INHIBITION OF AVIAN MYELOBLASTOSIS VIRUS REVERSE TRANSCRIPTASE BY ETHIDIUM BROMIDE

Effect on enzyme activity and primer tRNA recognition

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Received 17 September 1980
Revised version received 27 October 1980

1. Introduction

Following retrovirus infection of a permissive cell, a double-stranded circular DNA intermediate is believed to be reverse-transcribed from the viral RNA genome and subsequently integrated into the host nuclear genome [1]. Reverse transcription is carried out by a viral-coded RNA-dependent DNA polymerase (reverse transcriptase) which copies the viral genome into DNA using a partially base-paired specific tRNA as primer [2–4]. The integrated viral sequences are transcribed by the cell machinery giving rise to viral RNA. It has been shown that the intercalating drug ethidium bromide (EtBr) can inhibit the integration of retrovirus into the nuclear genome [5,6], as well as the expression of the provirus already integrated [7]. The same drug inhibits reverse transcriptase activity in vitro [8].

We have been interested in the recognition of primer $tRNA^{Trp}$ by avian myeloblastosis virus (AMV) reverse transcriptase [9,10]. Evidence has been presented that the acceptor stem of primer tRNA is partially unwound by reverse transcriptase, and we have proposed that this new function of reverse transcriptase can mediate the binding of primer tRNA to the viral genome [11]. Here, we provide evidence that EtBr and the non-intercalating analogous tetramethylEtBr inhibit both the polymerase activity as well as the ability of reverse transcriptase to unwind the acceptor stem of primer tRNA, while the formation of a stable complex between the enzyme and tRNA primer is abolished only by the intercalating drug.

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2. Materials and methods

2.1. *Materials*

AMV reverse transcriptase and virus were obtained from Dr J. W. Beard (Life Sciences, FL) and viral 35 S RNA was obtained from the viral pellets by phenol extraction [11]. Primer $tRNA^{Trp}$ from beef liver was purified as in [9]. Radioactive products were purchased from NEN. Synthetic polynucleotides were bought from Boehringer. EtBr and RNase T_1 were from Sigma. EtBr dimer and tetramethylEtBr were kind gifts of Dr J. Paoletti and Dr J. B. LePecq (Villejuif). Polynucleotide kinase purified from T_4 infected *E. coli* was a kind gift of Dr G. Keith (Strasbourg).

2.2. *Reverse transcriptase assay*

(A) With poly(A–$dT)_{12}$ or poly(dA–$dT)_{12}$ templates: In a final volume of 100 μl the incubation mixture contained: 50 mM Tris–HCl (pH 8.3); 40 mM KCl; 6 mM $MgCl_2$; 0.5 mM [^{3}H]TTP (70 cpm/pmol); 0.2 A_{260}/ml of template and different amounts of enzyme.

(B) With 35 S RNA–$tRNA^{Trp}$ template: The incubation mixture (100 μl) contained Tris buffer, KCl and $MgCl_2$ as above, including: 0.5 mM dATP, dCTP, dTTP and 5 μM d[^{32}P]GTP (2000 cpm/pmol); 0.15 A_{260} of 35 S AMV RNA and different amounts of enzyme.

In both cases the incubation was carried out at 37°C for different lengths of time. The incubation was stopped by addition of 0.1 ml 20% cold trichloroacetic acid plus 0.1 M sodium pyrophosphate. The radioactive material was collected onto nitrocellulose membranes

and counted in a PPO–POPOP–toluene scintillation mixture.

2.3. *3'-or 5'-End labeling of tRNA*

Dephosphorylation and labeling of the 5'-end of $tRNA^{Trp}$ with polynucleotide kinase was done as described; labeling of the 3'-end adenosine of tRNA was done as in [11].

