Initiation of HIV-2 reverse transcription: a secondary structure model of the RNA–tRNA\textsuperscript{Lys3} duplex

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ABSTRACT

Human immunodeficiency virus type 2 (HIV-2) reverse transcription is initiated from cellular tRNA\textsuperscript{Lys3} partially annealed to the RNA viral genome at the primer binding site (PBS). This annealing involves interactions between two highly structured RNA molecules. In contrast to HIV-1, in which the reverse transcription initiation complex has been thoroughly studied, there is still little information regarding a possible model to describe the secondary structure of the template–primer complex in HIV-2. To determine whether HIV-2 RNA sequences flanking the PBS may specifically interact with the natural primer tRNA, we performed site-directed mutagenesis and enzymatic footprinting. An RNA fragment corresponding to the HIV-2 U5 RNA domain and tRNA\textsuperscript{Lys3} were probed either in their free form or in the binary complex. Important reactivity changes to nucleases were obtained upon complex formation. In addition to the canonical contacts between the viral PBS and the 3' end acceptor stem of tRNA Lys3, we identified two additional interacting domains: (i) the U-rich region of the anticodon loop with the A-rich sequence of the internal loop within the PBS(ΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨΨPsi
of alternative strategies may be useful to demonstrate the existence of loop–loop interactions and to characterise their role in the initiation of reverse transcription. Using an antisense approach we have recently shown that the HIV-2 A-rich internal loop (287AAAA290) is able to interact with the U-rich anticodon of the tRNALys3 primer (15).

In the present study we combined site-directed mutagenesis and enzymatic probing of both partners engaged in the template–primer initiation complex to obtain further information about the structural rearrangements taking place upon complex formation. The results showed other secondary structure elements in interaction, besides those concerning the acceptor stem of tRNALys3 and the PBS involved in the stricto sensu priming. These new interactions were formed between the A-rich internal prePBS loop and the U-rich anticodon, as well as between a region of HIV-2 U5 and the stem of the TΨC stem–loop from tRNA Lys3. All these data taken together with sequence comparison between different HIV-2 isolates has allowed us to establish a secondary structure model of the HIV-2 tRNA Lys3–viral RNA primer–template initiation complex.

MATERIALS AND METHODS

Proteins

HIV-2 RT RNase H was expressed in transformed Escherichia coli (15) and purified as previously described (16).

T7 RNA polymerase was from Stratagene; DNase I, NcoI and mung bean nuclease (MBN) from Gibco BRL Life Technologies Inc.; human placental RNase inhibitor (RNasin) from Promega; bacteriophage T4 polynucleotide kinase from New England Biolabs Inc.; ribonuclease V1 and RNA ligase from Pharmacia.

Mutagenesis

The QuickChange™ Site-Directed Mutagenesis Kit was from Stratagene.

Nucleotides

Radioisotopes [α-32P]dATP (3000 Ci/mmol), [α-32P]dCTP (3000 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol) and [α-32P]pCp (3000 Ci/mmol) were purchased from Amersham Laboratories. Unlabelled nucleotides [deoxyribonucleoside triphosphates (dNTPs) and ribonucleoside triphosphates (rNTPs)] were from Gibco BRL Life Technologies Inc.

Oligodeoxynucleotides (ODNs)

The nucleotide sequence of the HIV-2ROD strain (13) was used to design all the antisense ODNs. ODNs 303–320 and 372–390 were purchased from MGW-Biotech AG.

tRNA Lys3

Beef liver tRNA Lys3 was purified as previously described (16).

In vitro HIV-2 RNA synthesis

The HIV-2ROD RNA fragment containing nucleotides 1–545 (a kind gift from Dr J.-L. Darlix, INSERM U-412, Lyon, France) was sub-cloned in a pBluescript vector. Escherichia coli XL-1 Blue was used for plasmid amplification. After digestion of the HIV-2ROD clone with NcoI (position 545) and in vitro transcription using the T7 RNA polymerase, we obtained RNA starting at position +1. Three micrograms of linearised plasmid DNA were transcribed in a reaction mixture (final volume 0.1 ml) containing 40 mM Tris–HCl (pH 8.0), 8 mM MgCl2, 10 mM spermidine, 25 mM NaCl, 10 mM dithiothreitol (DTT), 0.5 mM of each rNTP, 100 U of T7 RNA polymerase and 20 U of RNasin, for 1.5 h at 37°C. After treatment with 12 U of RNase-free DNase I for 10 min at 37°C, RNA transcripts were extracted with 1 vol of phenol/chloroform/isoamyl alcohol (24/24/1) and 1 vol chloroform, and precipitated by addition of 2.5 vol of ethanol and 0.3 M ammonium acetate.

