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Initiation of HIV-1 Reverse Transcription Is Regulated by a Primer Activation Signal*

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Reverse transcription of the human immunodeficiency virus type 1 (HIV-1) RNA genome appears to be strictly regulated at the level of initiation. The primer binding site (PBS), at which the $tRNA_3^{Lys}$ molecule anneals and reverse transcription is initiated, is present in a highly structured region of the untranslated leader RNA. Detailed mutational analysis of the U5 leader stem identified a sequence motif in the U5 region that is critical for activation of the PBS-bound $tRNA_3^{Lys}$ primer. This U5 motif, termed the primer activation signal (PAS), may interact with the TΨC arm of the $tRNA_3^{Lys}$ primer, similar to the additional interaction proposed for the genome of Rous sarcoma virus and its $tRNA^{Trp}$ primer. This suggests that reverse transcription is regulated by a common mechanism in all retroviruses. In HIV-1, the PAS is masked through base pairing in the U5 leader stem. This provides a mechanism for positive and negative regulation of reverse transcription. Based on structure probing of the mutant and wild-type RNAs, an RNA secondary structure model is proposed that juxtaposes the critical PAS and PBS motifs.

Infection of the host cell by a retroviral particle results in reverse transcription of the viral RNA genome into double-stranded DNA, which subsequently becomes integrated into the host cell genome (1). Reverse transcription is mediated by the virion-associated enzyme reverse transcriptase (RT),[1] and a cellular tRNA molecule is used as a primer (2). The tRNA primer binds with its 3′-terminal 18 nts to a complementary sequence, the primer binding site (PBS), that is located in the 5′-untranslated leader region of the viral RNA genome. Retroviral particles are competent to initiate reverse transcription shortly after budding from the producer cell, but there is also evidence that reverse transcription in virions is limited (3–7). This suggests that initiation of reverse transcription is restricted until a new host cell is infected. The mechanism that regulates reverse transcription is not known, but sequence motifs and RNA secondary structures in the region flanking the PBS have been implicated (8–13). Alternatively, reverse transcription may be restricted in extracellular virions by the low concentration of dNTP molecules in virus particles.

In the genome of human immunodeficiency virus type I (HIV-1), the PBS is predicted to be part of an extended RNA structure. Several RNA secondary structure models have been proposed for this region of the 5′-untranslated leader (14–17), and there is recent evidence that this region can adopt alternate conformations (18, 19). The model depicted in Fig. 1*A* shows the U5-PBS hairpin that occludes part of the PBS and the extended U5 leader stem, which is formed by base pairing of sequences in the upstream U5 and the downstream leader region. Similar RNA secondary structures have been predicted for other retroviruses (16, 20–23). For the avian Rous sarcoma virus (RSV), these structures have been reported to regulate initiation of reverse transcription (8–10, 24). In RSV, reverse transcription is stimulated by an additional vRNA-tRNA interaction between a sequence motif in the U5 region and the TΨC arm of the $tRNA^{Trp}$ primer (22). For HIV-1 reverse transcription, initiation is thought to be stimulated by other template-primer contacts, including a base-pairing interaction between the A-rich loop of the U5-PBS hairpin and the anti-codon loop of the $tRNA_3^{Lys}$ primer (12, 13, 25–30).

In this study, we present a detailed mutational analysis of the HIV-1 U5 leader stem. We measured the replication capacity of the mutant viruses and performed *in vitro* reverse transcription assays with the mutant RNA templates. Analysis of HIV-1 mutants with large deletions suggested that the U5 region contains a motif that is critical for $tRNA_3^{Lys}$-mediated initiation of reverse transcription but not for reactions that are initiated by a DNA primer. A second set of mutants was designed to map this HIV-1 RNA motif in more detail. We identified an eight-nucleotide sequence in the U5 region that is not involved in tRNA annealing but that is important for initiation of reverse transcription. We propose that this motif interacts with the TΨC arm of $tRNA_3^{Lys}$, thereby triggering initiation of HIV-1 reverse transcription. This U5 motif, termed primer activation signal (PAS), is masked in the wild-type RNA through base pairing in the U5 leader stem. Interestingly, reverse transcription can be activated by exposure of the PAS through mutation of the "opposing" leader sequence. The presence of the PAS enhancer element and a repressive RNA structure provides a mechanism for positive and negative regulation of reverse transcription. In addition, we performed structure probing of the mutant and wild-type RNAs to resolve the secondary structure of the PBS domain. An RNA secondary structure model is proposed in which the PAS and PBS motifs are juxtaposed. Overall, regulation of reverse transcription in HIV-1 appears to be very similar to that in RSV, suggesting that reverse transcription is regulated by a common mechanism in all retroviruses.

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[1] The abbreviations used are: RT, reverse transcriptase; PBS, primer binding site; HIV-1, human immunodeficiency virus type 1; RSV, Rous sarcoma virus; vRNA, viral RNA; PAS, primer activation signal; LTR, long terminal repeat; PCR, polymerase chain reaction; DEPC, diethyl pyrocarbonate; DMS, dimethyl sulfate; NC, nucleocapsid; nt, nucleotide(s).

