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Switching the in vitro tRNA usage of HIV-1 by simultaneous adaptation of the PBS and PAS

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ABSTRACT

Reverse transcription of the HIV-1 RNA genome is primed by the cellular $tRNA^{lys3}$ molecule that anneals to a complementary sequence in the viral genome, the primer-binding site (PBS). Additional interactions between the tRNA primer and the viral RNA were proposed to play a role in reverse transcription. We recently identified an 8-nt element in the U5 region upstream of the PBS that is critical for initiation and processive elongation of reverse transcription. This motif was termed the primer activation signal (PAS), and is proposed to interact with the "antiPAS sequence" in the TΨC arm of $tRNA^{lys3}$. In this study, we demonstrate that the efficiency of initiation of reverse transcription can be modulated by PAS mutations that strengthen or weaken the interaction with antiPAS. These results provide further evidence for a direct base-pairing interaction between the PAS in the viral RNA and the antiPAS in the $tRNA^{lys3}$ molecule. A broad phylogenetic survey indicated that a PAS element is present in all retroviral RNA genomes, suggesting that the process of reverse transcription is regulated by a common mechanism in all retroviridae. It has proven very difficult to change the identity of the tRNA primer for HIV-1 reverse transcription by changing the PBS sequence. Using in vitro reverse transcription assays, we demonstrate that the identity of the priming tRNA species can be switched by simultaneous alteration of the PBS and PAS motifs to accommodate a new tRNA primer. These results indicate that the PAS–antiPAS interaction is important for both primer selection and efficient reverse transcription.

Keywords: HIV-1; primer activation signal; reverse transcription; tRNA primer; tRNA usage and selection; U5-leader stem; viral RNA-tRNA interaction

INTRODUCTION

The replication cycle of retroviruses is characterized by reverse transcription of the viral RNA genome into double-stranded DNA, which subsequently integrates into the host cell genome. This process is mediated by the viral reverse transcriptase (RT) enzyme and a specific cellular tRNA molecule is used as primer. The tRNA primer binds with its 3′-terminal 18 nt to a complementary sequence, the primer-binding site (PBS), which is located in the 5′-untranslated leader region of the viral RNA genome. Reverse transcription of the retroviral RNA genome is a highly specific process that is regulated by multiple interactions between the viral RNA, the tRNA primer, and the RT enzyme. Several levels of specificity restrict aberrant reverse transcription from non-self tRNA primers. The self-tRNA primer is selectively packaged into virions (Peters et al., 1977; Waters & Mullin, 1977; Levin & Seidman, 1979; Huang et al., 1994; Mak et al., 1994; Liang et al., 1997b; Mak & Kleiman, 1997), and self-primers are favored over non-self-primers by the viral RT enzyme (Li et al., 1994; Das et al., 1995; Wakefield et al., 1995; Oude Essink et al., 1996). Besides the tRNA–PBS interaction, additional interactions between the tRNA primer and the viral RNA (vRNA) were proposed to play a role in reverse transcription (Murphy & Goff, 1989; Aiyar et al., 1992; Isel et al., 1995; Liang et al., 1997a; Miller et al., 2001).

HIV-1 uses $tRNA^{lys3}$ as primer for reverse transcription. We recently identified an 8-nt motif in the U5 region of the untranslated leader RNA that stimulates $tRNA^{lys3}$-mediated initiation and processive elongation of reverse transcription in HIV-1 (Fig. 1; Beerens et al., 2001; Beerens & Berkhout, 2002). This U5 motif is not required for tRNA annealing, but rather for activation of the PBS-bound tRNA primer to initiate reverse transcription, and is therefore referred to as primer activation signal (PAS). Mutation of the PAS element severely impairs reverse transcription that is initiated from the tRNA primer, but not from DNA oligonucleotide primers.

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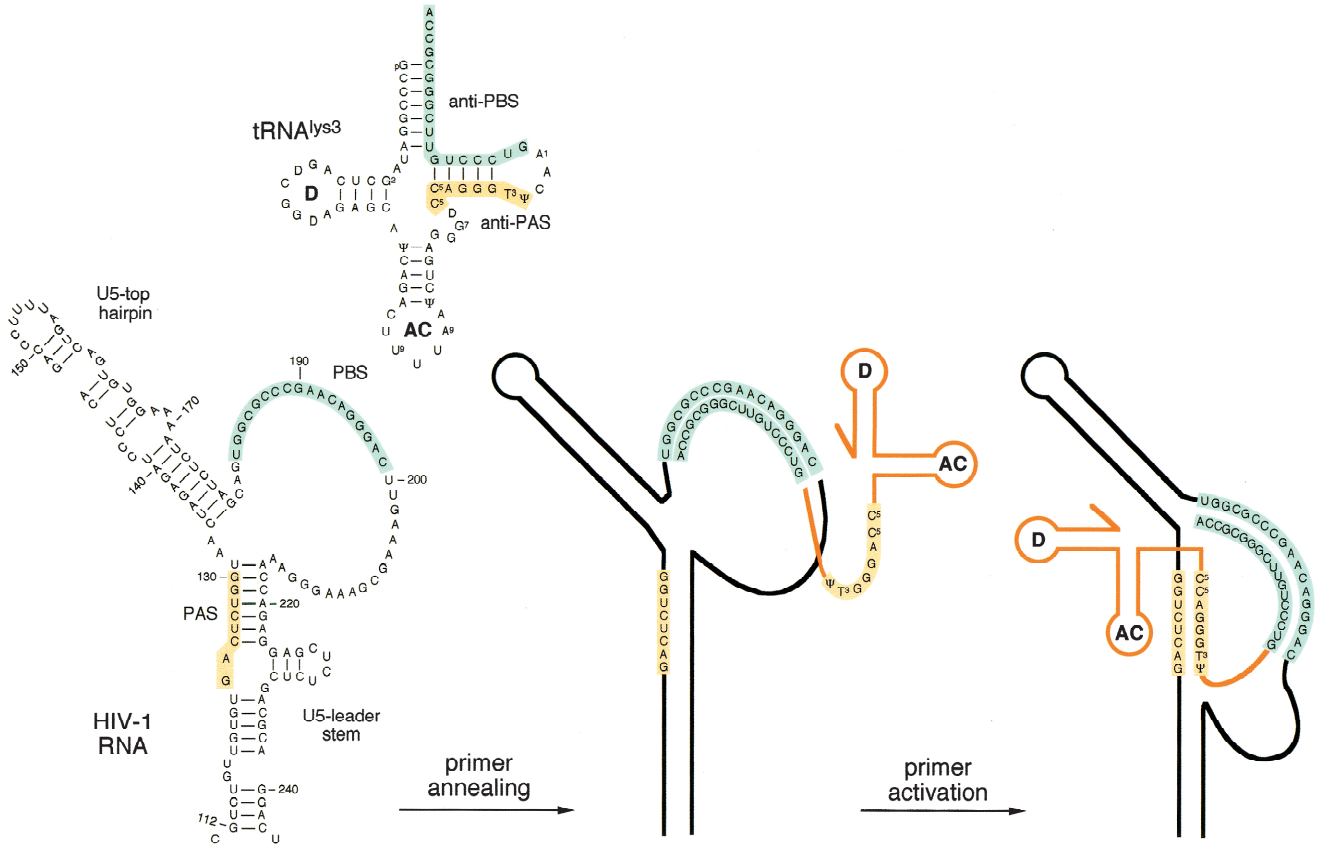


FIGURE 1. Mechanistic model of HIV-1 reverse transcription. Shown is the proposed secondary structure in the PBS region of the HIV-1 RNA genome and the $tRNA^{lys3}$ molecule (AC: anticodon loop, D: D loop). The HIV-1 RNA folds the U5-top hairpin just upstream of the PBS, and the U5-leader stem. The $tRNA^{lys3}$ molecule is used as a primer for reverse transcription by HIV-1 and binds to the PBS in the viral genome. The PBS and the complementary antiPBS sequence in the tRNA primer are marked in green. An additional interaction between the PAS element in the vRNA and the complementary antiPAS sequence in the tRNA primer (both marked in orange) is required to activate the PBS-bound tRNA primer for reverse transcription (Beerens et al., 2001; Beerens & Berkhout, 2002).

The PAS element thus modulates the efficiency of reverse transcription, although this motif is not absolutely essential for initiation of reverse transcription. We proposed that the PAS interacts with the antiPAS motif in the TΨC arm of $tRNA^{lys3}$ (Fig. 1, both motifs are marked in orange), similar to the interaction proposed for the Rous sarcoma virus (RSV) genome and the corresponding $tRNA^{trp}$ primer (Cobrinik et al., 1988, 1991; Aiyar et al., 1992, 1994; Leis et al., 1993; Miller et al., 1997; Morris & Leis, 1999). This interaction was also previously proposed for HIV-2 (Berkhout & Schoneveld, 1993), and recently confirmed by biochemical probing experiments (Freund et al., 2001). Thus, a common mechanism for the regulation of initiation of reverse transcription may be operating for different retroviruses. Interestingly, the HIV-1 PAS motif is occluded by base pairing in the U5-leader stem (Fig. 1), and we demonstrated that reverse transcription is greatly up-regulated by exposure of the PAS through mutation of the "opposing" leader sequence. The presence of the PAS enhancer element in a repressive RNA structure may provide a mechanism to regulate HIV-1 reverse transcription (Beerens et al., 2001).

