BH10 yielded two bands, corresponding to the two forms of $tRNA_3^{Lys}$. However, in mutated virus BH10-PR, most of the $tRNA_3^{Lys}$ was present in unextended form, whereas most of the $tRNA_3^{Lys}$ in wt virus was present in extended form. To more clearly observe the 2-base-extended form of $tRNA_3^{Lys}$ in BH10-PR, more viral RNA template (Fig. 4D) was employed in the same type of unextended-extended reverse transcription reaction whose results are shown in Fig. 4C. The results in Fig. 4E show that lower levels of cDNA products were generated by BH10-PR than by BH10 when the same amounts of viral RNA template were used. Thus, less $tRNA_3^{Lys}$ was placed in mutated virus BH10-PR than in wt BH10 virus for the amount of viral genomic RNA.

Importance of the 3′ end of the PBS in the placement of $tRNA_3^{Lys}$. Deletions of 2, 4, and 8 nt at the 3′ terminus of the PBS were enacted to generate mutated viruses BH10-PBS2, BH10-PBS4, and BH10-PBS8, respectively (Fig. 5A). Western blots with anti-CA p24 MAb showed that these mutated viruses exhibited the same protein profile (pr 55, p40, and p24) as did wt BH10 (Fig. 5B); therefore, the mutations did not affect the production of viral proteins.

Viral RNA was isolated from purified virus particles and used in cell-free reverse transcription reactions that included only [α-^{32}P]dCTP and [α-^{32}P]dGTP to label both the unextended and 2-base-extended forms of $tRNA_3^{Lys}$. The results in Fig. 5C show that both of these species were present in mutated viruses BH10-PBS2 and BH10-PBS4 and in wt BH10 and that the intensities of these bands diminished as more nucleotides were deleted. In BH10-PBS8, only one weak band, corresponding to the unextended form of $tRNA_3^{Lys}$, was observed. Primer extension experiments revealed that similar levels of viral RNA template were present in all of these reactions (Fig. 5D). Furthermore, in vitro reverse transcription experiments with viral RNA as the template in the presence of dNTPs revealed less cDNA product as more nucleotides were deleted (Fig. 5E). Thus, deletions of nucleotides from the 3′ end of the PBS resulted in less efficient placement of $tRNA_3^{Lys}$ but did not appear to affect primer extension.

Replication capacities of viruses with deletions at the 3′ end of the PBS. Consistent with the results discussed above, Fig. 5F shows that mutated viruses BH10-PBS2, BH10-PBS4, and BH10-PBS8 replicated less efficiently than did wt virus BH10 over 8 days in MT-2 cells. The extent of replication incapacity