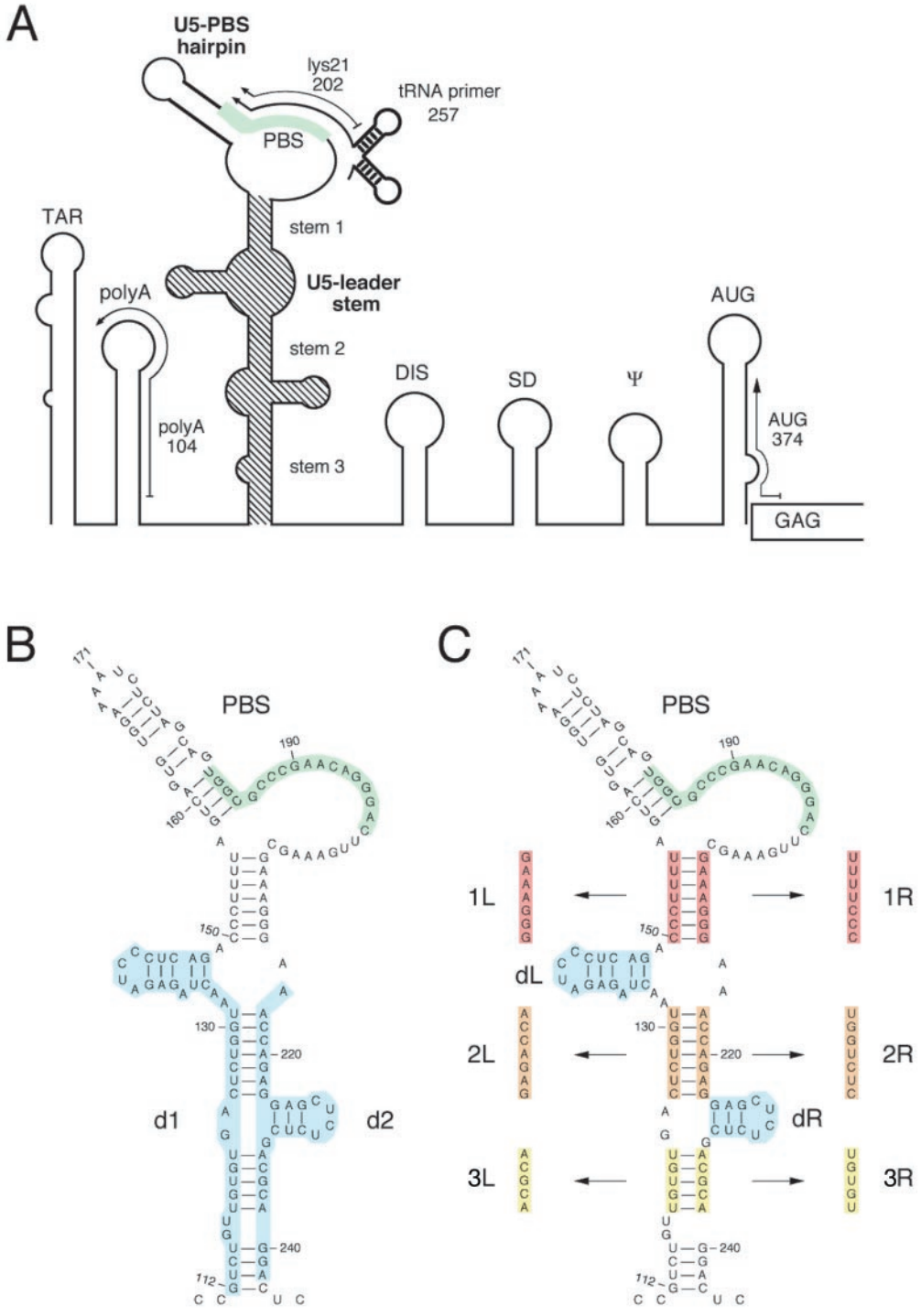

FIG. 1. **Schematic of the HIV-1 5′-untranslated leader RNA.** *A*, RNA secondary structure model of the HIV-1 leader region. See Ref. 14 for further details on the individual hairpin motifs. The $tRNA_3^{Lys}$ primer binds to the PBS (marked in *green*) that is part of an extended RNA structure,. This structure consists of a small U5-PBS hairpin that occludes part of the PBS and a large stem formed by upstream U5 and downstream leader sequences. This U5 leader stem (*shaded*) contains three distinct stem segments, the upper segment 1, the middle segment 2, and the lower segment 3. The position of several primers used in the reverse transcription assays is indicated. *B*, shown are the deletions d1 and d2 (marked in *blue*) in the U5 leader stem. The double mutant d1/2 contains both deletions. *C*, a second set of more subtle U5 leader stem mutants was constructed. Mutant 1L has a 7-nt substitution on the left side of stem 1, and mutant 1R has a 7-nt substitution on the right side (marked in *red*). Mutations 1L and 1R are complementary, and base pairing is restored in the double mutant. Mutations were also introduced in stem 2 (2L, 2R, and 2LR, marked in *orange*) and stem 3 (3L, 3R, and 3LR, marked in *yellow*). In addition, we deleted the left and right arms of the U5 leader stem in mutant dL and dR, respectively (shown in *blue*). The deletions were combined in the double mutant dLR.

EXPERIMENTAL PROCEDURES

DNA Constructs—A derivate of the full-length proviral HIV-1 clone pLAI was used to produce wild-type and U5 leader stem-mutated viruses. This construct pLAI-R37 has been described previously and contains a unique U5 region in the 5′-LTR (31). Nucleotide numbers refer to positions on HIV-1 genomic RNA, with +1 being the capped G residue. For mutation of the U5 leader stem, we used the construct Blue-5′-LTR (32), which contains a *Xba*I-*Cla*I fragment of HIV-1, encompassing the 5′-LTR, PBS, leader, and the 5′ end of the *gag* gene (positions −454 to +376) cloned into pBluescript KS+ (Stratagene). The U5 leader stem was mutated by oligonucleotide-directed *in vitro* mutagenesis with a Muta-Gene Phagemid *in Vitro* mutagenesis kit (Bio-Rad). For sequence analysis, the 5′-LTR leader region was PCR-amplified with the sense R region primer T7-1 (positions −54 to −34)

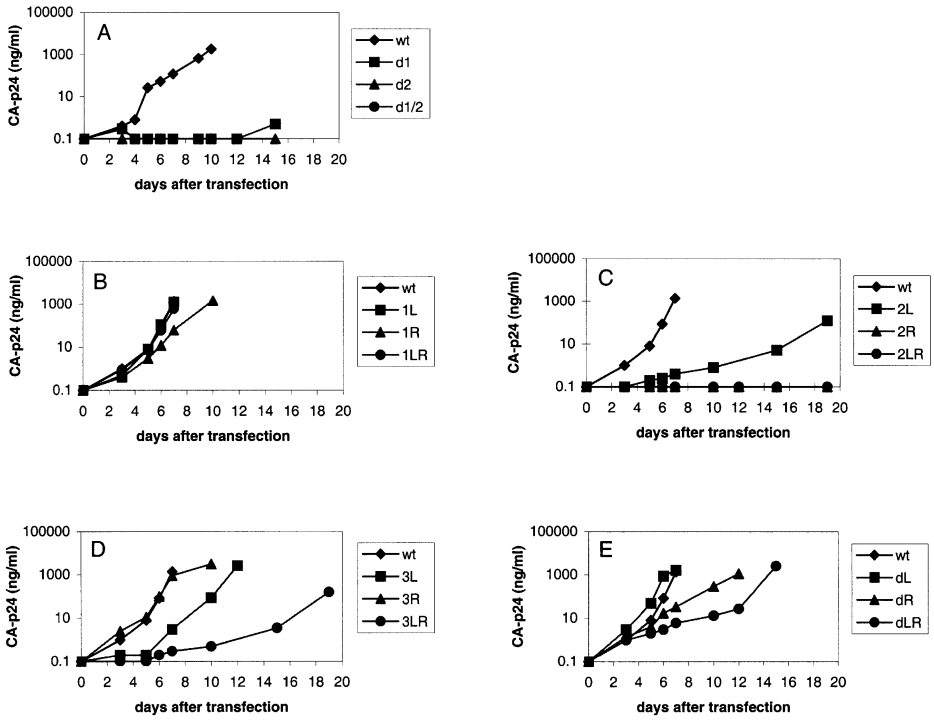

FIG. 2. **Replication of wild-type HIV-1 LAI (*wt*) and U5 leader stem mutants.** SupT1 cells were transfected with 1 μg of the proviral constructs. CA-p24 production was measured in the culture medium at several days post-transfection. The replication capacity of the mutants d1 and d2 with large deletions in the U5 leader stem and the double mutant d1/2 is shown in *A*. Also shown is the replication capacity of viruses with mutations in stem 1 (*B*), stem 2 (*C*), and stem 3 (*D*). The replication of the arm-deletion mutants dL, dR, and dLR is shown in *E*.

with the 5′-flanking T7 RNA polymerase promoter sequence and the antisense primer AUG (positions +348 to +368, with 6 additional nucleotides at its 5′-end). These PCR products were sequenced with the DYEnamic™ Direct cycle sequencing kit (Amersham Pharmacia Biotech) and an Applied Biosystems 373 DNA sequencer. Subsequently, the mutated *Xba*I-*Cla*I fragments were introduced into the proviral clone pLAI-R37, which was checked by sequence analysis of the mutated domain.

Cells, Transfection, and Virus Replication—SupT1 T cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum at 37 °C and 5% CO_2. SupT1 cells (5×10^6) were transfected with 1 μg of the HIV-1 proviral constructs by electroporation (250 V, 960 microfarads). Fresh SupT1 cells (0.5×10^6) were added after transfection to support virus replication. Cells were split 1 to 10 twice a week. CA-p24 levels in the culture medium were determined by enzyme-linked immunosorbent assay (33).

Synthesis of RNA Templates—The wild-type and mutant pBlue-5′-LTR plasmids were used as the template for PCR amplification and subsequent *in vitro* transcription. The 5′-LTR-leader region was PCR-amplified with the sense primer T7–2 (positions +1 to +20) with 5′-flanking T7 RNA polymerase promoter sequence and the antisense primer AUG (positions +348 to +368). The PCR fragments were phenol-extracted, precipitated and dissolved in water. *In vitro* transcription was performed with the T7-MegaShortscript kit (Ambion). Upon DNase treatment and phenol extraction, the unincorporated free nucleotides were removed by passage through a Sephadex G-50 column. Subsequently, the RNA was ethanol-precipitated and dissolved in renaturation buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl). The RNA was renatured by incubation at 85 °C for 2 min followed by slow cooling to room temperature, and the RNA was stored at −20 °C. The RNA concentration was measured by UV spectroscopy.

