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Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcription require the post-transcriptional modifications of primer $tRNA_3^{Lys}$

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Initiation of RNA-dependent DNA synthesis by retroviral reverse transcriptases is generally considered as unspecific. In the case of human immunodeficiency virus type 1 (HIV-1), the natural primer is $tRNA_3^{Lys}$. We recently found evidence of complex interactions between $tRNA_3^{Lys}$ and HIV-1 RNA that may be involved in the priming process. In this study, we compare the ability of natural and unmodified synthetic $tRNA_3^{Lys}$ and 18mer oligoribo- and oligodeoxyribonucleotides complementary to the viral primer binding site to initiate replication of HIV-1 RNA using either homologous or heterologous reverse transcriptases. We show that HIV-1 RNA, HIV-1 reverse transcriptase and primer $tRNA_3^{Lys}$ form a specific initiation complex that differs from the unspecific elongation complex formed when an oligodeoxyribo-nucleotide is used as primer. Modified nucleosides of $tRNA_3^{Lys}$ are required for efficient initiation and transition to elongation. Transition from initiation to elongation, but not initiation of reverse transcrip-tion itself, is facilitated by extended primer–template interactions. Elongation, but not initiation of reverse transcription, is inhibited by Mn^{2+}, which further differentiates these two different functional states of reverse transcriptase. These results define initiation of reverse transcription as a new target to block viral replication.

Keywords: AIDS/HIV-1/polymerase/replication/retrovirus

Introduction

Reverse transcriptase (RT) is a key enzyme of the retroviral cycle that converts the single-stranded RNA genome of retroviruses into double-stranded DNA (Baltimore, 1970; Temin and Mizutani, 1970). Since its discovery, RT has become an important tool in molecular biology, i.e. RTs from different origins are used to reverse transcribe RNAs of almost any sequence by using oligodeoxyribonucleotides to prime the reaction (Maniatis *et al.*, 1982). Given its efficiency with a large number of templates and primers, initiation of reverse transcription has been tacitly assumed to be an unspecific process.

However, during the retroviral replication cycle, specific tRNAs are used to prime (–) strand DNA synthesis (Harada *et al.*, 1975, 1979; Marquet *et al.*, 1995). $tRNA^{Trp}$ (Harada *et al.*, 1975) and $tRNA^{Pro}$ (Harada *et al.*, 1979) are the natural primers of avian and most murine retroviruses, respectively, while reverse transcription of the genomic RNA of human immunodeficiency type 1 virus (HIV-1) is primed by $tRNA_3^{Lys}$ (Ratner *et al.*, 1985). In these cases, the primer tRNA binds to the viral RNA by its 3′-terminal 18 nucleotides which are complementary to the so-called primer binding site (PBS) (for review, see Marquet *et al.*, 1995). Moreover, additional interactions between the genomic RNA of avian sarcoma and leukaemia virus (Aiyar *et al.*, 1992, 1994) and HIV-1 (Isel *et al.*, 1993, 1995), and their respective primer tRNAs were found outside of the PBS. In the case of HIV-1, as much as 31 nucleotides of $tRNA_3^{Lys}$ interact with the viral genome (Isel *et al.*, 1995). In particular, the anticodon loop of $tRNA_3^{Lys}$ interacts with an A-rich loop (GUAAAA) located 12–17 nucleotides upstream of the PBS. This loop–loop interaction requires the post-transcriptional modifications of natural $tRNA_3^{Lys}$ to be stable: its stability is strongly reduced when nucleoside 5-methoxycarbonylmethyl-2-thiouridine34 located in the anticodon loop of $tRNA_3^{Lys}$ is dethiolated into 5-methoxycarbonylmethyluridine34, and it cannot be observed when natural $tRNA_3^{Lys}$ is replaced by an unmodified $tRNA_3^{Lys}$ ($utRNA_3^{Lys}$) obtained by *in vitro* transcription (Isel *et al.*, 1993). The sequences involved in the extended interactions between $tRNA_3^{Lys}$ and the viral template are conserved in all HIV-1 isolates (Isel *et al.*, 1993, 1995), suggesting that initiation of (–) strand DNA synthesis requires specific interactions involving RNA tertiary structure.

Even though a large number of studies are devoted to reverse transcription, little is known about the detailed mechanism of initiation of (–) strand synthesis. This is primarily due to the fact that oligodeoxyribonucleotides (e.g. Kati *et al.*, 1992; Reardon, 1993; Spence *et al.*, 1995, and references therein) or $utRNA_3^{Lys}$ (Kohlstaedt and Steitz, 1992) are usually used to prime reverse transcription. In the few cases in which natural $tRNA_3^{Lys}$ was used (Arts and Wainberg, 1994; Arts *et al.*, 1994), the disappearance of the unelongated primer, and hence the initiation of reverse transcription, was not monitored. In this study, we compared the ability of $tRNA_3^{Lys}$, $utRNA_3^{Lys}$, 18mer oligodeoxyribonucleotide (ODN) and oligoribonucleotide (ORN) complementary to the PBS to initiate replication of HIV-1 RNA using either homologous or heterologous RTs. We show that initiation of reverse transcription is a specific process, and provide evidence for a functional transition from initiation to elongation of reverse transcription. This transition relies on the extended primer–template interactions, while proper initiation of reverse transcription does not. However, both processes require the post-transcriptional modifications of $tRNA_3^{Lys}$.