2.4. *RNase T_1 digestion of 5'-labeled tRNA*

In a final volume of 0.1 ml the digestion mixture contained: 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 45 pmol tRNA labeled as above, 0.6 units RNase T_1, and 60 units reverse transcriptase. Incubation was done at 37°C and the acid-precipitable material determined as for the polymerase assay. This experiment was also done in the presence of 200 μg/ml of bovine serum albumin to eliminate a possible adsorption of RNase T_1 to the walls of the tube.

2.5. *Sephadex G-100 chromatography of $tRNA^{Trp}$–reverse transcriptase complex*

The column (0.8 × 51 cm) was equilibrated in 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM Mg-acetate, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1% Triton X-100 and 10% glycerol. V_0 was determined with dextran blue. Fractions of 0.5 ml were counted in 5 ml Bray's scintillation mixture.

3. Results

3.1. *Inhibition of polymerase activity*

The inhibitory effect of EtBr, EtBr dimer and the non-intercalating drug tetramethylEtBr are shown in fig.1. Both polymerizing activities of reverse transcriptase, using an RNA or a DNA template are affected by the drugs. The most powerful inhibitor is the EtBr dimer which has been proven to have a very high affinity for nucleic acids. The same pattern of inhibition is obtained with EtBr or tetramethylEtBr using poly(A) or poly(dA) as templates, although higher concentrations of inhibitors are necessary to reach a given degree of inhibition with poly(A) than with poly(dA).

In order to define the type of inhibition of reverse transcription by EtBr we have studied the effect of the drug at different concentrations of TTP. As seen in fig.2 it is shown that the inhibition by EtBr is non-competitive. Similar results were obtained with tetramethylEtBr.

3.2. *Inhibition of primer tRNA recognition by reverse transcriptase*

The partial unwinding of the acceptor stem of [^{32}P]5'-labeled primer tRNA was followed by assaying the accessibility to nuclease digestion of the 5'-terminal phosphate of $tRNA^{Trp}$ under different conditions. As seen in fig.3, neither reverse transcriptase alone nor

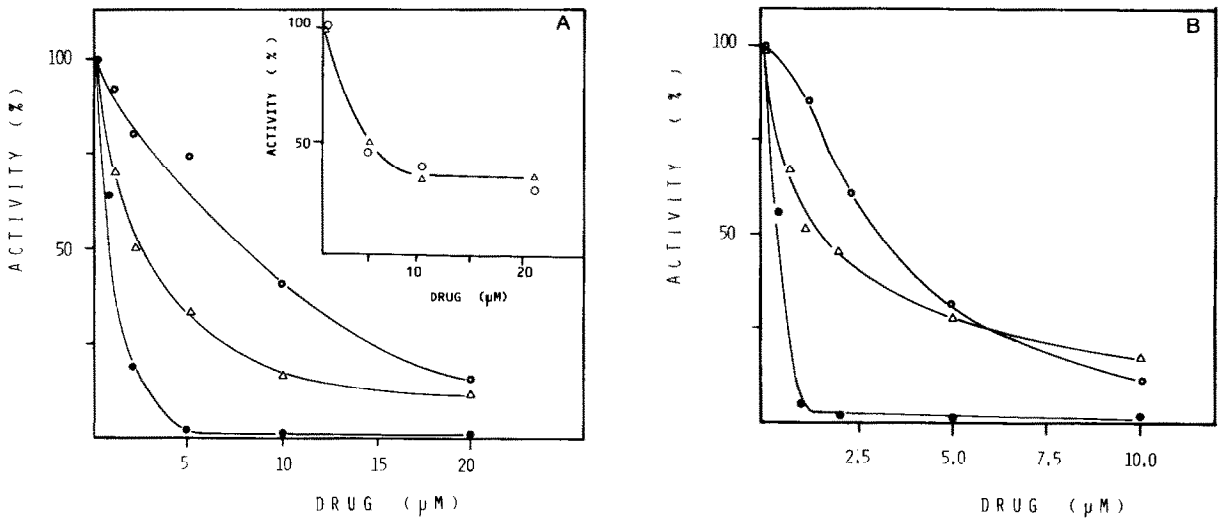

Fig.1. Effect of EtBr, tetramethylEtBr and EtBr dimer on poly(dT) synthesis using poly(A) [A] or poly(dA) [B] as template. The inset plot in [1A] corresponds to drug inhibition with the viral genome 35 S RNA as template. (•) EtBr dimer; (△) EtBr and (○) tetramethylEtBr. Amounts of enzyme used, 2 units (spec. act. 6 × 10^4 units/mg). Other conditions are described in section 2.