Reverse Transcription Assays—The *in vitro* synthesized RNA template (10 ng) was incubated either with 1.5 μg of calf liver tRNA (6 pmol total tRNA, of which ~1.2 pmol $tRNA_3^{Lys}$, Roche Molecular Biochemicals) or with 20 ng of DNA primer in 12 μl of annealing buffer (83 mM Tris-HCl, pH 7.5, 125 mM KCl) at 85 °C for 2 min, 65 °C for 10 min, followed by cooling to room temperature over a 1-h period. We previously demonstrated that there is selective priming by $tRNA_3^{Lys}$ in this system (6). Even the related $tRNA^{Lys1,2}$ molecules do not act as a primer. The primer was extended with 1 nt by the addition of 6 μl of RT(−) buffer (9 mM $MgCl_2$, 30 mM dithiothreitol, 150 μg/ml actinomycin D), 1 μl of [α-^{32}P]dCTP, and 0.5 units of HIV-1 RT (Medical Research Council AIDS Reagent Project). Reverse transcription was performed for 30 min at 37 °C. Complete cDNA synthesis was accomplished in RT(+) buffer (RT(−) buffer with 30 μM dATP, dGTP, and dTTP and 1.5 μM dCTP), 0.3 μl of [α-^{32}P]dCTP, and 0.5 units of HIV-1 RT. In the PBS occupancy assay, the RNA template was incubated simultaneously with 1.5 μg of calf liver tRNA and 20 ng of AUG primer, and reverse transcription was performed in RT(+) buffer with avian myeloblastosis virus RT enzyme (Roche Molecular Biochemicals). The cDNA products were precipitated in 0.3 M sodium acetate, pH 5.2, and 70% ethanol at −20 °C, dissolved in formamide-loading buffer, heated, and analyzed on a denaturing 6% polyacrylamide-urea-sequencing gel. The antisense primers used are poly(A) (positions +77 to +104), Lys-21 (positions +182 to +202), and AUG (positions +348 to +368, with 6 additional nucleotides at its 5′ end). Sequence reactions with the BB-3 (positions +215 to +245) and AUG primer were performed with the Sequenase kit 2.0 (Amersham Pharmacia Biotech) and included on the sequencing gels to determine the exact length of the cDNA products.

Structure Probing of U5 Leader Stem—In vitro synthesized HIV-1 leader RNA (positions +1 to +368) (50 ng) was treated with diethyl pyrocarbonate (DEPC, 0.5%/2.5%), dimethyl sulfate (DMS, 0.1/0.5%), RNase T1 (0.004 units/0.02 units), RNase S1 (2 units/10 units), or RNase One (10^{-5} units/5×10^{-5} units) in 10 mM Tris, pH 8.5, 10 mM $MgCl_2$, 50 mM NaCl for 10 min at 37 °C. The samples treated were phenol-extracted and recovered by ethanol precipitation. The antisense primers AD-SD (positions + 270 to +290) and Lys-21 (positions +182 to +202) were used to map the sites of modification or cleavage. Primers were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Roche Molecular Biochemicals). The labeled oligonucleotide (2 ng) was mixed with the RNA sample in a total volume of 10 μl of annealing buffer, incubated for 2 min at 85 °C and for 10 min at 65 °C, and slowly cooled to 25 °C. The primer was extended by the addition of 5 μl of RT(+) buffer and 12.5 units of avian myeloblastosis virus RT enzyme (Roche Molecular Biochemicals) in a 15-min incubation at 42 °C. The samples were mixed with formamide-loading buffer and analyzed on a denaturing 6% polyacrylamide-urea-sequencing gel. Sequence reactions initiated by the primers AD-SD or Lys-21 were included on the sequencing gels to determine the exact positions of modification or cleavage.

RESULTS

Design of U5 Leader Stem Mutants—To study the role of the U5 leader stem in reverse transcription, we constructed two deletion mutants (Fig. 1*B*). Mutant d1 contains a large deletion on left side of the U5 leader stem (positions +112 to +148), and mutant d2 contains a deletion on the right side of the stem (positions +216 to +242). The double mutant d1/2 combines both deletions. A second set of more subtle mutants was designed to change the individual stem segments of the U5 leader structure as illustrated in Fig. 1*C*. The upper stem segment 1