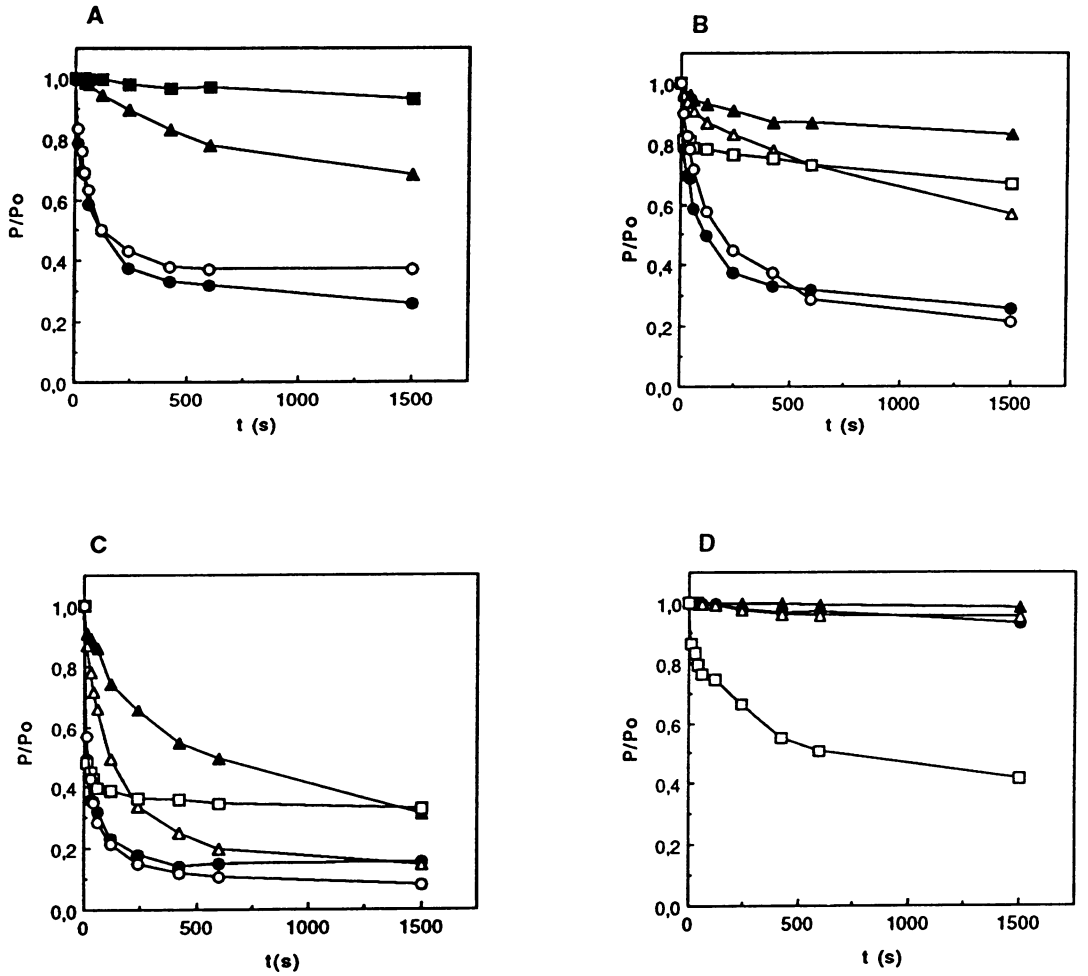

Fig. 1. Priming efficiency. The fraction of unextended primer is plotted versus time. P_0 and P are the initial amount and remaining amount of the unextended primer, respectively. P_0 was 10 nM in all experiments. (**A**) $tRNA_3^{Lys}$ was extended with 3 nM of AMV RT (■), MLV RT (▲), RNase H(–) HIV-1 RT (○) or HIV-1 RT (●). (**B**) and (**C**) $utRNA_3^{Lys}$ (▲), ORN (△) and $tRNA_3^{Lys}$ (●) were extended with HIV-1 RT, and ODN (□) was extended with RNase H(–) HIV-1 RT. $tRNA_3^{Lys}$ was also extended with HIV-1 RT using a mutant viral RNA as template (○) (see text). RT concentration was 3 nM (B) or 27 nM (C). (**D**) $tRNA_3^{Lys}$ (●), $utRNA_3^{Lys}$ (▲), ORN (△) and ODN (□) were elongated with 3 nM AMV RT.

Results and discussion

Specific initiation versus non-specific elongation

The specificity of initiation of HIV-1 reverse transcription was tested by analysing the synthesis of (–) strong-stop DNA, using the 5′ portion of the HIV-1 RNA genome containing the PBS as template. Evaluation of the priming efficiency was achieved by using labelled primers that allow direct quantification of the unextended primer and the extension products.

First, we compared the priming efficiency of the homologous RT [HIV-1 RT or a RNase H(–) HIV-1 RT] and heterologous RTs from Moloney murine leukaemia virus (MLV) and avian myeloblastosis virus (AMV), using $tRNA_3^{Lys}$ as primer (Figure 1A). $tRNA_3^{Lys}$-primed initiation of reverse transcription using HIV-1 RT is fast and efficient, as compared with heterologous RTs, e.g. 50% of $tRNA_3^{Lys}$ sustains initiation of reverse transcription with HIV-1 RT within 1 min, while <5% of initiation is obtained with MLV or AMV RT. After 4 min, ~60% of $tRNA_3^{Lys}$ is extended by HIV-1 RT, while <15% or 5% of primer is extended when using MLV or AMV RT, respectively. In contrast, RNase H(–) HIV-1 RT is almost as efficient as the wild-type HIV-1 RT in priming DNA synthesis (Figure 1A).

