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PRIMER $tRNA^{Trp}$ ENHANCES THE INHIBITION OF AVIAN MYELOBLASTOSIS VIRUS REVERSE TRANSCRIPTASE BY PYRIDOXAL-5'-PHOSPHATE

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SUMMARY: In the presence of $tRNA^{Trp}$ the inhibition of avian myeloblastosis virus reverse transcriptase by pyridoxal-5'-phosphate is greatly enhanced. This effect of tRNA is specific. While $tRNA^{Trp}_{beef}$ (the primer of the in vitro DNA synthesis with 35 S viral RNA as template) gives the maximal effect, $tRNA^{Val}_{beef}$ will not affect the enzyme activity. In the presence of $tRNA^{Trp}_{beef}$ two additional lysines are titrated with pyridoxal-5'-phosphate as measured by reduction of the enzyme-pyridoxal-5'-phosphate complex with tritiated $NaBH_4$. The effect of tRNA is dependent on the presence of the β subunit of avian myeloblastosis virus reverse transcriptase, since the enzymatic activity of the α subunit, although inhibited by pyridoxal-5'-phosphate, is not affected by tRNA.

INTRODUCTION.

RNA tumor viruses contain a DNA polymerase (reverse transcriptase) which can copy the genome RNA into complementary DNA, using a low molecular weight RNA as primer (1). Avian myeloblastosis virus (AMV) reverse transcriptase is composed of an α subunit (60 Kdalton) containing the DNA polymerase and ribonuclease H activities and a β subunit (90 Kdalton) enhancing template binding (2). In the case of AMV reverse transcriptase the primer has been shown to be cellular tryptophan tRNA ($tRNA^{Trp}$) (3). The primer is bound by base pairing along the 3' end of the tRNA to a site very near the 5' end of the genome (4). Cellular $tRNA^{Trp}$ has two binding specificities that allow it to function as a primer: it hybridizes to a site on 35 S viral RNA (5,6) and it binds specifically to the reverse transcriptase (7-9). AMV reverse transcriptase is inactivated by preincubation with pyridoxal-5'-phosphate (PLP)(10,21). This reaction is reversible, but can be made irreversible by reduction with sodium borohydryde. The presence of deoxynucleoside triphosphates protects the enzyme from inactivation. In this report we show that the inhibition of AMV reverse transcriptase by PLP is greatly enhanced by primer $tRNA^{Trp}$. A preliminary account of this work has been presented (22).

MATERIALS AND METHODS.

Homogeneous AMV reverse transcriptase, as judged by polyacrylamide gel electrophoresis (PAGE) was obtained from Dr. J.W.Beard (Life Sciences Inc.

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Florida, USA), or purified from viral pellets obtained from the same source (11). When necessary, a further phosphocellulose step was performed. Tryptophan tRNA from beef liver was purified as described before (12). The ability of $tRNA^{Trp}$ to serve as primer of the 35 S directed DNA synthesis by AMV reverse transcriptase has been reported previously (13).

Pyridoxal-5'-phosphate inhibition of AMV reverse transcriptase. AMV reverse transcriptase (35 µg/ml) was preincubated with different amounts of PLP for 15 minutes at 37°C in the dark. Then, 5 µl aliquots were analyzed for enzyme activity in assays containing the same final concentration of PLP. Assays were performed always in the dark. Incubation mixtures contained in a final volume of 0.1 ml: 50 mM Na borate pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, 100 µg/ml bovine serum albumin (BSA), 0.5 mM $|^3H|$-TTP (100 mC/mmole, Amersham), 1 O.D.$^{260\ nm}$/ml poly A-dT_{12} (5:1). When the effect of tRNA was studied, 1 µM $tRNA^{Trp}_{beef}$ was added (calculated on the basis of 1800 pmoles per O.D. 260). Reaction was carried out for 10 minutes at 37°C and stopped by addition of one volume of 20% cold TCA plus 0.1 M sodium pyrophosphate carrier. The samples were filtered on nitrocellulose membranes (Schleicher and Schuell), washed with 5% cold TCA, dried and counted in 5 ml of a PPO-POPOP-toluene scintillation mixture.