Other interactions between the HIV-1 RNA and $tRNA^{lys3}$ have been proposed based mainly on biochemical probing studies (Isel et al., 1995, 1996, 1998, 1999; Lanchy et al., 2000). The A-rich loop (A168–A171) in the vRNA may interact with the U-rich anticodon loop of the tRNA primer (U33–U36), and the 3′ portion of the anticodon stem of the tRNA molecule may interact with vRNA sequences in the U5 region. Mutation of the A-rich loop affects initiation and elongation of reverse transcription (Isel et al., 1996; Li et al., 1997; Liang et al., 1997a, 1998; Zhang et al., 1998; Lanchy et al., 2000). Deletion of the A-rich loop also affects virus replication, and the A-rich sequence is restored upon long-term culturing (Liang et al., 1997a). However, the presence of overlapping sequence motifs for integration of the viral DNA genome complicates the interpretation of these results (Vicenzi et al., 1994; Esposito & Craigie, 1998; Brown et al., 1999). Other members of the lentivirus genus

that utilize $tRNA^{lys3}$ as primer do not possess an A-rich loop in the region 5′ to the PBS, suggesting that the putative A-loop interaction, unlike the PAS interaction, is not conserved among retroviruses.

In this study, we introduced mutations in the HIV-1 PAS element to strengthen or weaken the interaction with the antiPAS sequence in $tRNA^{lys3}$. We demonstrate that the efficiency of in vitro reverse transcription correlates with the strength of the PAS–antiPAS interaction. Modified nucleotides in the tRNA molecule appear not important for the PAS–antiPAS interaction. We also demonstrate that the identity of the priming tRNA species can be modulated in vitro by simultaneous alteration of the PBS and PAS motifs. These results demonstrate that the PAS–antiPAS interaction is important for primer selection and reverse transcription. Phylogenetic analysis indicates that a similar PAS–antiPAS interaction is possible for all retroviruses, suggesting that the underlying mechanism to regulate reverse transcription has been conserved during evolution of the retrovirus family.

RESULTS AND DISCUSSION

Modulation of the PAS–antiPAS interaction

To critically test the role of the HIV-1 PAS element in reverse transcription, we introduced mutations in the vRNA template that strengthen or weaken the interaction with the antiPAS motif in the $tRNA^{lys3}$ primer (Fig. 2). In mutant N1, we substituted the U at position 126 by C, thus replacing the U-G base pair in the putative PAS–antiPAS interaction by a more stable C-G base pair. In mutant N2, the C residues at position 125 and 127 were changed into U residues, thus replacing two C-G base pairs by weaker U-G base pairs. In mutant N3, 2 nt were inserted at the 3′ end of the 8-nt PAS motif to obtain 10-nt complementarity to the antiPAS in $tRNA^{lys3}$. In mutant N4, 2 nt were inserted near the 5′ end of the PAS to extend the interaction with the tRNA primer with 1 nt, and to replace the G123-Ψ base pair with the more stable A123-Ψ base pair (Ψ = pseudouridine). Thus, we designed three vRNA mutants with an optimized PAS–antiPAS interaction (N1, N3, and N4) and one mutant template with decreased interaction potential (N2). The number of hypothetical base pairs and hydrogen bonds and the thermodynamic stability of the PAS–antiPAS duplex is indicated in Figure 2. M-fold analysis demonstrated that the mutations do not affect the overall structure of the PBS region, although the mutations destabilize base pairing in the upper part of the U5-leader stem.

anti-PAS

$tRNA^{lys3}$	A C Ψ T G G G A C C D G	PAS-antiPAS interaction		
HIV-1 RNA	PAS	bp	H	ΔG
wt	121 G U G A C U C U G G U A 132	8	18	- 9.1
N1	G U G A C C C U G G U A	8	19	-11.6
N2	G U G A U U U U G G U A	8	16	- 4.3
N3	G U G A C U C U G G A C U A	10	23	-16.7
N4	G U G G A A C U C U G G U A	9	21	-12.2

FIGURE 2. Design of the HIV-1 PAS mutants. The PAS is marked by an open box and mutations are marked in gray. For reference, the antiPAS sequence of the tRNA is shown on top (marked by a box). Mutants N1, N2, N3, and N4 strengthen or weaken the interaction with the antiPAS. Indicated are the number of base pairs (bp) and the number of hydrogen bonds (H) of the PAS–antiPAS duplex, and the thermodynamic stability of the PAS–antiPAS interaction (ΔG in kilocalories per mole).

Reverse transcription is modulated by the strength of the PAS–antiPAS interaction

We performed in vitro reverse transcription assays to study the effect of the PAS mutations. These assays were performed with in vitro-transcribed wild-type and PAS-mutated HIV-1 templates and the natural $tRNA^{lys3}$ primer or DNA primers to initiate reverse transcription. The primers were heat annealed onto the different RNA templates and reverse transcription was initiated by addition of dNTPs and HIV-1 RT enzyme. A representative experiment is shown in Figure 3. Full-length reverse transcription products were quantitated and corrected for the amount of input RNA template as determined with the DNA primer AUG (Fig. 3, lanes 1–5). The results of three independent assays were quantitated and are summarized in Figure 4.

Extension of the DNA primer lys21 that is complementary to the PBS results in a 98-nt full-length cDNA product on the wild-type and mutant N1 and N2 templates (Fig. 3, lanes 6–8). The cDNA product of the N3 and N4 templates has a length of 100 nt due to the 2-nt insertion in the PAS element (Fig. 3, lanes 9 and 10). The efficiency of DNA-primed reverse transcription is similar for all templates (Fig. 4A). Extension of the natural $tRNA^{lys3}$ primer results in a 153-nt cDNA product on the wild-type template (Fig. 3, lane 11). tRNA-primed reverse transcription was enhanced fourfold on the N1 template with an improved PAS–antiPAS interaction (Fig. 3, lane 12, and Fig. 4B), and reverse transcription was reduced fivefold on the N2 template with a weakened interaction (Fig. 3, lane 13, and Fig. 4B). Mutant N4 that extends the PAS–antiPAS interaction with 1 bp enhanced reverse transcription 3.5-fold (Fig. 3, lane 15, and Fig. 4B), but reverse transcription was reduced fourfold on the N3 template that extends the PAS–antiPAS interaction with 2 bp (Fig. 3, lane 14, and Fig. 4B).

We previously demonstrated that the PAS functions specifically to enhance the initiation of reverse transcription from the $tRNA^{lys3}$ primer. We therefore per-

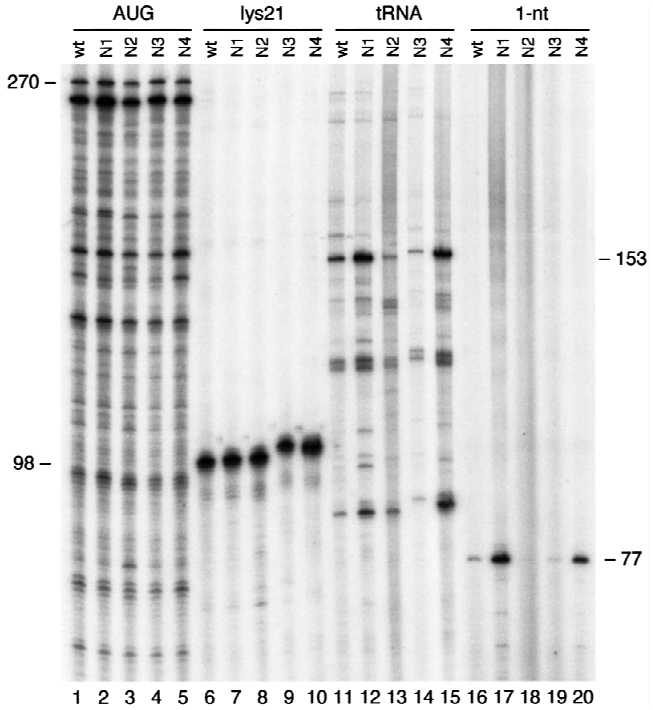


FIGURE 3. Reverse transcription assays on wild-type and PAS-mutated RNA templates. The DNA and $tRNA^{lys3}$-primers were heat annealed onto the HIV-1 RNA templates (positions +105 to +368) and extended by the HIV-1 RT enzyme in the presence of all dNTPs (lanes 1–15). The amount of input viral RNA was quantitated by DNA-primer extension with the AUG primer that produces a 270-nt product (lanes 1–5). Extension of lys21 results in a 98-nt cDNA product (lanes 6–10) and the tRNA primer produces a 153-nt tRNA-cDNA product (lanes 11–15). These sizes refer to reactions with the wild-type, N1, and N2 templates, and are longer for the N3 and N4 templates due to the 2-nt insertion in the PAS element. Several shorter cDNA products are visible that result from RT pausing. We also performed 1-nt incorporation assays with the $tRNA^{lys3}$ primer (lanes 16–20). The extension of the 76-nt tRNA by dCTP produces a radiolabeled 77-nt product.

formed initiation assays, in which reverse transcription was performed in the presence of ^{32}P-dCTP, but without the other dNTPs. This results in extension of the 76-nt $tRNA^{lys3}$ primer with 1 nt (Fig. 3, lanes 16–20). The phenotype of the mutant templates in this initiation assay is similar to that in tRNA-primed full-length cDNA synthesis. Reduced initiation was observed for the N2 and N3 templates, whereas enhanced initiation was measured for the N1 and N4 templates (Fig. 4C). These results suggest that the initiation efficiency of reverse transcription is determined by the strength of the PAS–antiPAS interaction. The point mutations introduced in the PAS element will not only affect the PAS–antiPAS interaction, but also base pairing of the PAS in the U5-leader stem. We previously showed that reverse transcription is greatly up-regulated by exposure of the PAS through mutation of the "opposing" leader sequence. Thus, the stimulation observed for the mutants N1 and N4 may be partially due to destabilization of the U5-leader stem. However, mutation N2 that weakens the PAS–antiPAS interaction also weakens the base pairing of the PAS in the U5-leader stem, and was found to reduce reverse transcription. This suggests that the effects on reverse transcription are mainly caused by modulation of the strength of the PAS–antiPAS interaction.