We used HIV-1 RT to compare the efficiency of $tRNA_3^{Lys}$, $utRNA_3^{Lys}$ and ORN in the initiation of (–) strong-stop DNA synthesis. In most studies (Kati *et al.*, 1992; Reardon, 1993; Spence *et al.*, 1995), RT is pre-incubated with the primer–template in the absence of divalent cations, which are added together with the nucleotides. Since we anticipated that the structure of the primer–template complex may be crucial for any specific initiation, we chose to include divalent cations during the pre-incubation step to allow folding of the native RNA structure (see Materials and methods). However, this protocol allows RNase H activity during pre-incubation when using ODN. This phenomenon was quite limited with AMV RT and MLV RT, but significant with HIV-1 RT. Therefore, in the case of ODN, we used RNase H(–) HIV-1 RT rather than HIV-1 RT in order to prevent cleavage of the template. This was justified by the fact that HIV-1 RT and RNase H(–) HIV-1 RT equally initiate reverse transcription when using $tRNA_3^{Lys}$ (Figure 1A), as well as $utRNA_3^{Lys}$ and ORN (data not shown) as primers.

Natural $tRNA_3^{Lys}$ is clearly the best primer while

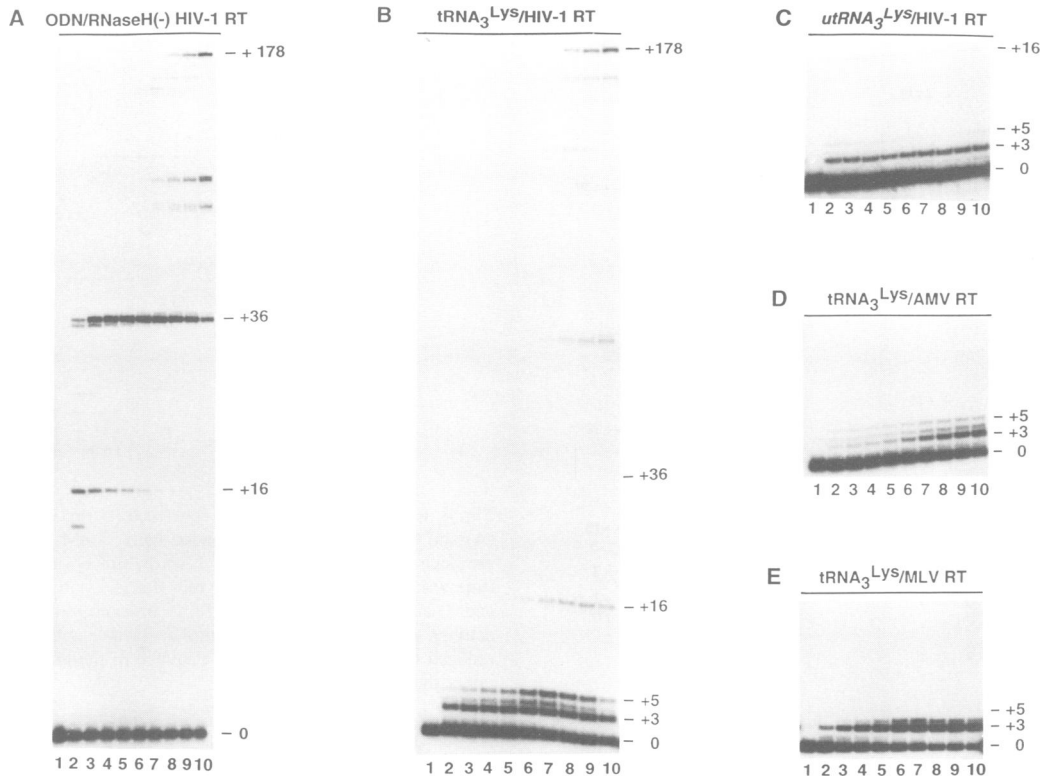

Fig. 2. Formation of short intermediate products during initial extension of primers. (**A**) ODN was extended with 27 nM of RNase H(–) HIV-1 RT. (**B**) $tRNA_3^{Lys}$ was extended with 3 nM of HIV-1 RT. (**C**) $utRNA_3^{Lys}$ was extended with 27 nM of HIV-1 RT. (**D**) and (**E**) $tRNA_3^{Lys}$ was extended with 27 nM of AMV RT and MLV RT, respectively. The amount of RT was adjusted to decrease differences in primer elongation. All extension products, up to (–) strong-stop DNA (+178), are shown in (A) and (B), while only the lower part of the gels are shown in (C), (D) and (E). Lanes 1–10 correspond to reverse transcription for 0 s, 15 s, 30 s, 45 s, 1 min, 2 min, 4 min, 7 min, 10 min and 25 min. The initial primer concentration was 10 nM in all experiments.

$utRNA_3^{Lys}$ is the worse (Figure 1B). Differences in priming efficiency observed with the different primers and RTs are partially reduced in the presence of an excess of enzyme, implying that the affinity of HIV-1 RT for the primer–template complex is an important parameter in the initiation of reverse transcription (Figure 1B and C). In most experiments, we used a 3/10 RT/primer–template (M/M) ratio, while HIV-1 particles contain 20–50 RT molecules. However, the viral sequences act as an internal trap for RT and the RT/template (W/W) ratio is more critical than the RT/template (M/M) ratio. The genomic RNA represents about half of the nucleic acid found in retroviruses; thus, the RT/template (W/W) ratio in our experiments and in the viral particles is similar. Also, since we used a 3.3-fold excess of primer–template compared with the RT concentration, and since the viral template itself acts as an internal trap, the data of Figure 1 correspond to multiple turnover kinetics.

