NEW POLYPEPTIDE CHAINS ASSOCIATED WITH HIGHLY PURIFIED RNA POLYMERASE II OR B FROM *PHYSARUM POLYCEPHALUM*

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Received 20 January 1981

1. Introduction

Three classes of DNA-dependent RNA polymerase (ribonucleoside-triphosphate: RNA-nucleotidyltransferase, EC 2.7.7.6) are present in the nuclei of eukaryotic cells. These isozymes differ from one another in subunit structure, intranuclear location, and transcriptional function (reviewed [1]). One of these polymerases (II or B) has been shown to be localized in the nucleoplasm, and to be responsible for the transcription of pre-messenger RNA [2,3]. This enzyme, therefore, plays a central role in eukaryotic gene expression.

In [4] we described a rapid, high-yield method for its preparation from exponentially growing microplasmodia of *Physarum polycephalum*. Here we describe the results of structural studies on the enzyme.

2. Materials and methods

2.1. *Enzyme purification and gel electrophoresis*

The methods used for the growth and harvest of *Physarum polycephalum* microplasmodia have been described [4,5]. A detailed description of the methods employed in the purification and assay of RNA polymerase II from this organism, as well as the electrophoretic and protein assay techniques used, is given in [4]. For gel scanning, appropriate lanes were cut from slab gels and scanned at 578 nm using a Beckman ACTA III spectrophotometer.

2.2. *Fractionated glycerol gradients*

Purified RNA polymerase II from the phosphocellulose column step [4] was dialyzed against 10% glycerol in buffer I (50 mM Tris (pH 7.9), 0.1 mM EDTA, 200 mM $(NH_4)_2SO_4$, and 10 mM 2-mercaptoethanol). Linear glycerol gradients of 25–50% in buffer I were poured in polyallomer centrifuge tubes [6]. Each gradient had a total volume of 4.5 ml. Dialyzed RNA polymerase (25 μg) was layered on top, and the gradients were centrifuged at 48 000 rev./min for 20 h at 4°C in a SW50.1 rotor using a Beckman L2-65B ultracentrifuge. Equal sized fractions were collected from these gradients by puncturing the bottom of the tube and assays were performed on them.

2.3. *Polyacrylamide gel-fixed glycerol gradients*

We developed a method for fixing and staining these gradients after sedimentation, similar to that used in [7] for fixing sedimented nucleic acids. For this purpose both stock solutions used to prepare 25–50% linear glycerol gradients contained 5% (w/v) acrylamide, 1.25% (w/v) bisacrylamide, 0.06% (v/v) *N,N,N',N'*-tetramethyl ethylenediamine and 0.05 mg riboflavin/ml. Glycerol gradients were poured in a darkroom under photographic safety lights, and protein samples were layered on top of the gradients as before. Once the gradients were capped and thus protected from light, sedimentation was carried out as above. After sedimentation, the gradients were fixed by allowing them to stand upright for 2 h in front of a strong fluorescent white lamp. The solidified gradients thus formed, were stained with Coomassie brilliant blue R250 as in [8].

3. Results

RNA polymerase II (or B) from microplasmodia in mid-exponential growth was purified through the

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phosphocellulose column step [4] and characterized by glycerol gradient sedimentation and SDS–polyacrylamide gel electrophoresis.

In one series of experiments sedimentation was carried out in 25–50% linear gradients of glycerol containing acrylamide monomer. After sedimentation, the gradients were fixed by exposure to light (see section 2) and stained with Coomassie blue. Fig.1 shows that a broad, often skewed, peak of protein was detected by this technique.

In another series of experiments, the acrylamide was omitted and the gradients were fractionated after sedimentation. When such gradients were assayed for total protein and enzyme activity, results like those shown in fig.2 were obtained. The curve for enzyme activity and protein follow one another quite closely. Taken together, these observations suggest that the preparation is homogeneous, with respect to sedimentation.