We plotted the tRNA-primed reverse transcription activity versus the thermodynamic stability of the PAS–antiPAS duplexes (Fig. 4D). Weakening of the wild-type PAS–antiPAS duplex reduces the reverse transcription activity (N2) and stabilization enhances the reverse transcription activity (N1 and N4). However, further stabilization of the PAS interaction appears to inhibit reverse transcription (N3). Thus, there seems to be an optimum in the stability of the PAS–antiPAS interaction, and reverse transcription may be inhibited if the duplex gets too stable. An excessively stable PAS–antiPAS interaction may interfere with the correct assembly and/or maturation of the tRNA-vRNA-RT initiation complex. These combined results provide further evidence for a direct base-pairing interaction between the PAS in the viral RNA and the antiPAS in the $tRNA^{lys3}$ molecule.

Modified $tRNA^{lys3}$ nucleotides are not required for PAS-mediated activation

The additional interaction between the A-rich loop in the HIV-1 vRNA and the $tRNA^{lys3}$ primer has been reported to depend on modified nucleotides within the anticodon loop of $tRNA^{lys3}$ (Isel et al., 1996). The anti-PAS motif in $tRNA^{lys3}$ does also contain several modified nucleotides (Fig. 5A). We therefore studied the requirement of modified nucleotides for PAS-mediated activation of reverse transcription. We compared the natural $tRNA^{lys3}$ primer and an in vitro synthesized $tRNA^{lys3}$ transcript as primer in reverse transcription reactions. Reverse transcription was performed on the wild-type template and a control template with a mutated PAS motif (2L template, Fig. 5B). The PAS–antiPAS interaction for these two vRNA templates and the natural and synthetic tRNA primer is shown in Figure 5B. The natural and synthetic $tRNA^{lys3}$ primers were heat annealed onto the RNA templates and reverse transcription was initiated by addition of dNTPs and HIV-1 RT enzyme. Extension of the natural tRNA primer on the wild-type template results in a profound 257-nt full-length tRNA-cDNA product (Fig. 6, lane 2). As expected, reverse transcription was reduced 10-fold on the PAS-minus template 2L (Fig. 6, lane 8). Because reverse transcription is less efficiently initiated from synthetic tRNA primers (Barat et al., 1991; Arts et al., 1996), we used five times more RNA template (50 ng) and two amounts of synthetic tRNA primer (0.5 and 1 μg). Reverse transcription primed by synthetic tRNA was also reduced 10-fold for the 2L template (Fig. 6, lanes 9 and 10) compared to reverse transcription on the wild-type

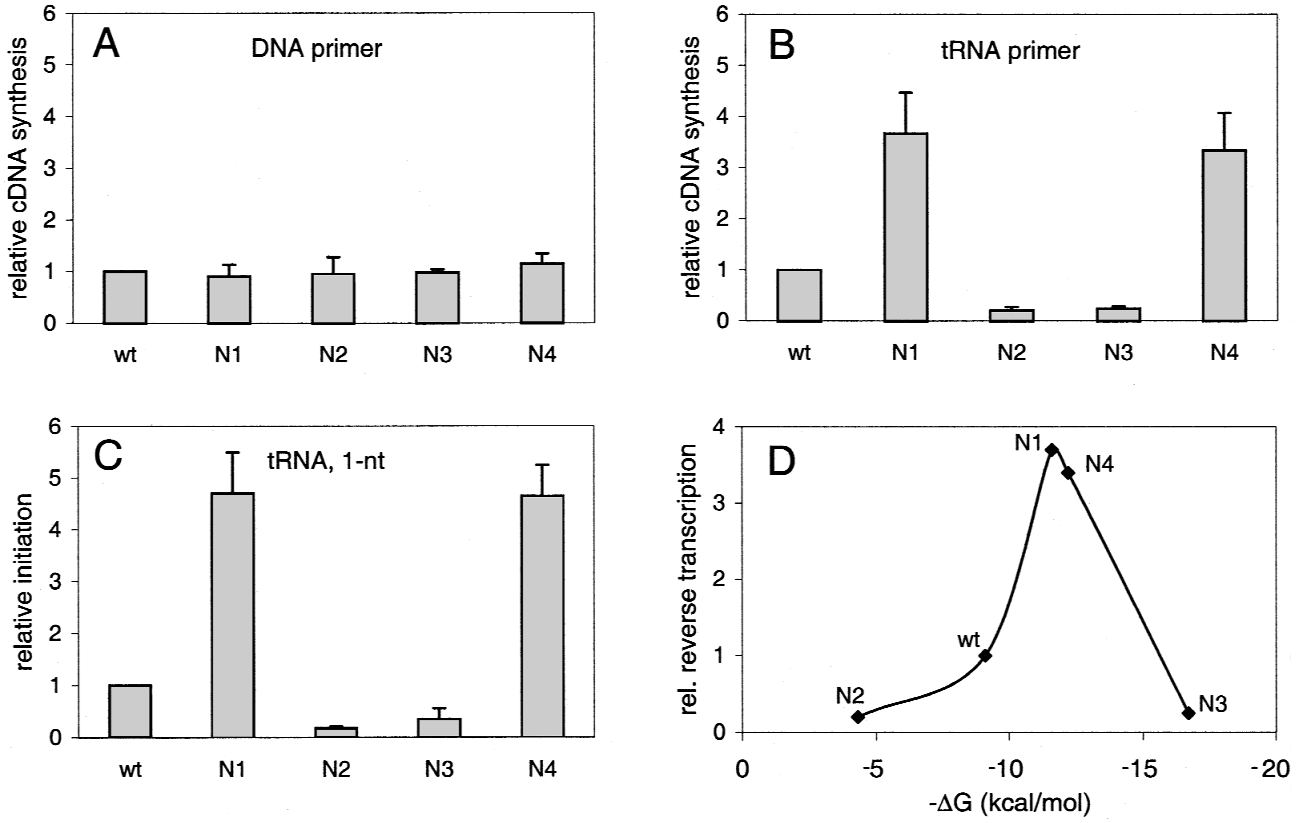


FIGURE 4. Relative reverse transcription activities of wild-type and PAS mutant templates. The results of three independent experiments were quantitated and all values were corrected for variation in the input RNA as measured with the AUG primer. The activity of the wild-type template was arbitrarily set at 1. **A**: DNA-primed reverse transcription with the lys21 primer. **B**: tRNA-primed reverse transcription. **C**: tRNA-primed 1-nt incorporation. **D**: We plotted the tRNA-primed reverse transcription activity (from **B**) versus the thermodynamic stability (ΔG) of the PAS–antiPAS interaction.

template (Fig. 6, lanes 3 and 4). Thus, reverse transcription primed by natural and synthetic $tRNA^{lys3}$ primers is stimulated by the PAS mechanism, indicating that the PAS–antiPAS interaction does not depend on modified nucleotides within the $tRNA^{lys3}$ molecule. However, we measured reduced reverse transcription activity with the synthetic tRNA primer as compared to the natural primer. This suggests that modified nucleotides, although not critical for the PAS–antiPAS interaction, do contribute to optimal reverse transcription.

Mutation of the PAS cannot be complemented by adaptation of the antiPAS

The finding that synthetic $tRNA^{lys3}$ can be used to study the PAS-mediated enhancement of reverse transcription allowed us to test a mutant form of the $tRNA^{lys3}$ primer. To study whether mutation 2L in the PAS can be complemented by a corresponding change in the antiPAS, we made the synthetic 2L-$tRNA^{lys3}$ primer (Fig. 5A,B). Reverse transcription on the 2L template was indeed enhanced twofold for the mutant 2L-$tRNA^{lys3}$ compared with the wild-type $tRNA^{lys3}$ (Fig. 6, lanes 11–12 and 9–10, respectively). However, the same effect was observed on the wild-type template (Fig. 6, lanes 5–6 and 3–4, respectively). This result indicates that the mutant 2L-$tRNA^{lys3}$ is a better primer on both templates. Thus, it seems that the PAS–antiPAS interaction cannot simply be replaced by another set of complementary sequences. The observation that the mutant 2L-$tRNA^{lys3}$ has improved priming activity may be due to opening of the TΨC stem, which liberates part of the antiPBS sequence for interaction with the PBS in the vRNA template (Fig. 5A).

Switch to usage of a non-self tRNA primer by simultaneous adaptation of the PBS and PAS motifs

It has proven very difficult to change the identity of the tRNA primer for reverse transcription by changing the HIV-1 PBS sequence. Such virus mutants show delayed replication kinetics and rapidly revert to the wild-type PBS-lys3 sequence (Li et al., 1994; Wakefield et al., 1994; Das et al., 1995). This result indicates that the PBS is not the only viral determinant of tRNA usage.