The above data indicate that a specific complex of initiation of HIV-1 reverse transcription results from the mutual adaptation of HIV-1 RNA, $tRNA_3^{Lys}$ and HIV-1 RT. Supporting this view, $tRNA_3^{Lys}$ is the most efficient primer only in combination with HIV-1 RT, while ODN is an efficient primer for heterologous RTs. DNA synthesis with AMV RT is primed efficiently only with ODN (Figure 1D) and the priming efficiency with MLV RT is ODN>>$tRNA_3^{Lys}$>ORN = $utRNA_3^{Lys}$ (Figure 1A and data not shown). Thus, differences observed among RTs in the $tRNA_3^{Lys}$-primed reaction (Figure 1A) do not simply reflect differences in the intrinsic activity of these enzymes (see Materials and methods). A parallel study of reverse transcription of MLV and Rous sarcoma virus RNAs yields similar observations, except that ODNs are also very efficient primers for the homologous RTs (C.Isel *et al.*, in preparation).

Due to the intrinsic RNase H activity of the retroviral RTs, ODNs cannot be used as primers *in vivo*, since they would induce cleavage of the PBS, which must be intact for the second strand transfer (Marquet *et al.*, 1995). However, RNA–DNA hybrid is the usual substrate of the RT during elongation of the (–) strand DNA. Therefore, the ODN–viral RNA–RT complex most likely corresponds to an unspecific complex of elongation of reverse transcription. Thus, our results evidence two efficient primers of reverse transcription, $tRNA_3^{Lys}$ and ODN, which probably form very different primer–template–RT complexes.

Transition from initiation to elongation

Analysis of the intermediate products during (–) strong-stop DNA synthesis indicates that some are common to all RTs, while others are specific to each enzyme. The number of intermediate products varies greatly from one RT to the other, reflecting large differences in the processivity of the RTs: processivity increases in the order

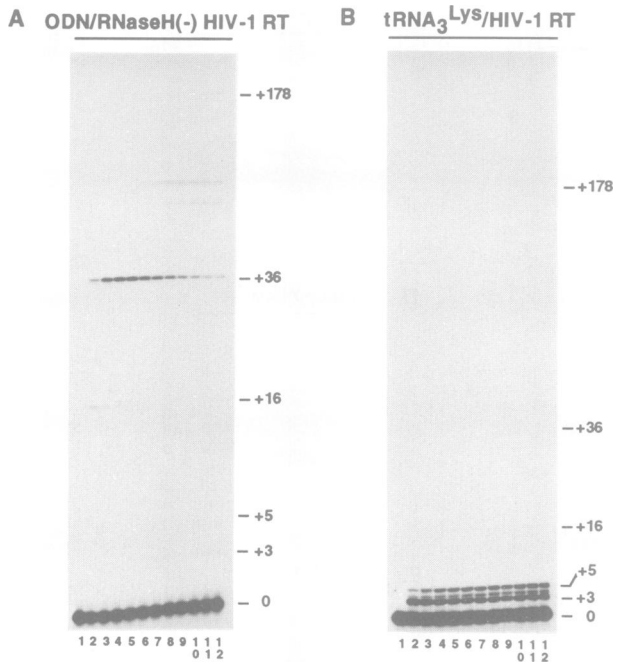

Fig. 3. Extension of ODN and $tRNA_3^{Lys}$ during a single RT turnover. Ten nM of ODN (**A**) and $tRNA_3^{Lys}$ (**B**) were pre-incubated with 9 nM of RNase H(–) HIV-1 RT (A) or HIV-1 RT (B), and the polymerization reaction was initiated by the addition of the mixture of deoxyribonucleoside triphosphates together with a large excess of poly(rA)·$(dT)_{15}$ that prevented recycling of the enzyme. Lanes 1–12 correspond to reverse transcription for 0 s, 15 s, 30 s, 45 s, 1 min, 3 min, 5 min, 7 min, 10 min, 15 min, 20 min and 25 min.

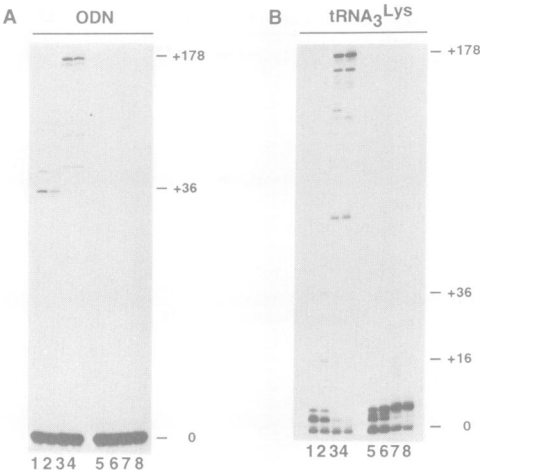

Fig. 4. Effects of manganese on reverse transcription. Ten nM of ODN (**A**) and $tRNA_3^{Lys}$ (**B**) were extended with 27 nM of HIV-1 RT in the presence of 4 mM $MgCl_2$ (lanes 1–4) or $MnCl_2$ (lanes 5–8). Reaction time was 30 s (lanes 1 and 5), 1 min (lanes 2 and 6), 5 min (lanes 3 and 7) and 15 min (lanes 4 and 8). In order to minimize RNase H activity, pre-incubation of the template–primer with HIV-1 RT was reduced to 1 min. No important cleavage of the template was observed under these conditions.