Reverse transcription with an HIV-2 RNA template

As described before (17), reverse transcription was performed in a total volume of 10 µl containing 50 mM Tris–HCl pH 8.0, 6 mM MgCl2, 2 mM DTT, 10 mM NaCl, 100 nM HIV RNA, 0.3 nM of each dNTP, 1 µM of synthetic ODN primer complementary to HIV-2 RNA and 5 µCi [α-32P]dCTP (3000 Ci/mmol). Template and ODN primer were incubated for 15 min at 37°C. Then, the dNTP mixture, [α-32P]dCTP, 150 nM RT (RNase H-) and 0.2 U RNasin were added. Incubation was carried out for 45 min at 37°C and the reaction was stopped by adding 3 µl of electrophoresis loading buffer (95% formamide, 0.5 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol, 0.025% SDS). The reaction products were analysed by electrophoresis on 6% polyacrylamide–TBE–7 M urea denaturing gels. After the run, gels were autoradiographed and films scanned using the NIH Image Program (Macintosh).
Directed mutagenesis of HIV-2 RNA

The QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) was used. A mutated HIV-2 ROD RNA (nucleotides 1–545) containing 275CUU276 [called mut(274–276)RNA] instead of the wild-type 275AA276 fragment, or 287AGU290 [called mut(287–290)RNA] instead of the wild-type 287AA290 fragment was obtained by transcription of the mutated plasmid.

tRNA_{Lys}^3 labelling

3′ End labelling. The tRNA_{Lys}^3 (1 µg) was labelled at its 3′ end with 100 µCi of [α-32P]pCP (3000 Ci/mmol) in ligation buffer (50 mM HEPES pH 7.5, 15 mM MgCl₂ and 3.3 mM DTT), in the presence of 1 mM ATP, 0.1 mg/ml of bovine serum albumin and 1300 U/ml T4 RNA ligase in 30 µl final volume. The reaction was incubated overnight at 4°C and stopped with 10 µl of loading buffer. The labelled [α-32P]pCPtRNA_{Lys}^3 was purified by electrophoresis on 8% denaturing polyacrylamide gel.

5′ End labelling. The tRNA_{Lys}^3 (1 µg) was mixed with 100 µCi of [γ-32P]ATP (3000 Ci/mmol) in the exchange buffer (50 mM imidazole–HCl pH 6.4, 12 mM MgCl₂, 1 mM 2-mercapto-ethanol, 70 µM ADP), in the presence of 5 U T4 polynucleotide kinase in a final volume of 25 µl. The reaction was performed for 1 h at 37°C, stopped with 10 µl of loading buffer and the labelled [32P]tRNA_{Lys}^3 was extracted as described above.

Enzymatic probing of tRNA_{Lys}^3

Either 5′ end labelled 32P-pRNA_{Lys}^3 or 3′ end [α-32P]pCPtRNA_{Lys}^3 (0.1 pmol) was hybridised to the RNA_{ROD} fragment (1 pmol) by heating at 95°C for 2 min, followed by slow cooling. The enzymatic digestion of the 3′2P-pRNA_{Lys}^3-viral RNA complex by MBN was performed in 30 µl at 37°C for 7 min, in the presence of 10 mM sodium acetate pH 5.0, 0.01 mM zinc acetate, 0.1 mM L-cysteine, 3 mM NaCl, 6 mM MgCl₂ and different concentrations of MBN. Nuclease digestion of the [α-32P]pCPtRNA_{Lys}^3-viral RNA complex was performed in 30 µl at 37°C for 7 min, in the presence of 20 mM Tris–HCl pH 7.2, 200 mM NaCl, 6 mM MgCl₂ and different concentrations of ribonuclease V1. The reaction was stopped with 1 vol of phenol/chloroform/isoamyl alcohol (24/24/1 v/v/v) and 3 µl of loading buffer were added to the aqueous phase previously recovered. Samples were analysed on 15% denaturing polyacrylamide gel.

