Structure–function relationships of the initiation complex of HIV-1 reverse transcription: the case of mutant viruses using tRNA^{His} as primer

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ABSTRACT

Reverse transcription of HIV-1 RNA is initiated from the 3' end of a tRNA₃^{Lys} molecule annealed to the primer binding site (PBS). An additional interaction between the anticodon loop of tRNA₃^{Lys} and a viral A-rich loop is required for efficient initiation of reverse transcription of the HIV-1 MAL isolate. In the HIV-1 HXB2 isolate, simultaneous mutations of the PBS and the A-rich loop (mutant His-AC), but not of the PBS alone (mutant His) allows the virus to stably utilize tRNA^{His} as primer. However, mutant His-AC selects additional mutations during cell culture, generating successively His-AC-GAC and His-AC-AT-GAC. Here, we wanted to establish direct relationships between the evolution of these mutants in cell culture, their efficiency in initiating reverse transcription and the structure of the primer/template complexes *in vitro*. The initiation of reverse transcription of His and His-AC RNAs was dramatically reduced. However, His-AC-GAC RNA, which incorporated three adaptative point mutations, was reverse transcribed more efficiently than the wild type RNA. Incorporation of two additional mutations decreased the efficiency of the initiation of reverse transcription, which remained at the wild type level. Structural probing showed that even though both His-AC and His-AC-GAC RNAs can potentially interact with the anticodon loop of tRNA^{His}, only the latter template formed a stable interaction. Thus, our results showed that the selection of adaptative mutations by HIV-1 mutants utilizing tRNA^{His} as primer was initially dictated by the efficiency of the initiation of reverse transcription, which relied on the existence of a stable interaction between the mutated A-rich loop and the anticodon loop of tRNA^{His}.

INTRODUCTION

Reverse transcription of the single-stranded genomic RNA into a double-stranded DNA with duplicated long terminal repeats is a key event in the retroviral replication cycle (1,2). This process is performed by reverse transcriptase (RT), an RNA- and DNA-dependent DNA polymerase that also harbors an RNase H domain. RT utilizes a cellular tRNA that is selectively encapsidated into the viral particles to prime DNA synthesis (3–5). The natural primer used by most immunodeficiency viruses, including the type 1 human immunodeficiency virus (HIV-1), is tRNA₃^{Lys} (3–5).

In all retroviruses, the 18 3' terminal nucleotides of the primer tRNA become annealed to the complementary primer binding site (PBS), located in the 5' region of the genomic RNA. Additional 'virus-specific' interactions have been described in several retroviruses and LTR-containing retrotransposons including avian retroviruses (6–8), HIV-1 (9–20), HIV-2 (21,22), FIV (23), Ty1 (24,25) and Ty3 (26).

In HIV-1, chemical and enzymatic probing of the viral RNA (vRNA) and of the primer tRNA₃^{Lys} (9,10,15), combined with site-directed mutagenesis of the former (14,19,27) provided strong evidence for the existence of additional interactions between tRNA₃^{Lys} and the genomic RNA *in vitro*. These experimental data were used to construct secondary (10) and tertiary (15) structure models of the initiation complex of the HIV-1 MAL isolate. In these models, parts of the anticodon arm and of the variable loop of tRNA₃^{Lys} interact with viral sequences upstream of the PBS (Fig. 1A). Of particular importance is the interaction between the anticodon loop of tRNA₃^{Lys} and a viral A-rich loop. The same interactions were observed when the primer–template complex was annealed by heat or by the nucleocapsid protein (28).

Evidence suggesting that these interactions also take place in cell culture was obtained from studies in which the identity of the primer tRNA was switched. Several groups showed that mutant viruses whose PBS had been changed to be complementary to a variety of non-cognate primer tRNAs are unstable and rapidly revert to the wild type (WT) PBS

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Figure 1. The initiation complex of reverse transcription. (A) Secondary structure model of the complex formed between the HIV-1 MAL RNA and tRNA₃^{Lys}, as deduced from structural probing (10). The PBS is highlighted in yellow and the sequences of the viral A-rich loop and of the anticodon loop of tRNA₃^{Lys} that form an intermolecular interaction are in green. (B) Sequence alignment of the HIV-1 MAL RNA and of the WT and mutant RNAs derived from HIV-1 HXB2 used in this study. The PBS complementary to tRNA₃^{Lys} and tRNA^{His} are in green and violet, respectively, and the mutations selected during cell culture are in blue. Numbering according to the MAL and HXB2 sequences is shown above and below the sequence alignment, respectively. (C) Secondary structure of tRNA^{His}. Nucleotide numbering follows international conventions, so that the anticodon corresponds to nucleotides 34–36.

sequence (29–31). However, HIV-1 can replicate by stably using either tRNA^{His} (32–35), tRNA^{Met} (36,37) or tRNA_{1,2}^{Lys} (38) as primers, provided that the viral PBS and A-rich loop are simultaneously mutated to be complementary to these tRNA species.

However, the implications of these results are not as straightforward as it might seem. As already pointed out (18,39), their interpretation is complicated by the presence of overlapping sequence motifs involved in the integration of the viral DNA (40–42). Even though the mutant viruses stably use the non-cognate primer tRNAs, they replicate more slowly than the WT virus (32,33,35,36,38). In addition, proviruses with deletions in the U5 region are replication competent (42).

Therefore, it was proposed that mutations of the A-rich loop allowed long term usage of non-cognate tRNAs by disfavoring tRNA₃^{Lys}, rather than favoring the alternate primers (43). Finally, even though mutant viruses in which the PBS and the A-rich loop were made complementary to tRNA^{His}, tRNA^{Met} or tRNA_{1,2}^{Lys} maintained these tRNA species as primer, they rapidly accumulated additional mutations that improved their replication capabilities (32,33,36,38,44). These observations suggest that the interaction between the anticodon loop of the primer tRNA and the viral A-rich loop may not be sufficient for optimal initiation of reverse transcription.

Here, we studied the *in vitro* reverse transcription of HIV-1 mutants in which either the PBS alone or both the PBS and the

A-rich loop were complementary to tRNA^{His}. In the latter case, we compared the initial mutant with mutants having accumulated adaptative mutations during prolonged cell culture. In order to better understand the structure–function relationships of the reverse transcription initiation complex, we also investigated its structure by chemical and enzymatic probing.

