DNA DEPENDENT-RNA POLYMERASES FROM *PHYSARUM POLYCEPHALUM*

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1. Introduction

Considerable work on *Physarum* has dealt with the biochemistry of the life cycle and was recently reviewed [1, 2]. RNA synthesis has been studied at various stages of development. Differential transcription might play a role during S phase [3], before nuclear division [4], in spherulation [5] and sporulation [6]. Analyses of extracted RNA by density gradient centrifugation [7], gel electrophoresis [8] and DNA–RNA hybridization [9], however, have not provided evidence for selective transcription at distinct stages of the cell cycle (for review see [10]). Variable permeability to precursors and drugs might have interfered in *in vivo* experiments and changes in RNAase activity [11] with RNA extractions.

Isolated nuclei from *Physarum* incorporated nucleoside phosphates into RNA [12] and further experiments gave evidence for a nucleolar and nucleoplasmic RNA polymerase activity [13]. It appeared to us that the isolation of RNA polymerases might provide a new approach to study the gene regulation in the life cycle of *Physarum*, in addition to studies on non-histone proteins [14].

In this paper we report the purification and partial characterization of two DNA-dependent RNA polymerases which differ in their sensitivity to α-amanitin, subunit structures and chromatographic behavior.

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2. Methods

We used published methods to grow *Physarum* [15] and two procedures to isolate nuclei from plasmodia [16]. RNA polymerase was extracted with buffer A (50 mM Tris–HCl, pH 7.5, 0.2 mM EDTA, 5 mM mercaptoethanol, 20% glycerol) and assayed as described previously [17, 18] with some modifications experienced in the yeast system [19] and A. Hildebrandt, unpublished results). The assay mixture contained in a final volume of 300 μl: 50 mM Tris–HCl, pH 7.5, 0.5 mM dithioerythritol, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.15 mM each of ATP, GTP, and CTP, 0.005 mM UTP, 1 μCi of [^{3}H]UTP (1000 cpm/pmol), 15 μg calf thymus DNA or 0.025 A_{260} units of artificial template and 50 μl enzyme preparation.

DNA cellulose was prepared as described [20] using calf thymus DNA. SDS gel-electrophoresis was done according to published methods [21]. *Physarum* DNA was prepared as described previously [8]. DNA was denatured by boiling for 10 min, followed by rapid cooling with ice. RNAase activity was tested by incubating 200 μl of each fraction eluted from DEAE-Sephadex A 25 (Pharmacia) and [^{3}H]*Physarum* RNA for 30 min at 37°C and TCA precipitation. Uridine-5-[^{3}H]-5′-triphosphate (10 000 mCi/mmol) was purchased from Amersham-Buchler, native thymus DNA, poly-d-(A–T), poly-dA·poly-dT), (poly-A·$d(pT)_{10}$) and rifampicin from Boehringer. *E. coli* RNA polymerase was a gift from Professor Bautz (Heidelberg), α-amanitin from Professor Wieland (Heidelberg), and rifampicin derivative AF/013 from Professor Silvester (Milano).

3. Results

3.1. *Purification of RNA polymerase A and B from Physarum*

Plasmodia were frozen in liquid nitrogen prior to homogenization in 10 vol of buffer A plus 0.5 M NH_4Cl, followed by sonication and ultracentrifugation. Addition of a nonionic detergent (Nonidet P 40 Shell, 0.2%) to buffer A had no effect on the yield of RNA polymerase activity. No RNA polymerase activity was measured in the pellets after ultracentrifugation. Nuclei and nucleoli were treated like whole plasmodia. The soluble extracts (100 000 *g* supernatant) were dialysed against or diluted to 0.1 M NH_4Cl with buffer A and applied to columns of DEAE-Sephadex A 25 at 4°C. Bound material was eluted by a linear or stepwise increase in NH_4Cl concentration. Fig. 1 shows the elution profile of an extract from whole plasmodia. With denatured thymus DNA as template two peaks of RNA polymerase activity were found at concentrations of 0.25 M and 0.3 M NH_4Cl respectively, also on rechromatography. No further active fractions were seen with native DNA or poly-d(A–T) as template nor was any measurable RNAase activity in the eluted fractions. Extracts from crude nuclear pellets, where centrifugation through 1 M sucrose was omitted, resulted in a profile of enzyme activity very similar to that shown in fig. 1. The yield of enzyme activity, however, was less than 50% of the enzymes extractable from whole plasmodia. Extracts from crude nucleolar preparations contained both peaks of activity from fig. 1. The second peak was the prominent fraction of enzyme activity although a considerable amount of the first enzyme peak was also present (approx. one fourth of that seen in fig. 1). Extracts from pure nuclei or nucleoli contained no RNA polymerase activity. From these observations it seemed that RNA polymerases of *Physarum* were not tightly bound to chromatin and therefore further purifications were done with extracts from whole plasmodia.