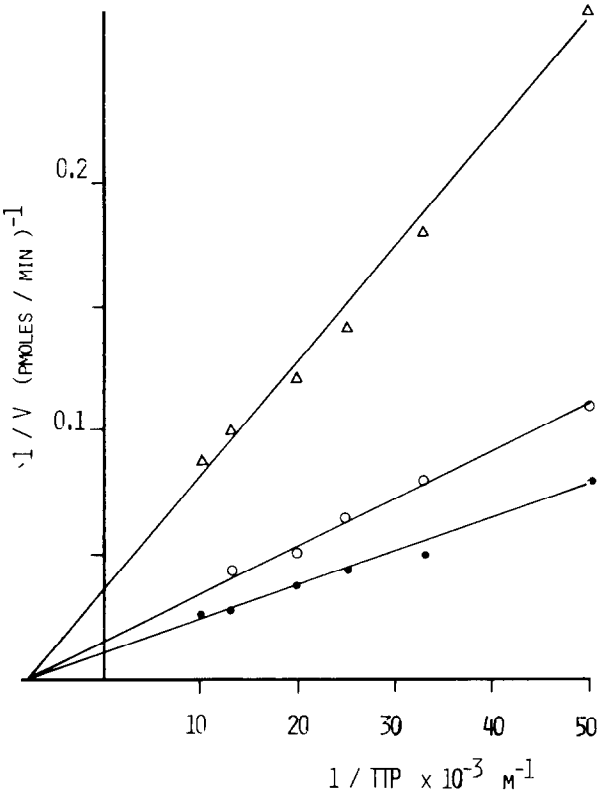

Fig.2. Effect of EtBr (△) and tetramethylEtBr (○) on enzyme activity at various concentrations of TTP: drug concentration, 5 μM; (●) control. Poly(A–dT)$_{12}$ was used as template. Reciprocal plot according to Lineweaver and Burk.

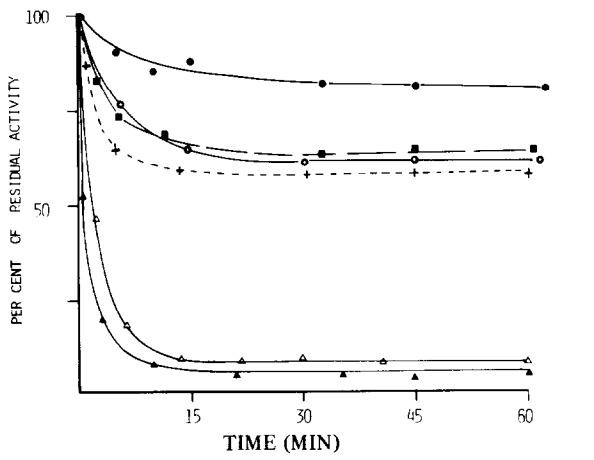

Fig.3. Nuclease digestion of 5′-labeled tRNA^{Trp} with RNase T_1: (●) plus 60 units reverse transcriptase; (■) plus 0.6 units RNase T_1; (△) plus reverse transcriptase, plus RNase T_1; (▲) plus EDTA 1 mM, plus RNase T_1; (○) plus reverse transcriptase, plus RNase T_1, plus EtBr 5 μM; (+) plus reverse transcriptase, plus RNase T_1, plus tetramethylEtBr 10 μM. Other conditions we described in section 2. When EDTA was used no Mg^{2+} was added.