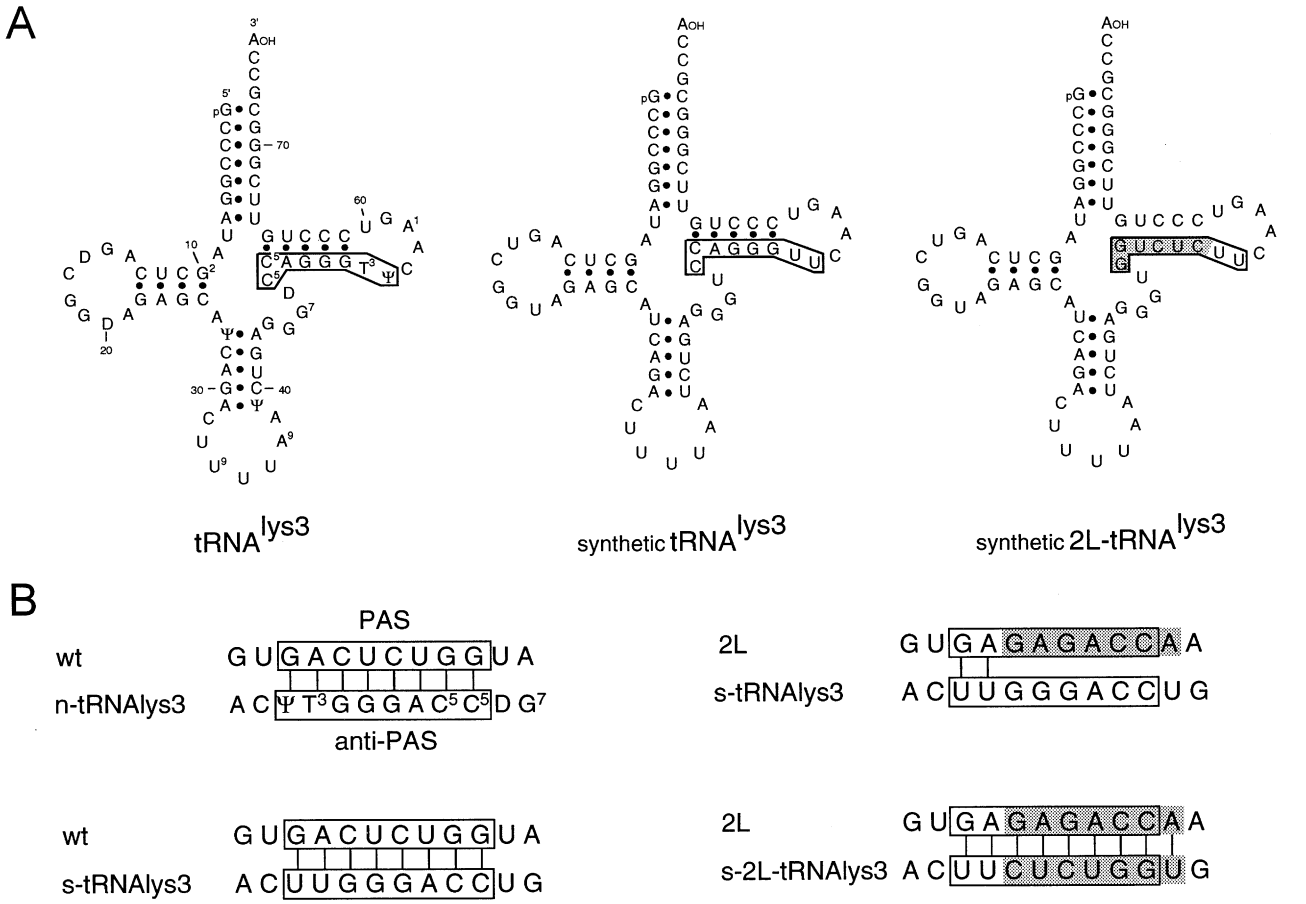


FIGURE 5. Interaction between vRNA templates and the natural or synthetic tRNA^{lys3} molecules. **A**: The cloverleaf structures of the natural tRNA^{lys3} and the synthetic tRNA^{lys3} and the mutant 2L-tRNA^{lys3}. Base modifications are indicated according to standard nomenclature (Sprinzl et al., 1989). The synthetic tRNA^{lys3} molecule does not contain modified bases, but was shown to adopt the correct secondary cloverleaf structure and L-shaped tertiary structure (Wohrl et al., 1993). The antiPAS motif is marked by an open box, and the mutations introduced in 2L-tRNA^{lys3} are marked in gray. **B**: The PAS–antiPAS interaction of the wild-type vRNA template with natural (n) and synthetic (s) tRNA^{lys3} is shown on the left. The interaction of the mutant 2L vRNA template with the synthetic tRNA^{lys3} and the complementary 2L-tRNA^{lys3} is shown on the right. The PAS and antiPAS elements are marked by an open box, the 2L mutation in the vRNA template and in the 2L-tRNA^{lys3} primer is marked in gray.

Additional vRNA–tRNA interactions may contribute to tRNA primer selection. Recently HIV-1 variants that use alternative tRNA primers were described. These viruses have sequence changes in both the PBS and the A-rich loop (Kang et al., 1996, 1997, 1999; Wakefield et al., 1996), although various PBS mutants could not be stabilized in this manner (Kang et al., 1996). Because the PAS is involved in tRNA primer recognition, we made a transcript in which both the PBS and PAS were replaced by sequences complementary to the non-self tRNA^{lys1} and tRNA^{lys2} molecules (Fig. 7B). These tRNAs have identical antiPBS and antiPAS sequences, and will be referred to as tRNA^{lys1,2} (Fig. 7A). As a control we used the PBS-lys1,2 mutant without PAS adaptation (Fig. 7B).

Three concentrations of the wild-type, PBS-lys1,2, and PBS/PAS-lys1,2 templates were incubated at 85 °C with a calf-liver tRNA preparation that contains tRNA^{lys3}, tRNA^{lys1,2}, and all other tRNA species. Reverse transcription was initiated from the annealed tRNA primer by addition of ^{32}P-dCTP and HIV-1 RT enzyme, resulting in the extension of the tRNA primer with 1 nt. The radiolabeled tRNA^{lys3}-cDNA product runs at a higher position on the denaturing gel than the tRNA^{lys1,2}-cDNA product due to different base modifications within the tRNA backbone (Das et al., 1995; Oude Essink et al., 1996). The wild-type template produces an intense tRNA^{lys3}-primed cDNA, and no tRNA^{lys1,2} signal is apparent (Fig. 8, lanes 7–9). The tRNA^{lys3} signal is significantly reduced on the PBS-lys1,2 template, and the tRNA^{lys1,2} signal is of approximately similar intensity (Fig. 8, lanes 1–3). The additional change of the PAS in the PBS-PAS-lys1,2 double mutant template markedly increased the tRNA^{lys1,2} signal, with a concomitant decrease of the tRNA^{lys3} signal (Fig. 8, lanes 4–6). The amount of input viral RNA was measured by DNA-primer extension with the AUG primer that produces a 270-nt product (Fig. 8, lanes 10–12). Quantitation of

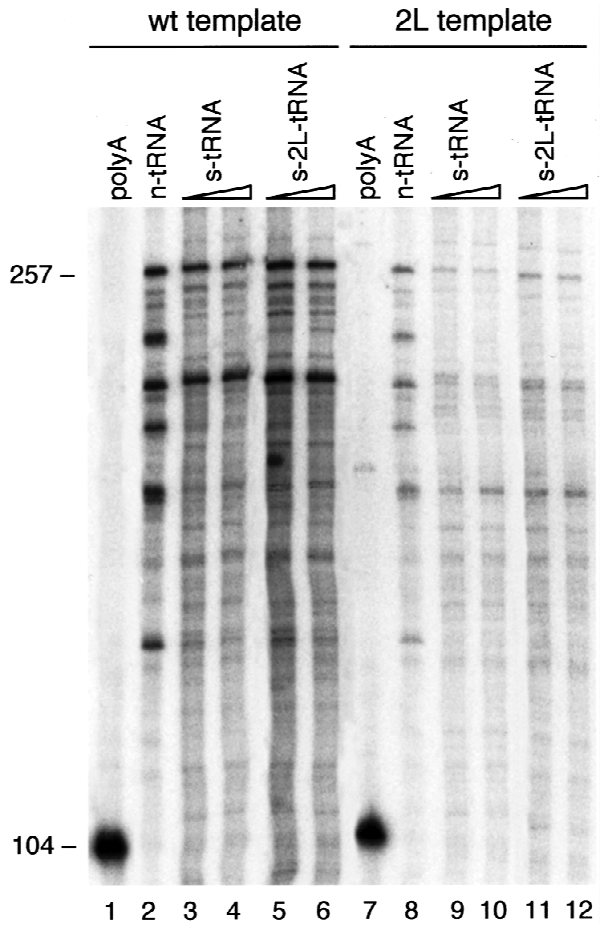


FIGURE 6. Reverse transcription primed by natural and synthetic $tRNA^{lys3}$. tRNA-primed reverse transcription on the wild-type HIV-1 template (lanes 2–6) was compared with that on the PAS-minus template 2L (lanes 8–12). The natural $tRNA^{lys3}$ (n-tRNA, lanes 2 and 8), synthetic $tRNA^{lys3}$ (s-tRNA, lanes 3–4 and 9–10), or mutant 2L-$tRNA^{lys3}$ (s-2L-tRNA, lanes 5–6 and 11–12) were heat annealed onto the RNA templates (positions +1 to +368) and extended by the HIV-1 RT enzyme in the presence of all dNTPs. We tested two amounts of synthetic tRNA primer (0.5 and 1 μg). Extension of the tRNA primers results in a 257-nt tRNA–cDNA product. Several shorter cDNA products are visible that result from RT pausing. 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 and 7).

the results indicates that the PAS adaptation enhances $tRNA^{lys1,2}$ usage approximately sixfold. Usage of the non-self $tRNA^{lys1,2}$ primer on the PBS-PAS-lys1,2 double mutant template is still relatively poor, with only 5% activity compared to usage of the self $tRNA^{lys3}$ primer on the wild-type template.

