"IN VITRO" BINDING OF A CELLULAR, α-AMANITIN SENSITIVE, RNA POLYMERASE TO INFECTIOUS, MENGOVIRUS-INDUCED DOUBLE-STRANDED RNA.

by R. Perez-Bercoff, G. Carrara, A. Dolei, G. Conciatori and G. Rita

The Institute for Virology - University of Rome.
Viale di Porta Tiburtina 28. - 00185 ROME (ITALY)

Received December 27,1973

SUMMARY

A cellular, α-Amanitin sensitive, RNA polymerase from mouse L cells binds "in vitro" to Mengovirus-induced, double-stranded (ds) RNA. Formation of the "enzyme-ds RNA" complex was studied by the nitrocellulose filter technique.
Reaction strongly depends on $K^+/(NH_4)^+$ and Mn^{++}/Mg^{++} concentration, occurs optimally at 37°C, is linear with time up to 10 minutes, and is inhibited by a rifamycin derivative AF/013.
Competition experiments demonstrated that neither heat-denatured Mengovirus RF nor single-stranded ribonucleotides interfere with complex formation, whereas the double-helical form o[illegible] same RNAs (native RF, Poly I:C; Poly A:U) efficiently compete with Mengovirus RF for cellular polymerase. These results seem to indicate that the double-helical nature of the template is essential for binding to occur.

DNA-directed RNA synthesis is a precisely regulated, multiple step process beginning with the attachement (binding) of the specific enzyme (RNA polymerase) to template DNA. Binding implicates the ability of the enzyme to recognize some special structure on the template molecule; even though initiation and termination of RNA synthesis require in addition the presence of specific signals in the DNA.

"In vitro" formation of the complex "enzyme-template" and the effect of drugs on the reaction "polymerase-DNA" have been extensively studied by the nitrocellulose filter techniques, that is: by taking advantage of the property of the complex to be retained by nitrocellulose filters under conditions which allow unbound template to pass through (1-3).

Labeled DNAs of different origins (viral and cellular) were

used to study this first step of transcription, but the possibility of a cellular RNA polymerase able to transcribe with a ds RNA as template was not explored. This was of interest because the replicative form (RF) of picornaviruses is infective, but the mechanism of its infectivity still remains obscure. In previous studies (4) we concluded that: i) cellular transcription must be conserved for RF to replicate and, ii) a cellular macromolecule is involved in the early steps of the infectious cycle initiated by RF. Analysis of the modifications in the structure of the incoming molecule of RF led us to postulate that the first viral messengers are transcribed by a cellular RNA polymerase with RF (ds RNA) as template and we are now reporting that a cellular, α-Amanitin sensitive, RNA polymerase from mouse L cells efficiently binds "in vitro" to Mengovirus-induced ds RNA.

MATERIALS AND METHODS

a) Preparation and purification of ^{3}H-labeled Mengovirus RF have been described in a previous paper (5).
b) Cellular, α-Amanitin sensitive RNA polymerase was extracted from nuclei of mouse L cells grown in suspended cultures by a modification of the method of Jacob et al. (6) : addition of NP-40 1% (v/v) and SDS 0.2% (w/v) during the incubation step resulted in an increased yield of the enzyme. Alternatively cellular polymerase was obtained as described in (7). In both cases purification was accomplished as in (8). Other technical details in Text Table I.

RESULTS

a) RNA polymerase activity was searched for in fractions that elute from DEAE-Cellulose at 300 mM $(NH^4)^2SO^4$. The properties of the enzyme (specific activity: 6990 pmol UMP/mg protein in our assay conditions) summarized in Table I are those of a cellular α-Amanitin sensitive RNA polymerase (cell.Pol.).
b) Fig. 1 (A and B) shows that in optimal conditions cellular RNA polymerase from mouse L cells efficiently binds to ^{3}H-Mengovirus RF. Recovery on filters is about 90% of input radioactivi

TABLE I
Properties of α-Amanitin sensitive RNA polymerase from mouse L cells

Reaction mixture	^{3}H-UMP incorporated	
Complete	7497	cpm
Omitted DNA	722	"
" Polymerase	93	"
" Phosphoenolpyruvate	5456	"
Complete + Actinomycin D	709	"
" + Daunomycin	772	"
" + α-Amanitin	647	"
" + Rifampicin	7894	"
" + AF/013	742	"

Text Table I
Assay of RNA-Polymerase activity: the reaction mixture (final volume: 150 µl) was: Tris. HCl (pH: 8.2) 80 mM; KCl 16 mM; $(NH^4)^2$ SO^4 80 mM; Mg Acetate 3 mM; MnCl 2 mM; NaF 3 mM: EDTA 1 mM; β-Mercaptoethanol 3 mM; Phosphoenolpyruvate 4 mM; Pyruvate kynase 26.6 µg/ml; Spermine 3 mM; ATP, CTP, GTP, o.4 mM each, UTP 0.013 mM; ^{3}H-UTP (spec. act. 40 Ci/mM) 6 µCi/ml; heat-denatured calf thymus DNA 133 µg/ml. Incubation (30 minutes, 37°C) was terminated by adding Na pyrophosphate (to 20 mM) and yeast RNA (100 µg). TCA was made 7.5%. After 30 minutes at 0°C, precipitates were collected, washed 3 times with ice-cold TCA 5%, twice with methanol, digested with Hyamine (0.2 ml) and counted with a toluene-based scintillation fluor.

