Regulation of HIV-1 reverse transcription
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Chapter 8
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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<sub>lys3</sub> 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 T<sup>Ψ</sup>C arm of tRNA<sub>lys3</sub>. 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<sub>lys3</sub> 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/ reverse transcription/ tRNA primer/ viral RNA-tRNA interaction/ U5-leader stem/ primer activation signal/ tRNA usage and selection

**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 enzyme (RT) and a specific cellular tRNA molecule is used as primer. The tRNA primer binds with its 3'-terminal 18 nucleotides 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 (Huang et al., 1994; Levin & Seidman, 1979; Liang et al., 1997a; Mak et al., 1994; Mak & Kleiman, 1997; Peters et al., 1977; Waters & Mullin, 1977), and self-primers are favoured over non-self-primers by the viral RT.
Chapter 8

Fig 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<sub>lys3</sub> 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<sup>lys3</sup> 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 (Beeren et al., 2001; Beerens & Berkhout, 2002).

HIV-1 uses tRNA<sub>lys3</sub> as primer for reverse transcription. We recently identified an 8-nucleotide motif in the U5 region of the untranslated leader RNA that stimulates tRNA<sub>lys3</sub>-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. 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<sub>lys3</sub> (Fig 1, both motifs are marked in orange), similar to the interaction proposed for the Rous sarcoma virus (RSV) genome and the corresponding tRNA<sup>tp</sup> primer (Aiyar et al., 1992; Aiyar et al., 1994; Cobrinik et al., 1988; Cobrinik et al., 1991; 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 basepairing in the U5-leader stem (Fig 1), and we demonstrated that reverse transcription is greatly upregulated 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\textsubscript{lys3} have been proposed based mainly on biochemical probing studies (Isel et al., 1995; Isel et al., 1996; Isel et al., 1998; Isel et al., 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; Lanchy et al., 2000; Li et al., 1997; Liang et al., 1997b; Liang et al., 1998; Zhang et al., 1998). Deletion of the A-rich loop also affects virus replication, and the A-rich sequence is restored upon long-term culturing (Liang et al., 1997b). However, the presence of overlapping sequence motifs for integration of the viral DNA genome complicates the interpretation of these results (Brown et al., 1999; Esposito & Craigie, 1998; Vicenzi et al., 1994). Other members of the lentivirus genus that utilize tRNA\textsubscript{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\textsubscript{lys3}. We demonstrate that the efficiency of \textit{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 \textit{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\textsubscript{lys3} primer (Fig 2). In mutant N1, we substituted the U at position 126 by C, thus replacing the U-G basepair in the putative PAS-antiPAS interaction by a more stable C-G basepair. In mutant N2, the C residues at position 125 and 127 were changed into U residues, thus replacing two C-G basepairs by weaker U-G basepairs. In mutant N3, two nucleotides were inserted at the 3'-end of the 8-nt PAS motif to obtain 10-nt complementarity to the antiPAS in tRNA\textsubscript{lys3}. In mutant N4, two nucleotides were inserted near the 5' end of the PAS to extend the interaction with the tRNA primer with one nucleotide, and to replace the G123-Ψ basepair with the more stable A123-Ψ basepair (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 basepairs 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.

![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 basepairs (bp) and the number of hydrogen bonds (H) of the PAS-antiPAS duplex, and the thermodynamic stability of the PAS-antiPAS interaction (ΔG in kcal/mol).](image-url)
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<sub>lys3</sub> 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 two nucleotide insertion in the PAS element (lanes 9,10). The efficiency of DNA-primed reverse transcription is similar for all templates (Fig 4A). Extension of the natural tRNA<sup>lys3</sup> primer results in a 153-nt cDNA product on the wild-type template (lane 11). tRNA-primed reverse transcription was enhanced 4-fold on the N1 template with an improved PAS-antiPAS interaction (Fig 3, lane 12 and Fig 4B), and reverse transcription was reduced 5-fold 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 basepair enhanced reverse transcription 3.5-fold (Fig 3, lane 15 and Fig 4B), but reverse transcription was reduced 4-fold on the N3 template that extends the PAS-antiPAS interaction with 2 basepairs (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<sub>lys3</sub> primer. We therefore performed initiation assays, in which reverse transcription was performed in the presence of <sup>32</sup>P-dCTP, but without the other dNTPs. This results in extension of the 76-nt tRNA<sup>lys3</sup> primer with one nucleotide (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 upregulated 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 full-length reverse transcription activity (shown in panel B) 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 antiPAS 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-
Fig. 5. Interaction between vRNA templates and the natural or synthetic tRNA<sup>lys3</sup> molecules. (A) The cloverleaf structures of the natural tRNA<sup>lys3</sup> and the synthetic tRNA<sup>lys3</sup> and the mutant 2L-tRNA<sup>lys3</sup>. Base modifications are indicated according to standard nomenclature (Sprinzl et al., 1989). The synthetic tRNA<sup>lys3</sup> molecule does not contain modified bases, but was shown to adopt the correct secondary cloverleaf structure and L-shaped tertiary structure (Wohle et al., 1993). The anti-PAS motif is marked by an open box, and the mutations introduced in 2L-tRNA<sup>lys3</sup> are marked in gray. (B) The PAS-antiPAS interaction of the wild-type vRNA template with natural (n) and synthetic (s) tRNA<sup>lys3</sup> is shown on the left. The interaction of the mutant 2L vRNA template with the synthetic tRNA<sup>lys3</sup> and the complementary 2L-tRNA<sup>lys3</sup> 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<sup>lys3</sup> primer is marked in gray.