When the apparently homogeneous activity was analyzed by dodecylsulphate–polyacrylamide gel electrophoresis, 10 polypeptide chains were detected. The molecular weights (or M_r values) of these polypeptide chains were determined by electrophoresis in dodecylsulphate–polyacrylamide gels. Gels of 7.5%, 12.5% and 15% polyacrylamide were used in these determinations. Fig.3 shows a densitometer tracing of such a polyacrylamide gel. At the gel concentration shown (15%) all polypeptides present in the preparation are resolved from the buffer front. The M_r values given are the results of determinations at each of the 3 gel concentrations above.

As noted in [4] the activity from the phosphocellulose column peak, which appears homogeneous by several criteria can be resolved into two major subforms by electrophoresis under non-denaturing conditions. These subforms, termed B_0 and B_1, differ from one another in that the largest polypeptide in form B_0 has M_r ~215 000; while the largest polypeptide in form B_1 has M_r ~170 000.

In an attempt to purify each of the two subforms, we isolated the enzyme activity from microplasmodia in the early and late exponential phases of growth. Electrophoresis of the purified products under non-denaturing conditions revealed that form B_0 was the dominant subform isolated in early exponential growth, and form B_1 was the dominant subform isolated in late exponential growth. In some experiments a pure preparation of a single form was obtained. In these cases only one band was observed in the non-

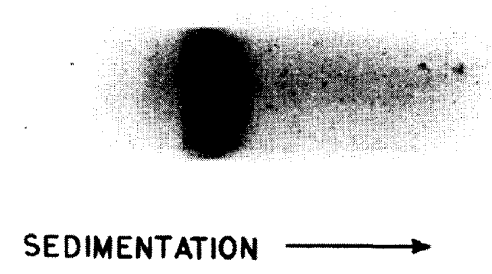

Fig.1. Glycerol gradient sedimentation profile of purified RNA polymerase II. RNA polymerase II (25 μg) was layered onto a 25–50% linear glycerol gradient containing uniform concentrations of acrylamide, bisacrylamide, *N,N,N',N'*-tetramethyl ethylenediamine, and riboflavin in buffer I. After sedimentation, the gradient was fixed by light promoted polymerization of the acrylamide, and stained with Coomassie blue.

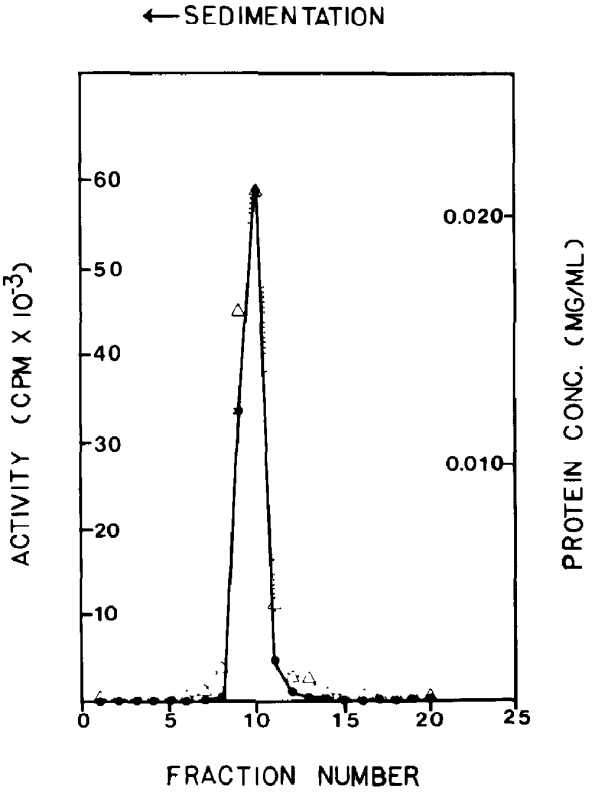

Fig.2. Co-sedimentation of total protein and RNA polymerase II activity. A 25–50% linear gradient of glycerol in buffer I was prepared and run as in section 2. The gradient was fractionated into 20 equal fractions by collecting drops from the bottom of the tube. Protein concentration and enzyme activity were determined.