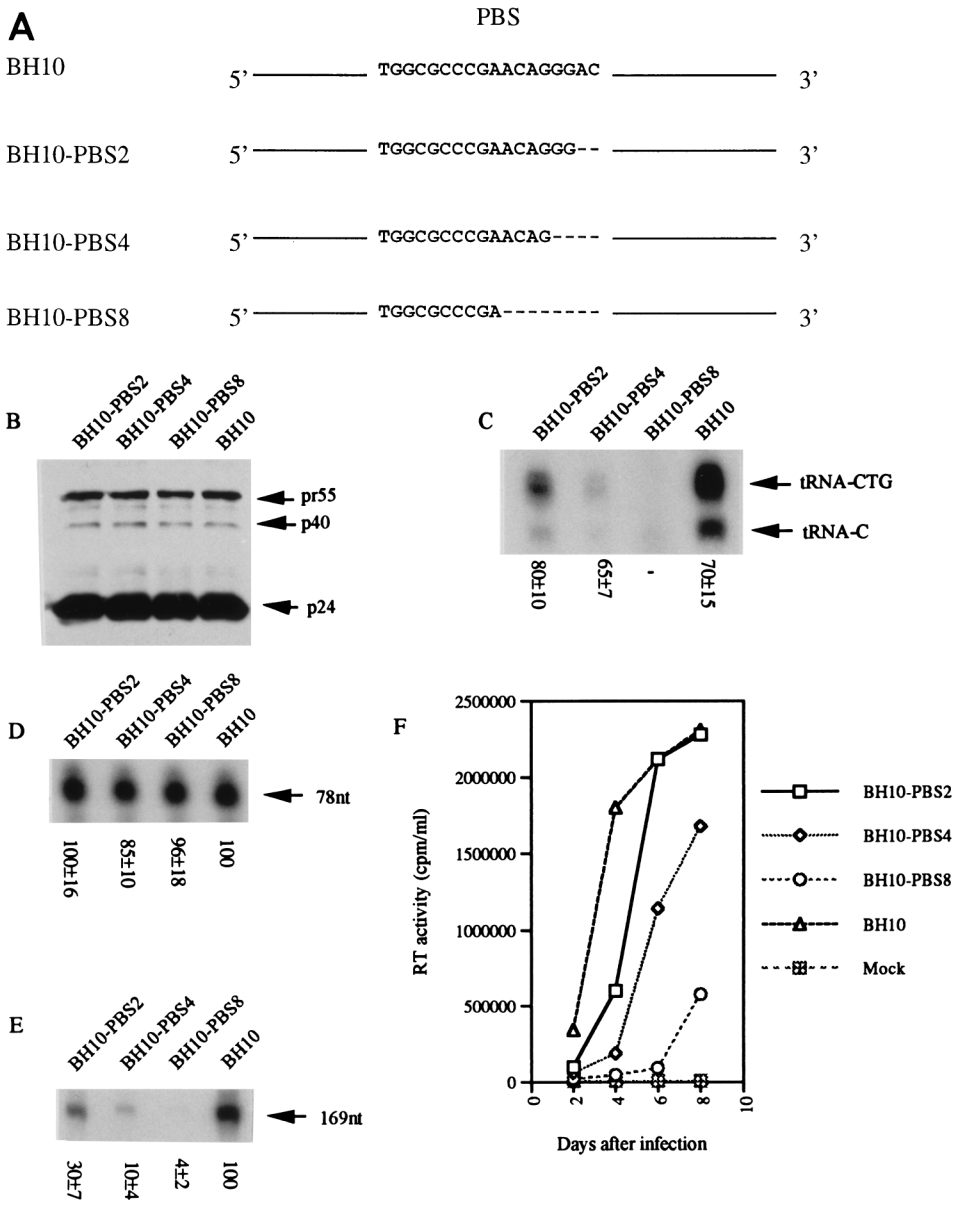


FIG. 5. Effects of deletions at the 3′ end of the PBS on the placement of $tRNA_3^{Lys}$. (A) Schematic depictions of deletions (dashes) at the 3′ end of the PBS. BH10-PBS2, BH10-PBS4, and BH10-PBS8 contain deletions of 2, 4, and 8 nt, respectively. (B) Western blot analysis of wt virus and viruses mutated in the PBS with MAbs to CA p24. (C) In vitro labelling of the unextended and 2-base-extended forms of primer $tRNA_3^{Lys}$ placed onto mutated and wt viral templates. The mean ± SD of extended $tRNA_3^{Lys}$ for each virus is shown below the gel. (D) Analysis of viral RNA templates by primer extension experiments. Experiments were performed three times, and the SD are indicated below each gel in panels D and E. The relative amounts of products are shown below each lane, with the wt level set at 100. (E) Analysis of negative-strand ssDNA products synthesized from primer $tRNA_3^{Lys}$ placed onto viral RNA templates. The relative amounts of products are shown below each lane, with the wt level set at 100. (F) Viral replication capacities of mutated and wt viruses. MT-2 cells were infected with the same amounts of mutated and wt virus based on RT activity (4×10^5 cpm/10^6 cells). The production of viral progeny was monitored by RT assay of culture fluids. Mock, infection with heat-inactivated viruses.

seemed to correlate with the number of nucleotides deleted and was especially pronounced with the BH10-PBS8 virus. The diminished replication abilities of these mutated viruses could have resulted from the lower efficiency of $tRNA_3^{Lys}$ placement onto viral genomic RNA; however, another possibility is that deletions in the 3′ end of the PBS resulted in decreased efficiency of extension of positive-strand ssDNA after the second template switch.

By 8 days after infection, high RT activities were observed with mutated viruses BH10-PBS2 and BH10-PBS4; the BH10-PBS8 virus also showed signs of restored replicative capacity. To analyze whether the PBS and sequences that flank the PBS might have been changed in these mutated viruses, cellular DNA was extracted from infected MT-2 cells and the PBS and its flanking region were amplified by PCR and cloned into vector pSP72. The sequencing results show that 5 of 12 clones of mutated virus BH10-PBS2 possessed wt PBS sequences after 8 days but had a deletion of 2 nt just downstream of the PBS (Table 1). In 2 of 12 clones, the BH10-PBS4 virus likewise possessed a wt PBS along with a 4-nt deletion downstream of the PBS. Finally, 1 of 11 clones of BH10-PBS8 possessed a wt PBS but had lost 18 nt just downstream of the PBS.

TABLE 1. Sequencing results of mutated viruses (BH10-PBS2, BH10-PBS4, and BH10-PBS8) after each of two rounds of infection

Construct	Passage no.[a]	Sequence[b]	No. of times mutated sequence observed/no. of clones sequenced
BH10		TGGCGCCCGAACAGGGACTTGAAAGCGAAAGGGAAACCAGA	
PBS2	0	TGGCGCCCGAACAGGG--TTGAAAGCGAAAGGGAAACCAGA	
	1	TGGCGCCCGAACAGGG--TTGAAAGCGAAAGGGAAACCAGA	7/12
	1	TGGCGCCCGAACAGGGAC--GAAAGCGAAAGGGAAACCAGA	5/12
	2	TGGCGCCCGAACAGGGAC--GAAAGCGAAAGGGAAACCAGA	8/8
PBS4	0	TGGCGCCCGAACAG----TTGAAAGCGAAAGGGAAACCAGA	
	1	TGGCGCCCGAACAG----TTGAAAGCGAAAGGGAAACCAGA	10/12
	1	TGGCGCCCGAACAGGGAC----AAGCGAAAGGGAAACCAGA	2/12
	2	TGGCGCCCGAACAGGGAC----AAGCGAAAGGGAAACCAGA	7/7
PBS8	0	TGGCGCCCGA-------TTGAAAGCGAAAGGGAAACCAGA	
	1	TGGCGCCCGA-------TTGAAAGCGAAAGGGAAACCAGA	10/11
	1	TGGCGCCCGAACAGGGAC----------CCAGA	1/11
	2	TGGCGCCCGAACAGGGAC----------ACCAGA	1/6
	2	TGGCGCCCGAACAGGGAC----------CCAGA	3/6
	2	TGGCGCCCGAACAGGGAC----------CAGA	2/6

[a] 0, original sequence.
[b] Dashes indicate deletions.

To further verify that these reversions had occurred, culture fluids were collected from infected MT-2 cells at 8 days and another round of infection was initiated. This time, all of the viruses replicated well (Fig. 6). Cellular DNA was extracted from infected MT-2 cells, and sequencing was performed. Table 1 shows that eight of eight clones of BH10-PBS2 virus possessed a wt PBS and a 2-nt deletion just downstream of this region. The BH10-PBS4 virus had a wt PBS and a 4-nt deletion just downstream of the PBS in each of seven clones. Finally, BH10-PBS8 possessed a wt PBS in six of six instances along with a deletion of 17, 18, or 19 nt just downstream of the PBS.

