EVIDENCE FOR TWO α-AMANITIN-RESISTANT RNA POLYMERASES IN VEGETATIVE AMOEBAE OF DICTYOSTELIUM DISCOIDEUM

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SUMMARY

Three DNA-dependent RNA polymerases (EC 2.7.7.6), P-I, P-II and P-III, have been isolated from the sonicated nuclear extract of vegetative amoebae of Dictyostelium discoideum by phosphocellulose chromatography. P-I was inhibited by α-amanitin, while P-II and P-III were not. Rifampicin did not prevent all the polymerase activities. These polymerases were more active in the presence of Mg^{2+} than Mn^{2+}. P-III was reduced in the enzyme activity by being passed through DEAE-Sephadex column and not obtained from the nuclear extract of amoebae at the culmination stage during morphogenetic development.

INTRODUCTION

D. discoideum amoebae have been shown to contain two RNA polymerases separable from each other by DEAE-Sephadex chromatography (1,2). One of them is resistant to α-amanitin and the other sensitive to it. Recently, Jacobson et al., (3) who studied RNA synthesis in isolated nuclei of this organism, reported the results suggesting the existence of two nuclear RNA polymerases resistant to α-amanitin; one of them produces RNA (probably rRNA) not bound to polyuridylic acid immobilized on filter papers, whereas the other synthesizes polyadenylic acid-containing RNA. Similarly, two types of α-amanitin-resistant RNA syntheses were observed with isolated nuclei of this slime mold (4).

In this paper, we report that α-amanitin-resistant RNA polymerase in the nuclei of D. discoideum vegetative amoebae exists as two discrete forms which can be separated by phosphocellulose chromatography. However, one (P-III) of the two α-amanitin-resistant polymerases was not detected in the amoebae at the culmination stage during morphogenetic development.

MATERIALS AND METHODS

The amoebae of D. discoideum NC-4 were grown with Escherichia coli at 23°C and harvested at the vegetative and culmination stages followed by washing to remove bacterial cells as described previously (2,5). The isolation of nuclei was carried out according to minor modifications (2) of the method of Soll and Sussman (6) and the solubilization of RNA polymerase was by the method of Roeder and Rutter (7) with slight modifications (2). The isolated nuclei suspended in buffer A (50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM dithiothreitol, 25 % (v/v) glycerin) were sonicated for 1 min (15 sec x 4 with 1-min intervals) in the presence of 0.3 M $(NH_4)_2SO_4$, immediately diluted with buffer A so that the concentration of $(NH_4)_2SO_4$ may be 0.1 M and then centrifuged at 45 000 rpm for 2 h in a Spinco #65 rotor. The supernatant fluid obtained (termed sonicated nuclear extract) was dialyzed for 6 h against buffer B (buffer A minus $MgCl_2$) containing 0.1 M KCl and then loaded on phosphocellulose (Whatman P-II) column, followed by the elution with a linear gradient of 0.1-0.6 M KCl in buffer B. For DEAE-Sephadex chromatography of RNA polymerases isolated by P-cellulose chromatography, the fractions having the polymerase activity were pooled, dialyzed against buffer A containing 0.05 M $(NH_4)_2SO_4$, absorbed to DEAE-Sephadex A-25 column and then eluted with a linear gradient of 0.05-0.3 M $(NH_4)_2SO_4$ in buffer A.

The RNA polymerase activity was assayed, as previously described (2), in 0.15 ml of the reaction mixture containing 50 mM Tris-HCl, pH 7.9, 2 mM $MnCl_2$, 5 mM $MgCl_2$, 10 mM dithiothreitol, ATP, GTP and CTP each 0.5 mM, 0.05 mM UTP, 0.001 mM [^{3}H]UTP (13 Ci/mmol), 20 μg of calf thymus DNA, 150 μg of bovine serum albumin and 50 μl of the enzyme fraction. The reaction mixture was incubated for 40 min at 23°C with a mild shaking and the incorporation of [^{3}H]UTP into trichloroacetic acid-insoluble fraction was measured (2).

RESULTS

As Fig. 1A shows, three RNA polymerase activities were obtained after P-cellulose chromatography of the sonicated nuclear extract of vegetative amoebae. These have been tentatively disignated as P-I, P-II and P-III in their order of elution; P-I was eluted at 0.28 M of KCl, P-II at 0.36 M, and P-III at 0.5 M. As shown in Fig. 2, when each of these polymerases was rechromatographed on DEAE-Sephadex column, P-I was eluted at 0.17 M of $(NH_4)_2SO_4$ and P-II and P-III at 0.11 M. The P-cellulose profile of the