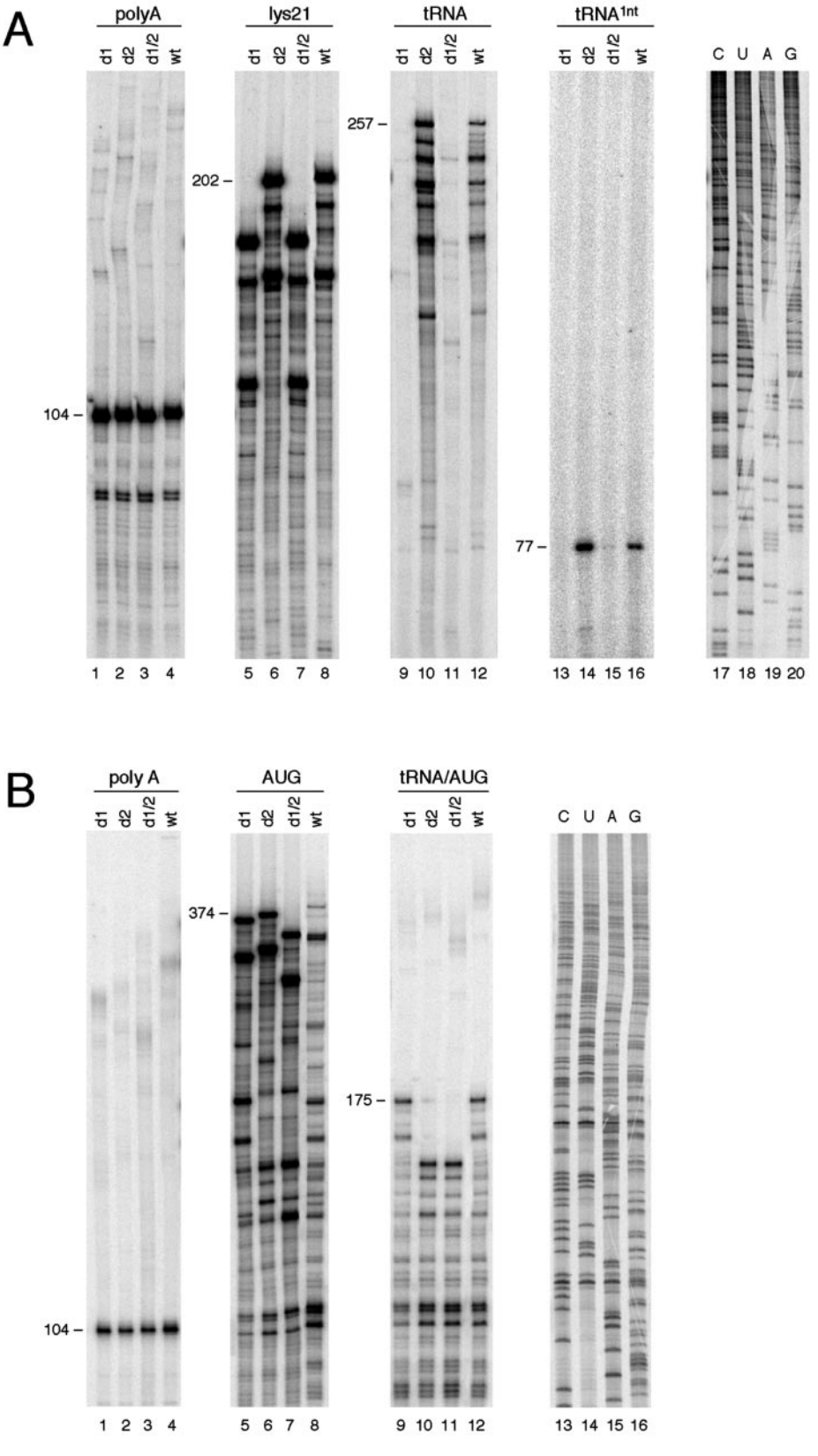

FIG. 3. **Reverse transcription assays on wild-type and mutant HIV-1 RNA templates.** *A*, the amount of input viral RNA was quantitated by DNA-primer extension with the poly(A) primer that produces a 104-nt product (*lanes 1–4*). The PBS-primer Lys-21 (*lanes 5–8*), and $tRNA_3^{Lys}$ (*lanes 9–12*) were heat-annealed onto the HIV-1 RNA templates and extended by the HIV-1 RT enzyme in the presence of all dNTPs. Extension of Lys-21 results in a 202-nt cDNA product, and the tRNA primer produces a 257-nt tRNA-cDNA product. These sizes refer to reactions with the wild-type template and are shorter for the d1 and d1/2 templates due to the d1 deletion in the U5 region. Several shorter cDNA products are visible that result from RT pausing. We also performed 1-nt incorporation assays with the $tRNA_3^{Lys}$ primer (*lanes 13–16*). The extension of the 76-nt tRNA by dCTP produces a 77-nt radiolabeled product. To accurately determine the length of the cDNA products, a sequencing reaction was analyzed in parallel (*lanes 17–20*). Part of the gels in *panel A* and *B*, including the wild type (*WT*) and marker *lanes*, was published previously (34). *B*, PBS occupancy test in which the PBS-bound $tRNA_3^{Lys}$ primer was visualized by selective extension of the downstream AUG primer (*lanes 9–12*). When the extension of the AUG primer is blocked by the PBS-bound tRNA, a 175-nt cDNA was produced. Free RNA templates will produce a full-length cDNA product of 374 nt (*lanes 5–8*). Control reactions were performed with the poly(A) primer (*lanes 1–4*). To accurately determine the position of the stop product, a sequence reaction was analyzed in parallel (*lanes 13–16*).

was mutated by a 7-nt substitution either on the left side in mutant 1L or on the right side in mutant 1R. Mutations 1L and 1R are complementary, and base pairing will be restored in the double mutant 1LR, at least according to the RNA secondary structure model in Fig. 1. Similar type of mutations were introduced in stem segment 2 (2L, 2R, and 2LR) and segment 3 (3L, 3R, and 3LR). In addition, the left arm (positions +134 to +148) and the right arm (positions +224 to +233) of the U5 leader structure were deleted in mutants dL and dR, respectively. Both deletions were combined in the double mutant dLR.

Replication Capacity of U5 Leader Stem Mutants—To study the replication potential of viruses with U5 leader stem mutations, we transfected wild-type and mutant proviral genomes

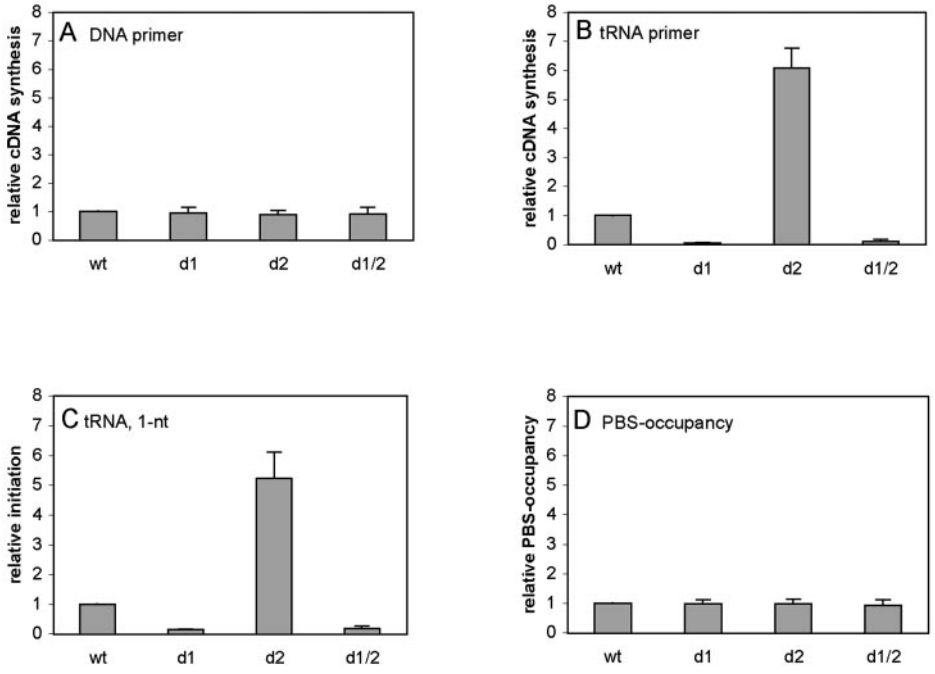

FIG. 4. **Relative reverse transcription activities of wild-type and d1, d2, and d1/2 mutant templates.** The results of three independent experiments were quantitated, and the activity of the wild-type template was arbitrarily set at 1. Shown is DNA-primed reverse transcription with the Lys-21 primer (*A*), tRNA-primed reverse transcription (*B*), and tRNA-primed 1-nt incorporation (*C*). The tRNA occupancy of the PBS is shown in *D* and was also set at 1 (100% occupancy) for the wild-type template.

into the SupT1 T cell line. These cells express the CD4-CXCR4 receptors and are fully susceptible for replication of the HIV-1 LAI strain. Virus replication was followed by measuring the accumulation of CA-p24 in the culture medium at several days post-transfection. Transfection with 1 μg of the proviral constructs d1, d2, and d1/2 demonstrated that these deletions completely impair virus replication (Fig. 2*A*), indicating that the U5 leader stem or sequence elements encoded by this region are important for viral replication. To study the contribution of distinct sequence or structure elements in more detail, we tested the second set of more subtle mutants. Mutation of stem 1 had a minor effect on virus replication (Fig. 2*B*). However, both mutations 2L and 2R in the middle segment severely impaired virus replication, and the double mutant did not restore replication (Fig. 2*C*). Mutation 3L in the lower stem segment also reduced viral replication significantly, whereas mutation 3R showed only a minor effect on viral replication (Fig. 2*D*). The combination of both mutations in mutant 3LR further reduced the replication capacity. Deletion dL did not significantly affect virus replication, deletion dR showed a modest defect, and combination of the mutations in mutant dLR further reduced replication (Fig. 2*E*). These combined results indicate that sequences within stem 2 of the U5 leader structure are most important for virus replication.

Reverse Transcription on the Mutant HIV-1 Templates—We next performed *in vitro* reverse transcription reactions with the wild-type and mutant HIV-1 RNA templates. RNA templates encompassing the complete untranslated leader region (positions +1 to +368) were used with the natural $tRNA_3^{Lys}$ primer or DNA primers to initiate reverse transcription. The position of the different primers is shown in Fig. 1*A*. The primers were heat-annealed onto the different RNA templates, and reverse transcription was initiated by the addition of dNTPs and HIV-1 RT enzyme. Full-length reverse transcription products were quantitated and corrected for the amount of input RNA template, as determined with the DNA primer poly(A) (Fig. 3*A*, *lanes 1–4*). Representative experiments are shown in Fig. 3. The results of three independent assays were quantitated and are summarized in Fig. 4.

Extension of the DNA primer Lys-21 that is complementary to the PBS resulted in a 202-nt full-length cDNA product on the wild-type template, with a predicted change in cDNA length for the d1 and d1/2 templates due to the deletion in the U5 region (Fig. 3*A*, *lanes 5–8*). The efficiency of DNA-primed reverse transcription is equal on all templates (Fig. 4*A*). In contrast, extension of the natural tRNA primer, which results in a 257-nt cDNA product on the wild-type template, was abolished on the d1 and d1/2 templates (Fig. 3*A*, *lanes 9–12* and Fig. 4*B*). Surprisingly, reverse transcription on the d2 template was stimulated 6-fold over the wild-type level. These differences in tRNA-primed reverse transcription efficiency on the mutant templates could result from differences in tRNA annealing, initiation, or elongation. To study initiation of tRNA-primed reverse transcription, the reaction was performed in the presence of [^{32}P]dCTP but without the other dNTPs. This will result in the extension of the 76-nt $tRNA_3^{Lys}$ primer with 1 nt on all templates (Fig. 3*A*, *lanes 13–16*). The results of this initiation assay are similar to the results of tRNA-primed full-length cDNA synthesis. No initiation was observed on the d1 and d1/2 templates, whereas a 6-fold stimulation was measured on the d2 template (Fig. 4*C*). This indicates that the inhibitory effect of deletion d1 and the stimulatory effect of deletion d2 are apparent at the level of initiation.