Reduction of the enzyme-PLP complex by $|^3H|$-$NaBH_4$ in the presence of tRNA and (or) TTP. Conditions as above for the preincubation with PLP. After 15 minutes incubation with PLP, the reaction mixture was treated with 28 nmoles of $|^3H|$-$NaBH_4$ (9 Ci/mmole, C.E.A. Saclay, France), the pH was brought to 4.5 with acetic acid and left 30 minutes at room temperature. The radioactivity incorporated into protein was measured by TCA precipitation as described above, except that BSA (0.1 mg/ml) was present. BSA and nitrocellulose filters were pretreated with unlabeled $NaBH_4$.

Effect of PLP and tRNA on the enzymatic activity of AMV reverse transcriptase α subunit. The α subunit of AMV reverse transcriptase was purified as summarized in the text (14). Conditions of incubation are those described above, except that Nonidet P-40 0.2% was included since, in its absence, 90% of the enzyme activity is lost after 15 minutes incubation.

RESULTS AND DISCUSSION.

Reverse transcriptase was inactivated by preincubation with PLP. The enzyme was preincubated and incubated in the presence of the reagent to avoid dissociation of the enzyme-PLP complex when measuring DNA polymerase activity. In Figure 1A it is shown that about 70% of inactivation is obtained with 2 mM PLP. If the preincubation is carried out in the presence of $tRNA^{Trp}_{beef}$, the same value of inactivation is obtained with 0.6 mM PLP. Fig. 1B shows that the α subunit of AMV reverse transcriptase can be inhibited similarly by PLP but $tRNA^{Trp}$ in this case does not enhance the effect of the inhibitor.

Fig. 2 shows that the inactivation of AMV reverse transcriptase in the presence of 1.2 mM PLP is a function of $tRNA^{Trp}$ concentration, a plateau of 90% being attained between 2 and 5 µM tRNA. In the presence of the α subunit no effect of tRNA was observed. As seen in Table I, tRNA alone will not affect the activity of AMV reverse transcriptase when poly A-dT_{12} is used as template. In the pre-

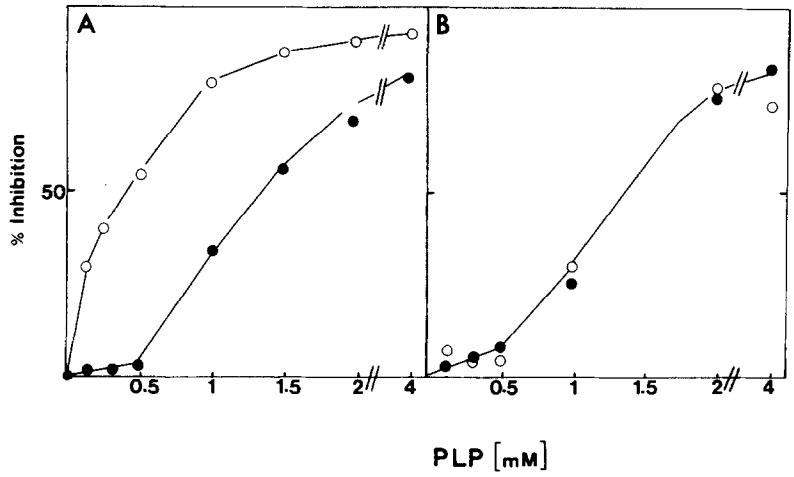

Figure 1: PLP inhibition of AMV reverse transcriptase activity. In this Figure, the value corresponding to 0% inactivation preincubated in the absence of PLP was 2250 pmoles TMP incorporated in 10 minutes for the holoenzyme (Fig. 1A) in the presence (O) or absence of tRNA (●) and 283 pmoles TMP incorporated by the α subunit (Fig. AB) in the presence (O) or absence (●) of the primer. The concentration of tRNA was 1 µM. Before the test the enzyme was dialysed against 50 mM buffer Borate pH 8.3 at 4° C for a minimum of 5 hours.