MATERIALS AND METHODS

Primers, templates and RT

Natural tRNA₃^{Lys} was purified from beef liver as described (45). Its sequence and post-transcriptional modifications are identical to those of human tRNA₃^{Lys}. The gene coding for human tRNA^{His} was constructed under the control of the T7 RNA polymerase and inserted into the EcoRI and XmaI sites of plasmid pUC18 using oligonucleotides 5'-AAT TCT AAT ACG ACT CAC TAT AGG CCG TGA TGA TCG TAT AGT-3', 5'-GGT TAG TAC TCT GCG TTG TGG CCG CAG CAA-3', 5'-CCT CGG TTC GAA TCC GAG TCA CGG CAC CAT GCA TC-3', 5'-TAC TAA CCA CTA TAC GAT CAC GGC CTA TAG TGA GTC GTA TTA G-3', 5'-AAC CGA GGT TGC TGC GGC CAC AAC GCA GAG-3' and 5'-CCG GGA TGC ATG GTG CCG TGA CTC GGA TTC G-3'. The resulting pFBHIS plasmid was cut with NsiI and transcribed *in vitro* with the T7 RNA polymerase as described (46). Alternatively, tRNA^{His} was purified from beef liver to >60% homogeneity using established methods (47). When required, tRNAs were dephosphorylated with calf intestine phosphatase and labeled at the 5'-end with [γ -³²P]ATP and phage T4 polynucleotide kinase according to published procedures (15).

In order to synthesize the WT and mutant RNA templates used in this study, nucleotides 1–732 of plasmids pHXB2, pHXB2(His), pHXB2(His-AC), pHXB2(His-AC-GAC) and pHXB2(His-AC-AT-GAC) (35), were amplified using oligonucleotides 5'-ACG TGA ATT CTA ATA CGA CTC ACT ATA GGT CTC TCT GGT TAG ACC A-3' and 5'-TGC ACC CGG GTA ATT TTG GCT GAC CTG-3'. The PCR products were cleaved with EcoRI and XmaI, and inserted into the corresponding site of pUC18. The resulting plasmids pHXB2(1–732)WT, pHXB2(1–732)His, pHXB2(1–732)His-AC, pHXB2(1–732)His-AC-GAC and pHXB2(1–732)His-AC-AT-GAC were linearized with RsaI and transcribed using previously described conditions (46), yielding vRNAs encompassing nucleotides 1–295 of HXB2.

The plasmid used for production of the heterodimeric WT HIV-1 RT was kindly provided by Dr Torsten Unge (Uppsala, Sweden), together with the protocols for protein overexpression and purification.

Annealing of tRNA₃^{Lys} and tRNA^{His} to the vRNAs

Viral RNA and primer tRNA₃^{Lys} or tRNA^{His} were first denatured in water for 2 min at 90°C and chilled on ice. Annealing was performed at 70°C for 20 min in sodium cacodylate (pH 7.5) 50 mM, KCl 300 mM. An aliquot of each annealing reaction was analyzed on a native 8% polyacrylamide gel to determine the annealing efficiency. The reaction products were discarded if <95% of the primer were annealed to the vRNA. A functional and structural comparison of the WT MAL initiation complexes formed using this protocol or

nucleocapsid protein mediated annealing at 37°C revealed no differences between the two complexes (28). In addition, we previously showed that this protocol allows quantitative annealing of both native and synthetic tRNAs (9).

Minus strand strong stop DNA synthesis

In a standard experiment, vRNA (100 nM final concentration) was annealed with ³²P-labeled tRNA₃^{Lys} or tRNA^{His} (2 nM) as described above, and pre-incubated for 4 min at 37°C with 25 nM RT in 50 mM Tris–HCl pH 8.0, 50 mM KCl, 6 mM MgCl₂, 1 mM DTE. Reverse transcription was initiated by adding a mixture of the four deoxynucleoside triphosphates (50 μ M each) pre-incubated at 37°C in the same buffer. Formamide containing 50 mM EDTA was added to aliquots of the reaction mixture at times ranging from 15 s to 30 min, and the reaction products were analyzed on 8% denaturing polyacrylamide gels, and quantified with a BioImager BAS 2000 (Fuji) using the MacBas software.

In order to compare the native and synthetic tRNA^{His}, a 5-fold molar excess of unlabeled tRNA^{His} was annealed to 10 nM of His-AC-GAC RNA as described above. DNA synthesis was performed as described above except for the following modifications. The RT concentration was 27 nM, the dATP, dGTP and dTTP concentrations were 150 μ M, and the dCTP concentration was 15 μ M (10 μ Ci in a final volume of 100 μ l).

Chemical probing of vRNA

After hybridization, the vRNA/tRNA complexes were incubated at 20°C for 15 min in the annealing buffer supplemented with 5 mM MgCl₂ before probing with dimethyl sulfate (DMS). Hybridization and probing buffers were the same for 1-cyclohexyl 3-(2 morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMCT), except that they contained 50 mM sodium borate (pH 8.0) instead of sodium cacodylate (pH 7.5).

After addition of 1 μ g of yeast total tRNA, RNA was modified by addition of 1 μ l of 10-fold diluted DMS in ethanol for 5 or 10 min, or 2 μ l of CMCT (40 mg/ml in water) for 10 or 20 min. RNA modification was stopped with 200 μ l ethanol and 50 μ l sodium acetate 0.3 M (pH 5.3) containing 1 μ g glycogen. Modified bases between positions 120 and 220 were detected by primer extension with RT as previously described (48).

Enzymatic and chemical probing of tRNA^{His}

Viral RNA (12 pmol) and 50 000 c.p.m. of 5'-end-labeled tRNA^{His} (0.4 pmol) were hybridized as described above. The tRNA^{His}, in its free form or involved in the binary complexes, was cleaved with 0.06 and 0.1 U of RNase VI and RNase T1, respectively, for 7.5 or 15 min at room temperature. Free and hybridized tRNA^{His} was cleaved with 200 U of nuclease S1 from *Aspergillus oryzae* for 7.5 or 15 min at room temperature. After phenol/chloroform and ethanol precipitation, tRNA₃^{Lys} fragments were analyzed on 15% denaturing polyacrylamide gels.

For chemical probing, 12 pmol of vRNA and 0.4 pmol of tRNA^{His} were hybridized as described above, except for the pH of the sodium cacodylate buffer, which was decreased to 7.0. After addition of 1 μ g of yeast total tRNA, tRNA^{His} in its free form or involved in the binary complexes was modified with 3 μ l of kethoxal (20 mg/ml) diluted in ethanol 20% for 7

Table 1. Characteristics of the viral RNAs used in this study

Viruses and clones used in cell culture	RNAs used <i>in vitro</i>	PBS	A-rich loop	Additional mutations	Evolution during cell culture
pHXB2	WT	Lys3	GAAAAT Lys3	–	stable
pHXB2(His)	His	His	GAAAAT Lys3	–	reversion → WT
pHXB2(His-AC)	His-AC	His	CCACAA His	–	evolution → His-AC-GAC
pHXB2(His-AC-GAC)	His-AC-GAC	His	CCACAA His	T174G, G181A, T200C	stable, may evolve → His-AC-AT-GAC
pHXB2(His-AC-AT-GAC)	His-AC-AT-GAC	His	CCACAA His	Idem plus C152A, C160T	stable

The names of clones used by Zhang *et al.* (35) and the corresponding RNAs used in this study are indicated in the first two columns. The following two columns indicate whether the PBS and A-rich loop sequences are complementary to tRNA₃^{Lys} or tRNA^{His}. The mutations selected by mutant pHXB2(His-AC) upon cell culture and the fate of the viruses are indicated in the last two columns.

and 15 min. Reactions were stopped with 200 µl ethanol and 50 µl sodium acetate 0.3 M (pH 5.3) containing 1 µg glycogen. Modified bases were detected by primer extension with RT as previously described (48).