DISCUSSION

The placement of primer tRNA onto the PBS is essential for viral reverse transcription to efficiently occur. Our studies show that the presence of an intact Pol protein is important in this process and that the RT portion of Pol is especially significant. However, the placement of primer $tRNA_3^{Lys}$ was found to be independent of RT polymerase activity but dependent on protease. A deletion of 8 nt at the 3′ end of the PBS also caused severe impairment of $tRNA_3^{Lys}$ placement.

The placement of primer tRNA is facilitated by viral proteins, especially RT. HIV-1 RT possesses a higher affinity for its cognate $tRNA_3^{Lys}$ primer than for other tRNAs and is specifically able to bind to the anticodon loop of $tRNA_3^{Lys}$ (1, 2, 17, 21, 22, 25, 27, 30, 33, 35, 37, 38). Others have previously studied the role of RT in the placement of $tRNA_3^{Lys}$ by means of a large deletion (~2 kb) that included both RT and integrase (22). However, large deletions may affect both the stability and secondary structure of genomic RNA and hence alter placement. Therefore, we introduced mutations into RT to generate stop codons that yielded truncated RT proteins; however, these changes did not result in marked changes in viral genomic RNA. Our studies revealed that a truncation of RT protein caused a significant decrease in the placement of primer $tRNA_3^{Lys}$, whereas the elimination of enzymatic function through a mutation at the active site did not affect this process. Therefore, placement of $tRNA_3^{Lys}$ is presumably de-

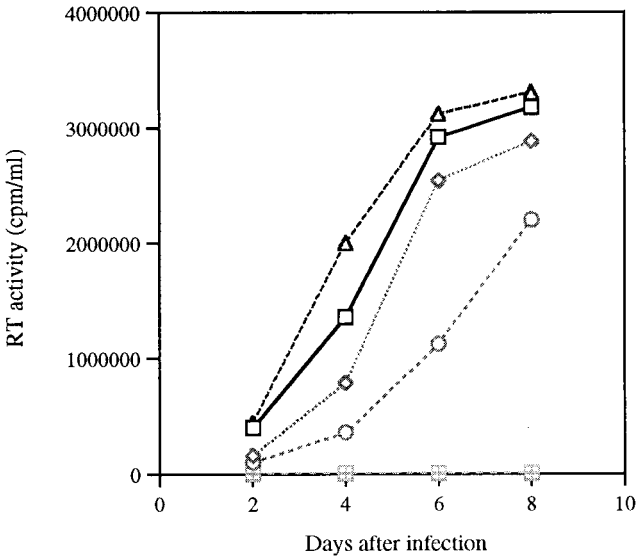


FIG. 6. Viral replication capacities of mutated (BH10-PBS2, BH10-PBS4, and BH10-PBS8) and wt (BH10) viruses in a second round of infection. Culture fluids were collected from infected MT-2 cells and used to initiate a second round of infection with a quantity of virus equivalent to an RT activity of 4×10^5 cpm/10^6 cells. Mock, infection with heat-inactivated viruses. The symbols are the same as those used in Fig. 5F.