α-Amanitin (5 μg/ml) inhibited 95% of enzyme activity in the first peak while the enzyme activity of the second peak was insensitive. With reference to the terminology of RNA polymerases in other cells [22], the first peak contained an enzyme of the B-type and the second an enzyme of the A-type.

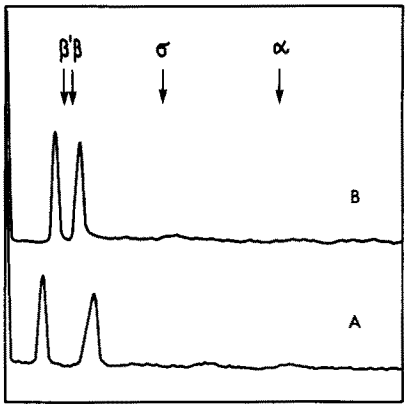

Fig. 2. Densitograms of SDS acrylamide gels. Enzymes A and B from *Physarum* were purified from the DEAE-Sephadex eluate by DNA cellulose chromatography and glycerol gradient centrifugation. Electrophoresis of approximately 10 μg protein per gel was done in 0.1% SDS on 10% polyacrylamide gels at 4 mA/gel. Coomassie blue stained gels were scanned at 550 nm. A = enzyme A, B = enzyme B, RNA polymerase from *E. coli* served as marker.

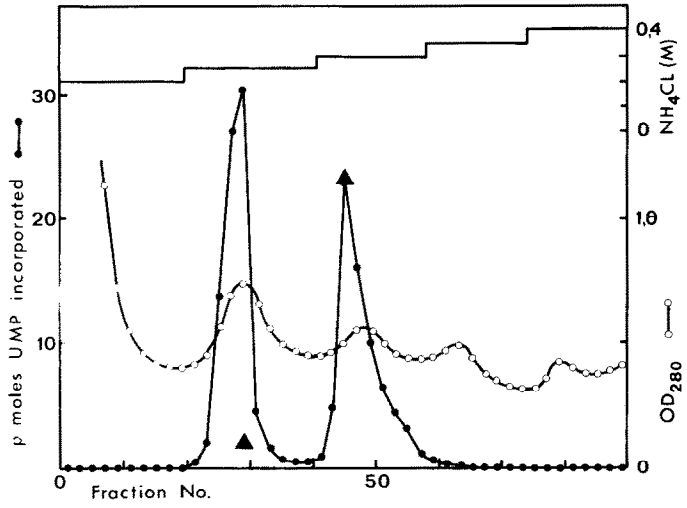

Fig. 1. Chromatography of RNA polymerases on DEAE-Sephadex A 25. Plasmodia (25 g wet weight) were homogenized in 250 ml buffer A plus 0.5 M NH_4Cl, sonicated in portions of 15 ml (5 × 15 sec, setting 4, Branson). The homogenate was centrifuged for 60 min at 100 000 *g*, the supernatant was diluted to 0.1 M NH_4Cl with buffer A and applied to 100 ml of DEAE-Sephadex A 25 equilibrated with buffer A in a column kept at 4°C. Elution was done with 50 ml for each step of NH_4Cl concentration, pumped at 100 ml/hr. The eluate was measured at 280 nm (o—o—o) and 50 μl from each fraction was tested for RNA polymerase activity (•—•—•). Peak fractions of RNA polymerase activity were checked with 5 μg/ml α-amanitin in the assay mixture (▲).

Enzymes A and B from the elution profile of fig. 1 were diluted to 0.1 M NH_4Cl and applied to a column with prewashed DNA-cellulose. Up to 80% of enzyme activity was bound to the column and could be eluted as a sharp peak with buffer A plus 0.3 M NaCl. The enzymes were concentrated on a 1 ml column of DEAE-Sephadex A 25 in 5% glycerol and layered over linear gradients of glycerol (20–50% glycerol in buffer A with or without 0.4 M NH_4Cl) centrifuged at 32 000 rpm for 20 hr in SW 39 rotor at 0°C and fractionated. Both enzymes had reached about the same position in the middle of the gradient (results not shown).

