DNA synthesis primed by mononucleotides (de novo synthesis) catalyzed by HIV-1 reverse transcriptase: $tRNA^{Lys,3}$ activation

Ol'ga D. Zakharova[a], Laura Tarrago-Litvak[b], Michel Fournier[b], Simon Litvak[b], Georgyi A. Nevinsky[a,*]

[a]*Institute of Bioorganic Chemistry, Siberian Division of the Academy of Sciences of Russia, Novosibirsk 630090, Russian Federation*
[b]*Institut de Biochimie et Génétique Cellulaires du CNRS 1, rue Camille Saint-Saëns, 33077 Bordeaux cedex, France*

Received 28 June 1995; revised version received 1 September 1995

Abstract HIV-1 RT is able to catalyze DNA synthesis starting from mononucleotides used both as minimal primers and as nucleotide substrates (de novo synthesis) in the presence of a complementary template. The rate of this process is rather slow when compared to the polymerization primed by an oligonucleotide. The addition of $tRNA^{Lys,3}$ to this system increased the de novo synthesis rate by 2-fold. Addition of low concentrations of agents able to modify protein conformation, such as urea, dimethylsulfoxide and Triton X-100, can activate the de novo synthesis by a factor 2 to 5. A dramatic synergy is observed in the presence of the three compounds since the stimulating effect of tRNA increases 10–15 times. These results suggest that compounds activating RT are able to induce a conformational change of the enzyme which results in a higher specific activity. Primer tRNA seems to play an important role in HIV-1 RT modification(s) leading to a polymerase having a higher affinity for the primer or the dTTP, but not for the template. The specificity of RT for the template is not influenced by changes in the kinetics or in the thermodynamic parameters of the polymerization reaction.

Key words: HIV-1; Reverse transcriptase; $tRNA^{Lys,3}$ induced activation; Synthesis (de novo)

1. Introduction

The retroviral encoded RNA-dependent DNA polymerase (reverse transcriptase, RT) plays an essential role in the life cycle of HIV-1 [1]. In virions HIV-1 RT has been isolated as a heterodimer consisting of 66 and 51 kDa polypeptides [2,3]. HIV-1 utilizes $tRNA^{Lys,3}$ as a primer for the initiation of cDNA synthesis, as deduced from the retroviral genome nucleotide sequence [4]. A complex between $tRNA^{Lys,3}$ and HIV-1 RT has been demonstrated (for a recent review see [5]). The regions of the primer tRNA in close contact with the enzyme have been identified as the anticodon region, the dihydrouridine loop and the TΨC loop [6–8]. Complex formation with primer tRNA induces significant structural changes in HIV-1 RT, as followed by fluorescence emission or by the accessibility of the RT p66/p51·tRNA complex to chymotrypsin [9]. Preincubation of HIV-1 RT with either $tRNA^{Lys,3}$ or oligonucleotides mimicking the anticodon loop sequence of $tRNA^{Lys,3}$ leads to an increase of the catalytic activity on poly(A)-oligo(dT) [10]. Each subunit of the heterodimeric form of HIV-1 RT (p66/p51) interacts with $tRNA^{Lys,3}$ showing different affinities: K_d (p66) = 23 nM, K_d (p51) = 140 nM. Our previous results indicate that the activation of DNA polymerase activity associated to the p66 subunit would result from the interaction of primer tRNA with the p51 subunit of p66/p51 HIV-1 RT [10]. Most DNA polymerases initiates DNA synthesis from a short oligonucleotide. However under certain conditions DNA synthesis catalyzed by these enzymes may initiate from a monomeric primer [11]. Contrary to several DNA-dependent DNA polymerases, retroviral reverse transcriptases cannot use a nucleoside monophosphate as a primer while a triphosphate precursor gives a significant level of initiation [12]. DNA synthesis catalyzed by HIV-1 RT and primed by a mononucleotide triphosphate is referred throughout this work as de novo synthesis.

Here we show that in the presence of compounds such as DMSO, urea and Triton X-100, the natural primer of HIV-1, $tRNA^{Lys,3}$, is able to activate 10 to 15 times the de novo DNA synthesis catalyzed by HIV-1 RT. A possible mechanism of RT activation by primer tRNA is discussed.