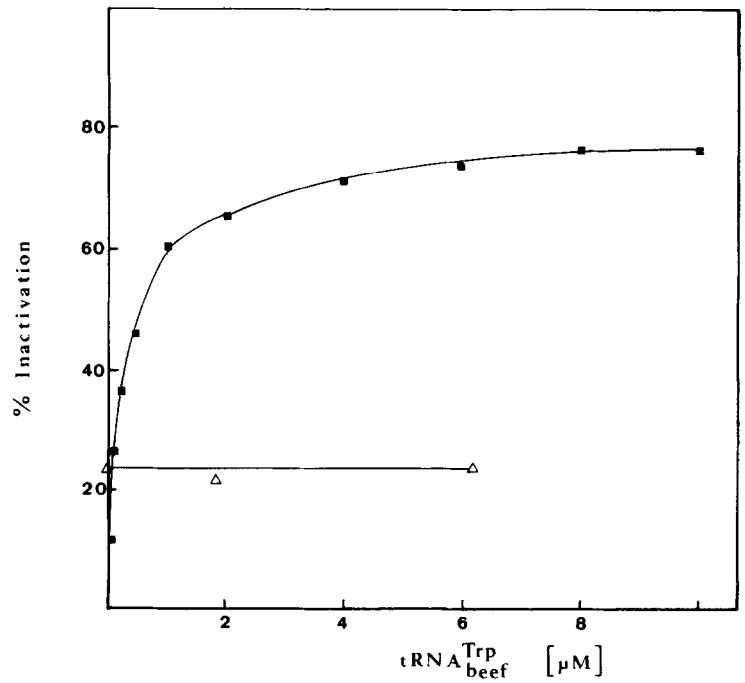

Figure 2 : Effect of $tRNA^{Trp}$ on the inhibition by PLP of the holoenzyme (■) and the α subunit (△) of AMV reverse transcriptase. PLP was used at 1.2 mM.

TABLE I. Inhibition of reverse transcriptase by PLP in the presence of tRNA and (or) TTP.

PLP mM	Addition	pmoles TMP/10 min	% Inactivation
0	None	1,184	0
0	4 µM tRNA	1,190	0
1.2	None	901	24
1.2	10 mM TTP	1,452	0
1.2	4 µM tRNA	295	85
1.2	4 µM tRNA + 10 mM TTP	580	51

sence of 1.2 mM PLP, 24% of inactivation is observed. Ten mM TTP will overcome the inhibition by PLP as shown before (10). When PLP and tRNA are preincubated together the inhibition attains 85%. If tRNA is present, TTP will overcome only partially the inhibition. Having observed that tRNA would enhance the inhibition by PLP, it was interesting to study whether there was some specificity in the effect of tRNA. As seen in Table II, $tRNA^{Trp}_{beef}$ was the most efficient in enhancing PLP inhibition of the enzyme. It is interesting to note that $tRNA^{Trp}_{yeast}$ gave a lower value than $tRNA^{Trp}_{beef}$, while $tRNA^{Val}_{beef}$ gave no effect at all. The sequences of tryptophan tRNAs from beef (15) and from yeast (16) are quite different, although some similarities must exist, since beef tryptophanyl tRNA synthetase will aminoacylate both tRNAs very efficiently (17), whereas $tRNA^{Val}_{beef}$ (20) is not aminoacylated by the enzyme (M.Fournier, personal communication).

TABLE II. Inhibition of AMV reverse transcriptase by PLP in the presence of beef and yeast tRNAs.

PLP mM	2 µM tRNA	pmoles TMP/10 min	% Inactivation
0	0	1,610	0
1.2	0	1,156	28
1.2	Trp/beef	485	70
1.2	Trp/yeast	781	51
1.2	Val/beef	1,154	28

TABLE III. Reduction of enzyme-PLP complex by $[^3H]NaBH_4$. Effect of TTP and tRNA.