RNase H(–) HIV-1 RT<HIV-1 RT<MLV RT<AMV RT (C.Isel and R.Marquet, unpublished data).

When comparing the intermediate products observed during reverse transcription of HIV-1 RNA with a particular RT and different primers, differences are found only during addition of the very first nucleotides (Figure 2).While no product shorter than +36 (and to a lesser extent +16) is observed during extension of ODN with RNase H(–) HIV-1 RT, significant amounts of +3 to +5 products are detected when $tRNA_3^{Lys}$ is extended with RNase H(–) HIV-1 RT or HIV-1 RT (Figure 2A and B). Similar patterns of intermediate products are also observed during extension of $utRNA_3^{Lys}$ and ORN with these RTs, and when the ribonucleotidic primers are extended with AMV and MLV RTs (Figure 2C–E, and data not shown). On the contrary, no +3 to +5 products are observed upon extension of ODN with heterologous RTs (data not shown). Thus, ODN is efficiently elongated by any RT, while the +3 to +5 products observed with the RNA primers may reflect a rate-limiting transition from initiation to elongation of reverse transcription.

In order to test this hypothesis, we extended $tRNA_3^{Lys}$ and ODN with HIV-1 RT and RNase H(–) HIV-1 RT, respectively, in the presence of a trap that prevents recycling of the enzyme (Figure 3). As expected, extension of both primers in this single turnover experiment is considerably reduced compared with the multiple turnover experiments shown in Figures 1 and 2. Even though the overall extension level is quite low in the presence of the trap, long extension products, including (–) strong-stop DNA, are observed when ODN is the primer (Figure 3A). Only a very faint band is observed in the region corresponding to the +3 to +5 products, indicating that almost all RT molecules can elongate ODN through this region without dissociating. The results obtained with $tRNA_3^{Lys}$ as primer are completely different: almost no products longer than +5 are observed, indicating than >95% of RT molecules dissociate from the complex during transition from initiation to elongation of reverse transcription (Figure 3B).

Since Mn^{2+} is known to modulate RT activities (Lazcano *et al.*, 1992; Zhan *et al.*, 1994; Patel *et al.*, 1995), we tested whether this ion may affect initiation and elongation of reverse transcription differently (Figure 4). In agreement with previous studies (Lazcano *et al.*, 1992), almost no elongation of the ODN by HIV-1 RT and RNase H(–) HIV-1 RT is detected when Mg^{2+} is replaced by 4 mM Mn^{2+} (Figure 4A). In the presence of Mn^{2+}, +3 to +5 products accumulate in the $tRNA_3^{Lys}$-primed reaction, but very few larger products are synthesized (Figure 4B). We interpret these results as a strong inhibition of the elongation process, but not of the initiation step, by Mn^{2+}.

Importance of the modified nucleosides of $tRNA_3^{Lys}$ and of the extended primer–template interactions

Since similar intermediate products are observed with all RNA primers, one must wonder why $tRNA_3^{Lys}$ is more efficient than ORN and $utRNA_3^{Lys}$. The modified nucleosides present in $tRNA_3^{Lys}$, but not in $utRNA_3^{Lys}$, favour initiation of reverse transcription, either directly or indirectly (Figure 1B and C). These nucleosides are required to stabilize the extended interactions between HIV-1 RNA and $tRNA_3^{Lys}$, which take place in addition to the 'standard' interaction of the 3′ end of $tRNA_3^{Lys}$ with the PBS (Isel *et al.*, 1993, 1995).