2. Materials and methods

Nucleotides and polynucleotides were obtained from Sigma or Pharmacia. Radioisotopes were purchased from ICN or Radioisotop (Russia). HIV-1 RT p66/p51 was expressed in the yeast *S. cerevisiae* and purified as described before [13]. Bovine liver tRNAs were prepared according to [14].

RT assay was performed as described in [12]. The standard reaction mixture (50–100 ml) contained 50 mM Tris-HCl buffer (pH 8.0), 40 mM KCl, 0.5 mM EDTA, 4 mM magnesium acetate, 1 mM DTT, 1.8 A_{260} units/ml polymer template, 25 mM [^{3}H]dNTP (150 mCi). The reaction mixture in the presence of RT was incubated at 20°C for several lengths of time.

The K_m and V_{max} values for dTTP substrate, $d(pT)_{10}$ primer and poly(A) template were estimated as in [15].

3. Results and discussion

It has been previously shown that nucleoside-5'-monophosphates and triphosphates may serve as minimal primers for pro-, eucaryotic and archaebacterial DNA polymerases, while AMV RT and HIV-1 RT can only use dNTP as a minimal primer [11,12]. The initial rate of the de novo synthesis primed by dTTP and catalyzed by HIV-1 RT is about 1 to 2 orders of magnitude lower than the rate obtained using a $d(pT)_{10}$ primer.

We have also shown that the preincubation of HIV-1 RT (either the homodimer p66/p66 [16] or the heterodimer p66/p51 [10]) with $tRNA^{Lys,3}$ led to conformational changes of the enzyme that increases its activity when the assay was performed with the classical poly(A)-oligo(dT) primer-template duplex. In

*Corresponding author.

Abbreviations: RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; DMSO, dimethyl sulfoxide.

SSDI 0014-5793(95)01056-4

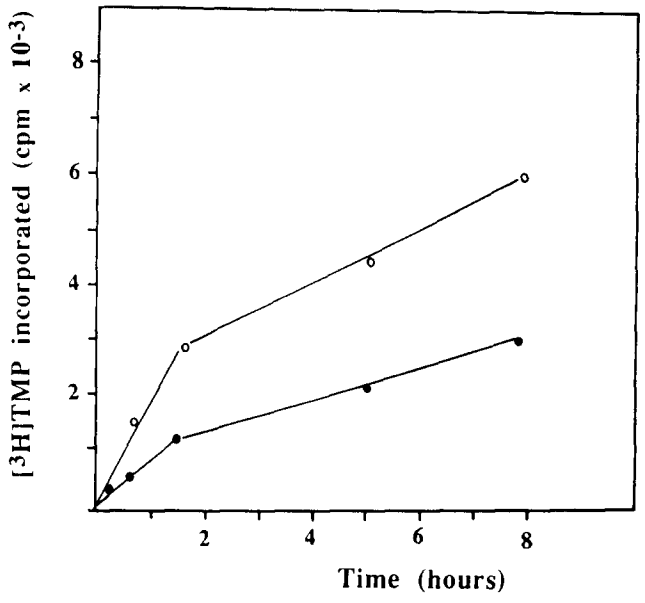

Fig. 1. Time course of poly(dT) synthesis. The poly(dT) synthesis, catalyzed by HIV-1 RT, was performed with a poly(A) template. [^{3}H]dTTP was used both as primer and as nucleotide precursor. Reactions were done in the absence (●) or in the presence of 300 nM $tRNA^{Lys,3}$ (○).

this work we have followed the stimulation of the de novo synthesis by preincubating the RT with $tRNA^{Lys,3}$. As it is shown in Fig. 1 the addition of tRNA led to a stimulation of de novo synthesis of about 2 times.

For some enzymes the transition from a constrained to a relaxed state (a more active enzyme) in the presence of specific ligands may occur as the result of the reorganization of new specific hydrophobic, electrostatic or hydrogen bonds resulting from the interactions between amino acid residues of the protein [17]. The rate of cleavage of one specific contact and the formation of a new one depends, besides other factors, on the type of enzyme, which may be allosteric or not, and the nature of the specific ligand. The effective reorganization of the amino acids involved in hydrogen bonds can be achieved in the presence of urea or dimethylsulfoxide, while hydrophobic interactions can be modified in the presence of non-ionic detergents such as Triton X-100.