RNase T_1 alone are able to efficiently digest the 5′-end of tRNA. Together they led to a dramatic loss of radioactivity. The digestion of the 5′-terminal phosphate of tRNA by RNase T_1 in the presence of reverse transcriptase is greatly diminished if EtBr or tetramethylEtBr are present. No difference was observed in the presence of bovine serum albumin. Moreover in the presence of EDTA total digestion was reached, RNase T_1 being the only protein present in the mixture.

When complex formation between primer tRNA and reverse transcriptase was followed by gel filtration in the presence of EtBr or tetramethylEtBr, the results shown in fig.4 were obtained. No stable complex was detected in the presence of EtBr while tetramethylEtBr did not affect complex formation a similar situation to the one obtained in the absence of drug.

4. Discussion

Genomic viral RNA extracted from AMV is partially base-paired to tRNA^{Trp}. DNA synthesis is initiated from the 3′-end of this primer tRNA [2,3]. Our studies of the specificity of interaction between AMV reverse transcriptase and primer tRNA^{Trp} led us to propose the hypothesis that partial unwinding of the acceptor stem of tRNA by the viral polymerase may be important in positioning the primer on the viral genome [11].

Strong intercalation of EtBr in the acceptor stem of tRNA has been described [14]. As this intercalation must lead to the stabilization of the acceptor stem, we felt it could be interesting to study the effect of EtBr on the recognition of primer tRNA by reverse transcriptase. Our results indicate that the partial unwinding of tRNA is strongly inhibited by EtBr and the non-intercalating drug tetramethylEtBr, while complex formation is only impaired by EtBr. The inhibitory effect of tetramethylEtBr on the polymerizing activities of the enzyme can be ascribed to a direct interaction of the drug and the polymerase, whereas the lack of complex formation in the presence of EtBr may be related to the intercalation of the drug and changes in the tertiary structure of tRNA^{Trp} by EtBr. Thus, tetramethylEtBr which is not intercalated, does not affect complex formation under the same conditions.

This in vitro inhibition of reverse transcriptase by EtBr may be related to the inhibition of virus integration by this drug, as in the case of avian sarcoma-