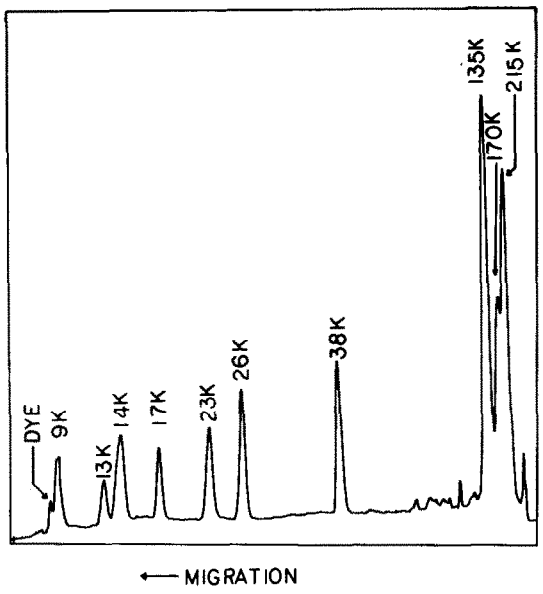

Fig.3. Densitometer tracing of *Physarum* RNA polymerase II after dodecylsulfate–polyacrylamide gel electrophoresis. The 15% polyacrylamide gel shown above resolves all polypeptide chains from the buffer front. Gel electrophoresis, staining and scanning were done as in section 2. The M_r values given were determined by comparison with a series of proteins of known M_r in separate experiments using 7.5%, 12.5% and 15% polyacrylamide gels.

denaturing gel (not shown). Both subforms were found to have about the same specific activity on denatured calf thymus DNA.

The activities purified from early and late exponential growth were analyzed by dodecylsulphate gel electrophoresis. Fig.4 shows 12.5% polyacrylamide gels of the two activities. The enzyme isolated from microplasmodia in early exponential growth (A) contained a polypeptide chain with app. M_r 215 000. The enzyme isolated from microplasmodia in late exponential growth (B) did not contain this polypeptide chain. In its place, a polypeptide chain with app. M_r 170 000 was found.

Since *Physarum* microplasmodia are known to express increased levels of several proteases in late exponential growth [9], proteolytic attack on the 215 000 M_r polypeptide could explain these data. As reported [4] we attempted to recover form B_0 from late-exponential phase microplasmodia by performing the isolation in the presence of 1 mM phenylmethylsulfonyl-fluoride. Form B_1, however, was still the predominant form recovered from these microplasmodia even in the presence of this serine esterase inhibitor.