Enzymatic probing of viral RNA

After prehybridising tRNA_{Lys}^3 (5 pmol) to the RNA_{ROD} fragment (0.5 pmol), the complex was digested in the same conditions as described above. The reaction products were extracted with 1 vol of phenol/chloroform/isoamyl alcohol and RNAs were precipitated with ethanol. RNA cleavage sites were identified by the reverse transcription reaction as follows: the digestion products were preincubated for 15 min at 37°C with two different primers, either an anti-PBS ODN (nucleotides 303–320) or an ODN primer (nucleotides 372–390), and then incubated for 5 min in the presence of 5 µCi [α-32P]dNTP, 150 mM RT and 0.2 U human placental RNase inhibitor (the labelled dNTP was dATP for the anti-PBS primer and dCTP for the other ODN primer). The incubation was performed for 45 min at 37°C in the presence of the four dNTPs, and the reaction was stopped by the addition of 3 µl loading buffer. Samples were analysed by electrophoresis on a 15% denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Our aim in this work was to obtain further information about the replication of HIV-2 and more specifically on the initiation of the reverse transcription step, a crucial target of antiviral agents. To analyse the structural changes occurring upon formation of the HIV-2 primer–template reverse transcription initiation complex, we probed tRNA_{Lys}^3 and the US HIV-2 ROD RNA fragment, both in the free state and the binary complex. Digestion patterns were obtained by using structure-specific nucleases, the MBN, a specific nuclease of single-stranded nucleic acids and ribonuclease V1, a specific enzyme of double-stranded structures.

Probing of tRNA_{Lys}^3

Labelled tRNA_{Lys}^3, either free or hybridised to HIV-2 ROD RNA (nucleotides 1–545), was subjected to limited digestion with RNase V1 and MBN. The positions of enzymatic cleavages within tRNA were identified by the size of the radioactive fragments. Figure 2A shows the cleavage products obtained when tRNA_{Lys}^3 alone (lanes 2–5) or annealed to the viral RNA (lanes 6–9) was digested with increasing amounts of ribonuclease V1. Figure 2B corresponds to the MBN digestion pattern of tRNA_{Lys}^3 alone or annealed to viral RNA (lanes 2–5 and 6–9, respectively). To compare the intensities of the cleavage bands obtained with either free or complexed tRNA, the concentration of RNA substrate was kept constant by adding an RNA which was non-complementary to the primer tRNA_{Lys}^3 (a 528 nt RNA fragment coding for the retroviral REV protein). Data obtained with both nucleases are summarised in Figure 2C.

When free tRNA_{Lys}^3 was probed with ribonuclease V1, strong cleavage bands (that became stronger with increasing enzyme concentrations) were observed at sites corresponding to the region C_{26–A_{74}}, as well as a weaker cleavage at nucleotide U_{14} (Fig. 2A, lanes 2–5). These nucleotides are present in the stem of the anticodon stem–loop. The treatment of free tRNA with MBN (Fig. 2B, lanes 2–5) produced single-stranded cleavages on the D-loop, the variable loop and the anticodon loop (Fig. 2C). The V1 and MBN digestion profiles were in agreement with the general cloverleaf structure of a native tRNA.

A striking result was obtained when tRNA_{Lys}^3 was probed with the nucleases after formation of the binary complex with tRNA. In this case, extremely important changes in reactivity were observed. As shown in Figure 2A (lanes 6–9) the annealed tRNA became more accessible to ribonuclease V1. The most dramatic changes between free and complexed tRNA were observed at positions 48–52 of the stem of the TΨC stem–loop. Strong bands appeared after ribonuclease V1 treatment. These corresponded to cleavage sites at m₅C₄₉ and A₅₀. Less intensive bands corresponding to nucleotides m₅C₄₈, G₅₁ and G₅₂ were also present.

Other changes observed after complex formation concerned the cleavage bands corresponding to the C_{26–A_{71}} region. These bands became more intense (Fig. 2, compare lane 3 with lane 4).
In addition, new weak bands appeared, corresponding to digestion at positions A23, G24, Ψ27, and C40.