The influence of DMSO, urea and Triton X-100 on poly(A)-dependent synthesis primed by dTTP was analyzed. As shown in Fig. 2 the effect of these compounds on RT activity was dose-dependent. Each compound when used separately at its optimal concentration increased the initial rate of polymerization by a factor 2–3. When the 3 compounds were used together the rate of activation was significantly higher (4- to 5-fold).

The reorganization of specific contacts between the amino acid residues of the RT in the presence of urea, DMSO and Triton X-100 can occur faster but they may be unspecific when compared to the effect of cellular or viral factors (tRNA, the *gag*-derived nucleocapsid protein, etc. [18,19]) involved in retroviral replication. Therefore, we analyzed the effect of RT conformation changes on DNA polymerase activity when the enzyme was simultaneously incubated with DMSO, urea and Triton X-100 in the presence or the absence of primer tRNA. Under such conditions the reorganization of the specific contacts of the enzyme could occur under the control of primer tRNA.

We have already described that the maximal activation of HIV-1 RT by $tRNA^{Lys,3}$ takes place at concentrations of tRNA allowing each subunit of the p66/p51 HIV-1 RT to form a complex with $tRNA^{Lys,3}$ [10]. The next step was to determine, in the presence of tRNA, the concentration of each compound allowing optimal activation. The values obtained were: urea (0.02–0.03%, or 3.3–5 mM), DMSO (0.5–0.6%) and Triton X-100 (0.03%). It is interesting to point out that the latter concentration of non-ionic detergent is the same used to obtain optimal endogenous DNA synthesis in semi-permeabilized virions.

As shown in Fig. 3, the stimulation of HIV-1 RT activity obtained in the presence of $tRNA^{Lys,3}$ together with urea, DMSO and Triton X-100 was time-dependent and much higher (10- to 15-fold) than if, independently, tRNA or the three compounds mentioned above were added.

The specificity of HIV-1 RT was not changed in the activated system. The reaction was completely dependent on the presence of the complementary template since no de novo synthesis from dTTP was observed when poly', poly(U), poly(G) and poly(dC) templates were used (Fig. 3).

As shown before HIV-1 RT can interact with other tRNAs besides $tRNA^{Lys,3}$ [14]. It is the case of $tRNA^{Trp}$ while $tRNA^{Val}$ cannot form a complex with RT. The activation effect of these tRNAs was tested on the de novo synthesis. $tRNA^{Trp}$ was able to activate the RT but to a lower extent than $tRNA^{Lys,3}$ whereas $tRNA^{Val}$ had practically no influence in the polymerization reaction (Fig. 3).

The effect of $tRNA^{Lys,3}$ together with DMSO, urea and Triton X-100 was tested using other templates and their corresponding mononucleotides. As shown in Fig. 4 also in this case an increase of the de novo synthesis was obtained.

To have a better understanding of the activation process the kinetic parameters of the polymerization reaction catalyzed by RT p66/p51 were determined in the presence or in the absence of $tRNA^{Lys,3}$. The preincubation of the RT with $tRNA^{Lys,3}$ only

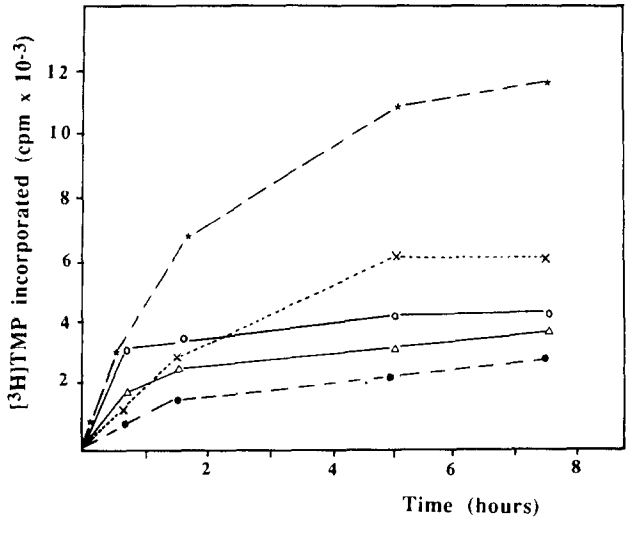

Fig. 2. Time course of poly(dT) synthesis. The de novo synthesis was done in the absence (●) or in the presence of: 0.0075% urea (△), 0.3% Triton X-100 (○), 0.6% DMSO (×), and 0.0075% urea, 0.3% Triton X-100, 0.6% DMSO together (★)