The observed differences in initiation efficiency may be caused by different amounts of tRNA primer annealed onto the PBS. This seems unlikely because the PBS motif itself is not altered in the mutant HIV-1 templates, and the primer was heat-annealed in these studies. To nevertheless rule out this possibility, we determined the tRNA occupancy of the PBS on the wild-type and mutant templates. The tRNA primer was annealed onto the template, and this complex was subsequently used for extension of the DNA primer AUG that is positioned downstream of the PBS (Fig. 1*A*). We used the avian myeloblastosis virus RT enzyme to selectively extend the DNA primer because this enzyme is unable to extend the tRNA primer (6, 34). When the PBS was occupied by the tRNA primer, AUG-mediated reverse transcription was blocked by the tRNA, yielding a cDNA product of ~175 nts. Free RNA templates will produce a full-length cDNA product of 374 nts on the wild-type template. All templates exclusively yield the stop product, indicating that the templates are fully occupied by the $tRNA_3^{Lys}$ primer (Fig. 3*B*, *lanes 9–12*). Control reactions were

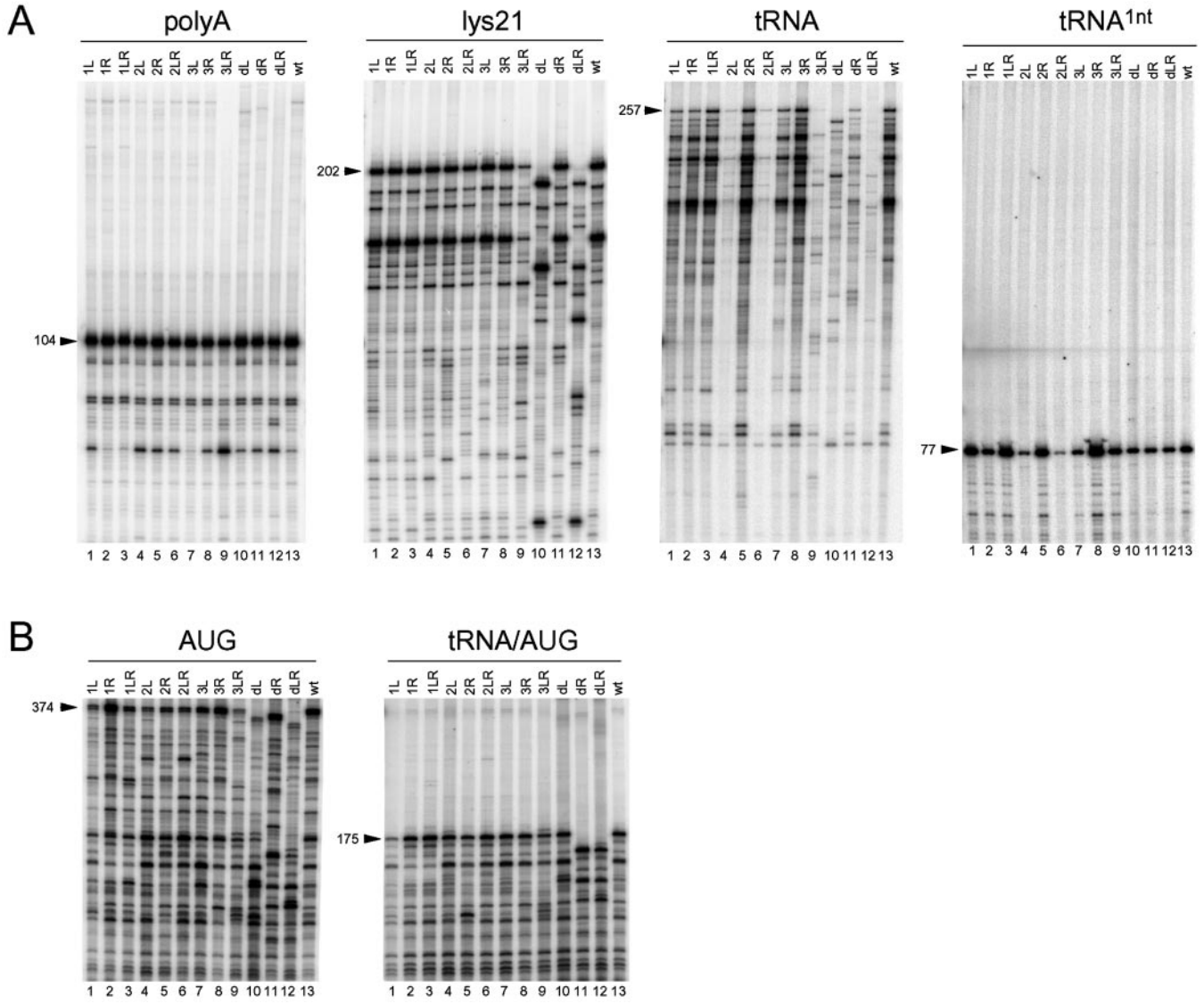

FIG. 5. **Reverse transcription assays on wild-type and mutant templates.** *A*, reverse transcription assays primed with the primers poly(A), Lys-21, and $tRNA_3^{Lys}$ (see Fig. 3*A* for further details). *B*, PBS-occupancy test (see Fig. 3*B* for further details).

performed with the upstream poly(A) primer and the AUG primer in the absence of tRNA (Fig. 3*B*, *lanes 1–4* and *5–8*, respectively). The results of the PBS occupancy test are summarized in Fig. 4*D*.

These combined results indicate a complex interplay of positive and negative regulation of HIV-1 reverse transcription. The left side of the U5 leader stem seems to encode a sequence motif that is involved in initiation of reverse transcription. Deletion of the right side of the stem may expose this sequence motif, thereby activating reverse transcription. These effects are observed exclusively with the natural $tRNA_3^{Lys}$ primer and not with a PBS-bound DNA primer, suggesting that additional tRNA-vRNA contacts may be involved.

The second set of stem mutants was designed to accurately map the sequence motifs that regulate HIV-1 reverse transcription. Reverse transcription assays were performed with these mutant templates and the $tRNA_3^{Lys}$ or Lys-21 DNA primer. All reverse transcription products were quantitated and corrected for the amount of input RNA template as determined by poly(A) primer extension (Fig. 5*A*, *polyA panel*). The results of three independent experiments are summarized in Fig. 6. We measured no difference among the templates in reverse transcription reactions initiated by the DNA primer Lys-21, whereas profound differences in cDNA synthesis were observed in tRNA-primed reactions. Most severe effects were observed with mutations in stem segment 2. Mutants 2L and 2LR showed 10-fold reduced tRNA-primed reverse transcription compared with the wild-type template (Fig. 5*A*, *tRNA panel*, *lanes 4* and *6*), whereas mutation 2R enhanced reverse transcription 2.5-fold (*lane 5*). Mutation 3R also stimulated reverse transcription (*lane 8*), and there is a modest 3-fold inhibitory effect on reverse transcription with the deletion mutants dL, dR, and dLR (*lanes 10–12*). Furthermore, the observed reverse transcription effects are specific for tRNA-primed reactions.