RESULTS

The vRNA mutants studied

Among all mutant that replicate using alternate tRNAs as primers, those using tRNA^{His} are the best characterized in cell culture (32–35). Unlike HXB2(His), which possessed a mutated PBS, HXB2(His-AC), in which both the PBS and A-rich loop were mutated, stably used tRNA^{His} as primer for reverse transcription (32) (Table 1, Fig. 1B and C). However, analysis of the U5-PBS region of this virus revealed multiple nucleotide changes in U5 and around the PBS (32,33). Three nucleotide substitutions predominated in the adapted HXB2(His-AC-GAC) virus: U174G, G181A and U200C (Table 1). Note that nucleotides 174 and 181 are located between the PBS and the A-rich loop, and nucleotide 200 is the first nucleotide 3' to the PBS (Fig. 1B). Importantly, substitution of a 200-nucleotide fragment encompassing the U5-PBS region of HXB2(His-AC) and HXB2(His-AC-GAC) into the WT proviral clone (pHXB2), conferred the capacity of the resulting viruses, named pHXB2(His-AC) and pHXB2(His-AC-GAC), respectively, to utilize tRNA^{His} to prime reverse transcription (33). These experiments provided evidence that the U5-R region of the genomic RNA plays a key role in the primer utilization. Following extensive cell culture of pHXB2(His-AC-GAC), two main additional nucleotide substitutions were selected: C152A and C160U (33) (Table 1 and Fig. 1B). Subcloning of the U5-PBS region of this virus into pHXB2 yielded pHXB2(His-AC-AT-GAC) (35).

We have cloned the R, U5, PBS and leader region, as well as part of the *gag* coding region of pHXB2, pHXB2(His), pHXB2(His-AC), pHXB2(His-AC-GAC) and pHXB2(His-AC-AT-GAC) immediately downstream of a T7 RNA polymerase promoter. *In vitro* transcription allowed us to synthesize RNAs corresponding to the first 295 nucleotides of the vRNAs and containing the mutations mentioned above (Table 1 and Fig. 1B).

Efficiency of (–) strand strong stop DNA synthesis

The (–) strand strong stop DNA results from the extension of a primer bound to the PBS up to the 5'-end of the genomic RNA. Its synthesis can be divided into two steps: initiation, which corresponds to the slow and distributive addition of the first six

nucleotides (in the case of the WT template) to the primer tRNA, followed by a fast and processive elongation (27,49,50).

Kinetics of (–) strand strong stop DNA synthesis from tRNA₃^{Lys} annealed to WT RNA, and from tRNA^{His} annealed to His, His-AC, His-AC-GAC and His-AC-AT-GAC RNAs are shown in Figure 2. When the WT RNA was reverse transcribed using tRNA₃^{Lys} as primer, (–) strand strong stop DNA appeared after 10 min and gradually increased to reach 21% of the initial amount of primer after 30 min. The amount of (–) strand strong stop DNA synthesized from tRNA^{His} on His RNA was much lower (Fig. 2) and only amounted to 5% after 30 min. (Table 2). More unexpectedly, the amount of (–) strand strong stop DNA was hardly increased on the His-AC RNA, but was dramatically raised by mutations U174G, G181A and U200C present in His-AC-GAC RNA (Fig. 2 and Table 2). Surprisingly, the amount of (–) strand strong stop DNA synthesized after 30 min was equal to 31%, corresponding to a 50% increased efficiency, as compared to the WT RNA (Table 2). This efficiency was not significantly affected by the additional mutations C152A and C160U that are present in His-AC-AT-GAC RNA (Fig. 2 and Table 2).

Kinetics of primer tRNA extension and pausing during reverse transcription

In order to directly access the efficiency of the initiation of reverse transcription, we determined the fraction of unextended primer remaining over time (Fig. 3). As previously observed, the kinetics of the initial tRNA extension are best described as a bi-exponential process (19,49). Indeed, previous pre-steady and steady-state kinetics conducted on the WT HIV-1 MAL RNA/tRNA₃^{Lys} complex indicated that the fast reaction corresponds to extension of the preformed primer-template:RT complex, whereas the slow step requires RT recycling and a conformational change of the primer-template complex (49,50).

When using the WT RNA, 71% of the primer tRNA₃^{Lys} was extended with a rate constant k_{fast} of 0.025 s^{–1} (Fig. 3 and Table 2). The remaining 29% of the primer were extended slowly, and 10% were not extended after 30 min (Fig. 3). The k_{fast} of tRNA^{His} extension on the His RNA was decreased 5-fold, and the amplitude of the fast phase (A_{fast}) was reduced to 20% (Fig. 3 and Table 2). Even though similar amounts of (–) strand strong stop DNA were generated on His and His-AC templates, the initiation of reverse transcription appeared more efficient with the latter RNA (Fig. 3). This was not due to an increase in k_{fast} , but to a 2-fold increase of A_{fast} (Table 2).

The efficiency of the initiation of reverse transcription was dramatically improved with His-AC-GAC (Fig. 3). Indeed, the

Figure 2. Kinetics of (–) strand strong stop DNA synthesis. Labeled native tRNA^{Lys} annealed to WT RNA (left panel in the upper row), and labeled synthetic tRNA^{His} annealed to the mutant templates indicated above the gels were extended for 0, 15, 30, 45 s, 1, 2.5, 5, 10, 15, 20, 25 and 30 min (lanes 1–12, respectively) as described in Materials and Methods. The extension products were analyzed on an 8% denaturing polyacrylamide gel. The labels on the right of the gels indicate the position of the primer and some of the extension products. The +181 product corresponds to the (–) strand strong stop DNA.

k_{fast} value measured on this template was ~4.5-fold higher than that for the WT RNA (Table 2). Surprisingly, k_{fast} was decreased to 1.25-fold the WT value with His-AC-AT-GAC, indicating that mutations C152A and C160U decreased the

efficiency of the initiation of reverse transcription (Fig. 3 and Table 2). Worth noticing, primer extension was complete on His-AC-GAT and His-AC-AT-GAC templates. At the opposite, ~10, 25 and 50% of the primer was not extended

Table 2. Efficiency of the (–) strand strong stop DNA synthesis and of the initiation of reverse transcription of WT and mutant RNA templates

Template RNA	Fraction of (–) strand strong stop DNA (%)	Rate constant of the fast phase (k_{fast})	Amplitude of the fast phase (A_{fast})
WT	21	$0.025 \pm 0.003 \text{ s}^{-1}$	$71 \pm 5\%$
His	5	$0.005 \pm 0.003 \text{ s}^{-1}$	$20 \pm 7\%$
His-AC	6	$0.004 \pm 0.001 \text{ s}^{-1}$	$43 \pm 7\%$
His-AC-GAC	31	$0.112 \pm 0.040 \text{ s}^{-1}$	$48 \pm 12\%$
His-AC-AT-GAC	29	$0.031 \pm 0.003 \text{ s}^{-1}$	$77 \pm 6\%$