increases the maximal rate of polymerization without changing the K_m values for dTTP (precursor) or poly(A)-oligo(dT) (template-primer). Thus, the next step was to determine the effect of the three activating compounds by measuring the K_m values for the precursor, primer and template of the reverse transcription reaction. With the template-primer poly(A)-d(pT)$_{10}$ the K_m values for the precursor dTTP were compared in the presence or in the absence of urea, DMSO and Triton X-100. In both cases the experiments were done in the presence of tRNA^{Lys,3}. In these conditions, besides increasing the maximal rate of polymerization, there was a 5-fold change in the K_m value of dTTP since we obtained a value of 10 mM in the absence, and 2 mM in the presence of DMSO, urea and Triton X-100.

Next, the K_m value for the primer d(pT)$_{10}$ was determined. The addition of DMSO, urea and Triton X-100 to the tRNA-activated system also led to an increase (5-fold) of the primer affinity. This was not the case for the template: the addition of tRNA together with the three activating compounds did not change the affinity of RT for poly(A).

A

Curve	tRNA	A. C.	Template	dNTP
1	Lys	+	poly (C), (dC), (U), (G)	dTTP
1	Lys	+	poly(A)	dATP or dCTP
2	Lys	+	poly(dA)	dTTP
3	Lys	-	poly(A)	dTTP
4	Val	+	poly(A)	dTTP
5	Trp	+	poly(A)	dTTP
6	Lys	+	poly(A)	dTTP

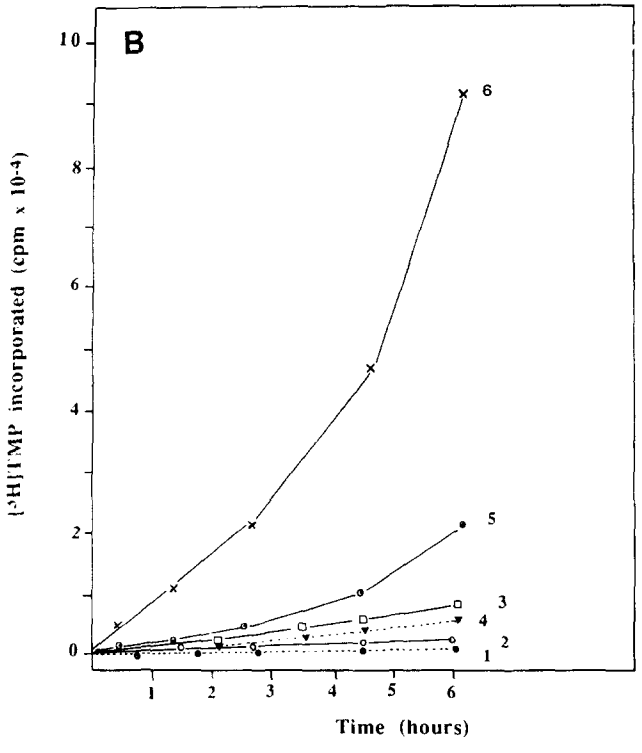

Fig. 3. De novo synthesis in the presence of different tRNAs, templates and primers. The following concentrations were used, tRNA: 300 nM; Activating compounds (A. C.) = urea: 0.0075%; Triton X-100: 0.3%; DMSO: 0.6%. Different conditions of incubation are given in (A), kinetic curves are presented in (B).