length tRNA-cDNA product (Fig 6, lane 2). As expected, reverse transcription was reduced 10-fold on the PAS-minus template 2L (lane 8). Because reverse transcription is less efficiently initiated from synthetic tRNA primers (Arts et al., 1996; Barat et al., 1991), we used 5 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 (lanes 9,10) compared to reverse transcription on the wild-type template (lanes 3,4). Thus, reverse transcription primed by natural and synthetic tRNA<sup>lys3</sup> primers is stimulated by the PAS-mechanism, indicating that the PAS-antiPAS interaction does not depend on modified nucleotides within the tRNA<sup>lys3</sup> 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<sup>lys3</sup> can be used to study the PAS-mediated enhancement of reverse transcription allowed us to test a mutant form of the tRNA<sup>lys3</sup> 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<sup>lys3</sup> primer (Fig 5A and 5B). Reverse transcription on the 2L template was indeed enhanced 2-fold for the mutant 2L-tRNA<sup>lys3</sup> compared with the
activity may due to opening of the TYC 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 (Das et al., 1995; Li et al., 1994; Wakefield et al., 1994). This result indicates that the PBS is not the only viral determinant of tRNA usage. 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; Kang et al., 1997; Kang et al., 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<sup>lys3</sup> and tRNA<sup>lys2</sup> molecules (Fig 7B). These tRNAs have identical antiPBS and antiPAS sequences, and will be referred to as tRNA<sup>lys1,2</sup> (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<sup>lys3</sup>, tRNA<sup>lys1,2</sup> and all other tRNA species. Reverse transcription was initiated from the annealed tRNA primer by addition of <sup>32</sup>P-dCTP and HIV-1 RT enzyme, resulting in the extension of the tRNA primer with one nucleotide. The radiolabeled tRNA<sup>lys3</sup>-cDNA product runs at a higher position on the denaturing gel than the tRNA<sup>lys1,2</sup>-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<sup>lys3</sup>-primed cDNA, and no tRNA<sup>lys1,2</sup> signal is apparent (Fig 8, lanes 7-9). The tRNA<sup>lys3</sup> signal is significantly reduced on the PBS-lys1,2 template, and the

![Fig 6. Reverse transcription primed by natural and synthetic tRNA<sup>lys3</sup>. RNA-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<sup>lys3</sup> (n-tRNA, lanes 2 and 8), synthetic tRNA<sup>lys3</sup> (s-tRNA, lanes 3-4 and 9-10) or mutant 2L-tRNA<sup>lys3</sup> (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 polyA primer that produces a 104-nt product (lanes 1 and 7).](image-url)
The anti-PAS motif is marked by an open box, nucleotides that differ from tRNA
lys1,2 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 double mutant template (4-6). The amount of input viral RNA was measured by DNA-primer extension with the AUG primer that produces a 270-nt product (lanes 10-12). Quantitation of the results indicates that the PAS adaptation enhances tRNA
lys1,2 usage approximately 6-fold. 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 non-random subset of the cellular tRNA population (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 (Das & Berkhout, 1995; Liang et al., 1997a; Mak et al., 1994; Oude Essink et al., 1995; Peters & Hu, 1980; Sawyer & Hanafusa, 1979). In addition, the viral NC protein has been implicated in placement of the tRNA onto the viral genome (Barat et al., 1993; De Rocquigny et al., 1992; Feng et al., 1999; Fu et al., 1997).

