RNA POLYMERASE ACTIVITIES IN *NEUROSPORA CRASSA*

María T. TELLEZ de IÑON, Patricia D. LEONI and Héctor N. TORRES
Instituto de Investigaciones Bioquímicas 'Fundación Campomar' and Facultad de Ciencias Exactas y Naturales, Obligado 2490, Buenos Aires 28, Argentina

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1. Introduction

DNA-dependent RNA synthesis in eucaryotic cells is catalyzed by RNA polymerases that can be found in multiple forms. The main differences between the several enzymes described resides in: localization within the cell, subunit structure, ionic strength and divalent cation requirement, template specificity, sensitivity to antibiotics and behaviour in different chromatographic systems (see ref. [1]). As a general feature, two types of polymerases are distinguishable: the mitochondrial enzyme with properties resembling that of bacteria [2, 3] and the nuclear enzyme with multiple molecular forms. In the latter case two principal forms have been described: a nucleolar enzyme apparently involved in the synthesis of ribosomal RNA, and the nucleoplasmic form which seems to be involved in the synthesis of messenger RNA [4–8]. Besides these enzymes, an additional type of polyribonucleotide synthetase has been described by Edmonds et al. [9, 10] which uses specifically ATP as substrate for polyadenylic synthesis.

Previous work from this laboratory indicated that in *Neurospora crassa* some metabolic parameters such as glycogenolysis and cyclic 3′5′adenosine monophosphate synthesis are under hormonal control [11–13]. Therefore the study in this ascomycete fungus of a similar control mechanism at the level of RNA metabolism seemed to be of interest. As a first approach some properties of the enzymes responsible for RNA synthesis were studied.

This paper reports the characterization of multiple forms of ribonucleotide polymerase in these fungal cells.

2. Materials and methods

2.1. *Cell cultures*

The slime mutant of *N. crassa* (strain F_2:Os-1, N1118-FGSC) was used. This mutant grows as isolated protoplasts surrounded by a plasma membrane. This fact simplifies considerably the procedure for cell lysis and the isolation of undamaged cytoplasmic organelles.

The organism was grown for 24 hr at 30°C in a liquid medium as previously described [14].

2.2. *Enzyme preparation*

The cells obtained from the liquid cultures were harvested by centrifugation for 10 min at 600 *g* and the cellular pellet was resuspended in a solution (5 ml/g of wet cells) containing 0.34 M sucrose, 0.25 mM spermine, 15 mM magnesium acetate and 10 mM Tris–HCl, pH 7.9. This suspension was homogenized with a glass-Teflon Potter homogenizer (tight-fitting) until no whole cells were observed under the phase contrast microscope. Thereafter the suspension was centrifuged for 10 min at 800 *g*; the pellet was slowly stirred and resuspended in the same solution (5 ml/g of cells) using a glass rod. The suspension was again centrifuged under the same conditions. The washing procedure was repeated once again. The preparation was carried out at 4°C.

Examination of the pellet from the latter centrifugation ('crude preparation') with the electron microscope indicated that the preparation containing undamaged nuclei and some cytoplasmic membranes.

Contamination with mitochondrial structures was not observed in any case. One the other hand RNA polymerase activity in this preparation is not sensitive to rifampicin.

When further purification was required this 'crude' preparation was treated as follows: the pellet was resuspended as described above in the sucrose–spermine–Mg^{2+} buffer solution, and the suspension was mixed with 2 vol of 2 M sucrose and centrifuged on a 2 M sucrose 'cushion' for 90 min at 40 000 *g* (SW 25 rotor, Spinco preparative ultracentrifuge, model L). The precipitate thus obtained, contained purified nuclei. No cytochrome *c* reductase activity was detected in this preparation ('purified' preparation). Extraction of RNA polymerase from 'crude' or 'purified' nuclear preparation was performed following the procedure of Roeder and Rutter [4]. The ammonium sulphate precipitate was resuspended in the glycerol–Mg^{2+}–EDTA–dithiothreitol buffer solution and stored at –80°C overnight.