3.2. *Structural properties of RNA polymerases from Physarum*

Aliquots of enzymes A and B from the glycerol gradient were treated with SDS and electrophoresed on SDS polyacrylamide gels. Each of the preparations consisted essentially of 2 subunits (fig. 2). With *E. coli* RNA polymerase (holoenzyme) as a reference marker, approximate molecular weights for the subunits of *Physarum* RNA polymerases were: 185 000 and 135 000 for enzyme A and 175 000 and 145 000 for enzyme B.

In order to purify one RNA polymerase of type A and one of type B from plasmodia of *Physarum*, ion exchange chromatography, affinity chromatography and density gradient centrifugation were necessary and adequate.

3.3. *Enzymatic properties of RNA polymerases from Physarum*

RNA polymerases from *Physarum* must be prepared in 20% glycerol at temperatures not exceeding 4°C at all steps. The enzyme pattern was not affected when the 100 000 *g* supernatant was kept at 4°C overnight before further fractionations. The enzymes were very sensitive to oxygen. Plasmodia can be kept in liquid nitrogen for weeks and at −20°C for several days. Both enzyme activities displayed linear dependence on the amount of protein added over a 10-fold range and reaction was linear for 20 min. The decrease of UMP incorporation thereafter seemed to be due to enzyme inactivation since addition of enzyme was followed by a burt of incorporation.

The activity of the purified enzymes was dependent on the addition of DNA and all 4 nucleoside triphosphates (results not shown). Sensitivity to α-amanitin served as the criterion to distinguish the two enzymes throughout the purification procedure. At 5 μg/ml Enzyme B was inhibited more than 95%, whereas enzyme A was not affected by 50 μg/ml. Both enzymes were insensitive to rifampicin but were sensitive to rifampicin derivative AF/013 (50% inhibition was observed with 20 μg/ml) like other cells [23]. Both enzymes required divalent cations, Mn^{2+} or Mg^{2+}. They were more active in the presence of Mn^{2+} as compared to Mg^{2+}, by a factor of 6 for enzyme B and 2 for enzyme A. Both enzymes had maximal activity at 2.5 mM Mn^{2+} or 10 mM Mg^{2+}. Both enzymes had the same broad pH optimum of pH 7–8. Only 10% of maximal activity was measured below pH 6 or above pH 9 (results not shown).

The following observations were made with various templates: Poly-dA·poly-dT and poly-A·$p(pT)_{10}$ were not accepted as templates. Denatured calf thymus DNA and poly-d-A-T were the most efficient templates for both enzymes and were approximately 20 times as active as native thymus DNA. Denatured *Physarum* DNA was about one half as active as denatured thymus DNA. Native *Physarum* DNA was a more active template than native calf DNA, by a factor of 7 or 3 for enzyme A and B respectively. However, the number of nicks has not been estimated in the DNA preparations.

4. Discussion

Like other cells [18, 22] *Physarum* contained multiple RNA polymerases. We have purified one enzyme of type A and one of type B from whole plasmodia. Under our conditions we did not observe more than two RNA polymerases. Similar results with nuclear extracts and from isolated nuclei [13] as well as rifampicin sensitive mitochondrial RNA synthesis in *Physarum* [24] indicated nuclear location for both enzymes. Increased activity of enzyme A in crude nucleolar extracts suggested location of that enzyme in nucleoli, in agreement with α-amanitin resistant UMP incorporation in nucleoli of *Physarum* [13] and other cells [25].

The two enzymes from *Physarum* had very similar enzymatic characteristics like enzymes from plant material [26] but were distinct from each other with respect to α-amanitin sensitivity and individual subunit patterns. Sensitivity to α-amanitin was almost as high

as seen in mammals [22] whereas *Physarum* enzymes consisted of two subunits only and each very similar in size to enzyme A and B from yeast [27].

The similarity of RNA polymerases from *Physarum* and yeast revealed by their subunit patterns will also be checked immunologically with purified antibodies prepared against yeast enzymes [28].

Despite similarities in ion requirements, enzyme B from *Physarum* was more active with Mn^{2+} than enzyme A (by a factor of 3). This observation confirms and explains the known stimulation of UMP incorporation by isolated nuclei with Mn^{2+} [12, 13].

Both enzymes from *Physarum* were easily removed from the nuclei which precluded their detection in and purification from clean nuclear preparations but will facilitate reconstitution experiments with enzymes and chromatin from various and distinct stages of the luft cycle of *Physarum*.

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