Fig 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 anti-PAS motif is marked by an open box, nucleotides that differ from tRNA
lys 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
lys. Nucleotides that differ from the wild-type sequence, which is complementary to tRNA
lys, are marked in gray.
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; Isel et al., 1996; Isel et al., 1998; Isel et al., 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\textsuperscript{ys3} primer (U33-U36) has been studied extensively. Mutation of the “A-rich loop” affects initiation and elongation of reverse transcription (Isel et al., 1996; Lanchy et al., 2000; Li et al., 1997; Liang et al., 1997b; Liang et al., 1998; Zhang et al., 1998). Deletion of the “A-rich loop” affects virus replication, and the A-rich sequence is restored upon long-term culturing (Liang et al., 1997b). However, the presence of overlapping sequence motifs for integration of the viral DNA genome severely complicates the interpretation of these results (Brown et al., 1999; Esposito & Craigie, 1998; Vicenzi et al., 1994). Moreover, this interaction appears to be specific for HIV, as other members of the lentivirus genus that utilize tRNA\textsuperscript{ys3} 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 basepair by a C-G basepair 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 basepair co-variation supports the 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 T\textsuperscript{Y}C arm of the corresponding tRNA\textsuperscript{DP} primer (Aiyar et al., 1992; Aiyar et al., 1994; Cobrinik et al., 1988; Cobrinik et al., 1991; 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\textsuperscript{ lys1,2} and tRNA\textsuperscript{ys} 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 basepaired, 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 regulate reverse transcription may be more widely conserved, we could e.g. identify a PAS-like element in the gypsy retrotransposon that uses tRNA\textsuperscript{ys} as primer. These combined results suggest that the process of reverse transcription is regulated by a common mechanism in all retroviridae.
Chapter 8

Lentivirus

HIV-1

PAS

HIV-2

FIV

EIAV

Alpha-retrovirus

RSV

ALV

Beta-retrovirus

MMTV

MPMV

MLV

GALV

Delta-retrovirus

HTLV-1

HTLV-2

Spumavirus

CFV

FFV

tRNA primers

Anti-PBS

Anti-PAS

tRNA\textsubscript{lys3}
tRNA\textsubscript{lys1,2}
tRNA\textsubscript{Trp}
tRNA\textsubscript{Pro}
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-nucleotide motif (positions +123 to +130), called PAS, that is important for tRNA\(^{\text{lys3}}\)-mediated initiation of HIV-1 reverse transcription (Beeren 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 (Beeren et al., 2001) and reverse transcription can be inhibited by stabilizing mutations in the U5-leader stem (Beeren et al., 2000). The PAS motif is also important for in vivo reverse transcription and virus replication (Beeren & 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\(^{\text{lys3}}\) 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 anti-PAS 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\(^{\text{lys3}}\) to the PBS may occur relatively early, e.g. 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\(^{\text{lys3}}\) to tRNA\(^{\text{val1,2}}\) by simultaneous alteration of the PBS and PAS motifs. However, reverse transcription primed by the non-self tRNA\(^{\text{lys3}}\) on the PBS-PAS double mutant template is relatively inefficient compared with usage of the self tRNA\(^{\text{lys3}}\) 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.

Fig 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\(^{\text{lys3}}\), tRNA\(^{\text{val1,2}}\), tRNA\(^{\text{val2}}\) and tRNA\(^{\text{val1}}\) 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 Ty3 arm of the tRNA primer used by that particular retrovirus. The PAS is predicted to be basepaired, 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). Alpharetrovirus genus: Rous sarcoma virus (RSV) and avian leukemia virus (ALV). Betaretrovirus genus: mouse mammary tumor virus (MMTV) and Mason-Pfizer monkey virus (MPMV). Gammaretrovirus genus: murine leukemia virus (MLV) and gibbon ape leukemia virus (GALV). Deltaretrovirus 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\(^{\text{val1,2}}\) indicates that this position is G in tRNA\(^{\text{val1}}\) and A in tRNA\(^{\text{val2}}\), and Y41 is C in tRNA\(^{\text{val1}}\) and U in tRNA\(^{\text{val2}}\).
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 & Biekhoi, 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-PBSloop (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<sup>lys1,2</sup> 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 templates:

To generate a template for the production of synthetic tRNA<sup>lys3</sup>, we PCR amplified the tRNA gene from the pUC-tRNA<sup>lys3</sup> 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 nucleotide 1 to 76. The compensatory 2L mutation was introduced by PCR mutagenesis with the mutagenic sense primer 3'-2L-tRNA (tRNA positions +39 to +76) and the sense 5'-7-T7-tRNA primer. The introduction of the mutations in the vRNA and tRNA sequence was verified by sequence analysis with the DYEnamic<sup>TM</sup> 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-MegaScript 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<sup>lys3</sup>, Roche), 0.5 or 1 μg of synthetic tRNA<sup>lys3</sup>, 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 one nucleotide by addition of 6 μl RT(-) buffer (9 mM MgCl₂, 30 mM DTT, 150 μg/ml actinomycin D), 1 μl [α-<sup>32</sup>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-<sup>32</sup>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 were: lys21 (positions +182 to +202) and AUG (positions +348 to +368, with 6 additional nucleotides at its 5'end).

Prediction of RNA secondary structure and thermodynamic stability with Mfold

The sequences of the different retroviruses for phylogenetic analysis were taken from the NCBI GenBank. Secondary structure predictions were performed using the Mfold version 3.0 algorithm (Mathews et al., 1999; Zuker & Turner, 1999). Sequences were sent to the Macfarlane Burnet Centre Mfold 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<sup>lys3</sup> primer was also determined using Mfold.

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