Figure 3. Kinetics of the initiation of reverse transcription. The amount of unextended and extended primer in the experiments shown in Figure 2 were quantified, and the fraction of unextended primer was plotted as a function of time. The experimental data obtained with WT (open circles), His (closed circles), His-AC (open squares), His-AC-GAC (closed squares) and His-AC-AT-GAC (open triangles) RNAs were fit with the Igor Pro Carbon software for Mac OS X using the equation $f_{\text{unextended}} = A_{\text{fast}} \cdot e^{-k_{\text{fast}} \cdot t} + A_{\text{slow}} \cdot e^{-k_{\text{slow}} \cdot t}$ where $f_{\text{unextended}}$ is the fraction of unextended primer, A_{fast} and A_{slow} are the amplitudes of the fast and slow phases, respectively, and k_{fast} and k_{slow} correspond to the rate constants of these phases. The values of A_{fast} and k_{fast} obtained from these fits are listed in Table 2.

after a 30 min reaction on the WT, His-AC and His templates, respectively (Fig. 3). The complete primer extension on His-AC-GAT and His-AC-AT-GAC RNAs suggests that the initiation complexes formed by annealing tRNA^{His} to these templates were structurally homogeneous, while a fraction of the WT complex adopted a non-functional structure. This feature likely contributes to the increased amount of (–) strand strong stop DNA observed with these mutant RNAs.

During (–) strand strong stop DNA synthesis, a distinct pattern of RT pausing sites was observed for each template (Fig. 2). As previously observed, the WT HIV-1 HXB2 RNA gave a pausing pattern similar to the WT HIV-1 MAL RNA (19,27,51), the main pausing sites being at position +3 and +5 upstream of the PBS and in the A-rich loop. The pausing patterns observed with the His and His-AC RNAs were unique, whereas those observed with His-AC-GAC and His-AC-AT-GAC were very similar. The only distinct feature was weak pausing at position +1 at long incubation times during reverse transcription of His-AC-AT-GAC RNA that was not detected with His-AC-GAC (Fig. 2). This observation suggested that the structures of the initiation complexes involving these templates were similar. On the contrary, the pausing patterns observed with the His-AC and His-AC-GAC RNAs were very different (Fig. 2). Since most of the differences were

Figure 4. Comparison of synthetic and natural tRNA^{His}. Minus strand strong stop DNA was synthesized using His-AC-GAC as template and either synthetic or natural unlabeled tRNA^{His}. A radioactive nucleotide was included in the reaction, which was stopped after 5, 10, 15, 20, 25 and 30 min (lanes 1–6, respectively), and the products were analyzed by polyacrylamide gel electrophoresis.

located at a distance from the three mutations that distinguish these RNAs, they indicated that the corresponding initiation complexes most likely adopted different secondary or/and tertiary structures (see below).

Effect of the post-transcriptional modifications of tRNA^{His}

Using the wild type HIV-1 MAL RNA, we previously showed that the efficiency of the initiation of reverse transcription was strongly dependent on the presence of the post-transcriptional modifications of tRNA₃^{Lys}. In their absence, the interaction between the viral A-rich loop and the tRNA anticodon loop was unstable (9,10,52), and initiation of DNA synthesis was inefficient (27,49).

Therefore, we compared the (–) strand strong stop DNA synthesis using His-AC-GAC RNA as template and tRNA^{His} that was either synthesized *in vitro* or purified from beef liver, as primer (Fig. 4). Since purification of the natural tRNA^{His} was not complete, we used unlabeled tRNA primers and included a radioactive dNTP in the DNA synthesis reaction. Under these conditions, only the (–) strand strong stop DNA and the longest pausing products were detected (Fig. 4). Interestingly, we noticed little difference between the two primers, both in the kinetics of synthesis and in the final amount of (–) strand strong stop DNA synthesized. By comparison, a 5- to 10-fold difference in the efficiency of (–) strand strong stop DNA synthesis could be observed between natural and *in vitro* synthesized tRNA₃^{Lys} when using the WT HIV-1 MAL RNA as template (27).

Probing of the His-AC and His-AC-GAC RNA templates in their initiation complexes

The kinetics of reverse transcription described in the previous sections indicated that the efficiency of the initiation step of reverse transcription, as well as that of the (–) strand strong stop synthesis, dramatically increased with the accumulation of mutations U174G, G181A and U200C in the RNA template. We therefore examined the structure of the initiation complexes formed with His-AC and His-AC-GAC RNAs, using chemical and enzymatic probes.

In order to compare the structure of His-AC and His-AC-GAC RNAs, either in their free form, or in complex with tRNA^{His}, we used DMS and CMCT (Fig. 5). DMS methylates positions N1 of adenines and N3 of cytosines, and CMCT

Figure 5. Chemical probing of His-AC and His-AC-GAC RNAs. His-AC and His-AC-GAC RNAs, either free or in complex with tRNA^{His}, were submitted to modification by DMS (A) or CMCT (B) for 0 (control lane), 5, 10 or 15 min, as indicated above the lanes. Lanes marked U, G, C and A correspond to dideoxy sequencing reactions. The position of the PBS and of the sequence complementary to the anticodon loop (AC) of tRNA^{His} is indicated on the right of the gels.

modifies positions N1 of guanines and N3 of uridines. Watson–Crick base pairs and non-canonical interactions involving these positions protect them from chemical modification.

Comparison of the reactivity profiles of His-AC and His-AC-GAC RNAs in their free form indicated that the U174G, G181A and U200C mutations affected their structure. Differences in the modification patterns could be observed in the vicinity of the mutated residues, but also at remote positions. For instance, U199 and U201 were modified by CMCT in His-AC RNA, but not in His-AC-GAC-RNA (Fig. 5B). Similarly, U174 was modified, but the corresponding G174 was not reactive in His-AC-GAC (Fig. 5B), and C179 was modified only in His-AC RNA (Fig. 5A).

As RNAs His-AC and His-AC-GAC both possess a sequence complementary to the anticodon loop of tRNA^{His}, we were particularly interested in potential modifications in the reactivity profile that could take place at and around nucleotides 167–172 upon annealing of tRNA^{His}. In the free form of these RNAs, several nucleotides were modified by CMCT and DMS. Upon formation of the tRNA^{His}-vRNA complexes, we observed several modifications in the reactivity patterns in this region. Regarding modifications of the U and G residues by CMCT, we observed no striking differences between His-AC and His-AC-GAC RNAs. In both templates, primer annealing increased the reactivity of U159 and U165 towards CMCT, while the reactivity of U163 decreased (Fig. 5B). However, marked differences appeared in the DMS reactivity patterns of His-AC and His-AC-GAC RNAs upon tRNA^{His} annealing. In His-AC RNA, the strong modifications of A171, A172 and A177, and the weak modifications of C173 and C175 were not affected by the primer binding. On the other hand, tRNA^{His} induced strong or complete protection of A169, C170, A171, A172 and C173, which were strongly modified in the free form of His-AC-GAC RNA (Fig. 5A).