Addition	Moles PLP/mole enzyme
1.2 mM PLP	9.4
1.2 mM PLP + 10 mM TTP	10.1
1.2 mM PLP + 2 μM $tRNA^{Trp}_{beef}$	12.6
1.2 mM PLP + 10 mM TTP + 2 μM $tRNA^{Trp}_{beef}$	10.1

Moreover, although $tRNA^{Trp}_{yeast}$ is partially recognized by AMV reverse transcriptase for complex formation, as measured by Sephadex G-150 chromatography, no DNA synthesis was observed in the 35 S viral RNA in vitro directed reaction when yeast $tRNA^{Trp}$ was used as primer (22).

PLP, in addition to its coenzyme function with certain enzyme systems, has become a useful active site labeling agent (18). The interaction of PLP with the enzyme has been demonstrated to involve the formation of a Schiff base between the reagent and amino groups of the protein. Studies with $NaBH_4$ reduction of pyridoxal inactivated enzymes affords supporting evidence for a primary base formation in the inactivation process. Reduction of PLP-enzyme complex with $[^3H]$-$NaBH_4$ allows the incorporation of tritium label into the stable pyridoxal group of the complex. The results in Table III indicate that when AMV reverse transcriptase is treated with $[^3H]$-$NaBH_4$ in the presence of 1.2 mM PLP, about 10 lysines residues are touched. This value is similar to the one reported for the same enzyme using the optical method to quantify the number of residues modified by PLP (10). In the presence of tRNA, two additional lysines are titrated. The presence of TTP will protect the new sites from being labeled with $[^3H]$. It has been shown that some tRNAs can also be labeled by $[^3H]$-$NaBH_4$ and PLP (23). This is not the case in our experiments since in the absence of enzyme no label was found in $tRNA^{Trp}$.

Some information is available on the role of both subunits functions in reverse transcriptase. By reducing the PLP-enzyme complex with $[^3H]$-$NaBH_4$ good indications that the α subunit carries the TTP site were obtained (10). In our laboratory we have found the same results by crosslinking $[^3H]$-TTP to AMV reverse transcriptase by ultraviolet irradiation (22). Good evidence has also been reported concerning the role of the β subunit in the binding of $tRNA^{Trp}$ to the enzyme (8, 19). Using the U.V. induced crosslinking of $tRNA^{Trp}_{beef}$ to AMV reverse transcriptase we have found that the primer becomes covalently

linked to both subunits. It seemed interesting then, to study whether the α subunit, having catalytic activity as mentioned before, was inhibited by PLP and if in the absence of β subunit, tRNA would be able to enhance the inhibition as shown for the complete enzyme. As seen in Fig. 1B, the α subunit purified through phosphocellulose and poly U-cellulose chromatography (14) is inhibited by PLP, but this inhibition is not affected by the presence of tRNA. No differences were observed in the inhibition by 1.2 mM PLP at different tRNA concentrations (Fig. 2). These results constitute another evidence of the involvement of the β subunit in the primer-enzyme interaction.

The recognition of the tRNA primer by reverse transcriptase may seem unnecessary, since tRNA is already able to form a stable complex with the 35 S RNA genome. However, several evidences mentioned in the text indicate that specific interactions take place between the enzyme and primer tRNA. These interactions may play an important role in reverse transcription. For instance: the formation of a complex between residues 2-17 (from the CCA end) of tRNA and 35 S AMV RNA (5,6) must be preceded by the opening of the very compact tRNA structure and may be mediated by the formation of an enzyme-tRNA complex. Moreover, as only 15 bases of tRNA seem to be involved in hydrogen bonding (about 20% of the total) with the viral genome, the rest of the structure may maintain some structural features that will help its recognition by reverse transcriptase. The 3' end of tRNA being the site of initiation of transcription, its recognition by the enzyme would increase the efficiency of the whole process. Recent results of G. Peters et al. (personal communication) show that in a polymerase defective avian sarcoma virus strain, $tRNA^{Trp}$ is absent. All these results suggest strongly that the viral DNA polymerase is involved in positioning the primer on the genome RNA.

The results presented in this communication indicate an important effect by $tRNA^{Trp}_{beef}$ on the enzyme structure as evidenced by the enhancement of PLP inhibition of AMV reverse transcriptase by tRNA.

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