DEAE-cellulose chromatography of the latter enzyme fraction was carried out as follows: 10–20 mg of the enzyme were diluted in glycerol–Mg^{2+}–EDTA–dithiothreitol buffer solution (about 0.5 mg protein/ml) and loaded on a 1 × 12 cm column equilibrated with the same buffer solution. The column was washed with 20 ml of this solution and eluted with 50 ml of a KCl linear gradient (0.025–0.50 M) made in the same buffer solution.

2.3. *Enzyme assays*

DNA-dependent RNA polymerase and polyadenylate synthetase were assayed at 30°C for 5 min. The incubation mixture for RNA polymerase contained: 56 mM Tris–HCl buffer, pH 7.9; 1.6 mM $MnCl_2$; 8 mM KCl; 6 mM NaF; 1.6 mM mercaptoethanol; 0.6 mM each of GTP, CTP and ATP; 0.10 mM [^{3}H]UTP (specific activity 16 cpm/pmole); calf thymus DNA (0.16 mg/ml) and 0.05 ml of enzyme (diluted in glycerol–Mg^{2+}–EDTA–dithiothreitol buffer solution) in a total volume of 0.125 ml. The reaction was stopped by the addition of cold 5% trichloroacetic acid containing 0.04 M $P_2O_7Na_4$. Precipitates were collected on nitrocellulose filters, dried and counted for radioactivity using 0.4% (w/v) Omnifluor (New England Nuclear) solution in toluene.

The incubation mixture for polyadenylate synthetase contained the same components of the assay for RNA polymerase, except that DNA, GTP, CTP and UTP were omitted. In addition the mixture contained 0.10 mM [α-^{32}P]ATP (specific activity 80–200 cpm/pmole) and 1.6 mM $MgCl_2$. The reaction was stopped and counting was carried out as described before for RNA polymerase.

Cytochrome *c* reductase was assayed in the nuclear preparation according to the procedure of Green and Ziegler [15].

2.4. *Protein determination*

Protein was determined by $A_{280\,nm}/A_{260\,nm}$ ratio or by the method of Lowry et al. [16].

2.5. *Reagents*

ATP, GTP, CTP, UTP, DNA (type V), cycloheximide and pancreatic RNAase were purchased from Sigma; [^{3}H]UTP from New England Nuclear. [α-^{32}P]ATP was prepared as described previously [14]. Actinomycin D was obtained from Merck, Sharp and Dohme and α-amanitin was a generous gift from Professor Th. Wieland (Heidelberg).

DNAase was purchased from Worthington.

3. Results and discussion

As is shown in fig. 1 RNA polymerase was eluted from the DEAE-cellulose column as four well defined peaks at KCl concentrations of 0.1–0.12, 0.17–0.18, 0.23–0.25 and 0.34–0.36 M (peak I, II, III and IV, respectively). The same pattern of elution was obtained with both, 'crude' or 'purified' nuclear preparation.

The activity was considerably inhibited by the addition of actinomycin D (table 1). This result suggests that the four peaks require a template for their activity. On the other hand, the activity of peaks III and IV was considerably reduced by the addition of α-amanitin to the assay mixture. These observations are in agreement with the results reported by Tata et al. [17], for rat liver nuclear polymerases. In both cases, liver and *Neurospora*, four peaks of RNA polymerase activity eluted from DEAE-cellulose columns and the peaks III and IV were inhibited by α-amanitin.