Thus, our probing data indicated that the interaction between the anticodon loop of tRNA^{His} and the mutated A-rich loop of His-AC RNA did not take place, or that it was very unstable, even though the mutations in the viral RNA were designed to allow it. In contrast, our data suggested that, in the case of His-AC-GAC RNA, this interaction was stable and conferred a strong protection against chemical modification to nucleotides 169–173.

In addition to the experiments described above, which were performed in the annealing buffer (see Materials and Methods), we also performed DMS probing of His-AC and His-AC-GAC RNAs, either free or in complex with tRNA^{His}, in the absence and in the presence of a 3-fold molar excess of RT, in the reverse transcription buffer (data not shown). Identical DMS modification patterns were obtained under all experimental conditions. The absence of an RT footprint on the initiation complex when using DMS is not new. We previously observed a footprint of RT on the WT MAL initiation complex when using enzymatic probes, but not chemical ones (15).

Probing of the primer tRNA^{His} in the initiation complexes formed by His-AC and His-AC-GAC RNA templates

In order to corroborate these conclusions, we analyzed the accessibility of tRNA^{His} in its free form and annealed to either

His-AC or His-AC-GAC RNA, using nuclease S1, RNase T1 and RNase V1 (Fig. 6A and data not shown). Nuclease S1 and RNase T1 cleave single-stranded regions of the RNA molecules, the latter being specific for G residues, while RNase V1 specifically cuts into base-paired and stacked RNA regions (53).

As expected, the cleavage pattern of tRNA^{His} was dramatically affected upon annealing to the viral templates. However, the cleavage patterns of tRNA^{His} hybridized either to His-AC RNA or to His-AC-GAC RNA looked very similar (Fig. 6A and data not shown). Nevertheless, minor differences in the cleavage pattern of the D arm of tRNA^{His} (nucleotides 10–25) by nuclease S1 (Fig. 6), RNase T1 and RNase V1 (data not shown) suggested that the initiation complexes formed by these viral RNAs adopted slightly different structures. Surprisingly, the anticodon loop of tRNA^{His}, which was cut preferentially 3' of nucleotides 34 and 33, and weakly at positions 31, 32 and 36 by nuclease S1 in the free form of the primer, was strongly protected by hybridization to either His-AC or His-AC-GAC RNA (Fig. 6). A similar observation was made with RNase T1 (not shown).

These results seem to contradict the DMS probing results indicating that only the mutated A-rich loop of His-AC-GAC RNA became protected upon tRNA^{His} annealing. Furthermore, when we annealed tRNA^{His} to His RNA and submitted the complex to nuclease S1 probing, we observed a strong protection of the anticodon loop of tRNA^{His} (Fig. 6A), even though the A-rich loop of His RNA cannot interact with this region of the primer (Fig. 1B and Table 1). Two alternative explanations can be put forward. Due to the size of the enzymatic probes, protection of the anticodon loop of tRNA^{His} might result from steric hindrance and might not be a reliable indicator of its interaction with the viral A-rich loop. Alternatively, the anticodon loop of tRNA^{His} might establish tertiary interactions with all the templates (His, His-AC and His-AC-GAC), but these interactions would differ from one vRNA to the other. Only with the His-AC-GAC RNA would the tRNA^{His} anticodon loop interact with the A-rich loop.

In order to test this possibility, we probed tRNA^{His} with kethoxal, which modifies positions N1 and N2 of unpaired guanines. Unlike nuclease S1 mapping, probing with kethoxal is not sensitive to steric hindrance. Interestingly, nucleotide G34 was strongly modified in the isolated tRNA^{His}, but protected in all tRNA^{His}/vRNA complexes (Fig. 6B). Thus, the tRNA^{His} anticodon loop did establish tertiary interactions in all complexes. A computer analysis using Mfold (54) indicated that nucleotides 0–57 of tRNA^{His} could fold into a stable intramolecular structure upon annealing its 3' end to the PBS. However, this structure is not consistent with the kethoxal probing data, since G34 is predicted to be unpaired (data not shown). This analysis strongly suggests that the tRNA^{His} anticodon loop interacted with the template RNAs. Indeed, several sequences partially complementary to the tRNA^{His} anticodon loop were identified in the vRNA sequences, outside the region probed in this study.

A complete analysis of the kethoxal modification patterns reveals that tRNA^{His} adopted different structures in the complexes with His RNA and His-AC-GAC RNA. For instance, tRNA^{His} G4 and G6 were more strongly modified in the complex with His-AC-GAC RNA than in the complex with His RNA, while the reverse situation was observed for

Figure 6. Enzymatic and chemical probing of tRNA^{His} . (A) 3'-end labeled tRNA^{His} , free or annealed to His-AC, His-AC-GAC or His RNA was submitted to hydrolysis with nuclease S1. Reactions were for 0 (control lane), 7.5 or 15 min, as indicated above the lanes. Lanes marked T1 and U2 correspond to sequencing reactions with T1 and U2 RNases, respectively. (B) Unlabeled tRNA^{His} , free or annealed to His, His-AC or His-AC-GAC RNA was modified with kethoxal for 0, 7 or 15 min. The modified guanines were identified by primer extension. The position of the anticodon loop (AC) and of the sequence complementary to the PBS (anti-PBS) are indicated.

G17, G18, G22 and G43 (Fig. 6B). An intermediate pattern was observed when tRNA^{His} was annealed to His-AC RNA (Fig. 6B), suggesting that this complex existed as a mixed population or equilibrium between two structures.

DISCUSSION

The mutant HIV-1 viruses that use alternate tRNAs as primer provide a unique opportunity to establish direct relationships between viral replication, efficiency of the initiation of reverse transcription and the structure of the initiation complex *in vitro*. This is particularly true for the mutants using tRNA^{His} because their evolution during prolonged cell culture is well documented (32–35).

The pHXB2(His) virus, in which a PBS complementary to tRNA^{His} has been substituted for the PBS complementary to $\text{tRNA}_3^{\text{Lys}}$ is unstable in cell culture and rapidly reverts to the WT virus (31,32). Here, we showed that the initiation of reverse transcription of the corresponding RNA (His RNA) is very inefficient. The k_{fast} value was decreased 5-fold as compared to the WT RNA, resulting in a 4-fold decrease of (–) strand strong stop DNA. These data explain why the pHXB2(His) virus is unstable in cell culture. Indeed, even

though the PBS in the WT and mutant PBS differ by seven nucleotides, reversion is a one step process (29–31). If a $\text{tRNA}_3^{\text{Lys}}$ molecule packaged into the virion is hybridized, although imperfectly, to the mutant PBS, its 18 3' terminal nucleotides are copied during synthesis of the (+) strand strong stop DNA, regenerating the WT PBS. Given the differences in the (–) strand strong stop DNA synthesis, the WT virus rapidly outgrows the pHXB2(His) virus.

