EHRLICH ASCITES CELLS DNA-DEPENDENT RNA POLYMERASES: EFFECT OF AMINO ACIDS AND PROTEIN SYNTHESIS INHIBITION

Silvia CEREGHINI and María T. FRANZE-FERNÁNDEZ
Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, Buenos Aires, Argentina

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1. Introduction

The rate of synthesis of ribosomal RNA (rRNA) is subject to a wide variety of physiological influences [1]. The biochemical mechanisms underlying this regulation, however, are in general unknown. We have found that in Ehrlich ascites cells, the rRNA synthesis and the activity of the enzyme most probably involved in its transcription (α-amanitin resistant RNA polymerase) are regulated by the amino acids concentration in the incubation medium [2] and that this control is mediated by the synthesis and decay of a short lived protein [3]. In the previous work, RNA polymerase activity had been assayed in isolated nuclei; consequently it has been possible to decide whether the enzyme itself or some protein component of the RNA polymerase system have a short half life.

As an approach to studying this problem we have isolated the RNA polymerases from Ehrlich ascites cells and compared the enzymes obtained from cells incubated in different conditions.

2. Methods

The preparation of Ehrlich ascites tumour cells and of the incubation media with and without amino acids, and incubation of the cells have been done as described previously [3]. Nuclei were purified as previously indicated [3] with the modification that all the solutions contained 0.1 mM spermidine. Nuclei isolated from 1 g of cells were resuspended in 3.5–5 ml of a solution containing: 0.02 M Tris–HCl, pH 7.90; 2 mM dithioerythritol (DTE); 5 mM $MgCl_2$; 1 M saccharose. The nuclear suspension was immediately used for RNA polymerase assays and for solubilization of the enzymes.

2.1. *Purification of RNA polymerases*

The RNA polymerases were solubilized from nuclei according to the procedure described by Roeder and Rutter [4]. The nuclear suspension was brought up to 0.32 M ammonium sulfate by the addition of 4 M stock solution adjusted to pH 7.90 with ammonia. Five to seven ml of this solution, were sonicated for 30–40 sec in 10 sec bursts (Branson sonifier model LS75, large probe, setting 2). Next, 2 vol. of a solution containing: 0.05 M Tris–HCl pH 7.90; 5 mM $MgCl_2$; 0.5 mM DTE; 0.1 mM EDTA; 0.3 mg/ml each of Dextrans No. T70, T150 and T250; and 30% glycerol, (Buffer R) was added and the solution was stirred for 15 min. The precipitate formed was removed by 30 min centrifugation at 12 000 *g* and discarded. The supernatant, containing all the enzymatic activity, was brought to near saturation with ammonium sulfate by the addition of 0.42 g of solid salt per ml of solution. The precipitate was collected by 30 min centrifugation at 12 000 *g*. The pellets were resuspended in buffer R (1–2 ml for a pellet from 1 g of cells). This solution (fraction F_2) was stored overnight at −70°C. Fraction F2 was diluted with buffer R to bring the ammonium sulfate concentration to 0.05 M and aliquots containing 14 mg protein were applied to a 0.9 × 7 cm DEAE-Sephadex A-25 column equilibrated

with buffer R containing 0.05 M ammonium sulfate. The enzymes were eluted with a gradient from 0.05 to 0.4 M ammonium sulfate in buffer R (30 ml total volume). The flow rate was 0.9 ml/min. Fractions of 0.8 ml were collected in tubes containing 1 mg of crystalline bovine serum albumin and RNA polymerase activity was immediately assayed. Although the nuclear suspension and fraction F2 can be stored at $-70^{\circ}C$ at least for 20 days without loss in activity, the purification procedure was always performed in two consecutive days.

2.2. *RNA polymerase assay*

The assay medium contained: 2.4 μmoles Tris–HCl pH 7.9; 0.08 μmoles $MnCl_2$; 0.1 μmoles $MgCl_2$; 0.3 μmoles NaF; 0.25 μmoles phosphoenolpyruvate; 0.25 μg pyruvate kinase; 0.05 μmoles DTE; 0.028 μmoles each of GTP, ATP and CTP and, unless otherwise indicated 0.0005 μmoles of H_3 UTP (specific activity 1000 μCi/μmol) and 5 μg of denatured calf thymus DNA, in a final volume of 50 μl. The ammonium sulfate concentration is indicated in each experiment. After an incubation period of 8 min at $37^{\circ}C$ the reaction tubes were placed in an ice bath and 30 μl of a solution containing 1% SDS and 0.05 M sodium pyrosphosphate was added. Aliquots of 60 μl were poured onto Whatman DE81 filter discs (2.1 cm). Filters were washed and counted as indicated by Lindell et al. [5]. In fraction F2 and afterwards, added DNA is absolutely required for the assay of RNA polymerase activity.