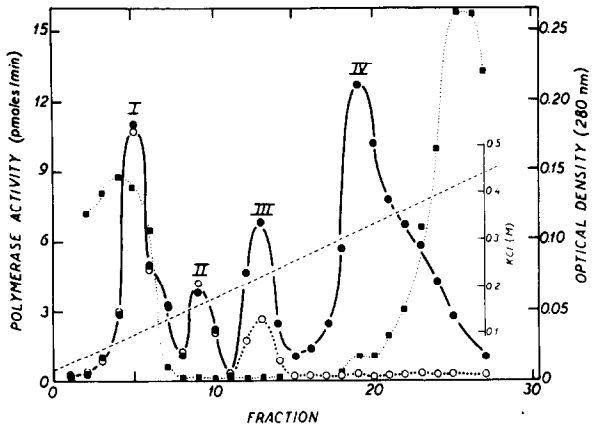

Fig. 1. DEAE-cellulose column chromatography of RNA polymerase from *Neurospora crassa* nuclei. RNA polymerase was assayed using [^{3}H]UTP as labelled substrate in the absence (●—●—●) or presence (○—○—○) of 12.5 μg/ml α-amanitin. (■—■—■): Optical density measured at 280 nm. The volume of each fraction was 1.7 ml. Other conditions were those indicated under Materials and methods.

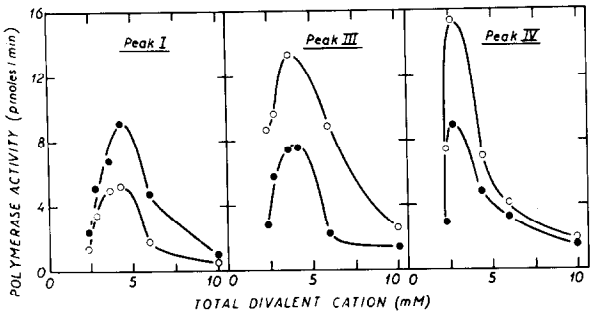

Fig. 2. RNA polymerase activity as a function of divalent cation concentration. The enzyme was assayed in the presence of 2 mM $MgCl_2$ plus an additional concentration of $MgCl_2$ (●—●—●) or $MnCl_2$ (○—○—○). In the abscissa the total concentration of divalent cation in each incubation mixture was indicated. Other conditions were those described under Materials and methods.

Some properties of *Neurospora* polymerases are indicated in table 1 and fig. 2. As it can be seen, in the presence of 2 mM $MgCl_2$ peaks III and IV were more active with increasing concentrations of Mn^{2+} (fig. 2). On the contrary, under the same conditions the α-amanitin-insensitive fraction of peak I was more active with Mg^{2+} than with Mn^{2+}.

This behaviour is the same as that of the rat liver system [17]. Optimum concentrations of the divalent cations were about 3–4 mM; in all cases a marked decrease in activity was observed with higher concentrations. Other requirements are shown in table 1. The four fractions needed ribonucleotide triphosphates and DNA for maximal activity. Denatured DNA was a better template than native DNA, only in the case of peak IV. Antibiotics such as rifampicin and cycloheximide did not affect the activity of the different enzyme peaks.

Table 1
Requirements of RNA polymerase of *Neurospora crassa*.

Conditions	Peak fractions (percent activities)			
	I	II	III	IV
Standard assay	100 (2)	100 (4)	100 (14)	100 (13)
Without CTP, GTP and ATP	18	5	12	4
Without DNA	28	–	20	18
Plus actinomycin D (125 μg/ml)	14	5	7	5
Plus α-amanitin (12.5 μg/ml)	95	105	44	3
Plus cycloheximide (80 μg/ml)	80	100	100	104
Plus rifampicin (4 μg/ml)	96	100	84	100
Plus DNAase (20 μg/ml)	1	–	5	–
Plus RNAase (0.5 μg/ml)	1	–	10	6
Without DNA plus denatured DNA	100	–	110	180

Conditions were as those indicated under Materials and methods. Numbers in parentheses indicate the specific activity of each fraction (nmoles/min per mg protein).