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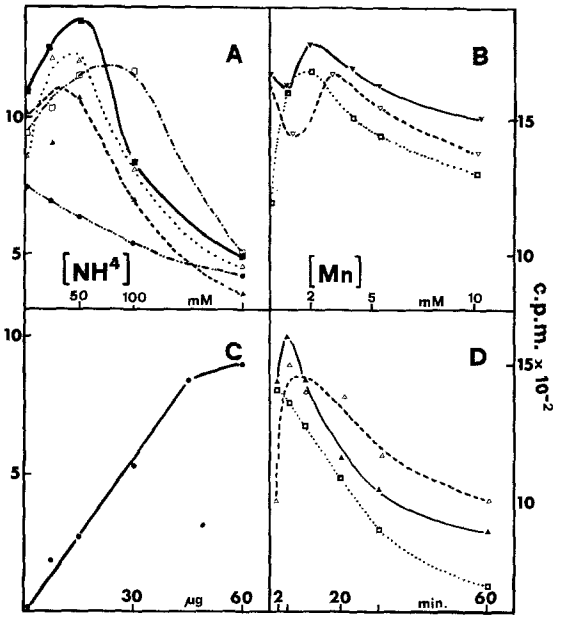
ty (values of blank deduced). The reaction strongly depends on K^+ and $(NH^4)^+$ concentrations (optimal: 25 mM and 50 mM respectively). Mn^{++} and Mg^{++} concentrations influence less markedly complex formation (optimal: 2 mM and 1 mM respectively; Mn/Mg ratio = 2).

c) Retention of ^{3}H-Mengovirus RF is linear with the amount of enzyme present in the reaction mixture (Fig. 1 C).



Text Fig. 1

Binding of cellular RNA-Polymerase to Mengovirus RF was performed in a final volume of 1 ml and the standard reaction mixture was: Tris. HCl (pH:8) 60 mM; β-Mercaptoethanol 5 mM; EDTA 1 mM; KCl 25 mM; $(NH^4)^2$ SO^4 50 mM; MnCl 2 mM; Mg Acet. 1 mM; Glycerol 20% (v/v); Polymerase 50 µl and 2,000 cpm ^{3}H-Mengovirus RF. Blanks containing either 40 µg SBA instead of Polymerase or no protein at all were included in each test. Reactions were incubated 10 minutes at 37°C, the volume was adjusted to 4 ml with the same buffer and filtration through Sartorious membrane filters (2.5 cm diameter) was performed at a constant rate of 1.3 ml/minute. Filters were dried and counted in a Packard Liquid Scintillation Spectrometer (counting time 20 minutes, accuracy level 0.7%). Values of blanks (usually 100-200 cpm) were deduced.

A: Effect of $(NH^4)^+$ concentration on binding. K^+ was held constant at 3.3 mM □•—•□; 25 mM ■——■; at 50 mM △•••△; at 100 mM ▲---▲; and 200 mM ●•••●.

B: Effect of Mn and Mg concentration on binding reaction. Mg was: 0.5 mM □••□; 1 mM ▲——▲ and 3 mM △---△.

C: The amount of partially purified Polymerase was modified as indicated.

D: Standard reaction mixtures were incubated at 37°C ▲——▲; 30°C △---△; and 45°C □•••□.

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d) Attachement of cellular RNA polymerase to ^{3}H-RF is maximal at 37°C (Fig. 1 D) and the reaction seems to be reversible, since incubation beyond 10 minutes results in a diminution of

TABLE II
Effect of inhibiting drugs on binding of cellular RNA-Polymerase to Mengovirus RF

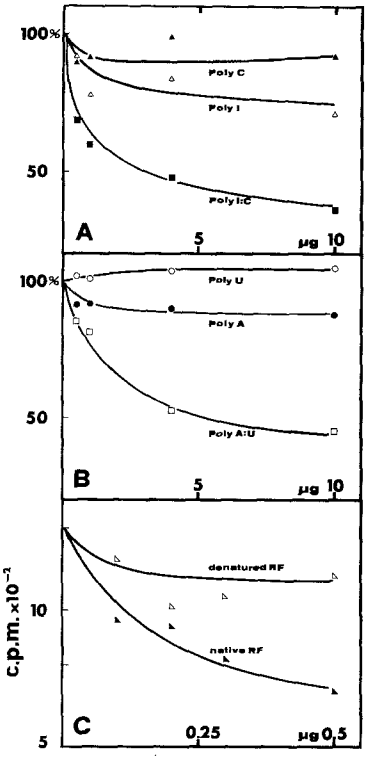
BLOCKING AGENT	BOUND c.p.m.	INHIBITION
. none	1398	-
. α-Amanitin	1227	12%
. AF/013	463	66%