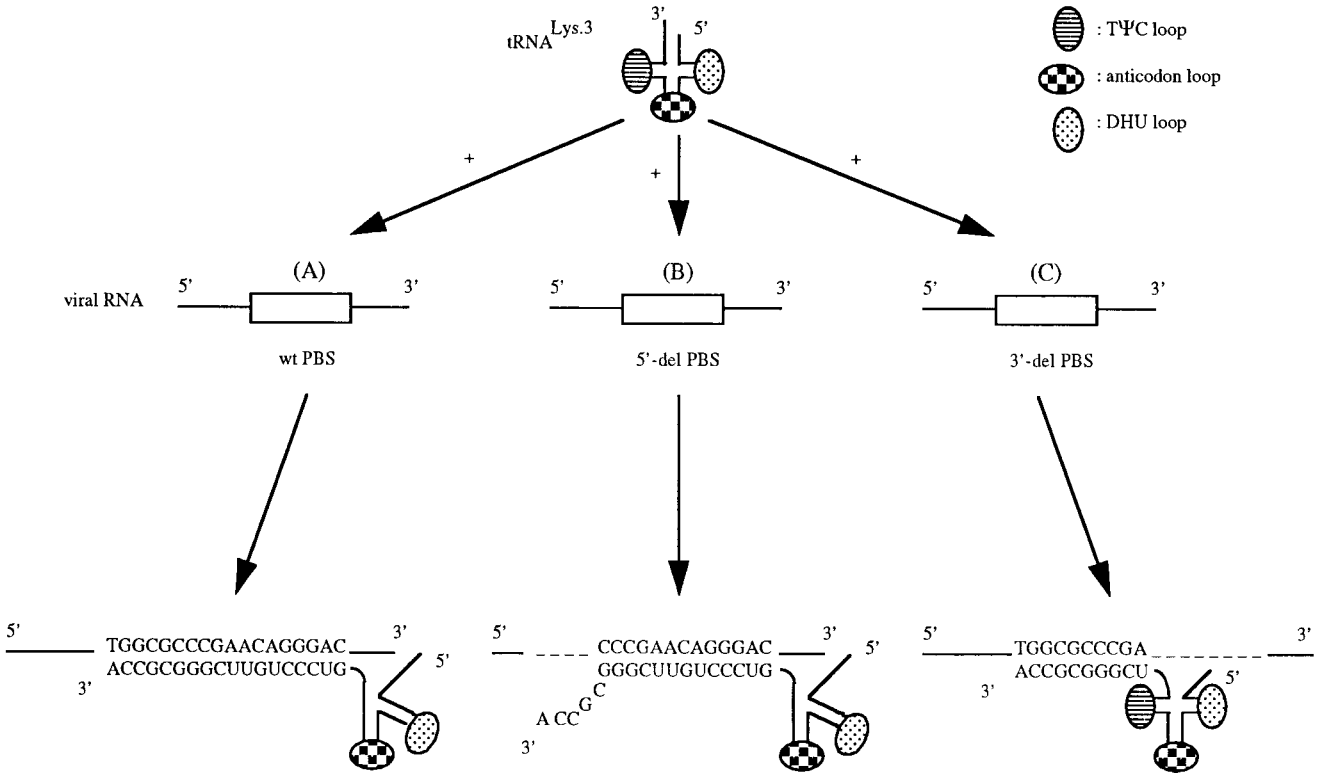


FIG. 7. Roles of the 5′ and 3′ ends of the PBS in the stability of the viral RNA-$tRNA_3^{Lys}$ complex. (A) $tRNA_3^{Lys}$ normally possesses a stable secondary structure, which is disrupted during interaction with viral genomic RNA to form a stable complex. This results from the annealing of 18 nt at the 3′ end of $tRNA_3^{Lys}$ with the wt PBS and causes destabilization of both the acceptor stem and the TΨC stem. (B) Deletion of the 5′ end nucleotides of the PBS (5′-del PBS) results in disruption of the acceptor and TΨC stems of annealed $tRNA_3^{Lys}$, even though several nucleotides at the 3′ terminus of $tRNA_3^{Lys}$ cannot be paired. (C) Deletion of 8 nt at the 3′ end of the PBS (3′-del PBS) permits the TΨC stem to be formed, since the 5′-GUCCCUGU-3′ stretch in $tRNA_3^{Lys}$ cannot bind to complementary sequences.

pendent on structural aspects of RT but independent of enzymatic function. In contrast, extension is clearly dependent on RT catalytic activity. However, a caveat must be introduced. Notably, the presence of stop codon mutations in mutated viruses BH10-RT1 and BH10-RT2 resulted in a loss of integrase as well as RT protein. While the loss of RT was most likely responsible for the decreased placement of $tRNA_3^{Lys}$, we cannot exclude a role for integrase protein in this regard.

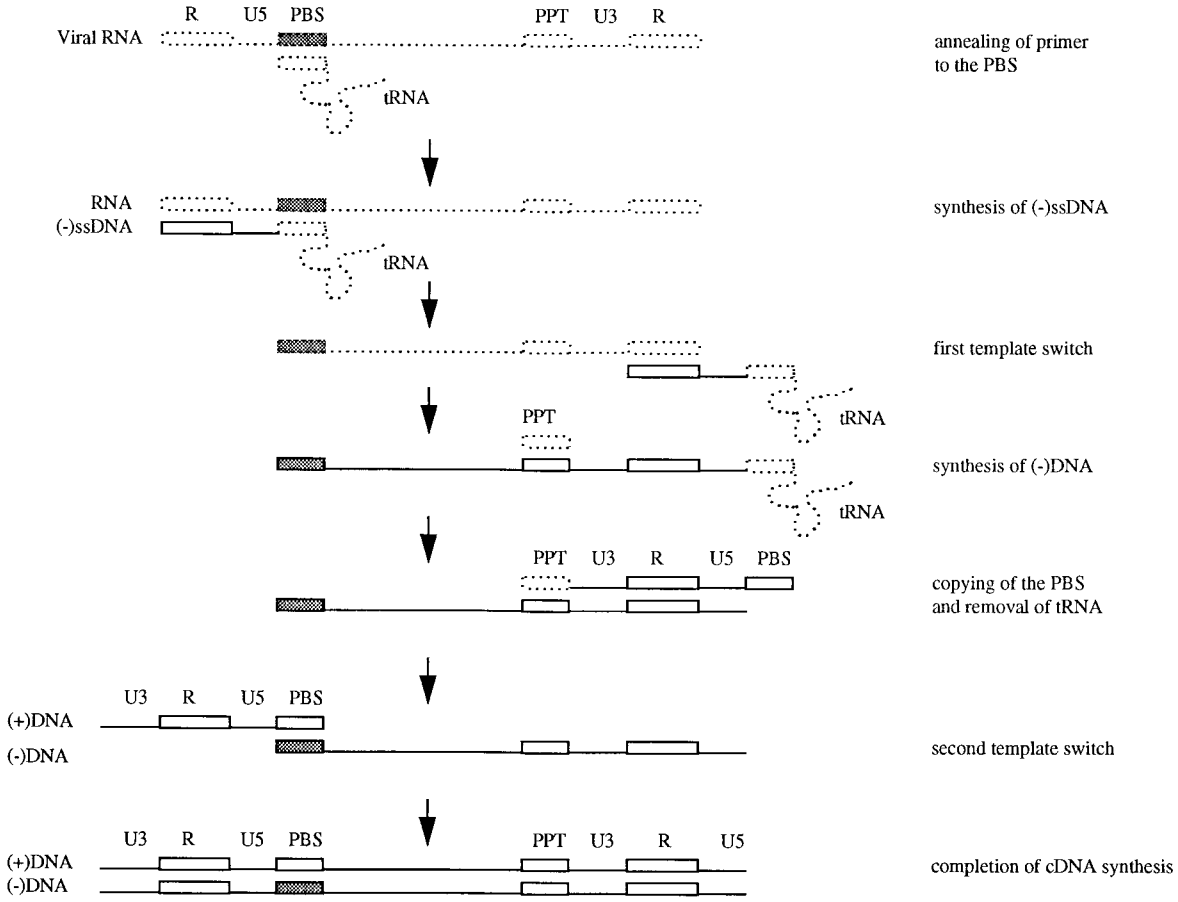
Experiments with in vitro-synthesized viral RNA template and recombinant RT showed that RT itself did not possess the ability to place $tRNA_3^{Lys}$ onto the viral RNA template (16). Hence, RT may function indirectly in this process. Mutations in the *pol* gene, especially within the RT region, resulted in diminished selective packaging of $tRNA_3^{Lys}$ into virions (12, 18, 19). The present study shows that deletions in RT compromise the placement of $tRNA_3^{Lys}$. Therefore, a correlation between selective packaging and efficient placement of primer $tRNA_3^{Lys}$ may exist, i.e., RT may indirectly influence placement by modulating the selective packaging of $tRNA_3^{Lys}$.