The pHXB2(His-AC) virus does not revert to the WT virus, but rapidly selects three additional mutations (T174G, G181A, T200C) (32,33). The resulting virus, pHXB2(His-AC-GAC) replicates more rapidly than pHXB2(His-AC). Our data showed that even though the initiation of reverse transcription of pHXB2(His-AC) is slightly more efficient than that of pHXB2(His) (Fig. 3), equal amounts of (–) strand strong stop DNA are synthesized. Thus, the reason why pHXB2(His) reverts to the WT virus, while pHXB2(His-AC) maintains tRNA^{His} as primer cannot be found in their relative efficiency in initiating reverse transcription. Rather, it stems from the fact that incorporation of only three point mutations in pHXB2(His-AC) allows this virus to initiate reverse transcription and synthesize (–) strand strong stop DNA more efficiently than the WT virus. At the opposite, reversion of

pHXB2(His-AC) to the WT virus would not only require the reversion of the PBS, but also of the four point mutations in the A-rich loop.

Even though we cannot exclude that other viral functions are affected, our data showed that the mutations introduced in pHXB2(His) and pHXB2(His-AC) dramatically affect the initiation of reverse transcription and the (–) strand strong stop DNA synthesis. More important, our work revealed that the first mutations selected by pHXB2(His-AC) restore these reverse transcription steps. Thus, despite the possible overlap with integration signals (40–42), viral evolution was dictated by the initiation of reverse transcription. Our data show that, contrary to previous proposals (43), the mutations in the A-rich loop allow maintaining tRNA^{His} as primer by increasing the efficiency of this primer, rather than solely disfavoring tRNA₃^{Lys}. The rapid selection of the T174G, G181A and T200C mutations reveals a strong selection pressure to restore the initiation of reverse transcription, and thus indicates that this process constitutes a valuable target for anti-HIV agents.

Upon prolonged cell culture, pHXB2(His-AC-GAC) slowly selects two additional mutations (33,44). However, our data revealed that these mutations did not increase reverse transcription. On the contrary, initiation of reverse transcription of His-AC-AT-GAC RNA was reduced >3-fold relative to His-AC-GAC RNA, but this resulted in no significant reduction of (–) strand strong stop DNA. Thus, our data indicate that the virus has no pressure to optimize initiation of reverse transcription above a given threshold, because this does not translate into a significant increase of the longer reverse transcription products. The data we obtained with His-AC-AT-GAC RNA also confirm that restoring the initiation of reverse transcription is the first problem pHXB2(His-AC) has to deal with, while optimizing other replication steps occurs at later stages. It is presently unclear whether mutations C152A and C160T affect other steps of the reverse transcription process, integration, or another replication step.

Even though both pHXB2(His-AC) and pHXB2(His-AC-GAC) viruses can potentially interact with the anticodon loop of tRNA^{His}, the latter replicate more efficiently (33) and initiate reverse transcription much faster. This observation raised the possibility that the interaction between the anticodon loop of tRNA^{His} and the viral RNA was not sufficient to allow efficient initiation of reverse transcription. However, enzymatic and chemical probing of the primer/template complexes revealed structural differences between the complexes containing His-AC and His-AC-GAC complexes. Most importantly, DMS probing of the vRNA revealed that tRNA^{His} interacts with the mutated A-rich loop of His-AC-GAC RNA, but not that of His-AC-RNA. Mutation T174G likely plays an essential role in this interaction by extending the complementarity between the mutated A-rich loop and the anticodon stem of tRNA^{His}.

Thus, in the mutant HXB2 viruses utilizing tRNA^{His} as primer, efficient replication and initiation of reverse transcription correlate with a stable interaction between the primer anticodon loop and the viral RNA. This finding extends the conclusions of our previous studies conducted on the HIV-1 MAL isolate which showed that efficient *in vitro* initiation of HIV-1 MAL RNA reverse transcription requires the interaction between the viral A-rich loop and the anticodon of tRNA₃^{Lys} (19,27,49,50). Collectively, our present and past results suggest that an intermolecular interaction between the

viral RNA and the primer anticodon loop is a widespread feature in natural HIV-1 isolates and mutant HIV-1 adapted to utilize alternate primers. However, this does not appear to be an absolute requirement, as mutant viruses with two PBS that lack any complementarity with the anticodon loop of the two potential primer tRNAs have been observed (55).

Berkhout and co-workers recently proposed an interaction between a viral sequence named Primer Activation Signal (PAS) and the complementary anti-PAS sequence located in the TΨC arm of tRNA₃^{Lys} (17,18,56). However, analysis of the *in vivo* evolution of HIV-1 mutants using either tRNA^{His} or tRNA^{Met} as primer revealed no adaptation of the PAS to the anti-PAS sequence of these primers (51).

Stabilization of the interaction between the A-rich loop of HIV-1 MAL RNA and the U-rich anticodon of tRNA₃^{Lys} (9,52,57), and hence efficient initiation of reverse transcription (27,49), was shown to require the post-transcriptional modifications of the tRNA, especially the mcm⁵s²U nucleotide at position 34. This was expected as the thio moiety of U34 was known to stabilize the otherwise unstable A-U base pairs (58). By comparison, His-AC-GAC RNA forms a G-C rich interaction with the anticodon stem of tRNA^{His} (Fig. 1B and C). Therefore, our finding that the post-transcriptional modifications of tRNA^{His} are not required to promote the efficient initiation of reverse transcription of His-AC-GAC RNA is not very surprising. However, this result is of great importance for future structural studies (X-ray crystallography or/and NMR) of the initiation complex, which will be greatly facilitated by the use of an *in vitro* transcribed tRNA, instead of natural tRNA purified from mammal cells.

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REFERENCES

1. Baltimore, D. (1970) Viral RNA-dependent DNA polymerase. *Nature*, **226**, 1209–1211.
2. Temin, H.M. and Mizutani, S. (1970) RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature*, **226**, 1211–1213.
3. Arts, E.J. and Le Grice, S.F.J. (1998) Interaction of retroviral reverse transcriptase with template-primer duplexes during replication. *Progr. Nucleic Acids Res. Mol. Biol.*, **58**, 339–393.
4. Mak, J. and Kleiman, L. (1997) Primer tRNAs for reverse transcription. *J. Virol.*, **71**, 8087–8095.
5. Marquet, R., Isel, C., Ehresmann, C. and Ehresmann, B. (1995) tRNAs as primer of reverse transcriptase. *Biochimie*, **77**, 113–124.
6. Aiyar, A., Cobrinik, D., Ge, Z., Kung, H.J. and Leis, J. (1992) Interaction between retroviral U5 RNA and the TΨC loop of the tRNA^{Trp} primer is required for efficient initiation of reverse transcription. *J. Virol.*, **66**, 2464–2472.
7. Cobrinik, D., Soskey, L. and Leis, J. (1988) A retroviral RNA secondary structure required for efficient initiation of reverse transcription. *J. Virol.*, **62**, 3622–3630.
8. Morris, S., Johnson, M., Stavnezer, E. and Leis, J. (2002) Replication of avian sarcoma virus *in vivo* requires an interaction between the viral RNA and the TΨC loop of the tRNA^{Trp} primer. *J. Virol.*, **76**, 7571–7577.