These effects are apparent at the level of initiation as determined in the single nucleotide incorporation assay (Fig. 5*A*, *$tRNA^{Int}$ panel*). The PBS occupancy test demonstrated that the tRNA primer is annealed onto each template with equal efficiency (Fig. 5*B*, *tRNA/AUG panel*). Thus, mutations in stem segment 2 affect reverse transcription in a similar way as the deletion mutants d1 and d2. Mutation of the left side of the stem inhibits reverse transcription, whereas mutation of the right side stimulates reverse transcription. These combined results suggest that the 2L sequence activates the PBS-bound tRNA molecule to initiate reverse transcription. We will there-

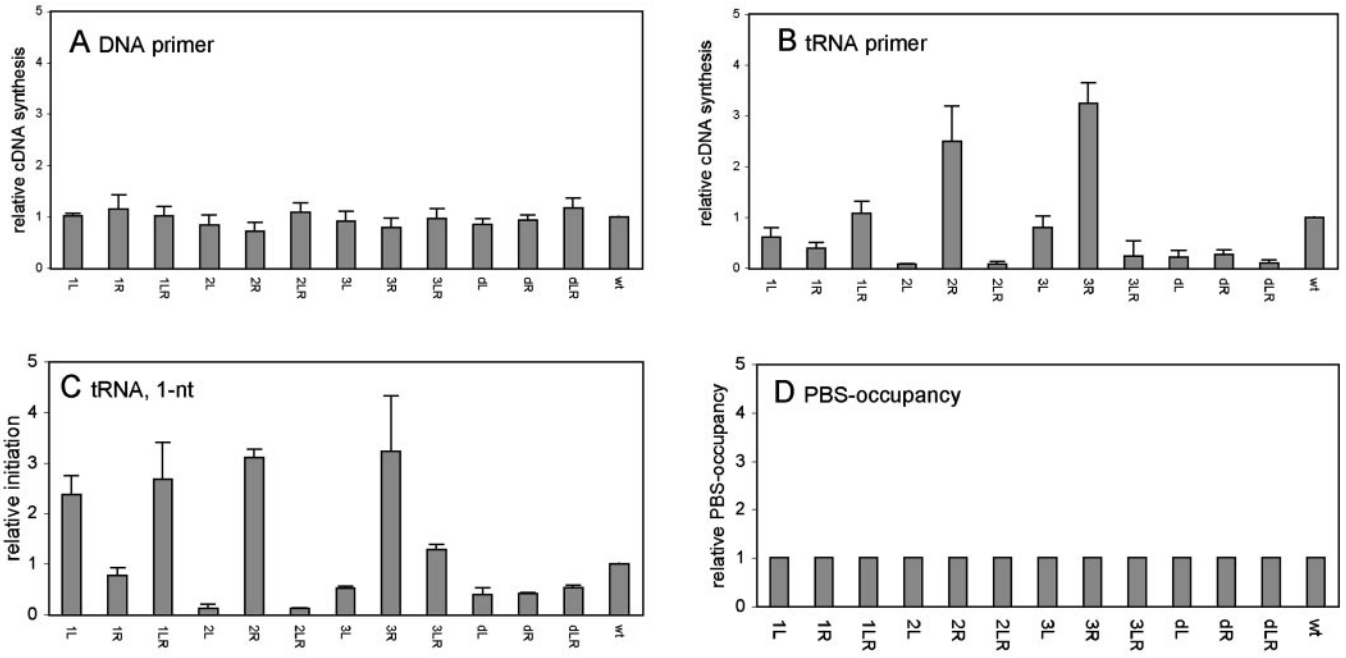

FIG. 6. **Relative reverse transcription activities of wild-type and mutant templates.** The average of three independent experiments was calculated, and the activity of the wild-type template was set at 1. Shown is DNA-primed reverse transcription (*A*), tRNA-primed reverse transcription (*B*), tRNA-primed 1-nt incorporation (*C*), tRNA occupancy of the PBS (*D*).

fore refer to this motif as the PAS. Mutation of the PAS in mutant 2L inhibits reverse transcription, whereas mutation 2R may stimulate initiation by making the PAS more accessible. Mutation 3R also stimulates reverse transcription, which may indicate that the opening of stem segment 3 weakens the stability of the adjacent stem segment 2.

Structure Probing of the U5 Leader Stem—The results presented above suggest an important biological role for the U5 leader stem, in particular for stem segment 2. We therefore determined the secondary structure of the U5 leader stem by treating wild-type and mutant HIV-1 templates with structure-specific probes (Fig. 7). Nucleotides sensitive to the chemicals DEPC or DMS and the RNases S1, T1, or One are assumed not to be involved in base pairing or base-stacking interactions. The sites of modification or cleavage were determined by primer extension analysis. Control experiments were performed in parallel to detect pauses of reverse transcription due to, for instance, stable RNA structure. The results of the probing experiments on the wild-type template are summarized in Fig. 8.

Probing of the wild-type template with RNase T1 resulted in cleavage of G residues at positions 202, 206, and 208 (Fig. 7*A*, *lanes 1* and *2*) that are single-stranded in the structure model of Fig. 1. However, the G residues that are proposed to be base-paired in stem 1 (G^{212}–G^{214}) are also sensitive to RNase T1, indicating that this part of the structure model is not correct. Probing of the wild-type template with the chemicals DEPC (Fig. 7*A*, *lanes 4* and *5*) and DMS (Fig. 7*A*, *lanes 6* and *7*) demonstrated that several A residues in the single-stranded PBS region are exposed. In addition, the A residues that are base-paired in stem 1 in Fig. 1 (A209-A211) were highly sensitive to both chemicals. Treatment with RNase S1 and RNase One (Fig. 7*B*) resulted in several cleavages in the single-stranded PBS region and the right arm of the U5 leader stem. RNase S1 also cleaves the U residues at position 153–156 that are proposed to be base-paired in stem 1 (Fig. 7*B*, *lanes 1* and *2*, and results not shown). These results indicate that the sequences proposed to be involved in base pairing in stem 1 (Fig. 1) are present in a single-stranded region, whereas the sequences in segment 2 and 3 are base-paired.

Additional evidence for the presence of stem 2 was obtained by probing of this segment in the mutant templates. The right side of stem 2 is not sensitive to DEPC treatment in the wild-type template (Fig. 7*C*, *lanes 1* and *2*), whereas mutation 2L on the left side exposed the sequence on the right side of stem 2. The nucleotides A^{225}, A^{222}, and A^{220} become accessible to DEPC, indicating that they are released from base pairing in mutant 2L (Fig. 7*C*, *lanes 9* and *10*). In addition, cleavage at several G residues in this G/A-rich stretch was observed. In the double mutant 2LR, these nucleotides are no longer accessible to DEPC (Fig. 7*C*, *lanes 13* and *14*), indicating that base pairing in stem 2 is restored. In all other mutants, stem 2 is not accessible to DEPC.

These combined results are consistent with a partially modified RNA secondary structure model for the U5 leader stem that is shown in Fig. 8. The U5 leader stem segments 2 and 3 are maintained in this model, but it shows the extended U5-top hairpin instead of stem 1 and the U5-PBS hairpin. The residues U^{153}–U^{156} that are accessible to RNase S1 are positioned in the loop of the U5-top hairpin, and the A-rich loop (positions 168–171) of the U5-PBS hairpin is present as an internal loop. The right side of stem 1 is present in the single-stranded PBS region that is highly sensitive to DEPC, DMS, RNase T1, and RNase S1. The bulged-out A residues at positions 124, 132, and 133 are sensitive to DEPC and/or DMS. Positions 228–230 that are highly accessible to RNase S1 and RNase One are present in loop of the right arm of the U5 leader stem.