The current in vitro results demonstrate that the identity of the priming tRNA species can be switched by simultaneous alteration of the PBS and PAS motifs. However, the new $tRNA^{lys1,2}$ primer is used relatively inefficiently. This result reinforces the idea that additional viral factors are important for selection of the tRNA primer. Other interactions between the tRNA primer and the viral RNA template and/or the RT enzyme may be involved in tRNA primer selection. The RT enzyme is involved in placement of the tRNA primer onto the PBS (Mak et al., 1994; Oude Essink et al., 1996), and the HIV-1 RT enzyme is strongly committed to the self-$tRNA^{lys3}$ primer for initiation of reverse transcription (Oude Essink et al., 1996). Retrovirus particles contain a nonrandom subset of the cellular tRNA pool (Huang et al., 1994; Mak et al., 1994; Mak & Kleiman, 1997), and there is accumulating evidence that the priming tRNA species is selectively packaged by the viral RT protein as part of the Gag-Pol precursor protein (Sawyer & Hanafusa, 1979; Peters & Hu, 1980; Mak et al., 1994; Das & Berkhout, 1995; Oude Essink et al., 1995; Liang et al., 1997b). In addition, the viral NC protein has been implicated in placement of the tRNA onto the viral genome (De Rocquigny et al., 1992; Barat et al., 1993; Fu et al., 1997; Feng et al., 1999).

A similar PAS–antiPAS interaction can be proposed for all retrovirus genera

For HIV-1, a detailed vRNA–tRNA interaction model was proposed, based primarily on biochemical probing studies (Isel et al., 1995, 1996, 1998, 1999; Lanchy et al., 2000). However, some of the proposed interactions are possible only for the HIV-1 MAL isolate that was probed, and cannot be formed by other viral isolates, including the HIV-1 LAI isolate used in this study. The putative interaction between the A-rich loop (A168–A171) in the viral RNA and the U-rich anticodon loop of the $tRNA^{lys3}$ primer (U33–U36) has been studied extensively. Mutation of the A-rich loop affects initiation and elongation of reverse transcription (Isel et al., 1996; Li et al., 1997; Liang et al., 1997a, 1998; Zhang et al., 1998; Lanchy et al., 2000). Deletion of the A-rich loop affects virus replication, and the A-rich sequence is restored upon long-term culturing (Liang et al., 1997a). However, the presence of overlapping sequence motifs for integration of the viral DNA genome severely complicates the interpretation of these results (Vicenzi et al., 1994; Esposito & Craigie, 1998; Brown et al., 1999). Moreover, this interaction appears to be specific for HIV, as other members of the lentivirus genus that utilize $tRNA^{lys3}$ as primer do not possess an A-rich loop in the region 5′ to the PBS. This indicates that the "A-loop" interaction is not a general property of retroviruses.

In contrast to the A-rich loop interaction, the PAS–antiPAS interaction appears to be a widely conserved interaction. The nucleotide sequence of the PAS is highly conserved in all HIV-1 subtypes. Subtype O encodes a C at position 126, thus replacing a U-G base pair by a C-G base pair in the PAS–antiPAS interaction. The same U126C mutation in mutant N1 was found to stimulate initiation of reverse transcription in vitro. Interestingly, the subtype O virus has a complementary change in the "opposing" leader sequence that restores base pairing in the U5-leader stem and masking of the PAS motif. This natural base-pair covariation supports the

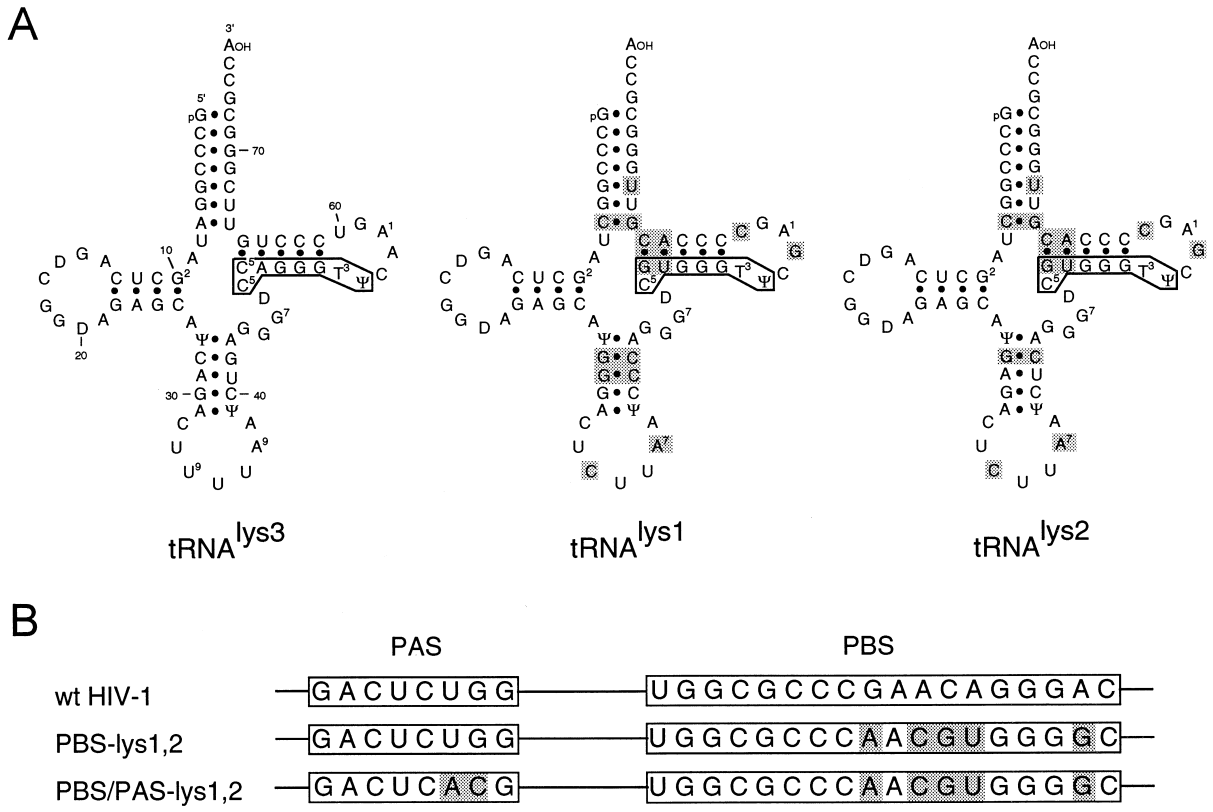


FIGURE 7. Secondary structure of alternative tRNA^{lys} primers and the PBS/PAS mutant HIV-1 template. **A**: Cloverleaf structures of the natural tRNA^{lys3}, tRNA^{lys1}, and tRNA^{lys2}. Base modifications are indicated according to standard nomenclature (Sprinzl et al., 1989). The antiPAS motif is marked by an open box; nucleotides that differ from tRNA^{lys3} are marked in gray. **B**: Schematic of the wild-type and mutant templates, in which the PBS or both the PBS and PAS are replaced by sequences that are complementary to tRNA^{lys1,2}. Nucleotides that differ from the wild-type sequence, which is complementary to tRNA^{lys3}, are marked in gray.