After treatment with MBN (Fig. 2B), practically no differences appeared in the digestion patterns of tRNA either alone or complexed to RNA, indicating that the stem–loop structure of the anticodon and the D-loop domains were maintained within the primer–template complex. Interestingly, while the bands obtained by MBN digestion were more intense in the presence of viral RNA, especially for nucleotides D47, A38, and R37, the reactivity of the nucleotide U36 seemed to be lower (Fig. 2B).

From these results it may be concluded that upon formation of the initiation complex, the region 48m5Cm5CAG51 of the TΨC loop must be engaged in a double-stranded secondary structure. Similarly, the relative protection of U36 in the anticodon loop could be due to its internal position within the complex. However, the increase in reactivity of nucleotides R37 and A38 does not favour this hypothesis. The anticodon could also be engaged in a double-stranded structure. Thus, these two regions could be in interaction with other domains of the viral RNA.

Probing of the U5 region from HIV-2\textsubscript{ROD} RNA

HIV-2 RNA was probed using the same nucleases and experimental conditions described above. In this case, however, the enzymatic cleavages within RNA were identified by using primer extension assays. Reverse transcription of the resulting prePBS fragments was performed with an anti-PBS ODN primer. The synthesis bands were visualised by radioactive labelling (see Materials and Methods). The full cDNA synthesis product before nuclease treatment is shown in Figure 3A (lane C). The intermediate bands correspond to pausing sites of reverse transcription. After nuclease treatment the cleavage sites were defined either by the appearance of new radioactive bands, or by an increase in the already existing ones.

Digestion of the prePBS region (nucleotides 244–305). The results of viral RNA digestion in the absence of tRNA\textsubscript{Lys} are presented and summarised in Figure 3. Treatment of viral RNA with MBN revealed highly accessible regions such as the triplets 279GAG281 and 287AAA289 and the nucleotides G285 and C294 (Fig. 3A, lanes 1–3). Although MBN is specific to single-stranded regions, the majority of the cleavage sites corresponded to nucleotides involved in a double-stranded region, according to the secondary structure model proposed by
Berkhout and Schoneveld (13). These authors also observed a sensitivity of certain double-stranded regions (including A280, A287 and A288) to dimethyl sulfate, diethyl pyrocarbonate and ribonuclease T1, which are normally specific agents of single-stranded molecules.

When the digestion was performed in the presence of RNase VI, cleavages were distributed throughout the prePBS region (Fig. 3B, lanes 7–10). Some cleavage sites coincided with those obtained with MBN. These results may be due to the fact that the U5 region of viral RNA (Fig. 3C), and more specifically the apical stem–loop (nucleotides 261–288), is a dynamic structure that can be transitorily destabilised. This is in agreement with the secondary structure of the 244–305 region described previously (13).

Digestion of the prePBS-PBS RNA region annealed to primer tRNAlys3. After annealing the viral RNA to tRNA, the binary complex was digested by MBN (Fig. 3A, lanes 4–6). Upon complex formation we observed a decreased sensitivity of all nucleotides that were reactive towards MBN in the free viral RNA, thus showing a protection of viral RNA by tRNAlys3.

Similarly, the presence of tRNAlys3 on the PBS protected the prePBS region from ribonuclease V1 attack (Fig. 3B, lanes 11–14). However, at the highest concentration of V1 used, weak but significant cleavage bands appeared as with the free template. This was the case with nucleotides U268, A284 and G285, thus indicating that the apical stem–loop structure is maintained. It was also the case for nucleotides C298, A299, G300 and G301, showing that the stem–loop structure adjacent to the PBS was also conserved.