In order to test whether the extended template–primer

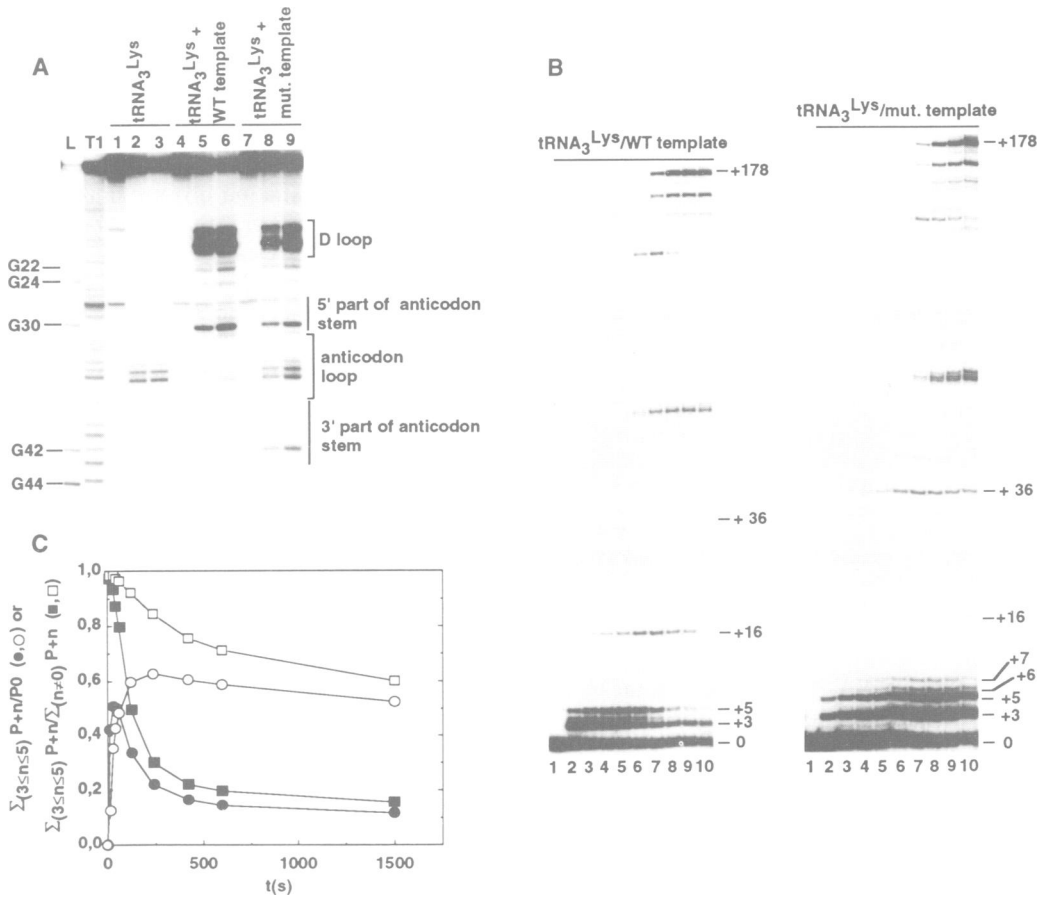

Fig. 5. Importance of the extended primer–template interactions for the switch from initiation to elongation of reverse transcription. (**A**) Enzymatic probing of $tRNA_3^{Lys}$. 3′ End-labelled $tRNA_3^{Lys}$ either free (lanes 1–3), hybridized to wild-type HIV-1 RNA (lanes 4–6) or hybridized to the mutant HIV-1 RNA (lanes 7–9) were treated with 350 units of nuclease S1 from *Aspergillus orizae* for 7 (lanes 2, 5 and 8) or 12 min (lanes 3, 6 and 9) at room temperature. Lanes 1, 4 and 7 were controls obtained by incubation for 7 min without enzyme. T1 and L refer to RNase T1 sequencing and to alkaline ladder, respectively. After phenol–chloroform extraction and ethanol precipitation, RNA fragments were separated on a 15% denaturing polyacrylamide gel. (**B**) Ten nM of $tRNA_3^{Lys}$ hybridized either to wild-type HIV-1 RNA (left panel) or to the mutant template (right panel) were extended with 9 nM of HIV-1 RT. For both templates, reaction time was for 0 s, 15 s, 30 s, 45 s, 1 min, 2 min, 4 min, 7 min, 10 min and 25 min. (**C**) Quantification of the +3 to +5 products. The sum of the +3 to +5 products is compared with the initial amount of primer (P_0) and to the total of extension products ($\Sigma_{(n\neq 0)}$ P+n) and the ratios ($\Sigma_{(3\leq n\leq 5)}$ P+n)/P_0: circles) and ($\Sigma_{(3\leq n\leq 5)}$ P+n)/$\Sigma_{(n\neq 0)}$ P+n: squares) are plotted as a function of the reaction time. The closed and open symbols correspond to the wild-type and mutant template, respectively. P+n corresponds to the primer extended by n nucleotides.

interactions, or the modified nucleosides by themselves, are important for initiation of reverse transcription, we mutated the viral A-rich loop (GUAAAA to CUAUG) that interacts with the anticodon loop of $tRNA_3^{Lys}$ (Isel *et al.*, 1993, 1995). As previously described (Isel *et al.*, 1993, 1995), hybridization of $tRNA_3^{Lys}$ to the wild-type HIV-1 RNA induces important structural rearrangements that can be evidenced by probing the primer tRNA with single strand-specific nucleases. Using nuclease S1, strong cuts are observed in the D-loop and in the 5′ part of the anticodon stem of the hybridized $tRNA_3^{Lys}$ (Figure 5A, lanes 4–6). These cuts are not observed with the free $tRNA_3^{Lys}$ (Figure 5A, lanes 1–3). They reflect the disruption of the tertiary interactions and the opening of the anticodon stem that occur upon hybridization of $tRNA_3^{Lys}$ and are also observed when the primer tRNA is annealed to the mutant viral RNA (Figure 5A, lanes 7–9). When $tRNA_3^{Lys}$ interacts with the wild-type viral RNA, the anticodon loop is protected from cleavage by nuclease S1 due to its interaction with the viral A-rich loop (Figure 5A, lanes 1–6). Similarly, the 3′ part of the anticodon stem of the hybridized $tRNA_3^{Lys}$ interacts with viral sequences upstream of the PBS (Isel *et al.*, 1995) and, hence, is protected from cleavage by nuclease S1 (Figure 5A, lanes 4–6). However, when $tRNA_3^{Lys}$ is hybridized to the mutant HIV-1 RNA, the anticodon is not protected from cleavage by nuclease S1, and a strong S1 cut is also observed in the 3′ part of the anticodon stem (Figure 5A, lanes 7–9). These data show that substitution of the viral A-rich loop allows annealing of the PBS with the 18 3′-terminal nucleotides of $tRNA_3^{Lys}$, but totally abolishes the interaction between the anticodon loop of $tRNA_3^{Lys}$ and the viral RNA, and alters the interactions of this RNA with the 3′ part of the anticodon stem of $tRNA_3^{Lys}$.