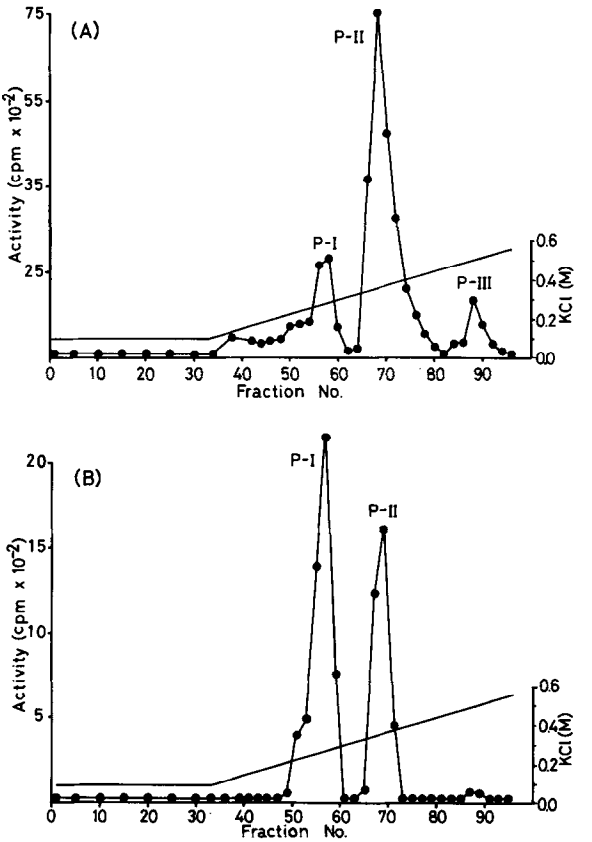

Fig. 1. Phosphocellulose profiles of D. discoideum RNA polymerase activity. The sonicated nuclear extract obtained as described in Methods was loaded on a phosphocellulose column (1x25 cm) equilibrated with buffer B containing 0.1 M KCl, washed with the above buffer, and eluted with a linear gradient of 0.1-0.6 M KCl in buffer B. The flow rate was at 10 ml/h. 4-ml fractions were collected and the enzyme activity was assayed as described in Methods. (A), Sonicated nuclear extract of vegetative amoebae; (B), sonicated nuclear extract of culminating amoebae.

sonicated nuclear extract of amoebae at the culmination stage during morphogenetic development shows a complete loss of P-III activity (Fig. 1B). Figs 1A and 1B also indicate that at the vegetative stage the activity of P-II is higher than that of P-I but this relationship is reversed at the culmination stage.

These polymerase activities were completely dependent on DNA as templates and four ribonucleoside 5'-triphosphates and not reduced during the

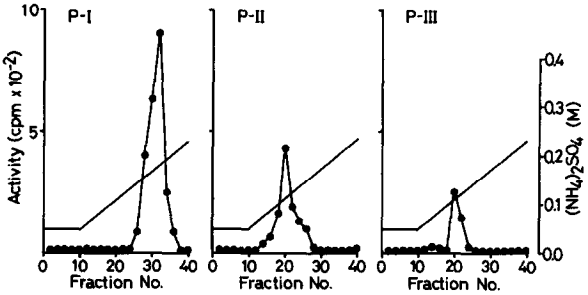

Fig. 2. DEAE-Sephadex chromatography of P-I, P-II and P-III. The sonicated nuclear extract of vegetative amoebae was subjected to phosphocellulose chromatography as in Fig. 1A. The fractions containing the polymerase activity were pooled, dialyzed against buffer A containing 0.05 M $(NH_4)_2SO_4$. The dialysates were applied to DEAE-Sephadex column (1x15 cm) equilibrated with the above buffer containing 0.05 M $(NH_4)_2SO_4$, washed with the same solution, and eluted with a linear gradient of 0.05-0.3 M $(NH_4)_2SO_4$ in buffer A. The flow rate was at 10 ml/h. 4-ml fractions were collected and the enzyme activity was assayed as described in Methods.

incubation at 23°C for at least 1 h (data not shown). As indicated in Table I, α-amanitin strongly inhibited the P-I activity, while P-II and P-III were resistant to the agent and rifampicin exhibited no inhibitory effect on these polymerase activities.

The experiments on the divalent metal ion requirements for these polymerase activities, shown in Figs. 3A-3C, indicate that these polymerases prefer Mg^{2+} to Mn^{2+}, though the degree of the metal ion requirement is different between them. The proportion of the polymerase activity at the optimum Mg^{2+} concentration to that at the optimum Mn^{2+} concentration was 11.4, 6.7 and 4.0 for P-I, P-II and P-III, respectively. Fig. 3D shows that the optimum concentration of $(NH_4)_2SO_4$ for the polymerase activity was 50 mM for P-I and 25 mM for P-II and P-III.

DISCUSSION

The results described in this paper indicate that the vegetative amoebae of D. discoideum additionally contain a special form of RNA polymerase which is apprently different from other two polymerases in respect to the chromatographic behavior on P-cellulose column and the requirement of Mg^{2+}.