3. Chemicals

α-Amanitin was a generous gift from Prof. Th. Wieland of the Max Planck Institute, Heidelberg (Germany); pactamycin was kindly supplied by the Upjohn Co. Glycerol redistilled extra pure was from Merck; DEAE-Sephadex A-25 from Pharmacia. Ammonium sulfate enzyme grade and sucrose from Schwarz-Mann; calf thymus DNA Type 1 was obtained from Sigma Chemical Co., and [^{3}H]UTP from New England Nuclear.

4. Results and discussion

4.1. *Isolation of RNA polymerases from Ehrlich ascites cells*

Fig. 1 shows the resolution of the solubilized RNA polymerases. By DEAE-Sephadex chromatography two peaks of activity appeared, designated I and II. No other peak has been found by eluting the column with up to 0.55 M ammonium sulfate.

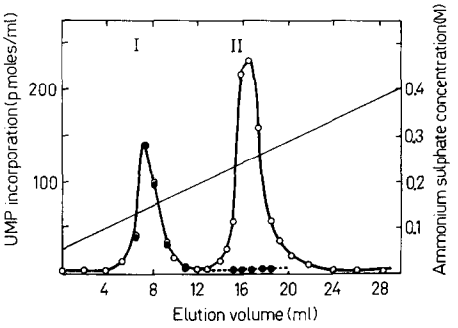

Fig. 1. Resolution of the RNA polymerases by DEAE-Sephadex chromatography: A sample of F2 was chromatographed as indicated in Methods. Fractions of 0.8 ml were collected and aliquots were taken for RNA polymerase activity so that the final ammonium sulfate concentration in the assay medium was 0.05–0.07 M. RNA polymerase activity is expressed as pmoles of UMP incorporated into RNA per ml per 8 min. The activity values of the peak tubes were 3–4 times higher when reassayed at 0.1 mM UTP. RNA polymerase activity in the absence (o–o–o) and in presence (•–•–•) of 0.1 μg of α-amanitin. (——) Ammonium sulfate concentration.

The peak I activity elutes at about 0.15 M ammonium sulfate, it is insensitive to α-amanitin and prefers native DNA as template (fig. 2). The peak II activity elutes approximately at 0.25 M ammonium sulfate, it is sensitive to α-amanitin and transcribes preferentially denatured DNA (fig. 2). With respect to these properties our peaks I and II correspond to the RNA polymerases I and II (A and B, following the nomenclature of Chambon et al. [6] from other eukaryotic organisms [6–8].

Our results differ from those of Blair and Dommasch [9] who reported the isolation by DEAE-Sephadex chromatography of two α-amanitin sensitive forms of RNA polymerase in Ehrlich ascites cells. One

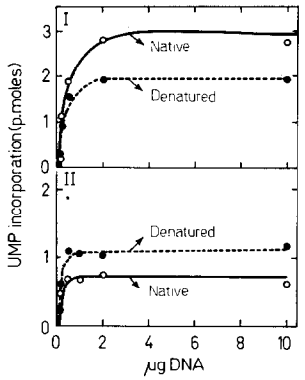

Fig. 2. Transcription of native (o–o–o) and heat-denatured (•–•–•) DNA by RNA polymerases I and II from the DEAE-Sephadex fraction. The assays were performed as indicated in Methods, with the amount of DNA indicated and at 0.07 M ammonium sulfate.

of these forms is unique to cancer cells. A possible explanation for this discrepancy is that a stepwise gradient was used by the authors to resolve the enzymes; it is known that stepwise gradients may produce elution artifacts [10].

In adult mice livers a second form of RNA polymerase 1 has been described [11]. Nevertheless, in mice tumour cells we did not find this second form of the enzyme that in the case of the liver cells elutes at 0.10 M ammonium sulfate from a DEAE-Sephadex column [11].

The purification procedure of the RNA polymerases is highly reproducible (see table 1). The enzyme yields compare well with those obtained in the purification of the RNA polymerases from other sources [12, 8].

4.2. *Effect of pactamycin and concentration of amino acids on RNA polymerase activity*

In fig. 3 the decay is shown of the form 1 enzyme activity in the isolated nuclei of Ehrlich ascites cells when protein synthesis is inhibited or when the cells are transferred to a medium with no amino acids. However, no differences in the amount of the soluble RNA polymerase I activity obtained from nuclei have been found (table 1). Form II enzyme activity in the isolated

Table 1
Yields of soluble RNA polymerases from Ehrlich ascites cells shifted to media with pactamycin or without amino acids

	RNA polymerase activity (pmoles/g cells)			
Fraction	Form I enzyme		Form II enzyme	
A	Control	+ Pactamycin	Control	+ Pactamycin
F2	2.046	2.148	7.670	7.200
DEAE-Sephadex chromatography	2.020	2.000	3.600	3.300
B	Control	No amino acids	Control	No amino acids
F2	3.560	3.570	5.200	5.100
DEAE-Sephadex chromatography	3.600	3.200	N.D.*	N.D.*

* N.D., Not determined.