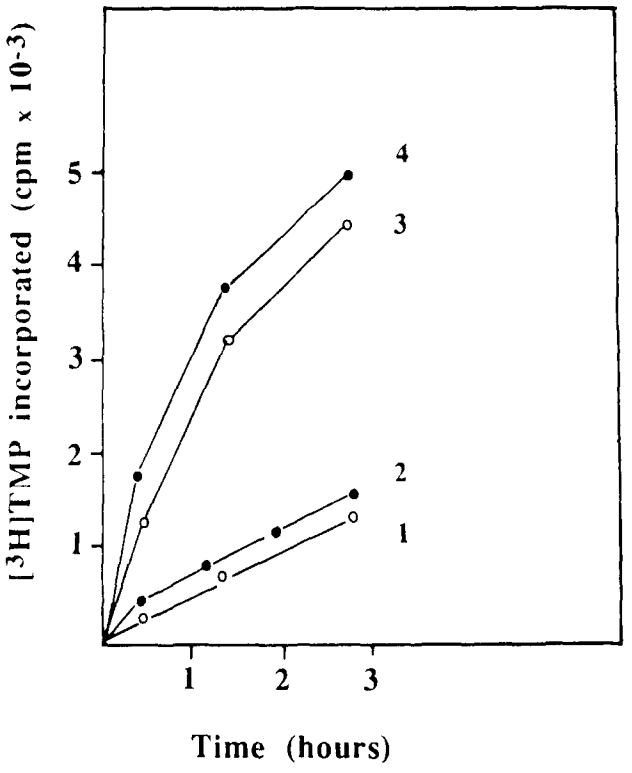

Fig. 4. Time course of the synthesis catalyzed by HIV-1 RT. Syntheses were performed with [^{3}H]dGTP and poly(C) (1) and with [^{3}H]dATP and poly(U) (2) in the absence of tRNA^{Lys,3} or from [^{3}H]dGTP and poly(C) (3) and [^{3}H]dATP and poly(U) (4) in the presence of 300 nM tRNA^{Lys,3}.

The enzyme activation by tRNA^{Lys,3} in the de novo reaction occurs slowly: the half time values of the RT activation are within 20–30 min. Thus, most probably activators not only accelerate the specific changes of the RT conformation induced by tRNA, but allow to reach even deeper conformational changes.

The recent determination of the three-dimensional structure of HIV-1 RT [19,20] may help to understand the ability of reverse transcriptase to use a monomeric primer unit in vitro. Crystallographic data has allowed to determine the importance that two clusters, regions Met-41 to Leu-74 and Thr-215 to Lys-219, play in the primer-template binding to HIV-1 RT [19]. No crystallographic data is currently available of a HIV-1 RT·dNTP complex, but UV-induced crosslinking of labelled dTTP to HIV-1 RT has shown that within the p66 subunit, dTTP appears to cross-link to a single residue, Lys-73 [21]. Thus, the precursor site and the primer-template one are very close in the catalytic subunit of the viral polymerase. This distance may allow the dNTP to slide from the precursor site to the template-primer one and initiate DNA synthesis but at a lower rate than an oligonucleotide primer which may bind directly to the RT template-primer binding site. The reason why reverse transcriptase, contrary to other DNA polymerases, is able to use a nucleoside triphosphate as a monomeric primer, but does not recognize a nucleoside monophosphate, remains to be established.

HIV-1 RT should be considered as an extremely conformationally active protein. It cannot be excluded that a set of different factors such as the basic nucleocapsid protein which

has been involved in genome dimerization, primer anealing, strand transfer etc., or other factors, which are present in the intracellular structure where retroviral replication takes place, can modify the RT activity during the polymerization reaction. The latter effect may take place when DNA synthesis starts either from the primer $tRNA^{Lys,3}$ or from oligonucleotide natural primers. Our results suggest that specific changes in the conformation of HIV-1 RT produced by a given cofactor can provide extremely high rates of polymerization.

Acknowledgements: This work was supported by the Siberian Division of Russian Academy of Sciences, the Agence Nationale de Recherches sur le SIDA (ANRS), and the Association des Recherches pour le Cancer (ARC). Expert technical assistance by Ms Christina Calmels (supported by SIDACTION) is acknowledged.