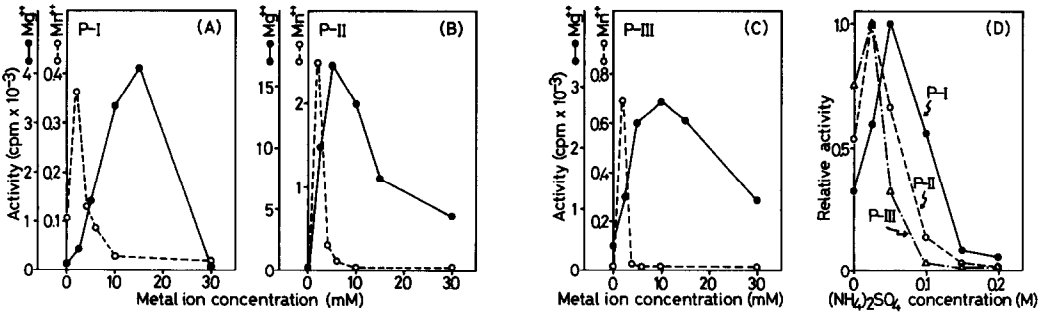

Fig. 3. Effect of divalent metal ions (Mn^{2+}, Mg^{2+}) and ionic strength on RNA polymerase activity. The polymerases (P-I, P-II and P-III) were isolated as described in the legend to Fig. 1 and dialyzed against buffer B. The assay conditions for the enzyme activity were the same as described in Methods except for the concentrations of $MnCl_2$, $MgCl_2$ and $(NH_4)_2SO_4$. For the test of the effect of Mn^{2+} (or Mg^{2+}), Mg^{2+} (or Mn^{2+}) was not contained in the reaction mixture, and for that of $(NH_4)_2SO_4$ the reaction mixture included 2 mM Mn^{2+} and 5 mM Mg^{2+}.

The possibility of the contamination of E. coli RNA polymerase into our enzyme preparations will be excluded for two reasons; (i) rifampicin, an inhibitor of bacterial RNA polymerases, did not influence these polymerase activities and (ii) under our experimental conditions E. coli RNA polymerase was eluted from P-cellulose column at the KCl concentration (0.31 M) different from that for D. discoideum RNA polymerase.

A question arises as to why P-III was not found in the experiment by Pong and Loomis (1) who isolated RNA polymerase from the amoebae in the axenic strain A3 of D. discoideum, a mutant derived from the wild-type strain NC-4. The answer for this is at present obscure but at least two reasons can be considered. One of them may probably lie in the difference of the chromatographic procedure for the isolation of polymerase, because in the experiment by Pong and Loomis (1) in contrast to ours, DEAE-cellulose column was employed in the initial step of the chromatographic purification of the enzyme. We have observed that the P-III activity was scarcely obtained when RNA polymerase was separated initially with DEAE-Sephadex, ir-

Table I. Sensitivity of RNA polymerases to inhibitors

Addition of inhibitors	P-I		P-II		P-III	
	Activity (cpm)	%	Activity (cpm)	%	Activity (cpm)	%
None	1699	100	4718	100	730	100
α-Amanitin (33 μg/ml)	0	0	4944	104.8	726	99.5
Rifampicin (27 μg/ml)	1611	94.8	4174	88.5	696	95.3

Three RNA polymerases were prepared from the sonicated nuclear extract of vegetative amoebae as described in the legend to Fig. 1A and their activities assayed as described in Methods. α-Amanitin or rifampicin was allowed to contact with the polymerases for 5 min at 0°C before their addition of the reaction mixture.

respective of further purification by P-cellusose chromatography. In fact, it is also apparent in this study that P-III was extremely reduced in the enzyme activity by being passed through DEAE-Sephadex column (Fig. 2). The second may be the difference of the strain used. According to Simpson et al.(8), vegetatively growing cells of the axenic strain A3 appear to be metabolically equivalent to the interphase cells of the strain NC-4. Since our unpublished data indicate that the level of the P-III activity is very low in the interphase amoebae of the NC-4 strain, it will supposedly be difficult to detect the P-III activity in the vegetative amoebae of the A3 strain.

When these three polymerase were compared with each other in respect to the chromatographic begavior on DEAE-Sephadex column (Fig. 2) and the sensitivity to α-amanitin (Table I), P-I will be thought to correspond to nucleoplasmic RNA polymerase as found in other eukaryotic organisms (9). In addition, P-II and P-III seem to be originated from nucleoli, since in

addition to the above criteria, it has been shown that nucleolar RNA polymerase of rat liver is separated into two different forms by P-cellulose chromatography but not by DEAE-Sephadex chromatography (10). However, it is at present unknown whether the two α-amanitin-resistant polymerases, P-II and P-III, are both involved in synthesis of rRNA or whether either of them can synthesize polyadenylic acid-containing RNA, as just suggested from the experimental results of Jacobson et al. (3). Experiments are now in progress to distinguish these two possibilities.

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