9. Isel, C., Marquet, R., Keith, G., Ehresmann, C. and Ehresmann, B. (1993) Modified nucleotides of transfer-RNA₃^{Lys} modulate primer/template loop-loop interaction in the initiation complex of HIV-1 reverse transcription. *J. Biol. Chem.*, **268**, 25269–25272.
10. Isel, C., Ehresmann, C., Keith, G., Ehresmann, B. and Marquet, R. (1995) Initiation of reverse transcription of HIV-1, secondary structure of the HIV-1 RNA/tRNA₃^{Lys} (template/primer) complex. *J. Mol. Biol.*, **247**, 236–250.
11. Arts, E.J., Ghosh, M., Jacques, P.S., Ehresmann, B. and Le Grice, S.F.J. (1996) Restoration of tRNA₃^{Lys}-primed (–) strand DNA synthesis to an HIV-1 reverse transcriptase mutant with extended tRNAs. Implications for retroviral replication. *J. Biol. Chem.*, **271**, 9054–9061.
12. Arts, E.J., Stetor, S.R., Li, X., Rausch, J.W., Howard, K.J., Ehresmann, B., North, T.W., Wohrl, B.M., Goody, R.S., Wainberg, M.A. and Grice, S.F.J.L. (1996) Initiation of (–) strand DNA synthesis from tRNA₃^{Lys} on lentiviral RNAs: Implications of specific HIV-1 RNA-tRNA₃^{Lys} interactions inhibiting primer utilization by retroviral reverse transcriptases. *Proc. Natl Acad. Sci. USA*, **93**, 10063–10068.
13. Skripkin, E., Isel, C., Marquet, R., Ehresmann, B. and Ehresmann, C. (1996) Psoralen crosslinking between human immunodeficiency virus type 1 RNA and primer tRNA₃^{Lys}. *Nucleic Acids Res.*, **24**, 509–514.
14. Isel, C., Keith, G., Ehresmann, B., Ehresmann, C. and Marquet, R. (1998) Mutational analysis of the tRNA₃^{Lys}/HIV-1 RNA (primer/template) complex. *Nucleic Acids Res.*, **26**, 1198–1204.
15. Isel, C., Westhof, E., Massire, C., Le Grice, S.F.J., Ehresmann, C., Ehresmann, B. and Marquet, R. (1999) Structural basis for the specificity of the initiation of HIV-1 reverse transcription. *EMBO J.*, **18**, 1038–1048.
16. Lanchy, J.M., Isel, C., Keith, G., Le Grice, S.F., Ehresmann, C., Ehresmann, B. and Marquet, R. (2000) Dynamics of the HIV-1 reverse transcription complex during initiation of DNA synthesis. *J. Biol. Chem.*, **275**, 12306–12312.
17. Beerens, N., Groot, F. and Berkhout, B. (2001) Initiation of HIV-1 reverse transcription is regulated by a primer activation signal. *J. Biol. Chem.*, **276**, 31247–31256.
18. Beerens, N. and Berkhout, B. (2002) The tRNA primer activation signal in the human immunodeficiency virus type 1 genome is important for initiation and processive elongation of reverse transcription. *J. Virol.*, **76**, 2329–2339.
19. Goldschmidt, V., Rigourd, M., Ehresmann, C., Le Grice, S.F.J., Ehresmann, B. and Marquet, R. (2002) Direct and indirect contributions of RNA secondary structure elements to the initiation of HIV-1 reverse transcription. *J. Biol. Chem.*, **277**, 43233–43242.
20. Iwatani, Y., Rosen, A.E., Guo, J., Musier-Forsyth, K. and Levin, J.G. (2003) Efficient initiation of HIV-1 reverse transcription *in vitro*: Requirement for RNA sequences downstream of the primer binding site abrogated by nucleocapsid protein-dependent primer-template interactions. *J. Biol. Chem.*, **278**, 14185–14195.
21. Boulme, F., Freund, F., Gryaznov, S., Nielsen, P.E., Tarrago-Litvak, L. and Litvak, S. (2000) Study of HIV-2 primer-template initiation complex using antisense oligonucleotides. *Eur. J. Biochem.*, **267**, 2803–2811.
22. Freund, F., Boulme, F., Litvak, S. and Tarrago-Litvak, L. (2001) Initiation of HIV-2 reverse transcription: a secondary structure model of the RNA-tRNA₃^{Lys} duplex. *Nucleic Acids Res.*, **29**, 2757–2765.
23. Miller, J.T., Ehresmann, B., Hubscher, U. and Le Grice, S.F. (2001) A novel interaction of tRNA₃^{Lys,3} with the feline immunodeficiency virus RNA genome governs initiation of minus strand DNA synthesis. *J. Biol. Chem.*, **276**, 27721–27730.
24. Friant, S., Heyman, T., Bystrom, A.S., Wilhelm, M. and Wilhelm, F.X. (1998) Interactions between Ty1 retrotransposon RNA and the T and D regions of the tRNA_{Met} primer are required for initiation of reverse transcription *in vivo*. *Mol. Cell. Biol.*, **18**, 799–806.
25. Friant, S., Heyman, T., Wilhelm, M.L. and Wilhelm, F.X. (1996) Extended interactions between the primer tRNA_{Met} and genomic RNA of the yeast Ty1 retrotransposon. *Nucleic Acids Res.*, **24**, 441–449.
26. Gabus, C., Ficheux, D., Rau, M., Keith, G., Sandmeyer, S. and Darlix, J.S. (1998) The yeast Ty3 retrotransposon contains a 5′-3′ bipartite primer-binding site and encodes nucleocapsid protein NCp9 functionally homologous to HIV-1 NCp7. *EMBO J.*, **17**, 4873–4880.
27. Isel, C., Lanchy, J.M., Le Grice, S.F.J., Ehresmann, C., Ehresmann, B. and Marquet, R. (1996) Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcription require the post-transcriptional modifications of primer tRNA₃^{Lys}. *EMBO J.*, **15**, 917–924.
28. Brulé, F., Marquet, R., Rong, L., Wainberg, M.A., Roques, B.P., Le Grice, S.F.J., Ehresmann, B. and Ehresmann, C. (2002) Structural and functional properties of the HIV-1 RNA-tRNA₃^{Lys} primer annealed by the nucleocapsid protein: Comparison with the heat annealed complex. *RNA*, **8**, 8–15.
29. Das, A.T., Klaver, B. and Berkhout, B. (1995) Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural tRNA₃^{Lys}. *J. Virol.*, **69**, 3090–3097.
30. Li, X.G., Mak, J., Arts, E.J., Gu, Z.X., Kleiman, L., Wainberg, M.A. and Parniak, M.A. (1994) Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication. *J. Virol.*, **68**, 6198–6206.
31. Wakefield, J.K., Wolf, A.G. and Morrow, C.D. (1995) Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to tRNA₃^{Lys}. *J. Virol.*, **69**, 6021–6029.
32. Wakefield, J.K., Kang, S.M. and Morrow, C.D. (1996) Construction of a type 1 human immunodeficiency virus that maintains a primer binding site complementary to tRNA^{His}. *J. Virol.*, **70**, 966–975.
33. Zhang, Z., Kang, S.M., LeBlanc, A., Hajduk, S.L. and Morrow, C.D. (1996) Nucleotide sequences within the U5 region of the viral RNA genome are the major determinants for an human immunodeficiency virus type 1 to maintain a primer binding site complementary to tRNA^{His}. *Virology*, **226**, 306–317.
34. Li, Y., Zhang, Z., Wakefield, J.K., Kang, S.M. and Morrow, C.D. (1997) Nucleotide substitutions within U5 are critical for efficient reverse transcription of human immunodeficiency virus type 1 with a primer binding site complementary to tRNA^{His}. *J. Virol.*, **71**, 6315–6322.
35. Zhang, Z.J., Kang, S.M., Li, Y. and Morrow, C.D. (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.
36. Kang, S.M., Zhang, Z.J. and Morrow, C.D. (1997) Identification of a sequence within U5 required for human immunodeficiency virus type 1 to stably maintain a primer binding site complementary to tRNA^{Met}. *J. Virol.*, **71**, 207–217.
37. Kang, S.M. and Morrow, C.D. (1999) Genetic analysis of a unique human immunodeficiency virus type 1 (HIV-1) with a primer binding site complementary to tRNA^{Met} supports a role for U5-PBS stem-loop RNA structures in initiation of HIV-1 reverse transcription. *J. Virol.*, **73**, 1818–1827.
38. Kang, S.M., Zhang, Z. and Morrow, C.D. (1999) Identification of a human immunodeficiency virus type 1 that stably uses tRNA^{Lys1,2} rather than tRNA^{Lys,3} for initiation of reverse transcription. *Virology*, **257**, 95–105.
39. Beerens, N., Klaver, B. and Berkhout, B. (2000) A structured RNA motif is involved in correct placement of the tRNA₃^{Lys} primer onto the human immunodeficiency virus genome. *J. Virol.*, **74**, 2227–2238.
40. Esposito, D. and 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.
41. Brown, H.E., Chen, H. and 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.
42. Vicenzi, E., Dimitrov, D.S., Engelman, A., Migone, T.S., Purcell, D.F., Leonard, J., Englund, G. and Martin, M.A. (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.
43. Huang, Y., Shalom, A., Li, Z., Wang, J., Mak, J., Wainberg, M.A. and Kleiman, L. (1996) Effects of modifying the tRNA₃^{Lys} anticodon on the initiation of human immunodeficiency virus type 1 reverse transcription. *J. Virol.*, **70**, 4700–4706.
44. Zhang, Z., Kang, S.M. and Morrow, C.D. (1998) Genetic evidence of the interaction between tRNA^{Lys,3} and U5 facilitating efficient initiation of reverse transcription by human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses*, **14**, 979–988.
45. Bénas, P., Bec, G., Keith, G., Marquet, R., Ehresmann, C., Ehresmann, B. and Dumas, P. (2000) The crystal structure of HIV reverse transcription primer tRNA^{Lys,3} shows a canonical anticodon loop. *RNA*, **6**, 1347–1355.
46. Marquet, R., Baudin, F., Gabus, C., Darlix, J.L., Mougél, M., Ehresmann, C. and Ehresmann, B. (1991) Dimerization of human immunodeficiency virus (type 1) RNA: stimulation by cations and possible mechanism. *Nucleic Acids Res.*, **19**, 2349–2357.