The kinetics of $tRNA_3^{Lys}$-primed initiation of reverse transcription of the mutant RNA are identical to those of the wild-type HIV-1 RNA (Figure 1B and C). Thus, the extended primer–template interactions are not crucial for initiation of DNA synthesis. Most likely, increased affinity of HIV-1 RT for the $tRNA_3^{Lys}$–HIV RNA complex compared with the *utRNA*$_3^{Lys}$–HIV RNA complex is due to direct contacts between the enzyme and some of the

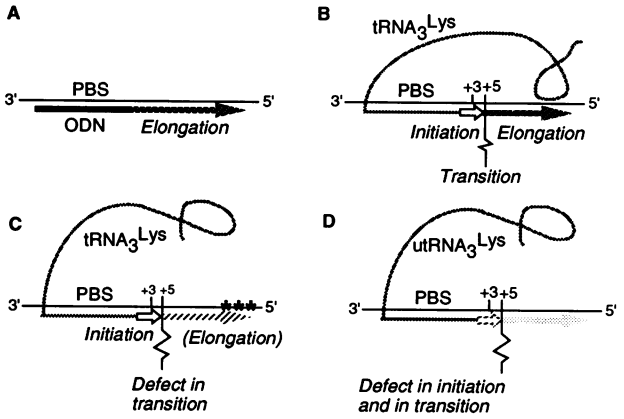

Fig. 6. Priming mechanism with different primers and templates. Template is represented by a thin line and cDNA by a thick arrow. Positions +3 and +5 are indicated. (**A**) ODN primer (**B**) $tRNA_3^{Lys}$ primer (**C**) $tRNA_3^{Lys}$ primer and a template mutated in the A-rich loop (the mutation is indicated by asterisks) (**D**) $utRNA_3^{Lys}$ primer. A similar scheme is valid for ORN.

modified nucleosides of $tRNA_3^{Lys}$. Accordingly, the higher efficiency of $tRNA_3^{Lys}$ compared with $utRNA_3^{Lys}$ in the initiation of reverse transcription is observed with HIV-1 RT but not with AMV RT (Figure 1D).

While primer–template interactions out of the PBS are not involved in initiation of reverse transcription, they are very important for efficient (–) strong-stop DNA synthesis. The (–) strong-stop DNA obtained when 10 nM of $tRNA_3^{Lys}$ hybridized to the wild-type template are extended with 9 nM of HIV-1 RT represents 6, 21, 27 and 37% of the initial amount of primer after extension for 4, 7, 10 and 25 min respectively, while after the same reaction time using the mutant template it amounts only to 1, 5, 8 and 16% (Figure 5B). Analysis of gels reveals that mutation of the viral A-rich loop produces a defect at the level of the transition from initiation to elongation of reverse transcription (Figure 5B). More +3 to +5 products are detected with the mutant template than with the wild-type RNA, and new weak bands corresponding to +6 and +7 products are observed. After an initial burst, the amount of the +3 to +5 products rapidly decreases during reverse transcription of the wild-type HIV-1 RNA (Figure 5B and C). On the contrary, the amount of +3 to +5 products obtained with the mutant viral RNA hardly decreases, even after prolonged incubation. Quantification of the gels indicates that, after 25 min, the +3 to +5 products represent ~15% of all extension products with the wild-type viral RNA, but still represent 60% of the products obtained with the mutant RNA (Figure 5C). Analysis of reverse transcription using ORN and $utRNA_3^{Lys}$ as primers revealed a similar defect (Figure 2C and data not shown). These results indicate that the switch from initiation to elongation of reverse transcription is a limiting step that is facilitated by extended interactions between $tRNA_3^{Lys}$ and HIV-1 RNA.

Concluding remarks

Our results are summarized in Figure 6. Taken together, they show that HIV-1 RNA, HIV-1 RT and $tRNA_3^{Lys}$ form a specific complex of initiation of reverse transcription that shares functional similarities with a promoter. Efficient initiation of reverse transcription requires the post-transcriptional modifications of $tRNA_3^{Lys}$ and transition to an elongation complex is facilitated by extended primer–template interactions that we previously evidenced by chemical probing (Isel *et al.*, 1993, 1995). This is the first evidence that initiation of retroviral reverse transcription is a specific process that can be distinguished from elongation. Our results explain why a strong selection pressure exists *in vivo* to maintain the wild-type PBS (Li *et al.*, 1994; Das *et al.*, 1995). Indeed, even though altered PBS allow hydridization of tRNAs other than $tRNA_3^{Lys}$, our results indicate that initiation of reverse transcription and transition to elongation probably remain very inefficient with the mutant viruses.

Elongation in the presence of a trap that prevents multiple turnovers of RT indicates that transition from initiation to elongation is a rate-limiting step. In all model systems studied so far, the limiting step of nucleotide addition by HIV-1 RT is a structural rearrangement of RT (Kati *et al.*, 1992; Reardon, 1993), at least in the absence of non-nucleoside inhibitors (Spence *et al.*, 1995). Since the chemical reaction is the same in initiation and elongation of reverse transcription, inhibition of the latter but not of the former step by Mn^{2+} further suggests that distinct conformations of RT are involved in these processes. Thus, not only the RNase H active site (Götte *et al.*, 1995), but also the polymerization site of HIV-1 RT is able to distinguish between RNA–RNA and DNA–RNA hybrids.

The mechanism by which the extended HIV-1 RNA–$tRNA_3^{Lys}$ interactions facilitate transition from initiation to elongation remains unclear. Since RT dissociates during this transition, these interactions may favour the re-binding of HIV-1 RT to the +3 to +5 products by preventing a steric clash between RT and $tRNA_3^{Lys}$ (Kohlstaedt *et al.*, 1992; Jager *et al.*, 1994). Moreover, they positively affect transition from initiation to elongation since this transition is more efficient with $tRNA_3^{Lys}$ than with ORN, which cannot produce 'steric clashes' with RT.