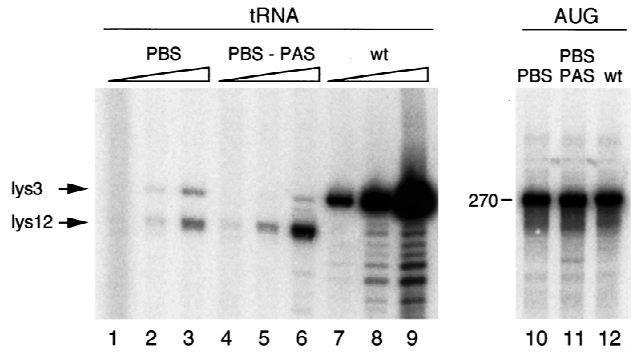


FIGURE 8. Switching the tRNA-usage of HIV-1. The PBS-lys1,2 (lanes 1–3) and PBS/PAS-lys1,2 (lanes 4–6) mutant templates, and the wild-type HIV-1 template (positions +105 to +368; lanes 7–9) were incubated with a calf-liver tRNA preparation that contains tRNA^{lys3}, tRNA^{lys1,2}, and all other tRNA species. We tested three amounts of template RNA (10, 50, and 250 ng). Reverse transcription was initiated from the annealed tRNA primer by addition of ^{32}P-dCTP and HIV-1 RT enzyme. This results in the extension of the tRNA primer with 1 nt. The tRNA^{lys3} product runs at a higher position in the gel than the tRNA^{lys1,2} product due to different base modification. The amount of input viral RNA was quantitated by DNA-primer extension with the AUG primer that produces a 270-nt product (lanes 10–12).

idea that the accessibility of the PAS motif, and thus the initiation of reverse transcription, is restricted by RNA secondary structure. Furthermore, a vRNA–tRNA contact similar to the HIV-1 PAS–antiPAS interaction was proposed previously for the Rous sarcoma virus (RSV) genome and the TΨC arm of the corresponding tRNA^{trp} primer (Cobrinik et al., 1988; Cobrinik et al., 1991; Aiyar et al., 1992, 1994; Leis et al., 1993; Miller et al., 1997; Morris & Leis, 1999). A similar interaction was also proposed for HIV-2 (Berkhout & Schoneveld, 1993; Freund et al., 2001). We performed an extensive phylogenetic analysis of different retrovirus genera, including retroviral species that use tRNA^{lys1,2} and tRNA^{pro} as a primer. This survey demonstrates that a PAS-like element is present in all retroviral RNA genomes (Fig. 9). The PAS elements are located in the U5 region upstream of the PBS. Interestingly, M-fold analysis of these retroviral sequences indicates that the PAS is usually base paired, either to the PBS or to other leader sequences. Thus, the PAS–antiPAS interaction has been conserved in evolution, despite diversity in tRNA usage of the different retroviruses. This mechanism to regu-

late reverse transcription may be more widely conserved, we could, for example, identify a PAS-like element in the gypsy retrotransposon that uses $tRNA^{arg}$ as primer. These combined results suggest that the process of reverse transcription is regulated by a common mechanism in all retroviridae.

CONCLUSIONS

Reverse transcription of the HIV-1 RNA genome appears a highly specific process that is regulated by interactions between the viral RNA, tRNA primer and the viral RT enzyme. We previously identified an 8-nt motif (positions +123 to +130), called PAS, that is important for $tRNA^{lys3}$-mediated initiation of HIV-1 reverse transcription (Beerens et al., 2001). Interestingly, the PAS element is occluded by base pairing in the U5-leader stem of the wild-type HIV-1 template. Reverse transcription can be activated by exposure of the PAS through mutation of the "opposing" leader sequence (Beerens et al., 2001) and reverse transcription can be inhibited by stabilizing mutations in the U5-leader stem (Beerens et al., 2000). The PAS motif is also important for in vivo reverse transcription and virus replication (Beerens & Berkhout, 2002). In this study, we provide further evidence for a direct base-pairing interaction between the PAS in the viral RNA and the antiPAS in the $tRNA^{lys3}$ molecule. We show that the efficiency of reverse transcription can be up- and down-regulated by mutations in the HIV-1 PAS element that strengthen or weaken the interaction with the antiPAS sequence in the tRNA primer. Thus, reverse transcription of the wild-type HIV-1 template appears restricted, both by imperfect complementarity of the PAS and antiPAS elements and by inclusion of the PAS in a repressive RNA secondary structure. 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. Although binding of $tRNA^{lys3}$ to the PBS may occur relatively early, for example, in the virus-producing cells, activation of the primer will require a structural rearrangement of the vRNA–tRNA complex to establish the PAS–antiPAS interaction. This conformational change may be facilitated by the viral nucleocapsid protein (NC), which acts as an RNA chaperone (Rein et al., 1998). Because NC is only released from the Gag precursor protein during virus maturation, this will ensure the proper timing for initiation of reverse transcription.

The additional PAS–antiPAS interaction may also increase the specificity of reverse transcription and restrict aberrant reverse transcription from non-self-primers. We demonstrate that the PAS motif is involved in selection of the tRNA primer for reverse transcription. The tRNA usage of HIV-1 can be switched from $tRNA^{lys3}$ to $tRNA^{lys1,2}$ by simultaneous alteration of the PBS and PAS motifs. However, reverse transcription primed by the non-self-$tRNA^{lys1,2}$ on the PBS-PAS double mutant template is relatively inefficient compared with usage of the self-$tRNA^{lys3}$ primer on the wild-type template. Therefore, additional viral RNA–tRNA interactions, including the previously discussed A-rich loop, and interactions between the viral RNA–tRNA complex and the RT and NC proteins may be required for efficient reverse transcription. It will be of interest to study the replication of viruses in which both the PBS and PAS motifs are altered to accommodate a new tRNA primer. These viruses may acquire adaptive changes in the viral RNA or the RT/NC proteins. The PAS-mechanism appears highly conserved in evolution, suggesting that the process of reverse transcription is regulated by a common mechanism in all retroviridae. Retroviruses may have evolved the additional PAS–antiPAS interaction to regulate initiation of reverse transcription and to increase the level of specificity of reverse transcription.

MATERIALS AND METHODS

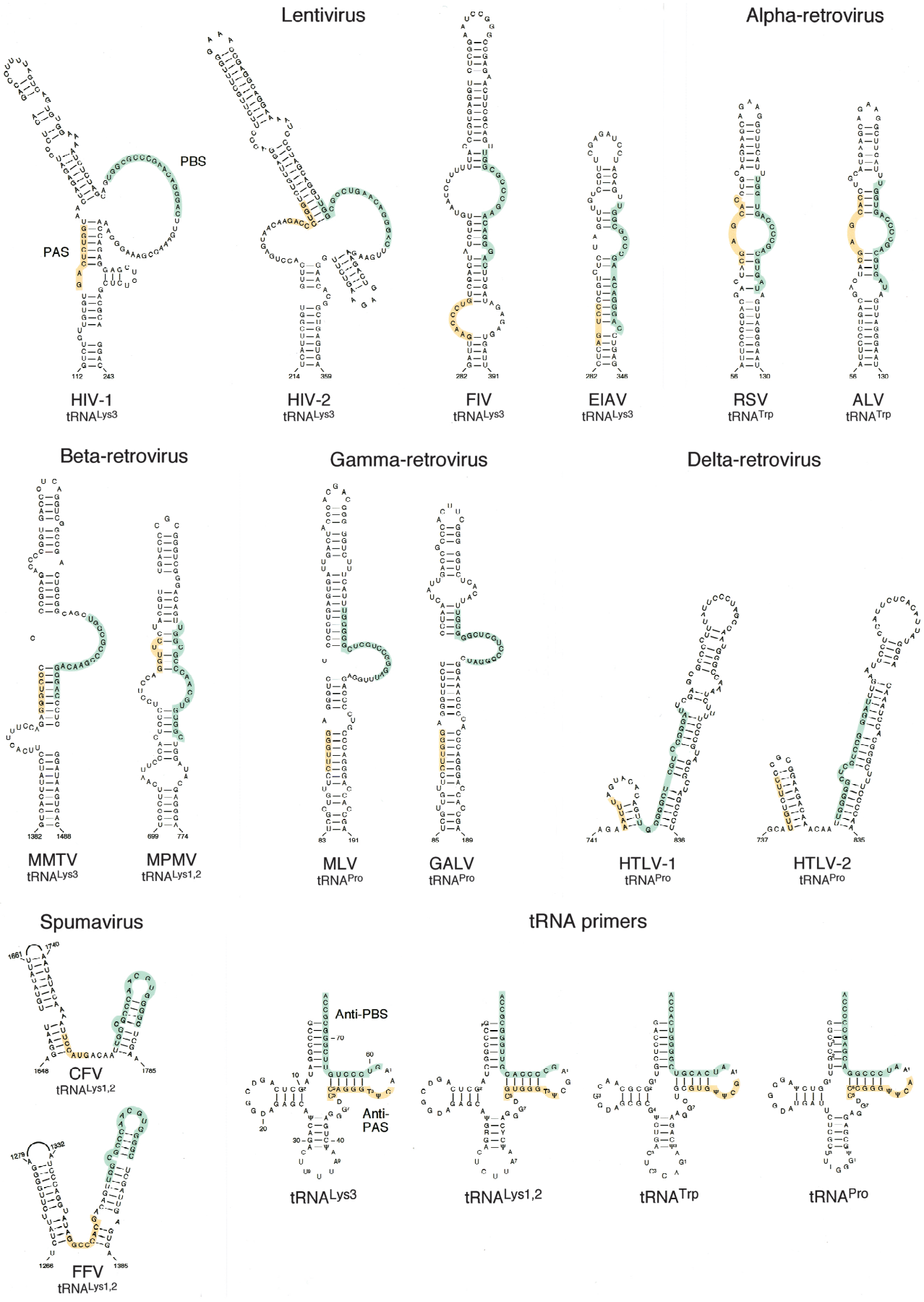
HIV-1 vRNA and tRNA constructs

HIV-1 templates

Mutations were introduced in the HIV-1 PAS element by PCR mutagenesis with the antisense primer AUG (positions +348 to +368) and the mutagenic sense primers T7-N1, T7-N2, T7-N3, or T7-N4 (positions +105 to +136, with 5′-flanking T7 RNA polymerase promoter sequence). The PCR reactions were performed on the HIV-1 pLAI construct. Nucleotide numbers refer to positions on HIV-1 genomic RNA, with +1 being the capped G residue. The PAS-minus template 2L was generated by PCR amplification of the construct pLAI(R37)-2L that was described previously (Beerens et al., 2001; Beerens & Berkhout, 2002) with the sense primer T7-2 (positions +1 to +20, with 5′-flanking T7 RNA polymerase promoter sequence) and the antisense primer AUG. The PBS-mutated template was generated by PCR amplification of the construct pLAI PBS-lys1,2 that was described previously (Das et al., 1995) with the sense primer T7-PBS loop (positions +105 to +125, with 5′-flanking T7 RNA polymerase promoter sequence) and the antisense primer AUG. The same pLAI PBS-lys1,2 construct was used to mutate the PAS to be complementary to $tRNA^{lys1,2}$ by PCR mutagenesis with the mutagenic sense primer PAS-lys1,2 (positions +105 to +136, with 5′-flanking T7 RNA polymerase promoter sequence) and the antisense primer AUG.