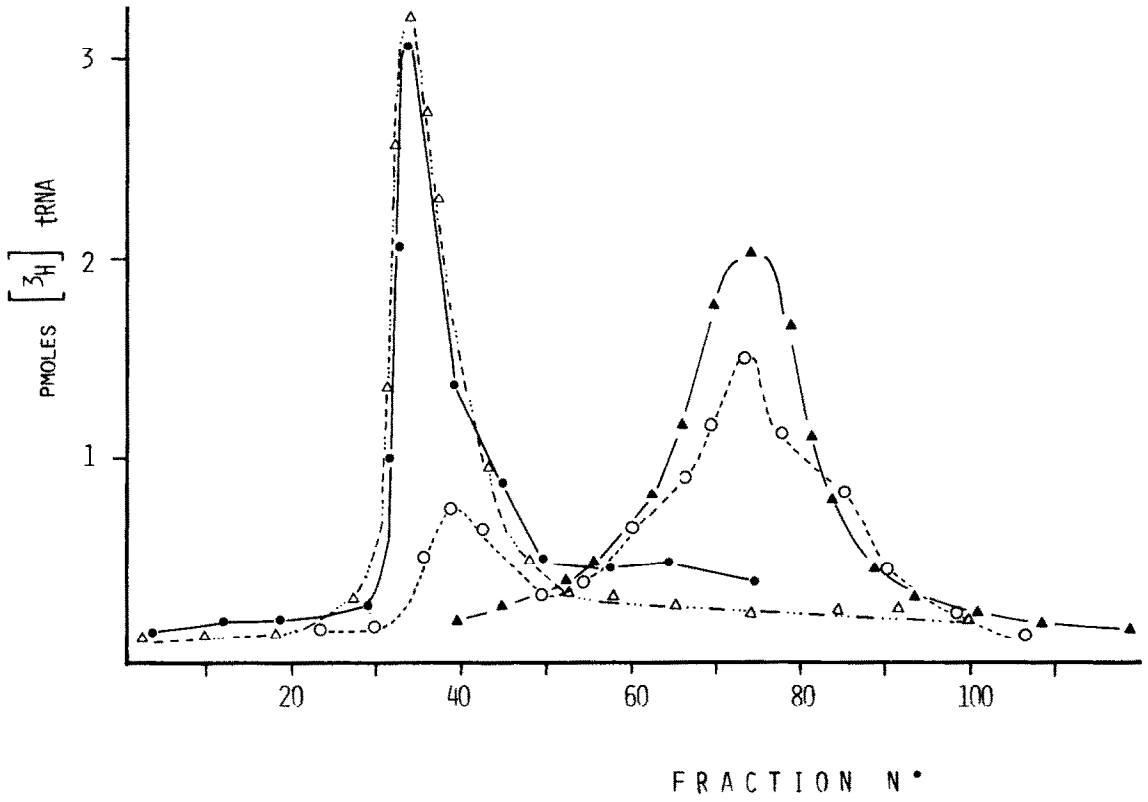

Fig.4. Sephadex G-100 chromatography of reverse transcriptase–$tRNA^{Trp}$ complex. Reverse transcriptase (150 units) and t[^{3}H]-RNA (labeled at the terminal adenosine, 10 pmol 1390 cpm/pmol) were submitted to gel chromatography as in section 2 in the presence or absence of 5 μM EtBr or 10 μM tetramethylEtBr: (•) enzyme plus tRNA; (△) plus tetramethylEtBr; (○) plus EtBr; (▲) tRNA alone.

infected cells [5,6]. The inhibition of the partial unwinding of the acceptor stem of tRNA by EtBr can be related to the in vivo inhibitory effect of the drug on infectious virus production in cells containing the integrated provirus [7]: the impairment of the interaction between tRNA primer and reverse transcriptase would lead to the encapsidation of viral RNA lacking the natural primer, which could then be incapable of integration into the nuclear genome.

The main characteristic of reverse transcriptase is to recognize a natural or synthetic RNA template. Since animal DNA polymerase γ is able to efficiently copy a poly(A)–oligo(dT) template, the two enzymes were at one time thought to be related. It is currently clear that the two polymerases are in fact different enzymes [12]. Yet reverse transcriptase and DNA polymerase γ, unlike other DNA polymerases, are both strongly inhibited in vitro by EtBr [8,13]. Although the degree of inhibition is comparable, a different mechanism seems to be involved.

Intercalation is essential in the inhibition of polymerase γ by the drug [13]; this is not the case for reverse transcriptase: the non-intercalating agent tetramethylEtBr strongly inhibited this enzyme (fig.1). We have confirmed that intercalated EtBr does not inhibit reverse transcriptase activity with a template poly(dC)–oligo(dG) [8] under the same conditions that DNA polymerase γ is strongly inhibited [13]. If we assume that the drug interacts with the enzyme, we can speculate that poly(dA)–oligo(dT) and poly-(dC)–oligo(dG) can interact differently with reverse transcriptase and that in the latter case the enzyme target site for EtBr is not accessible to the drug.

Furthermore in the case of reverse transcriptase, the inhibition obtained at varying concentration of TTP is non-competitive (fig.2) for EtBr and tetramethylEtBr, whereas the inhibition of DNA polymerase γ under the same conditions is clearly competitive [12].

Acknowledgements

The authors are greatly indebted to Drs A. Araya, L. Tarragó-Litvak and B. Ricard for helpful discussions. The help of the Biological Carcinogenesis Branch of the National Institutes of Health and Life Sciences Inc. as well as the kind gift of drugs by Dr J. Paoletti have been essential for performing this work. This research was supported by DGRST and INSERM.

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