Virus particles lacking protease contained unprocessed Gag and Gag-Pol proteins. Other workers have previously shown that primer tRNA can still be placed onto viral RNA under these conditions (28). Similar results were obtained in the present study with mutated BH10-PR virus; however, we also found that the amount of $tRNA_3^{Lys}$ placed onto viral genomic RNA was reduced in mutated BH10-PR virus compared with that in wt virus. This difference cannot be due to defects in the packaging of primer $tRNA_3^{Lys}$, since normal packaging has in fact been reported for protease-negative viruses (18). A more likely explanation is that viral NCp may not have been available for placement in viruses containing only unprocessed Gag and Gag-Pol proteins. Viral NCp7 has previously been shown to play an important role in the placement of $tRNA_3^{Lys}$ (5) and may have been able to participate in this process as part of Gag or Gag-Pol but with greatly reduced efficiency.

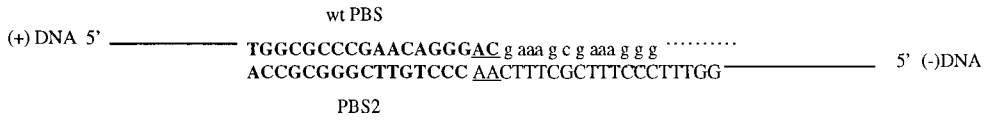
Previous studies have also been performed in regard to minimal sequences in the PBS required for efficient reverse transcription (31). Deletions or insertions of either 2 or 4 nt at the 5′ end of the PBS did not cause a significant decrease in the placement of $tRNA_3^{Lys}$ but had a more severe impact on the initiation of reverse transcription (6). In our study, nested deletions of 4 and 8 nt at the 3′ end of the PBS caused 90 and 96% decreases, respectively, in the placement of $tRNA_3^{Lys}$. These findings cannot be attributed to initiation effects in regard to the mutated PBS, since only 6 nt at the 5′ end of the PBS are required for this process (31). It is also unlikely that $tRNA_3^{Lys}$ molecules were lost during the process of viral purification, since wt and certain viruses mutated in RT maintained these elements under similar conditions. The results presented here also show that deletions at the 3′ end of the PBS had no effect on the production of viral proteins. However, we cannot rule out the possibility that these PBS mutations also affected the efficiency of the synthesis of viral positive-strand ssDNA after the second template switch.

An understanding of the tertiary structure of $tRNA_3^{Lys}$ can help to explain the different roles played by the 5′ and 3′ ends of the PBS in the placement of $tRNA_3^{Lys}$ (Fig. 7). The 4 nt (5′-GCCA-3′) at the 3′ end of $tRNA_3^{Lys}$ normally exist in a single-stranded state and do not contribute to the stability of the tertiary structure; however, the other 13 nt (5′-UCCCUG

A


R U5 PBS PPT U3 R
Viral RNA
annealing of primer to the PBS
tRNA
RNA
(-)ssDNA
synthesis of (-)ssDNA
tRNA
first template switch
PPT
tRNA
synthesis of (-)DNA
tRNA
PPT U3 R U5 PBS
copying of the PBS and removal of tRNA
U3 R U5 PBS
(+)DNA
(-)DNA
second template switch
U3 R U5 PBS PPT U3 R U5
(+)DNA
(-)DNA
completion of cDNA synthesis

B


wt PBS
(+) DNA 5'
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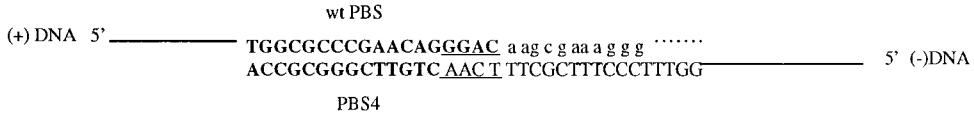
ACCGCGGGCTTGTCCC AACTTTCGCTTTCCCTTTGG
5' (-)DNA
PBS2

C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(+) DNA 5'
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ACCGCGGGCTTGTC AAC T TTCGCTTTCCCTTTGG
5' (-)DNA
PBS4

D

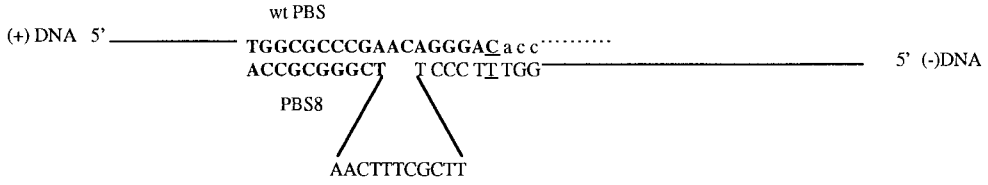
wt PBS
(+) DNA 5'
TGGCGCCCGAACAGGGAC a c c
ACCGCGGGCT T CCC TT TGG
5' (-)DNA
PBS8
AACTTTCGCTT