References

[1] Skalka, A.M. and Goff, S.P. (1993) Reverse transcriptase, Cold Spring Harbor Laboratory Press.
[2] Di Marzo Veronese, F.D., Copeland, T.D., De Vico, A.L., Rahman, R., Oroszlan, S., Gallo, R.C. and Sarngadharan, M.G. (1986) Science 231, 1289–1291.
[3] Wondrak, E.M., Lower, J. and Kurth, R. (1986) J. Gen. Virol. 67, 2791–2797.
[4] Wain Hobson, S., Sonigo, P., Danos, O., Cole, S. and Alizon, M. (1985) Cell 40, 9–17.
[5] Litvak, S., Sarih-Cottin, L., Fournier, M., Andreola, M.L. and Tarrago-Litvak, L. (1994) Trends Biochem. Sci. 19, 114–118.
[6] Barat, C., Lullien, V., Schatz, O., Keith, G., Nugeyre, M.T., Gruninger-Leitch, F., Barré-Sinoussi, F., Le Grice, S.F.J. and Darlix, J.L. (1989) EMBO J. 8, 3279–3285.
[7] Sarih-Cottin, L., Bordier, B., Musier-Forsyth, K., Andreola, M.L., Barr, P. and Litvak, S. (1992) J. Mol. Biol. 226, 1–6.
[8] Wöhrl, B.M., Ehresmann, B., Keith, G. and LeGrice, S.F.J. (1993) J. Biol. Chem. 268, 13617–13624.
[9] Robert, D., Sallafranque-Andreola, M.L., Bordier, B., Sarih-Cottin, L., Tarrago-Litvak, L., Graves, P.V., Barr, P.J., Fournier, M. and Litvak, S. (1990) FEBS Lett. 277, 239–242.
[10] Zakharova, O., Tarrago-Litvak, L., Fournier, M., Andreola, M.L., Repkova, M.N., Venyaminova, A.G., Litvak, S., Nevinsky G.A. (1995) FEBS Lett. 361, 287–290.
[11] Nevinsky, G.A., Veniaminova, A.G., Levina, A.S., Podust, V.N., Lavrik, O.I. and Holler, E. (1990) Biochemistry 29, 1200–1207.
[12] Nevinsky, G.A., Andreola, M.L., Jamkovoy, V.I., Levina, A.S., Barr, P.J., Tarrago-Litvak, L., Tharaud, D. and Litvak, S. (1992) Eur. J. Biochem. 207, 351–358.
[13] Sallafranque-Andreola, M.L., Robert, D., Barr, P.J., Fournier, M., Litvak, S., Sarih-Cottin, L. and Tarrago-Litvak, L. (1989) Eur. J. Biochem. 184, 367–374.
[14] Bordier, B., Tarrago-Litvak, L., Sallafranque-Andreola, M.L., Robert, D., Tharaud, D., Fournier, M., Barr, P.J., Litvak, S. and Sarih-Cottin, L. (1990) Nucleic Acids Res. 18, 429–436.
[15] Eisenthal, R. and Cornish-Bowden, A. (1974) Biochem. J. 139, 715–720.
[16] Andreola, M.L., Nevinsky, G.A., Barr, P.J., Sarih-Cottin, L., Bordier, B., Fournier, M., Litvak, S. and Tarrago-Litvak, L. (1992) J. Biol. Chem. 267, 19356–19362.
[17] Fersht, A. (1977) in: Enzyme Structure and Mechanism, W.H. Freeman and Company, Reading and San Francisco, pp. 226–241.
[18] Prats, A.C., Sarih, L., Gabus, C., Litvak, S., Keith, G. and Darlix, J.L. (1988) EMBO J. 7, 1777–1783.
[19] Kohlstaedt, L.A., Wang, J., Friedman, J.M., Rice, P.A. and Steitz, T.A. (1992) Science 256, 1783–1790.
[20] Jacobo-Molina, A., Ding, J., Nanni, R.G., Clark, A.D., Jr., Lu, X., Tantillo, C., Williams, R.L., Kamer, G., Ferris, A.L., Clark, P., Hizi, A., Hughes, S.H. and Arnold, E. (1993) Proc. Natl. Acad. Sci. USA 90, 6320–6324.
[21] Cheng, N., Merrill, B.M., Painter, G.R., Frick, L.W. and Furman, P.A. (1993) Biochemistry 32, 7630–7634.