A: Ehrlich ascites cells were preincubated for 90 min in medium with amino acids. At this time nuclei were isolated from an aliquot of the cells suspension and the RNA polymerases were purified as indicated in Methods. (Control). To the remainder incubate, 0.2 µg/ml of pactamycin was added and the incubation was continued for 60 min. At this time the RNA polymerases were purified. *B:* The control experiment was performed as in *A*. The cells of the remainder incubate were collected, resuspended in medium without amino acids and incubated for another 70 min. After this time the nuclei were prepared and the RNA polymerases were purified. RNA polymerase activity was determined at 0.1 mM UTP and at an ammonium sulfate concentration of 0.06–0.07 M. Forms I and II activities in F2 correspond to the ones non inhibited and inhibited by 1 µg of α-amanitin respectively.

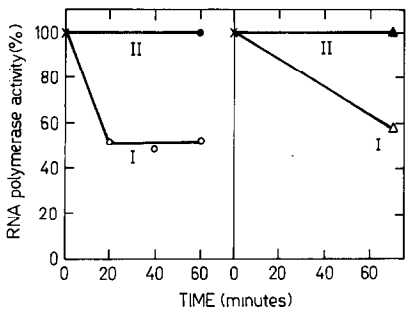

Fig. 3. RNA polymerase activity in the isolated nuclei after shifting Ehrlich ascites cells to media with no amino acids or with protein synthesis inhibitor. Ehrlich ascites cells were preincubated in medium with amino acids for 90 min. At this time the nuclei were isolated in an aliquot of the cells suspension and the RNA polymerase activity was assayed. This was the zero time value and was considered 100% (X). Left: 0.2 μg/ml of pactamycin was added to the remainder incubate and the incubation was continued for the indicated times. The inhibition of protein synthesis by that concentration of pactamycin was more than 98%. Right: the cells of the remainder incubate were collected by centrifugation, resuspended in medium without amino acids and incubated for 70 more minutes. At the times indicated the nuclei were isolated and the RNA polymerase activity was determined. The assays were performed with aliquots of the nuclear suspension containing 42 μg of DNA at 0.32 M ammonium sulfate, and with (1 μg) and without α-amanitin. ○, △, α-amanitin insensitive activity (Form I enzyme); ●, ▲, α-amanitin sensitive activity (Form II enzyme)

nuclei and the amount of the soluble enzyme have not been modified by protein synthesis inhibition or by shifting the cells to an amino-acids-deprived medium (fig. 3, table 1). By DEAE-Sephadex chromatography the two peaks of activity appeared regardless the incubation conditions of the cells (results not shown).

Our results indicate that the amount of the RNA polymerase I is not the factor responsible for the decay observed in enzyme activity in isolated nuclei. The control should be mediated, instead, by some rapidly turning over protein(s) whose regulating capacity is only detected in nuclei system. After solubilization of the enzymes either the regulatory properties of this protein(s), that might stimulate the RNA polymerase I, are not manifested in our assay system, or the protein(s) has been lost or inactivated during the purification procedure.

In connection with the former results it is worth noting that when Ehrlich ascites cells are incubated for 2 hr in a medium with amino acids there is 5–6-fold increase in the RNA polymerase I activity in the isolated nuclei compared to the activity in nuclei form non incubated cells. Nevertheless, there is only a 2-fold increase in the amount of the soluble enzyme activity* suggesting that there might be stimulation of the RNA polymerase I in addition to enzyme synthesis.

Yu and Feigelson [13] reported a decrease in the RNA polymerase activity in the intact rat liver nucleoli after cycloheximide administration to the animals and concluded from this finding that the RNA polymerase I has a rapid turning over time. Benecke et al. [14] however, found no change in the amount of the soluble RNA polymerase I several hours after the administration of cycloheximide to rats. In Ehrlich ascites cells we have found a rapid decay in RNA polymerase I activity in the isolated nuclei after protein synthesis inhibition or amino acids deprivation, but this decay is not the consequence of the rapid turning over time of the enzyme. Our results agree with those recently obtained in yeast [15].

In several experiments we have found that the RNA polymerase I peak from cells incubated with pactamycin eluted from a DEAE-Sephadex column at a slightly higher ammonium sulfate concentration than the enzyme from control cells. We are further characterizing the RNA polymerases to establish the cause of this behaviour.

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* S. Cereghini, M. T. Franze-Fernández, unpublished results.

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