tRNA transcripts

To generate a template for the production of synthetic $tRNA^{lys3}$, we PCR amplified the tRNA gene from the pUC-$tRNA^{lys3}$ construct that was described previously (Oude Essink et al., 1995) with the sense primer 5′-T7-tRNA (tRNA positions +1 to +20) and the antisense primer 3′-tRNA (tRNA positions +54 to +76). This yields the complete tRNA from nt 1 to 76. The compensatory 2L mutation was introduced by PCR mu-


Lentivirus
Alpha-retrovirus
PBS
PAS
HIV-1
tRNALys3
HIV-2
tRNALys3
FIV
tRNALys3
EIAV
tRNALys3
RSV
tRNATrp
ALV
tRNATrp
Beta-retrovirus
Gamma-retrovirus
Delta-retrovirus
MMTV
tRNALys3
MPMV
tRNALys1,2
MLV
tRNAPro
GALV
tRNAPro
HTLV-1
tRNAPro
HTLV-2
tRNAPro
Spumavirus
tRNA primers
CFV
tRNALys1,2
FFV
tRNALys1,2
Anti-PBS
Anti-PAS
tRNALys3
tRNALys1,2
tRNATrp
tRNAPro

tagenesis with the mutagenic antisense primer 3′-2L-tRNA (tRNA positions +39 to +76) and the sense 5′-T7-tRNA primer. The introduction of the mutations in the vRNA and tRNA sequence was verified by sequence analysis with the DYEnamic™ Direct cycle sequencing kit (Amersham) and an Applied Biosystems 377 DNA sequencer. The PCR products were used directly for T7-transcription.

Synthesis of RNA templates

In vitro transcription was performed on the PCR fragments with the T7-MegaShortscript kit (Ambion). Upon DNase treatment, the transcripts were purified on 4% denaturing polyacrylamide gels and visualized by UV shadowing. Transcripts were eluted from the gel by overnight incubation in TE buffer, ethanol-precipitated and redissolved 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. The RNA concentration was measured by UV absorbance measurement, and transcripts were stored at −20 °C.

Reverse transcription assays

The in vitro-synthesized vRNA template (10 ng) was incubated either with 1.5 μg calf liver tRNA (6 pmol total tRNA, of which approximately 1.2 pmol $tRNA^{lys3}$, Roche), 0.5 or 1 μg of synthetic $tRNA^{lys3}$, or 20 ng DNA primer in 12 μL 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. The primer was extended with 1 nt by addition of 6 μL RT(−) buffer (9 mM $MgCl_2$, 30 mM DTT, 150 μg/mL actinomycine D), 1 μL [α-^{32}P]-dCTP, and 0.5 U HIV-1 RT (MRC). 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 [α-^{32}P]-dCTP, and 0.5 U HIV-1 RT. The cDNA products were precipitated in 0.3 M sodiumacetate, 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: lys21 (positions +182 to +202) and AUG (positions +348 to +368, with 6 additional nt at its 5′ end).

Prediction of RNA secondary structure and thermodynamic stability with M-fold

The sequences of the different retroviruses for phylogenetic analysis were taken from the NCBI GenBank. Secondary structure predictions were performed using the M-fold version 3.0 algorithm (Mathews et al., 1999; Zuker & Turner, 1999). Sequences were sent to the Macfarlane Burnet Centre M-fold server (mfold.edu.burnet.au) and analyzed with standard settings. The thermodynamic stability of the interaction between the wild-type or mutant PAS elements and the antiPAS in the $tRNA^{lys3}$ primer was also determined using M-fold.

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REFERENCES

Aiyar A, Cobrinik D, Ge Z, Kung HJ, Leis J. 1992. Interaction between retroviral U5 RNA and the TYC loop of the tRNA(Trp) primer is required for efficient initiation of reverse transcription. *J Virol 66*:2464–2472.

Aiyar A, Ge Z, Leis J. 1994. A specific orientation of RNA secondary structures is required for initiation of reverse transcription. *J Virol 68*:611–618.

Arts EJ, Stetor SR, Li Y, Rausch JW, Howard KJ, Ehresmann B, North TW, Wohrl BM, Goody RS, Wainberg MA, Le Grice SFJ. 1996. Initiation of (−) strand DNA synthesis from $tRNA^{lys3}$ on lentiviral RNAs: Implications of specific HIV-1 RNA-$tRNA^{lys3}$ interactions inhibiting primer utilization by retroviral reverse transcriptases. *Proc Natl Acad Sci USA 93*:10063–10068.

Barat C, Le Grice SF, Darlix JL. 1991. Interaction of HIV-1 reverse transcriptase with a synthetic form of its replication primer, tRNA(Lys,3). *Nucleic Acids Res 19*:751–757.

Barat C, Schatz O, Le Grice S, Darlix JL. 1993. Analysis of the interactions of HIV-1 replication primer tRNA(Lys, 3) with nucleocapsid protein and reverse transcriptase. *J Mol Biol 231*:185–190.

FIGURE 9. The PAS–antiPAS interaction can be proposed for all retrovirus genera. The secondary structure prediction of part of the RNA genome surrounding the PBS of the different retroviruses and the cloverleaf structure of the $tRNA^{lys3}$, $tRNA^{lys1,2}$, tRNApro, and tRNAtrp primers are shown. A PAS-like motif (marked in orange) is present in the U5 region upstream of the PBS (marked in green) in all viral genomes. The PAS motif is complementary to the antiPAS sequence (marked in orange) in the TΨC arm of the tRNA primer used by that particular retrovirus. The PAS is predicted to be base paired, either to the PBS or to other leader sequences. Lentivirus genus: human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2), feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). Alpha-retrovirus genus: Rous sarcoma virus (RSV) and avian leukosis virus (ALV). Beta-retrovirus genus: mouse mammary tumor virus (MMTV) and Mason-Pfizer monkey virus (MPMV). Gamma-retrovirus genus: murine leukemia virus (MLV) and gibbon ape leukemia virus (GALV). Delta-retrovirus genus: human T-lymphotropic virus type 1 and 2 (HTLV-1 and HTLV-2). Spumavirus genus: chimpanzee foamy virus (CFV) and feline foamy virus (FFV). Base modifications in the tRNA molecules are indicated according to standard nomenclature (Sprinzl et al., 1989). R29 in $tRNA^{lys1,2}$ indicates that this position is G in $tRNA^{lys1}$ and A in $tRNA^{lys2}$, and Y41 is C in $tRNA^{lys1}$ and U in $tRNA^{lys2}$.

Beerens N, Berkhout B. 2002. The tRNA primer activation signal in the HIV-1 genome is important for initiation and processive elongation of reverse transcription. *J Virol 76*:2329–2339.

Beerens N, Groot F, Berkhout B. 2000. Stabilization of the U5-leader stem in the HIV-1 RNA genome affects initiation and elongation of reverse transcription. *Nucleic Acids Res 28*:4130–4137.

Beerens N, Groot F, Berkhout B. 2001. Initiation of HIV-1 reverse transcription is regulated by a primer activation signal. *J Biol Chem* 31247–31256.

Berkhout B, Schoneveld I. 1993. Secondary structure of the HIV-2 leader RNA comprising the tRNA-primer binding site. *Nucleic Acids Res 21*:1171–1178.

Brown HEV, Chen H, Engelman A. 1999. Structure-based mutagenesis of the human immunodeficiency virus type 1 DNA attachment site: Effects on integration and cDNA synthesis. *J Virol 73*:9011–9020.

Cobrinik D, Aiyar A, Ge Z, Katzman M, Huang H, Leis J. 1991. Overlapping retrovirus U5 sequence elements are required for efficient integration and initiation of reverse transcription. *J Virol 65*:3864–3872.

Cobrinik D, Soskey L, Leis J. 1988. A retroviral RNA secondary structure required for efficient initiation of reverse transcription. *J Virol 62*:3622–3630.

Das AT, Berkhout B. 1995. Efficient extension of a misaligned tRNA-primer during replication of the HIV-1 retrovirus. *Nucleic Acids Res 23*:1319–1326.

Das AT, Klaver B, Berkhout B. 1995. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNA(3Lys). *J Virol 69*: 3090–3097.

De Rocquigny H, Gabus C, Vincent A, Fournie-Zaluski M-C, Roques B, Darlix J-L. 1992. Viral RNA annealing activities of human immunodeficiency virus type 1 nucleocapsid protein require only peptide domains outside the zinc fingers. *Proc Natl Acad Sci USA 89*:6472–6476.