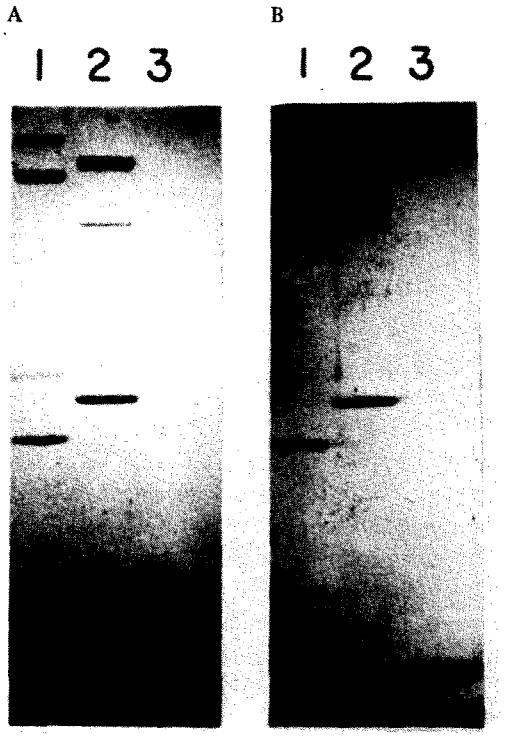

Fig.4. 12.5% polyacrylamide Tris–dodecylsulfate slab gel electrophoresis of RNA polymerase II isolated from microplasmodia in early and late exponential growth. (A) RNA polymerase II from microplasmodia in early exponential growth. The pooled activity from the phosphocellulose stage [4] was analyzed by dodecylsulfate gel electrophoresis as described: (1) RNA polymerase II (10 μg); (2) *E. coli* RNA polymerase marker, M_r 160 000 (average for β and β'), 84 000 (σ), 39 000 (α); (3) trypsin inhibitor marker (M_r 21 500). Migration is from top to bottom. (B) RNA polymerase II from microplasmodia in late exponential growth. The pooled activity from the phosphocellulose step [4] was analyzed by dodecylsulfate gel electrophoresis as described: (1) RNA polymerase II (10 μg); (2) *E. coli* RNA polymerase marker, M_r 160 000 (average for β and β'), 84 000 (σ), 39 000 (α); (3) trypsin inhibitor (M_r 21 500).

4. Discussion

Subforms of RNA polymerase II have now been isolated from a variety of organisms [10–13]. In each of these organisms, these enzyme activities are associated with either 9 or 10 polypeptide chains, and the subforms differ from one another in the app. M_r of their largest. Our data suggest that this is also true for the enzymes from *Physarum polycephalum*. Each form appears to have polypeptide chains of M_r 135 000, 38 000, 26 000, 17 000, 14 000, 13 000 and 9000. In

addition, form B_0 has another of $M_r \sim 215\,000$. This replaced in form B_1 by a polypeptide chain of M_r 170 000. In yeast and *Drosophila* these subforms are thought to arise via proteolysis of the largest subunit [10,11]. Our data on the origin of form B_1 is consistent with the proteolytic attack on the 215 000 M_r polypeptide found in B_0 to produce the 170 000 M_r polypeptide found in form B_1. We cannot offer direct evidence for proteolysis, however, since we were not able to inhibit the process with the serine esterase inhibitor phenylmethylsulfonyl-fluoride. This inhibitor is effective in preserving the B_0 form in yeast [11] but ineffective in preserving the analogous form in *Drosophila* larvae [10]. The microplasmodial enzymes from *Physarum* are similar to those from *Drosophila* in this regard, but the evidence for proteolysis remains circumstantial for both organisms.

Our data on the polypeptide composition of these enzymes extends that in [4,6,14,15]. The data presented suggests that the enzyme purified in [6,14,15] is similar to form B_1 described here. In [6,14,15] an app. M_r ~175 000 was reported for the largest polypeptide chain in the collection. Only in [15] have >2 polypeptide chains been reported associated with the enzyme. In [15], putative enzyme subunits were found to have M_r 175 000, 140 000, 24 000 and 17 000. Within experimental error, these M_r values correspond to those of 4 of the polypeptide chains observed in form B_1. We have thus isolated a form of B_1 containing 5 previously unobserved polypeptide chains, which bears a strong resemblance to that isolated from several other eukaryotic organisms [10–12].

Preliminary studies on the sedimentation properties of the two forms suggest that both sediment near the 14.5 S value observed in [15]. In addition, these studies suggest that the collection of small polypeptide chains co-sediment with the enzyme activity. At present, however, we cannot be certain that all of these polypeptide chains are functional subunits of RNA polymerase II. Some may be adventitious proteins which bind to RNA polymerase II during isolation. Others may be proteolytic cleavage products produced from the putative subunit of 215 000 M_r. Further work will be needed to clarify these points.

However, we would like to point out that as improvements in purification techniques have been made, more putative subunits have been found associated with RNA polymerase II from *Physarum* [4,6,14,15].

Acknowledgements

The authors thank K. Behrens for expert technical assistance, and the Swiss National Science Foundation, grant 3.501.75, for financial support.

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