FIG. 8. Synthesis of positive-strand DNA after the second template switch in mutated viruses BH10-PBS2, BH10-PBS4, and BH10-PBS8. (A) Schematic representation of reverse transcription. Dotted lines represent viral RNA, and thin lines indicate cDNA generated during reverse transcription. The PBS region of the viral genome is shaded to distinguish it from the PBS that is copied from primer tRNA. PPT, polypurine tract; R, repeat sequence. (B) Synthesis of positive-strand DNA after the second template switch in mutated virus BH10-PBS2. PBS sequences are shown in bold letters. Mismatched nucleotides, between the mutated PBS (generated from the viral genome) and the wt PBS (copied from primer tRNA) are underlined. Despite the mismatched sequences, DNA synthesis continues. (C) Synthesis of positive-strand DNA after the second template switch in mutated virus BH10-PBS4. (D) Synthesis of positive-strand DNA after the second template switch in mutated virus BH10-PBS8. The 3′ end sequence (AGGGA) of wt PBS in positive-strand DNA is paired with a stretch of homologous nucleotides (TCCCT) downstream of the mutated PBS in negative-strand DNA and can initiate the synthesis of positive-strand DNA. The sequences between the mutated PBS and TCCCT in negative-strand DNA are looped out and are not copied into positive-strand DNA, resulting in a deletion of approximately 17 nt.

UUCGGGC-3′) at the 3′ end can pair with other nucleotides within $tRNA_3^{Lys}$ to form the acceptor and TΨC stems. The formation of the primer $tRNA_3^{Lys}$-viral RNA complex is favored by RNA hybridization between the wt 18-nt PBS and the 18 nt located at the 3′ end of $tRNA_3^{Lys}$ and by disruption of the tertiary structure of $tRNA_3^{Lys}$ (the acceptor stem and the TΨC stem) (Fig. 7A). When deletions were introduced into the 5′ end of the PBS (Fig. 7B), the acceptor and TΨC stems of $tRNA_3^{Lys}$ were still disrupted because of binding to other nucleotides within the PBS, in spite of the fact that several bases at the 3′ end of $tRNA_3^{Lys}$ could no longer pair well with the mutated PBS. However, when as many as 8 nt were deleted from the 3′ end sequences of the PBS (Fig. 7C), the TΨC stem and part of the acceptor stem of $tRNA_3^{Lys}$ were still formed because of the absence of corresponding PBS sequences. This may favor the generation of $tRNA_3^{Lys}$ tertiary structure and greatly destabilize the placement of $tRNA_3^{Lys}$. This explains why the 3′ terminus of the PBS is more important than is the 5′ end in the placement of $tRNA_3^{Lys}$.

In wt viruses, $tRNA_3^{Lys}$ is placed onto viral RNA in either of two major forms, unextended and 2-base extended (9). Our study clearly demonstrates that the 2-base extension of primer $tRNA_3^{Lys}$ is performed by viral RT. For example, the lower levels of RT activity in the protease-negative virus BH10-PR yielded less of the extended form of $tRNA_3^{Lys}$. Viruses devoid of RT activity due to a point mutation at the polymerase-active site still placed but did not extend $tRNA_3^{Lys}$. The length of the PBS is also not involved in this process, since deletions of either 2 or 4 nt from the 3′ end did not prevent the 2-base-extended form of $tRNA_3^{Lys}$ from being generated. Recent studies have suggested that the initiation of reverse transcription is a distinct process from that of elongation and is a function of the viral RT itself (10). The 2-base extension of primer $tRNA_3^{Lys}$ in virus particles shown here is consistent with such an interpretation.

The regeneration of a wt PBS in cells infected by viruses with substitution mutations in this region has been previously documented. An 18-nt sequence at the 3′ end of primer tRNA was copied and used for the continuation of positive-strand synthesis after the second template switch (7, 15, 32, 34). In such cases, mismatches of nucleotides existed after the second template switch at the PBS region from which positive-strand DNA synthesis would normally continue. However, changes to sequences that flank the PBS were observed only infrequently in those studies; i.e., positive-strand synthesis was initiated from the first nucleotide downstream of the PBS after the second template switch in spite of the mismatches, although with possibly reduced efficiency. Studies have also previously been performed to rescue the mismatches by introducing complementary sequences downstream of the PBS; these changes increased both the efficiency of positive-strand ssDNA synthesis after the second template switch and viral replication capacity (31). In our study of deletions at the 3′ end of the PBS, the restoration of a wt PBS was accompanied by deletions just downstream of this region. This further supports the notion that the newly copied wt PBS requires an 18-nt sequence with which to align and continue the synthesis of positive-strand DNA.