Esposito D, Craigie R. 1998. Sequence specificity of viral end DNA binding by HIV-1 integrase reveals critical regions for protein–DNA interaction. *EMBO J 17*:5832–5843.

Feng Y-X, Campbell S, Harvin D, Ehresmann B, Ehresmann C, Rein A. 1999. The human immunodeficiency virus type 1 Gag polyprotein has nucleic acid chaperone activity: Possible role in dimerization of genomic RNA and placement of tRNA on the primer binding site. *J Virol 73*:4251–4256.

Freund F, Boulme F, Litvak S, Tarrago-Litvak L. 2001. Initiation of HIV-2 reverse transcription: A secondary structure model of the RNA-tRNA(Lys3) duplex. *Nucleic Acids Res 29*:2757–2765.

Fu W, Ortiz-Conde BA, Gorelick RJ, Hughes SH, Rein A. 1997. Placement of tRNA primer on the primer-binding site requires pol gene expression in avian but not murine retroviruses. *J Virol 71*: 6940–6946.

Huang Y, Mak J, Cao Q, Li Z, Wainberg MA, Kleiman L. 1994. Incorporation of excess wild-type and mutant tRNA(3Lys) into human immunodeficiency virus type 1. *J Virol 68*:7676–7683.

Isel C, Ehresmann C, Keith G, Ehresmann B, Marquet R. 1995. Initiation of reverse transcription of HIV-1: Secondary structure of the HIV-1 RNA/tRNA(3Lys) (template/primer). *J Mol Biol 247*: 236–250.

Isel C, Keith G, Ehresmann B, Ehresmann C, Marquet R. 1998. Mutational analysis of the tRNA3Lys/HIV-1 RNA (primer/template) complex. *Nucleic Acids Res 26*:1198–1204.

Isel C, Lanchy J-M, Le Grice SFJ, Ehresmann C, Ehresmann B, Marquet R. 1996. Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcriptase require the post-transcriptional modifications of primer $tRNA^{lys3}$. *EMBO J 15*:917–924.

Isel C, Westhof E, Massire C, Le Grice SFJ, Ehresmann B, Ehresmann C, Marquet R. 1999. Structural basis for the specificity of the initiation of HIV-1 reverse transcription. *EMBO J 18*: 1038–1048.

Kang S-M, Wakefield JK, Morrow CD. 1996. Mutations in both the U5 region and the primer-binding site influence the selection of the tRNA used for the initiation of HIV-1 reverse transcription. *Virol 222*:401–414.

Kang S-M, Zhang Z, Morrow CD. 1997. Identification of a sequence within U5 required for human immunodeficiency virus type 1 to stably maintain a primer binding site complementary to tRNAMet. *J Virol 71*:207–217.

Kang S-M, Zhang Z, Morrow CD. 1999. Identification of a human immunodeficiency virus type 1 that stably uses $tRNA^{lys1,2}$ rather than $tRNA^{lys3}$ for initiation of reverse transcription. *Virol 257*: 95–105.

Lanchy J-M, Isel C, Keith G, Le Grice SFJ, Ehresmann C, Marquet R. 2000. Dynamics of the HIV-1 reverse transcription complex during initiation of DNA synthesis. *J Biol Chem 275*:12306–12312.

Leis J, Aiyar A, Cobrinik D. 1993. Regulation of initiation of reverse transcription of retroviruses. In: Skalka AM, Goff SP, eds. *Reverse transcriptase*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 33–48.

Levin JG, Seidman JG. 1979. Selective packaging of host tRNA's by murine leukemia virus particles does not require genomic RNA. *J Virol 29*:328–335.

Li X, Mak J, Arts EJ, Gu Z, Kleiman L, Wainberg MA, Parniak MA. 1994. Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. *J Virol 68*: 6198–6206.

Li Y, Zhang Z, Wakefield JK, Kang S-M, Morrow CD. 1997. Nucleotide substitutions within U5 are critical for efficient reverse transcription of human immunodeficiency virus type 1 with a primer binding site complementary to tRNAHis. *J Virol 71*:6315–6322.

Liang C, Li X, Rong L, Inouye P, Quan Y, Kleiman L, Wainberg MA. 1997a. The importance of the A-rich loop in human immunodeficiency virus type 1 reverse transcription and infectivity. *J Virol 71*:5750–5757.

Liang C, Rong L, Gotte M, Li X, Quan Y, Kleiman L, Wainberg MA. 1998. Mechanistic studies of early pausing events during initiation of HIV-1 reverse transcription. *J Biol Chem 273*: 21309–21315.

Liang C, Rong L, Morin N, Cherry E, Huang Y, Kleiman L, Wainberg MA. 1997b. The roles of human immunodeficiency virus type 1 Pol protein and the primer binding site in the placement of primer $tRNA^{lys3}$ onto viral genomic RNA. *J Virol 71*:9075–9086.

Mak J, Jiang M, Wainberg MA, Hammarskjold ML, Rekosh D, Kleiman L. 1994. Role of Pr160gag-pol in mediating the selective incorporation of tRNA(Lys) into human immunodeficiency virus type 1 particles. *J Virol 68*:2065–2072.

Mak J, Kleiman L. 1997. Primer tRNAs for reverse transcription. *J Virol 71*:8087–8095.

Mathews DH, Sabina J, Zuker M, Turner DH. 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol 288*:911–940.

Miller JT, Ehresmann B, Hubscher U, Le Grice SFJ. 2001. A novel interaction of tRNALys,3 with the feline immunodeficiency virus RNA genome governs initiation of minus strand DNA synthesis. *J Biol Chem 276*:27721–27730.

Miller JT, Ge Z, Morris S, Das K, Leis J. 1997. Multiple biological roles associated with the Rous sarcoma virus 5′ untranslated RNA U5-IR stem and loop. *J Virol 71*:7648–7656.

Morris S, Leis J. 1999. Changes in Rous sarcoma virus RNA secondary structure near the primer binding site upon $tRNA^{Trp}$ primer annealing. *J Virol 73*:6307–6318.

Murphy JE, Goff S. 1989. Construction and analysis of deletion mutations in the U5 region of the Moloney murine leukemia virus: Effects on RNA packaging and reverse transcription. *J Virol 63*:319–327.

Oude Essink BB, Das AT, Berkhout B. 1995. Structural requirements for the binding of tRNA Lys3 to reverse transcriptase of the human immunodeficiency virus type 1. *J Biol Chem 270*:23867–23874.

Oude Essink BB, Das AT, Berkhout B. 1996. HIV-1 reverse transcriptase discriminates against non-self tRNA primers. *J Mol Biol 264*:243–254.

Peters G, Harada F, Dahlberg JE, Panet A, Haseltine WA, Baltimore D. 1977. Low-molecular-weight RNAs of Moloney murine leukemia virus: Identification of the primer for RNA-directed DNA synthesis. *J Virol 21*:1031–1041.

Peters GG, Hu J. 1980. Reverse transcriptase as the major determinant for selective packaging of tRNA's into avian sarcoma virus particles. *J Virol 36*:692–700.

Rein A, Henderson LE, Levin JG. 1998. Nucleic-acid-chaperone activity of retroviral nucleocapsid proteins: Significance for viral replication. *Trends Biochem Sci 23*:297–301.

Sawyer RC, Hanafusa H. 1979. Comparison of the small RNAs of polymerase-deficient and polymerase-positive Rous sarcoma virus and another species of avian retrovirus. *J Virol 29*: 863–871.

Sprinzl M, Hartmann T, Weber J, Blank J, Zeidler R. 1989. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res 17*:1–172.

Vicenzi E, Dimitrov DS, Engelman A, Migone T-S, Purcell DFJ, Leonard J, Englund G, Martin MA. 1994. An integration-defective U5 deletion mutant of human immunodeficiency virus type 1 reverts by eliminating additional long terminal repeat sequences. *J Virol 68*:7879–7890.

Wakefield JK, Kang S-M, Morrow CD. 1996. Construction of a type 1 human immunodeficiency virus that maintains a primer binding site complementary to tRNAHis. *J Virol 70*:966–975.

Wakefield JK, Rhim H, Morrow CD. 1994. Minimal sequence requirements of a functional human immunodeficiency virus type 1 primer binding site. *J Virol 68*:1605–1614.

Wakefield JK, Wolf AG, Morrow CD. 1995. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to $tRNA^{lys3}$. *J Virol 69*:6021–6029.

Waters LC, Mullin BC. 1977. Transfer RNA in RNA tumor viruses. *Prog Nucleic Acid Res Mol Biol 20*:131–160.

Wohrl BM, Ehresmann B, Keith G, Le Grice SF. 1993. Nuclease footprinting of human immunodeficiency virus reverse transcriptase/tRNA(Lys-3) complexes. *J Biol Chem 268*:13617–13624.

Zhang Z, Kang S-M, Li Y, Morrow CD. 1998. Genetic analysis of the U5-PBS of a novel HIV-1 reveals multiple interactions between the tRNA and RNA genome required for initiation of reverse transcription. *RNA 4*:394–406.

Zuker M, Turner DH. 1999. Algorithms and thermodynamics for RNA secondary structure prediction: A practical guide. In: Barciszewski J, Clark BFC, eds. *RNA biochemistry and biotechnology*. Dordrecht/Boston/London: Kluwer Academic Publishers. pp 11–43.