DISCUSSION

Reverse transcription of the HIV-1 RNA genome appears to be strictly regulated at the level of initiation. Mutational analysis demonstrated that the sequence on the left side of segment 2 of the U5 leader stem is critical for initiation of reverse transcription but is exclusively necessary in reactions primed by $tRNA_3^{Lys}$. Mutation of this motif in mutant 2L results in a 10-fold decrease in tRNA-primed reverse transcription. This sequence motif is not required for annealing of the tRNA primer onto the PBS but stimulates the incorporation of the

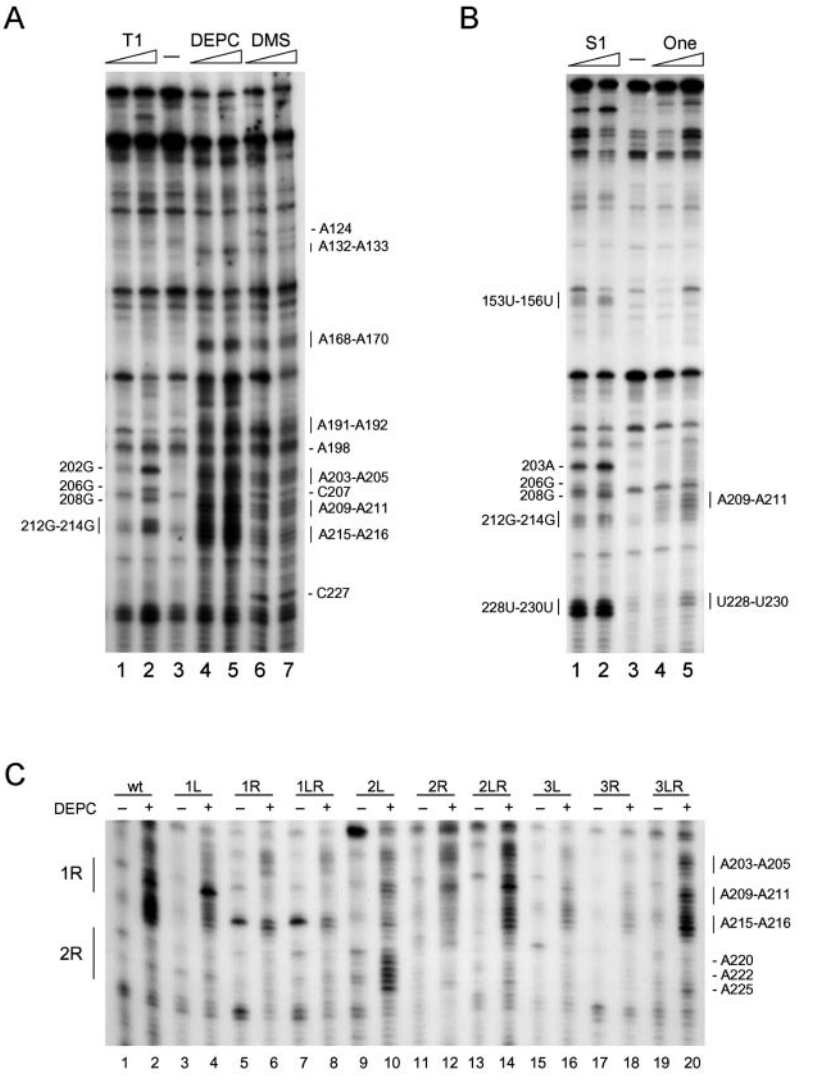

FIG. 7. **RNA structure probing of the U5 leader stem under native conditions.** The wild-type RNA template was treated with a limiting amount of the single strand-specific reagents. *Panel A*, RNase T1 (G-specific), DEPC (A-specific), DMS (A/C-specific). *Panel B*, RNase S1 and RNase One (both not sequence-specific). *Panel C*, wild-type and mutant templates (indicated on top of the *panel*) were treated with DEPC. Mock incubations were performed in parallel (−). Reactive sites were detected using primer extension analysis with the downstream primer AD-SD. The products were analyzed on a 6% polyacrylamide gel. Positions of reactive sites are indicated, and the results are summarized in Fig. 8.

first dNTP. We will therefore refer to this sequence as the PAS. We propose that the PAS sequence base pairs with the TΨC arm of the tRNA molecule (Fig. 8), similar to the additional interaction proposed for the RSV genome and the corresponding $tRNA^{Trp}$ primer. The PAS-anti-PAS interaction does not require additional melting of the tRNA because PBS-anti-PBS annealing will open both the acceptor en TΨC stems. However, the PAS sequence in the HIV-1 genome is occluded by base pairing in the U5 leader stem, as was demonstrated by biochemical probing experiments and, thus, needs to be unwound for the interaction with the tRNA primer. The presence of the PAS enhancer motif that is initially repressed by base pairing provides a unique mechanism for positive and negative regulation of HIV-1 reverse transcription. Consistent with this idea, reverse transcription is stimulated up to 6-fold by opening of the U5 leader stem in mutants d2 and 2R. Virus replication studies demonstrated that mutant 2L and, in particular, the deletion mutant d1 are replication impaired. Furthermore, faster-replicating revertant viruses of mutant 2L were obtained in five independent long term cultures. All these revertants were found to contain a G-to-A mutation at position 127 within the 2L motif (results not shown). This mutation partially repairs the PAS-anti-PAS interaction, suggesting that this interaction is important for virus replication.

A similar interaction between a U5 motif in the genome of RSV and the TΨC arm of the $tRNA^{Trp}$ primer has been demonstrated to stimulate initiation of reverse transcription (22), and a similar vRNA-tRNA interaction was also proposed for HIV-2 (20). These combined results suggest that retroviral reverse transcription is activated by a common mechanism. However, there are also some differences between the HIV-1 and RSV mechanisms. For instance, although the RSV genome folds a similar U5 leader stem, the PAS enhancer is not base-paired in RSV but rather positioned in a single-stranded region opposite the PBS (21). This suggests that initiation of RSV reverse transcription cannot be down-regulated by masking of the PAS motif, arguing that regulation of reverse transcription may be more complex in HIV-1. Mutations in RSV that disrupt the structure of either the U5 leader stem or the U5 IR hairpin, the latter the equivalent of the HIV-1 U5-top hairpin, were reported to impair the initiation of reverse transcription, whereas mutations that alter the sequence but that retain the structure had no effect (8, 9, 24). Similar mutations that disrupt the structure of the U5 leader stem in HIV-1 were found to stimulate reverse transcription, probably by exposure of the PAS enhancer. In a previous study, we demonstrated that stabilization of the U5 leader stem inhibited initiation of reverse transcription and virus replication (34). Analysis of a faster-replicating revertant virus demonstrated that opening of stem segment 2 by additional mutations on the right side restored reverse transcription. These combined results suggest that the efficiency of HIV-1 reverse transcription is determined by the accessibility of the PAS enhancer and that the structure