Mutated viral RNAs
To substantiate our results regarding the eventual interaction between the U-rich anticodon loop of tRNAlys3 and one of the two A-rich loops of the HIV-2 U5 region, we combined site-directed mutagenesis and probing with MBN. The two A-rich regions of the HIV-2 RNA prePBS region (stem–loop comprising nucleotides 244–305) were mutated independently (Fig. 4). Two fragments with different sequences in the A-rich regions were constructed: a mut274–276 RNA with a CUU instead of the wild-type triplet 274AAA276 on the apical loop, and a mut287–290 RNA with an AGUU instead of 287AAAA290 on the internal loop. The thermodynamic stability of each mutated RNA was then calculated and compared to that of wild-type RNA. Although the values obtained were similar for the three RNAs (Fig. 4) the substitution of the A nucleotides...
led to a small decrease in the stability of the stem–loop, especially for mut(274–276) RNA. Both mutants were then tested for their sensitivity towards MBN in the absence or in the presence of tRNALys3.

**Digestion of mutated RNA in the absence of tRNALys3.** Although the MBN digestion patterns of mut(274–276) RNA and mut(287–290) RNA were rather similar (Fig. 5), some differences in sensitivity of mutated RNAs were observed. For example, in mut(287–290) RNA, nucleotides U 264 and U 265 were more accessible than in the wild-type or in the mut(274–276) RNA. This is probably due to differences in the stability of the stem–loop caused by changes in the sequence, thereby making some regions of the RNA more accessible.

**Digestion of mutated RNAs in the presence of tRNALys3.** Like wild-type, mut(274–276) RNA became resistant to the attack by MBN in the presence of tRNALys3 (Fig. 5, lanes 5–8). The new cleavage sites (U 269, U 270, G 271 and G 272) at the highest nuclease concentration used can be explained by the decrease in stability of the prePBS stem–loop (ΔG = –111 kJ). The second mutated RNA, mut(287–290), also showed increased resistance to nuclease digestion in the presence of tRNALys3. However, contrary to wild-type and the mutated RNAs strongly supports the interaction between the tRNALys3 anticodon and the internal A-rich loop within the initiation complex.

**Study of the (321–371) region from viral RNA**

To determine whether the region 321–371 at the 3′ end of the PBS involved in a stem–loop interaction with the U5 region (Fig. 1) was implicated in the initiation complex, we used the same protocol described above (Fig. 3). This time the cleavage sites were identified by reverse transcription from a hybridised primer in the region 372–390. Figure 6A shows the MBN digestion pattern of the RNA region comprising nucleotides 321–371, either free or in the duplex with tRNA (lanes 1–3 and 4–6, respectively). Data are summarised in Figure 6B. The two digestion profiles were identical in the presence or absence of tRNALys3. The main cleavage sites A 368 and G 335 corresponded to the two loops in the secondary structure model. It may be concluded that there is no modification of the secondary structure of the fragment 206–226/321–371 upon formation of the template–primer complex, thus showing that the 321–371 region is not involved in an interaction with tRNALys3.

**A secondary structure model of the HIV-2 tRNALys3–RNA complex**

As described above we used structure-specific nucleases to map both viral RNA and tRNA primer secondary structures before and after formation of the HIV-2 RNA–tRNA complex. The reactivity of the prePBS region towards MBN and ribonuclease V1 does not allow the direct observation of a possible interaction between the anticodon loop of the natural primer and one of the two A-rich loops of the HIV-2 RNA. However, the comparison of the digestion patterns obtained with the wild-type and the mutated RNAs strongly supports the interaction between the tRNALys3 anticodon and the internal A-rich loop within the initiation complex.
Upon complex formation, the 28AAA290 region of the U5-prePBS internal loop was protected, thus showing an interaction with tRNA (Figs 3 and 5). Moreover, the 48m5Cm5CAG51 region was highly degraded by ribonuclease V1 when tRNA was annealed to the PBS (Fig. 2), thereby pointing to the existence of a double-stranded region between nucleotides 48 and 51 of tRNALys3 and a complementary sequence of the viral RNA.

The analysis of the U5 domain showed a sequence presenting a perfect match with the region 48–55 of the TΨC stem–loop. In view of these observations, an interaction may be predicted between the region 240–247 of HIV-2 RNA and the complementary region (nucleotides 48–55) of the TΨC domain upon formation of the initiation complex. In relation to this, it is important to point out that when Berkhout and Schonveld proposed their structural model of HIV-2 RNA, they already raised the possibility of having a base-paired region between the TΨC loop of tRNALys3 and this 5′ region upstream of the PBS (13).