47. Keith, G., Pixa, G., Fix, C. and Dirheimer, G. (1983) Primary structure of three tRNAs from brewer's yeast: tRNA^{Pro2}, tRNA^{His1} and tRNA^{His2}. *Biochimie*, **65**, 661–672.
48. Baudin, F., Marquet, R., Isel, C., Darlix, J.L., Ehresmann, B. and Ehresmann, C. (1993) Functional sites in the 5' region of human immunodeficiency virus type-1 RNA form defined structural domains. *J. Mol. Biol.*, **229**, 382–397.
49. Lanchy, J.M., Ehresmann, C., Le Grice, S.F.J., Ehresmann, B. and Marquet, R. (1996) Binding and kinetic properties of HIV-1 reverse transcriptase markedly differ during initiation and elongation of reverse transcription. *EMBO J.*, **15**, 7178–7187.
50. Lanchy, J.M., Keith, G., Le Grice, S.F.J., Ehresmann, B., Ehresmann, C. and Marquet, R. (1998) Contacts between reverse transcriptase and the primer strand govern the transition from initiation to elongation of HIV-1 reverse transcription. *J. Biol. Chem.*, **273**, 24425–24432.
51. Goldschmidt, V., Ehresmann, C., Ehresmann, B. and Marquet, R. (2003) Does the HIV-1 primer activation signal interact with tRNA^{Lys} during the initiation of reverse transcription? *Nucleic Acids Res.*, **31**, 850–859.
52. Bajji, A.C., Sundaram, M., Myszk, D.G. and Davis, D.R. (2002) An RNA complex of the HIV-1 A-loop and tRNA^{Lys,3} is stabilized by nucleoside modifications. *J. Am. Chem. Soc.*, **124**, 14302–14303.
53. Ehresmann, C., Baudin, F., Mougél, M., Romby, P., Ebel, J.P. and Ehresmann, B. (1987) Probing the structure of RNA in solution. *Nucleic Acids Res.*, **15**, 9109–9128.
54. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.*, **31**, 3406–3415.
55. Li, Y., Zhang, Z., Kang, S.M., Buescher, J.L. and Morrow, C.D. (1997) Insights into the interaction between tRNA and primer binding site from characterization of a unique HIV-1 virus which stably maintains dual PBS complementary to tRNA^{Gly} and tRNA^{His}. *Virology*, **238**, 273–282.
56. Beerens, N. and Berkhout, B. (2002) Switching the *in vitro* tRNA usage of HIV-1 by simultaneous adaptation of the PBS and PAS. *RNA*, **8**, 357–369.
57. Tisne, C., Rigourd, M., Marquet, R., Ehresmann, C. and Dardel, F. (2000) NMR and biochemical characterization of recombinant human tRNA^{Lys3} expressed in *Escherichia coli*: identification of posttranscriptional nucleotide modifications required for efficient initiation of HIV-1 reverse transcription. *RNA*, **6**, 1403–1412.
58. Houssier, C. and Grosjean, H. (1985) Temperature jump relaxation studies on the interactions between transfer RNAs with complementary anticodons. The effect of modified bases adjacent to the anticodon triplet. *J. Biomol. Struct. Dyn.*, **3**, 387–399.