The primer $tRNA_3^{Lys}$ forms a binary complex with HIV-1 RT in which the anticodon loop is in close contact with the polymerase (Barat *et al.*, 1989, 1991; Wöhrl *et al.*, 1993), and the anticodon of $utRNA_3^{Lys}$ has been cross-linked to RT in a $utRNA_3^{Lys}$–viral RNA–HIV-1 RT ternary complex (Mishima and Steitz, 1995). Thus, a possible role for the interaction between the anticodon loop of natural $tRNA_3^{Lys}$ and a conserved A-rich loop located upstream of the PBS (Isel *et al.*, 1993, 1995) may be to prevent interaction of RT with the anticodon of $tRNA_3^{Lys}$. Such an interaction may impair efficient initiation and/or switching to elongation of reverse transcription by blocking RT on the tRNA. To support this hypothesis, ORN is a better primer than $utRNA_3^{Lys}$.

Avian retroviruses present several analogies with HIV-1. First, as in the case of HIV-1, AMV RT specifically binds to primer $tRNA^{Trp}$ (Panet *et al.*, 1975; Hizi *et al.*, 1977), in a way that probably mimicks the interactions governing the specific encapsidation of the primer species (Peters and Hu, 1980; Mak *et al.*, 1994). Second, $tRNA^{Trp}$ also makes extensive interactions with viral RNA (Aiyar *et al.*, 1992, 1994), out of the PBS, that are required for efficient reverse transcription *in vivo* (Cordell *et al.*, 1979; Aiyar *et al.*, 1992). We suggest that in HIV-1 and in avian

retroviruses RT binds differently to the primer tRNA during encapsidation and initiation of reverse transcription, and that extended primer–template interactions allow RT to discriminate between the primer tRNA and the primer–template complex.

Finally, since we showed that initiation and elongation of reverse transcription are distinct processes that may be selectively inhibited, one may use this observation as the conceptual basis for the development of agents that would specifically block initiation of reverse transcription. Such agents probably might not affect DNA synthesis by cellular polymerases, and since one of the components of the initiation complex is not allowed to vary ($tRNA_3^{Lys}$), emergence of resistant strains may be limited.

Materials and methods

Templates, primers and RTs

In most experiments, the template was an RNA corresponding to nucleotides 1–311 of HIV-1 genomic RNA (Mal isolate) that was synthesized *in vitro* by transcription with RNA polymerase from phage T7 as previously described (Marquet *et al.*, 1991). A mutant template was obtained by transcription of a plasmid in which nucleotides 162GUAAAA167 in HIV-1 Mal were replaced by CUAUG using PCR. Natural $tRNA_3^{Lys}$ was purified from beef liver; its sequence and post-transcriptional modifications are identical to those of human $tRNA_3^{Lys}$ (Isel *et al.*, 1993). Synthetic $utRNA_3^{Lys}$, which lacks all post-transcriptional modifications, was obtained by *in vitro* transcription of the corresponding artificial gene inserted in pUC18, in the presence of 16 mM GMP, which favours the synthesis of 5′-monophosphorylated products (Isel *et al.*, 1993).

3′ End labelling of $tRNA_3^{Lys}$ and $utRNA_3^{Lys}$ with [α-^{32}P]ATP (Amersham) was as described (Isel *et al.*, 1993). ORN and ODN primers were chemically synthesized and 5′ end-labelled with [γ-^{32}P]ATP (Amersham) and polynucleotide kinase from phage T4 (USB). RTs from MLV and AMV were from Gibco and Life Sciences, respectively. Wild-type HIV-1 RT, and RNase H(−) HIV-1 RT bearing the E478Q mutation were purified essentially as previously described (Le Grice and Grueninger-Leitch, 1990).

RT assays

In a standard experiment, template RNA was hybridized as described (Isel *et al.*, 1993) with ^{32}P-labelled primer at a 2:1 molar ratio and pre-incubated at 37°C for 4 min with 3, 9 or 27 nM RT in 50 mM Tris–HCl pH 8.0, 50 mM KCl, 6 mM $MgCl_2$ and 1 mM dithioerythritol. Reverse transcription was initiated by adding the four deoxyribonucleoside triphosphates (50 μM each) in the same buffer. Formamide containing 50 mM EDTA was added to aliquots of the reaction mixture at times ranging from 15 s to 25 min, and the reaction products were analysed on 8 or 12% polyacrylamide–urea gels and quantified with a BioImager BAS 2000 (Fuji) using the whole band analyser software (Bio Image).

For the single turnover experiments, the experimental protocol was essentially the same, except that a poly(rA)·$(dT)_{15}$ at a final concentration of 1.66 μM of $(dT)_{15}$ was added together with the mixture of deoxyribonucleoside triphosphates. Prior to addition in the reaction mixture, poly(rA) and $(dT)_{15}$ (10:1 w/w) were hybridized at 70°C for 20 min.

Titration of the active site concentration of RTs using ODNs (Kati *et al.*, 1992; Reardon, 1993; Spence *et al.*, 1995), which is useful when studying unspecific elongation, cannot be applied when studying initiation of reverse transcription. RT activity depends on several parameters, including the size, sequence and chemical nature (RNA or DNA) of both primer and template and, hence, absolute comparison of the activity of HIV-1 RT, MLV RT and AMV RT was not possible (C.Isel and R.Marquet, unpublished data and results above), and we preferred to compare their activities in the homologous and heterologous systems.

Enzymatic probing of $tRNA_3^{Lys}$

Enzymatic probing of $tRNA_3^{Lys}$, either free or hybridized to wild-type or mutant viral RNA, with nuclease S1 from *Aspergillus orizae* (Pharmacia) was as previously described (Isel *et al.*, 1995).

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