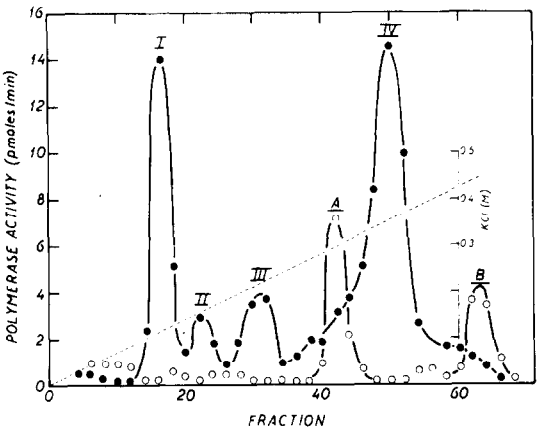

Fig. 3. DEAE-cellulose column chromatography of polyadenylate synthetase and RNA polymerase from *N. crassa* nuclei. Activities were assayed using [α-^{32}P]ATP as labelled substrate in the absence (o—o—o) or presence (•—•—•) of DNA, GTP, UTP and CTP (polyadenylate synthetase and RNA polymerase, respectively). The volume of each fraction was 0.7 ml. Other conditions were those indicated under Materials and methods.

The analysis of poly A synthetase on DEAE-cellulose columns showed two peaks of activity eluting at KCl concentrations of 0.30 and 0.45 M respectively (fig. 3). None of those eluted associated with any peak of RNA polymerase activity. This could suggest that polyadenylate synthetase is independent from the molecular entities of RNA polymerase described. The activity of the synthetase from both peaks was greater with Mg^{2+} than with Mn^{2+}. Besides, it was inhibited by the presence of the three other nucleoside triphosphates, but was not affected by actinomycin D (table 2). However, the fact that the addition of DNAase inhibits the synthesis of polyadenylate suggests that the reaction could require a template and/or primer. The addition of pancreatic RNAase lowered the incorporation only slightly, about 30%, in both peaks.

Table 2
Properties of polyadenylate synthetase.

Conditions	Peak fractions (percent activities) A*	B*
Standard assay	100	100
Plus CTP, GTP and UTP	16.7	18
Plus DNA	130	80
Plus DNA and UTP, CTP and GTP	30.1	28
Plus actinomycin D	90	100
Plus DNA, UTP, CTP, GTP and actinomycin D	0.4	0.5
Plus DNAase	30	26
Plus RNAase	75	60

Conditions were as those indicated under Materials and methods. Concentrations of CTP, GTP, UTP, DNA, actinomycin D, DNAase and RNAase were those corresponding to the standard assay for RNA polymerase (see Materials and methods) and those indicated in table 1, respectively.
* Eluted at 0.30 M KCl concentration.
** Eluted at 0.45 M KCl concentration.

The role of this enzyme should be important, considering that the eucaryotic cells contain a polyadenylic sequence bound to the 3′ end of the RNA molecules [18–20].

3.1. *Comments on enzyme purification and properties*

The elution pattern of RNA polymerase from the DEAE-cellulose columns is quite reproducible in terms of the number of peaks and their position in the KCl gradients but the enzymes are very unstable. Several attempts were made in order to stabilize the activity in the eluted fractions. Some of them were: increase in glycerol concentration up to 50% (v/v), addition of ammonium sulphate or spermine or albumin or increase of dithiothreitol concentration. None of them was successful. In all cases further dilution, dialysis or column chromatography of the different fractions leads to the complete inactivation of the enzyme.

The identity of the different peaks of RNA polymerase and polyadenylate synthetase activities remains unsolved. A good criterion to assert the authenticity of each peak should be a rechromatography of the different fractions. This operation was not possible due to the unstability of the enzymes as described above. However, in all the columns the activity and properties of peak I and II as well as III and IV remained similar. In addition, results not shown here indicated that when a nuclear preparation was chromatographed on a phosphocellulose column instead of the standard DEAE-cellulose column, four peaks of RNA polymerase and two of polyadenylate synthetase activity were also obtained. Furthermore, specific activities of RNA polymerase from peaks I to IV (about 2, 4, 14 and 13 nmoles min per mg protein, respectively) are similar to those reported for liver [4] and ten to one hundred times higher than those reported for yeast [7]. The

purity of these fractions could exclude a possible interference by protein aggregation in the column chromatography.

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