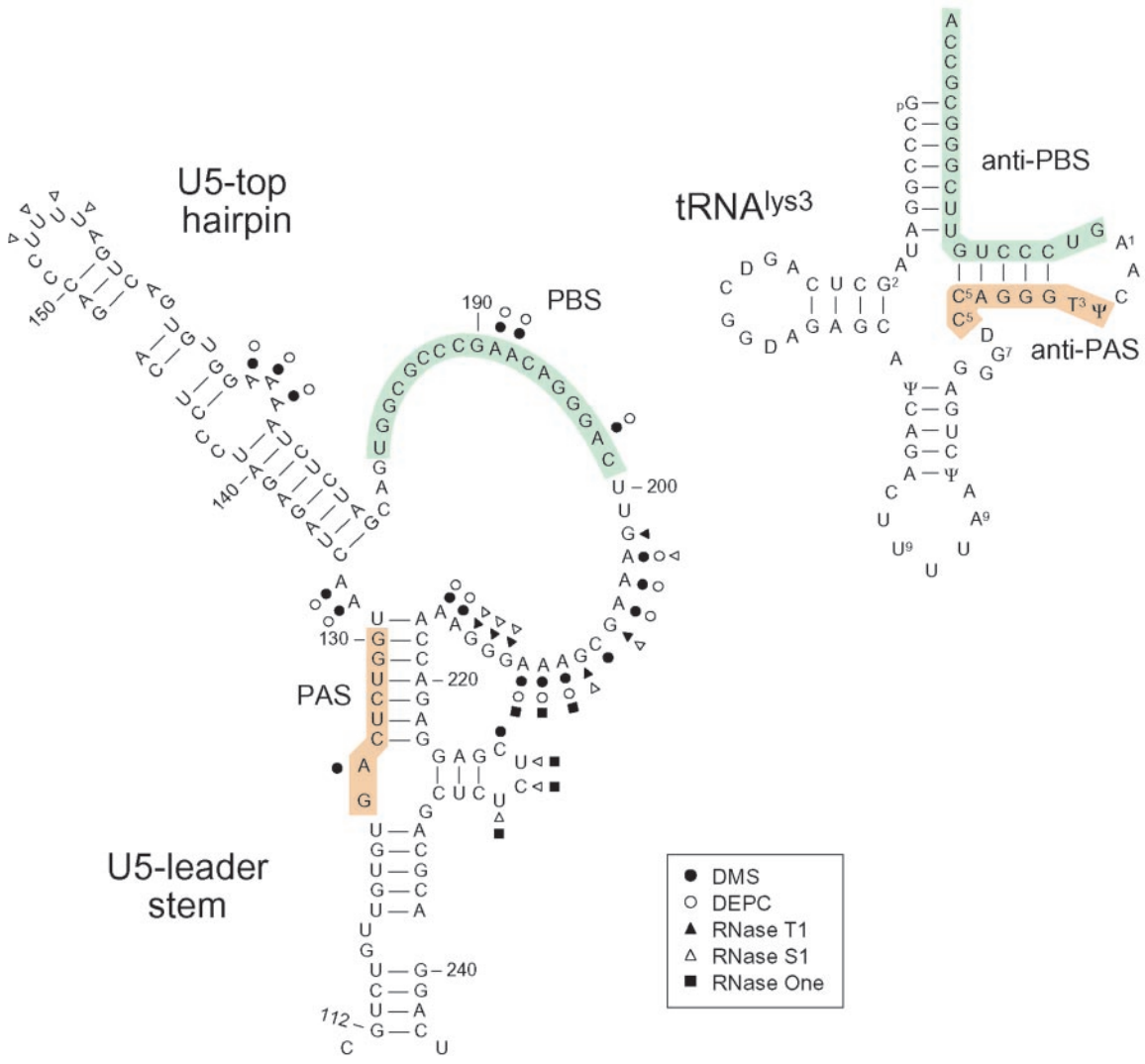

FIG. 8. **Modified RNA secondary structure model for the U5-PBS-leader region of HIV-1.** Based on structure probing results, this region is folded into the U5 leader stem, the U5-top hairpin, and the single-stranded PBS region. The reactive sites for the individual structure-specific probes are indicated. The proposed base pairing interaction between the HIV-1 PAS element and anti-PAS in the TΨC arm of the $tRNA_3^{Lys}$ primer is marked in *orange*. The PBS-anti-PBS interaction is marked in *green*.

of the U5 leader stem can negatively modulate reverse transcription.

Several alternative interactions between the $tRNA_3^{Lys}$ molecule and the HIV-1 RNA genome have been proposed on the basis of extensive biochemical probing experiments and modeling studies (12, 30, 35). The A-rich loop of the U5-PBS hairpin was proposed to interact with the U-rich anti-codon loop of $tRNA_3^{Lys}$ (12, 13, 25–30). In addition, the left arm of the U5 leader stem (positions +141 to +144) was suggested to interact with the 3′ portion of the anticodon stem of the tRNA molecule. Some of these interactions were also tested by mutational analysis in *in vitro* reverse transcription assays (36). Although our mutant set was not designed to test these interactions, the +141 to +144 motif is deleted in mutant dL, which shows a 3-fold defect in reverse transcription. The interaction between the PAS and the TΨC arm of $tRNA_3^{Lys}$ seems more important because deletion of the PAS motif results in a 10-fold inhibition. However, other vRNA-tRNA contacts, in addition to the PBS-anti-PBS and PAS-anti-PAS interactions, may contribute to the efficient initiation of reverse transcription. The critical role of the PAS element in tRNA usage is further supported by recent experiments in our laboratory. By simultaneous alteration of the PAS and PBS motifs to accommodate another tRNA molecule, we could modulate the identity of the priming tRNA species.[2] Furthermore, the PAS motif is absolutely conserved among all HIV-1 isolates.

The RNA secondary structure probing experiments performed in this study suggest an alternative folding of the upper part of the U5 leader stem (segment 1) and the U5-PBS hairpin to form the U5-top hairpin (Fig. 8). Nucleotides A^{168}–A^{171} that form the A-rich loop of the U5-PBS hairpin are present in an internal loop of the U5-top hairpin. This RNA secondary structure model was proposed previously (16, 17) and is very similar to the RNA structure model for HIV-2 (20). In this modified RNA structure model, the PAS and PBS sequences are juxtaposed, which may facilitate tRNA annealing to both motifs to trigger initiation of reverse transcription. It remains possible that both RNA conformations play a role in specific stages of the viral life cycle. For instance, there is some phylogenetic support for the U5-PBS hairpin (20, 23), and alternative RNA conformations have been reported to exist for other domains of the HIV-1 leader RNA (18, 19). In a previous study, we designed mutations in the HIV-1 leader RNA to specifically stabilize or destabilize the U5-PBS hairpin (11). Destabilization of the U5-PBS hairpin affects virus replication and the correct

[2] N. Beerens and B. Berkhout, manuscript in preparation.

placement of the tRNA primer onto the PBS. However, these mutations also destabilize the U5-top hairpin conformation. Stabilization of the U5-PBS hairpin affects virus replication and inhibits tRNA annealing onto the PBS. Fast replicating revertant viruses were selected with additional mutations that reduce the stability of the modified U5-PBS hairpin and, thus, may restore folding of the U5-top hairpin. Another complicating factor is that the region upstream of the PBS encodes a critical sequence element that is recognized as part of the proviral DNA by the viral integrase protein (37, 38). Further studies are required to provide more insight into the function of the U5-PBS and/or U5-top hairpin conformations.

A surprising result of this study is that the wild-type HIV-1 RNA template appears sub-optimal for *in vitro* reverse transcription, as the initiation step is actively suppressed by RNA secondary structure. We propose that this mechanism may preclude premature reverse transcription in the virus-producing cell such that the viral RNA genome is copied only after it is appropriately dimerized and packaged into virions. This mechanism would rigidly limit reverse transcription to the correct template and, hence, protect the host cell from potentially deleterious unrestricted reverse transcription (39). Replication studies with the wild-type and mutant viruses do suggest that this restriction of reverse transcription is also beneficial for virus replication. The mutations d2 and 2R stimulate reverse transcription *in vitro* but impair virus replication. We are currently testing whether these mutations do also activate reverse transcription *in vivo* in virus-producing cells and virion particles. A complicating factor is that these mutations may also affect other steps in viral replication, thereby causing a replication defect. The U5 leader region has been reported to encode signals important for viral replication (40, 41). For instance, this region was reported to encode DNA binding sites for transcription factors as part of the proviral LTR promoter (42–45). We therefore measured the effect of the mutations on viral gene expression by measuring virus production in transiently transfected cells. Most mutations did not affect transcription, translation, and virion assembly, except for mutations d2 and 2R, which reduced virus production 4- and 2-fold, respectively (results not shown). This production defect will also contribute to the observed replication defect of these mutants, which makes it difficult to extrapolate the defect that may be due to premature reverse transcription.

Occlusion of the PAS motif by base pairing may provide a mechanism to restrict early reverse transcription. Although binding of $tRNA_3^{Lys}$ to the PBS can occur relatively early, *e.g.* in virus-producing cells, primer activation will require a structural rearrangement of the vRNA-tRNA complex to establish the PAS-anti-PAS interaction. It is possible that the viral nucleocapsid (NC) protein, which acts as an RNA chaperone (reviewed in Ref. 46), can mediate this conformational change. Because NC is only released from the Gag precursor protein during maturation of virion particles, this mechanism will ensure the precise timing for activation of reverse transcription. We recently described a conformational switch in the HIV-1 leader RNA, which is mediated by NC (19). An extended form of the U5 leader hairpin was shown to mask the downstream RNA dimerization initiation signal, which can subsequently be exposed by NC. This raises the interesting possibility that a single RNA structure in the HIV-1 genome is used to mask the "late" RNA signals that control dimerization and primer activation. This mechanism will also ensure that both signals are activated simultaneously by the NC protein during virion assembly.

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