^{3}H-RF bound. No degradation of ^{3}H-RF was observed after 1 hour incubation with the enzyme.

e) The effect on the reaction of two inhibiting agents was studied: α-Amanitin and a rifamycin derivative AF/013. As shown in Table II, α-Amanitin has no action on binding of cellular RNA polymerase to ^{3}H-RF, whereas AF/013 drastically inhibits complex formation.

f) Treatment of ^{3}H-RF with RNase-free DNase (10 μg; 30°C, 25 minutes) does not alter its ability to bind to cellular RNA polymerase.

g) In order to study the structure responsible for binding of cellular RNA polymerase to Mengovirus RF, competition experiments were performed: to standard reaction mixtures containing fixed amounts of cell. Pol. and ^{3}H-RF, increasing amounts of unlabeled single - and double-stranded RNAs were added. As shown in Fig. 2 (A and B), synthetic, single-stranded polynucleotides (Poly I;Poly C;Poly A and Poly U) did not interfere with the complex formation,but the same ribopolymers in the double-helical form (Poly I:C;Poly A:U) efficiently compete with Mengovirus RF for cell. RNA polymerase.Fig.2 C shows that unlabe



Text Fig. 2
Competition between ^{3}H-Mengovirus RF and unlabeled, single - and double-stranded RNAs.
Increasing amounts of the unlabeled competitor were added to standard reaction mixtures.
A and B: abscissa = micrograms of added competitor; ordinate = ^{3}H-RF bound on filters (percentage of controls)
C : To standard reaction mixtures the indicated amounts of heat-denatured (△——△) or native (▲——▲) unlabeled Mengovirus RF were added. Ordinate = ^{3}H-RF bound onto filters.

led Mengovirus RF can efficiently compete for the enzyme only in its native (double-stranded) form.

DISCUSSION

The nitrocellulose filter technique has been widely used to check the "in vitro" formation of the complex "RNA polymerase-template DNA" and results presented in this communication indicate that a cellular RNA polymerase effectively binds to Mengo-

virus RF, provided that this abnormal (RNA) template is present in its double-helical form.

Evidence against the possibility of these results being a mere technical artifact is: i) high efficiency of recovery (up to 90% of input radioactivity in optimal conditions) and low level of blanks (100-200 cpm); ii) the inability of other proteins to retain ^{3}H-RF on filters; iii) the critical dependence of the reaction of temperature and cation concentration. Complex formation is inhibited by a rifamycin derivative, AF/013. Furthermore, binding appears to be strictly related with the double helical nature of the template: all single-stranded RNAs tested failed to compete with Mengovirus RF for cell. Pol. whereas the double-helical form of the same RNAs was an efficient competitor. It is tempting to postulate that recognition of the template (and subsequent attachement of the enzyme) depends (at least "in vitro") on the structure of the template.

A second question to be considered is whether a contaminant protein in the preparation of RNA polymerase might be actually responsible for the binding reaction with Mengovirus RF. Besides RNA polymerase, other proteins have been described that bind to nucleic acids: namely Stimulating Factors I and II (10) and IF3 (11). The former are basic proteins (totally excluded from anion exchangers) and therefore are very unlikely present in a preparation after DEAE-Cellulose chromatography. IF3, on the other hand, is a small protein, tenaciously bound to ribosomes, and eluting from DEAE-Cellulose at moderate (100 mM) $(NH^4)Cl$ concentrations (12). A third possibility to be taken into account is that the protein binding to Mengovirus RF might be a still unidentified one, involved perhaps in the synthesis of cellular ds RNAs (13,14). The existence of such structures has been repeatedly claimed and indirect evidence of their relationship with cellular mRNAs has been presented (14).

As far as the mechanism of infectivity of Mengovirus RF is concerned, these results give further support to the hypothesis

that early viral messengers are transcribed by a cellular RNA polymerase using as template the incoming molecule of RF. Since the complex "cellular Polymerase - Mengovirus RF" detected by the nitrocellulose filter technique might be a non-functional one, experiments are in course to test whether or not a polymerase of cellular origin may transcribe "in vitro" with double-stranded RNAs as template.

Acknowledgement: Authors wish to thank Prof. Th. Wieland, Heidelberg, for kindly supplying α-Amanitin - Prof. L. Silvestri, Lepetit Milano for AF/013 and Prof. G. Tocchini-Valentini, Rome for helpful discussion.
This work was partly supported by Consiglio Nazionale delle Ricerche, Rome - Grant N. 115/0107 72.00294/44.

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