Figure 8 presents a schema to explain the new observations. According to the generally accepted model of reverse transcription, the 3′ end of tRNA acts as a primer for the synthesis of negative-strand DNA and a polypurine tract just upstream of the 3′ long terminal repeat acts as a primer for the synthesis of positive-strand DNA prior to the first strand transfer. After the first template switch, the synthesis of positive-strand DNA continues from the 3′ end of an 18-nt DNA sequence that has been copied from the 3′ end of primer tRNA during the synthesis of positive-strand DNA (i.e., before the first template switch) (Fig. 8A). As is the case with PBS substitution mutations, the copying of an 18-nt stretch during positive-strand synthesis at the 3′ end of primer $tRNA_3^{Lys}$ leads to the use of copied sequences in the second template switch and the alignment of these sequences with the PBS generated from viral genomic RNA during the synthesis of negative-strand DNA (Fig. 8A). The BH10-PBS2 and BH10-PBS4 viruses (containing deletions of 2 and 4 nt at the 3′ end of the PBS, respectively) caused 2 (AC) and 4 (GGAC) nt, respectively, of the copied wt PBS in positive-strand DNA to align with the downstream nucleotides AA and AACT in negative-strand DNA (Fig. 8B and C); this resulted in deletions of two (AA) and 4 (AACT) nt, respectively, in the newly synthesized positive-strand cDNA. Thus, DNA synthesis must have been initiated (although possibly with decreased efficiency), despite the fact that the terminal 2 or 4 nt were mismatched.

In mutated virus BH10-PBS8, the AGGGA sequence at the 3′ end of the copied wt PBS in positive-strand DNA must have been paired with a homologous TCCCT sequence downstream of the mutated PBS in negative-strand DNA after the second template switch. This would have enabled the synthesis of positive-strand DNA to continue (Fig. 8D). Under these circumstances, the AACTTTCGCTT sequence in negative-strand DNA must have been looped out and therefore could not have been copied during the synthesis of positive-strand DNA. This would in turn give rise to a deletion of TCCCT complementary to the AGGGA to which it had initially been paired (Fig. 8D). It can also be inferred from our sequence data that the first nucleotide was incorporated at various positions; hence, deletions of slightly different lengths downstream of the PBS were generated in mutated virus BH10-PBS8.

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REFERENCES

1. **Andreila, M. L., L. Tarrago-Litvak, A. S. Levina, T. I. Kolocheva, R. el Dirani-Diab, V. I. Jamkovoy, N. L. Khalimskaya, P. J. Barr, and G. A.**