To further assess the biological importance of the interactions with U5, we analysed the sequences of this region from 16 HIV-2 isolates (Table 1). We considered only the isolates that have been completely sequenced and are available in Internet databases. From this analysis we can conclude the following. (i) The changes affecting the whole prePBS stem–loop (positions 244–305) are essentially distributed within the apical stem–loop (nucleotides 261–298). In fact, 13 isolates out of 16 presented at least one mutation in this hairpin. In contrast, only five isolates out of 16 showed the same change within the stem 244–256/293–305, in which the nucleotide G301 is replaced by an A. However, most of the changes altering the double strand did not lead to a modification of the RNA secondary structure, because they corresponded to compensatory mutations, e.g., the formation of U-A or C-G base pairs instead of U-G. This is in agreement with previous phylogenetic and mutagenesis data obtained from HIV-1 (18,19). It was suggested that mutations modifying the stability of the prePBS hairpin were able to disturb both tRNA annealing and initiation of reverse transcription. In HIV-2, the lower stem of prePBS seems to play a similar role. (ii) In six isolates out of 16, the 274AAA276 triplet is replaced by GAA or AAU, while the sequence 287AAAA290 is perfectly conserved. This observation underlines the importance of this region in the formation of the initiation complex. (iii) Similarly, five changes were found in the 230–250 region, (G231, A232, G233, A235 and A236), while no changes for nucleotides involved in
the interaction of viral RNA with the TΨC stem–loop were observed.

Since the HIV-2 RNA ROD isolate has a U instead of a C at position 310 within the PBS, some authors have raised the possibility that an alternative primer, tRNA_{Lys}^{3} (also called tRNA_{Lys}^{7}), may be used (20, 21). This variant differs from tRNA_{Lys}^{3} at five nucleotide positions. Interestingly, the change m_{5}C_{48}-U was found, which is involved in the interaction with viral RNA. This change leads to the formation of a base pair G_{247}/U_{48} instead of G_{247}/m_{5}C_{48} that should not influence the structure of the HIV-2 initiation complex established in this work.

By using enzymatic probing data and sequence comparison, we propose a structural model of the HIV-2 primer–template reverse transcription initiation complex (Fig. 7). This model comprises the secondary structure elements in interaction: (i) the viral PBS and the 3′ end acceptor stem of tRNA_{Lys}^{3} that serves as *stricto sensu* primer; (ii) the U-rich region of the anticodon loop and the A-rich sequence of the internal loop (287AAAA290) contained in the prePBS region; (iii) nucleotides 48–54 from the TΨC domain of tRNA with the region 240–247 of viral RNA.

This HIV-2 model is similar to that described for HIV-1 (4), mainly concerning the interaction between the U-rich sequence of the anticodon and the A-rich sequence of the viral prePBS. However, there are some significant differences. In contrast to HIV-1, no intramolecular changes were observed in the structure of the HIV-2 binary complex. In this sense, the HIV-2 complex is similar to that described for Rous sarcoma virus (RSV) (22), where only the TΨC domain of tRNA interacts with the U5 RNA.

The use of site-directed mutagenesis and nuclease footprinting approaches allowed us to define the secondary structure of two crucial RNA partners of the HIV-2 initiation complex. These results should help to overcome the absence of appropriate crystals allowing the study of the 3D structure of this complex. Furthermore, the interactions inferred from these studies are in good agreement with the kinetic results we reported previously for the *in vitro* study of HIV-2 reverse transcription (23). As in the case of HIV-1 and RSV (24, 25), tRNA primed cDNA synthesis in HIV-2 occurs in two steps: a slow and distributive initiation step, and a fast and processive elongation process. From these results obtained with these three retroviruses, it can be proposed a unifying model of the initiation of reverse transcription, which links the primer–template interactions with the emergence of the two kinetic steps mentioned above. However, although HIV-1, RSV and HIV-2 seem to have a similar mechanism for the initiation of
cDNA synthesis, they present different secondary structures of the initiation primer–template duplex.

Further work is necessary to ascertain the structure–function relationship of the nucleic acids and protein partners of the retroviral initiation complex. This approach will help to fully understand the mechanism of reverse transcription and to design new inhibitors able to interfere with the structure of the initiation complex, a critical target in the fight against HIV infection.

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