Nevinsky. 1993. Affinity labeling and functional analysis of the primer binding domain of HIV-1 reverse transcriptase. Biochemistry **32:**3629–3637.
2. **Arts, E. J., M. Ghosh, P. S. Jacques, B. Ehresmann, and S. F. Le Grice.** 1996. Restoration of tRNALys.3-primed (−)-strand DNA synthesis to an HIV-1 reverse transcriptase mutant with extended tRNAs. Implications for retroviral replication. J. Biol. Chem. **271:**9054–9061.
3. **Baltimore, D.** 1970. Viral RNA dependent DNA polymerase. Nature **226:** 1209–1211.
4. **Barat, C., O. Schatz, S. Le Grice, and J. L. Darlix.** 1993. Analysis of the interactions of HIV-1 replication primer tRNALys.3 with nucleocapsid protein and reverse transcriptase. J. Mol. Biol. **231:**185–190.
5. **Darlix, J. L., M. Lapadat-Tapolsky, H. de Rocquigny, and B. P. Roques.** 1995. First glimpses at structure-function relationships of the nucleocapsid protein of retroviruses. J. Mol. Biol. **254:**523–537.
6. **Das, A. T., and B. Berkhout.** 1995. Efficient extension of a misaligned tRNA primer during replication of the HIV-1 retrovirus. Nucleic Acids Res. **23:** 1319–1326.
7. **Das, A. T., B. Klaver, and B. Berkhout.** 1995. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural $tRNA_3^{Lys}$. J. Virol. **69:**3090–3097.
8. **Gheysen, D., E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde.** 1989. Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. Cell **59:**103–112.
9. **Huang, Y., J. Wang, A. Shalom, Z. Li, A. Khorchid, M. A. Wainberg, and L. Kleiman.** 1997. Primer $tRNA_3^{Lys}$ on the viral genome exists in unextended and two-base extended forms within mature human immunodeficiency virus type 1. J. Virol. **71:**726–728.
10. **Isel, C., J. M. Lanchy, S. F. J. Le Grice, C. Ehresmann, B. Ehresmann, and R. Marquet.** 1996. Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcription require the post-transcriptional modifications of primer tRNALys.3. EMBO J. **15:**917–924.
11. **Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus.** 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression. Nature **331:**280–283.
12. **Jiang, M., J. Mak, Y. Huang, and L. Kleiman.** 1994. Reverse transcriptase is an important protein for the primer tRNA selection in HIV-1. Leukemia **8**(Suppl. 1)**:**S149–S151.
13. **Larder, B. A., D. J. Purifoy, K. L. Powell, and G. Darby.** 1987. Site-specific mutagenesis of AIDS virus reverse transcriptase. Nature **327:**716–717.
14. **Leob, D. D., R. Swanstrom, L. Everitt, M. Manchester, S. E. Stamper, and C. A. Hutchison III.** 1989. Complete mutagenesis of the HIV-1 protease. Nature **340:**397–400.
15. **Li, X. G., J. Mak, E. J. Arts, Z. X. Gu, L. Kleiman, M. A. Wainberg, and M. A. Parniak.** 1994. Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. J. Virol. **68:**6198–6206.
16. **Li, X. G., Y. D. Quan, E. J. Arts, Z. Li, B. D. Preston, H. Rocquigny, B. P. Roques, J. Darlix, L. Kleiman, M. A. Parniak, and M. A. Wainberg.** 1996. Human immunodeficiency virus type 1 nucleocapsid protein (NCp7) directs specific initiation of minus-strand DNA synthesis primed by human $tRNA_3^{Lys}$ in vitro: studies of viral RNA molecules mutated in regions that flank the primer binding site. J. Virol. **70:**4996–5004.
17. **Litvak, S., L. Sarih-Cottin, M. Fournier, M. Andreola, and L. Tarrago-Litvak.** 1994. Priming of HIV replication by tRNALys.3: role of reverse transcriptase. Trends Biochem. Sci. **19:**114–118.
18. **Mak, J., M. Jiang, M. A. Wainberg, M. L. Hammarskjold, D. Rekosh, and L. Kleiman.** 1995. Role of Pr160gag-pol in mediating the selective incorporation of $tRNA_3^{Lys}$ into human immunodeficiency virus type 1 particles. J. Virol. **68:**2065–2072.
19. **Mak, J., A. Khorchid, Q. Cao, Y. Huang, I. Lowy, M. A. Parniak, V. R. Prasad, M. A. Wainberg, and L. Kleiman.** 1997. Effects of mutations in Pr160gag-pol upon tRNALys.3 and Pr160gag-pol incorporation into HIV-1. J. Mol. Biol. **265:**419–431.
20. **Marquet, R., C. Isel, C. Ehresmann, and B. Ehresmann.** 1995. TRNAs as primer of reverse transcriptases. Biochimie **77:**113–124.
21. **Mishima, Y., and J. A. Steitz.** 1995. Site-specific crosslinking of 4-thiouridine-modified human tRNALys.3 to reverse transcriptase from human immunodeficiency virus type 1. EMBO J. **14:**2679–2687.
22. **Oude Essink, B. B., A. T. Das, and B. Berkhout.** 1995. Structural requirements for the binding of tRNALys.3 to reverse transcriptase of the human immunodeficiency virus type 1. J. Biol. Chem. **270:**23867–23874.
23. **Prats, A. C., L. Sarih, C. Gabus, S. Litvak, G. Keith, and J. L. Darlix.** 1988. Small finger protein of avian and murine retroviruses has nucleic acid annealing activity and positions the replication primer tRNA onto genomic RNA. EMBO J. **7:**1777–1783.
24. **Quillent, C., A. M. Morman, S. Paulous, C. Dauguet, and F. Clavel.** 1996. Extensive regions of pol are required for efficient human immunodeficiency virus polyprotein processing and particle maturation. Virology **219:**29–36.
25. **Richter-Cook, N. J., K. J. Howard, N. M. Cirino, B. M. Wohrl, and S. F. Le Grice.** 1992. Interaction of tRNALys.3 with multiple forms of human immunodeficiency virus reverse transcriptase. J. Biol. Chem. **267:**15952–15957.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
27. **Sarih-Cottin, L., B. Bordier, K. Musier-Forsyth, M. L. Andreila, P. J. Barr, and S. Litvak.** 1992. Preferential interaction of human immunodeficiency virus reverse transcriptase with two regions of primer tRNALys.3 as evidenced by footprinting studies and inhibition with synthetic oligoribonucleotides. J. Mol. Biol. **226:**1–6.
28. **Stewart, L., G. Schatz, and V. M. Vogt.** 1990. Properties of avian retrovirus particles defective in viral protease. J. Virol. **64:**5076–5092.
29. **Temin, H. M., and S. Mizutani.** 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature **226:**1211–1213.
30. **Thrall, S. H., J. Reinstein, B. M. Wohrl, and R. S. Goody.** 1996. Evaluation of human immunodeficiency virus type 1 reverse transcriptase primer tRNA binding by fluorescence spectroscopy: specificity and comparison to primer/template binding. Biochemistry **35:**4609–4618.
31. **Wakefield, J. K., H. Rhim, and C. D. Morrow.** 1994. Minimal sequence requirements of a functional human immunodeficiency virus type 1 primer binding site. J. Virol. **68:**1605–1614.
32. **Wakefield, J. K., A. G. Wolf, and C. D. Morrow.** 1995. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to $tRNA_3^{Lys}$. J. Virol. **69:**6021–6029.
33. **Weiss, S., B. Konig, H. J. Muller, H. Seidel, and R. S. Goody.** 1992. Synthetic human tRNA(UUULys.3) and natural bovine tRNA(UUULys.3) interact with HIV-1 reverse transcriptase and serve as specific primers for retroviral cDNA synthesis. Gene **111:**183–97.
34. **Whitcomb, J. M., B. A. Ortiz-conde, and S. H. Hughes.** 1995. Replication of avian leukosis viruses with mutations at the primer binding site: use of alternative tRNAs as primers. J. Virol. **69:**6228–6238.
35. **Wohrl, B. M., B. Ehresmann, G. Keith, and S. F. Le Grice.** 1993. Nuclease footprinting of human immunodeficiency virus reverse transcriptase/tRNALys.3 complexes. J. Biol. Chem. **268:**13617–13624.
36. **Wilson, W., M. Braddock, S. E. Adams, P. D. Rathjen, S. M. Kingsman, and A. J. Kingsman.** 1988. HIV expression strategies: ribosomal frameshifting is directed by a short sequence in both mammalian and yeast systems. Cell **55:**1159–1169.
37. **Zakharova, O. D., L. Tarrago-Litvak, M. Fournier, M. L. Andreola, M. N. Repkova, A. G. Venyaminova, S. Litvak, and G. A. Vevinsky.** 1995. Interaction of primer tRNALys.3 with the p51 subunit of human immunodeficiency virus type 1 reverse transcriptase: a possible role in enzyme activation. FEBS Lett. **361:**287–290.
38. **Zakharova, O. D., L. Tarrago-Litvak, G. Maksakova, M. L. Andreola, E. Dufour, S. Litvak, and G. A. Vevinsky.** 1995. High-affinity interaction of human immunodeficiency virus type-1 reverse transcriptase with partially complementary primers. Eur. J. Biochem. **233:**856–863.