DNA-DEPENDENT RNA POLYMERASES FROM YEAST. PARTIAL CHARACTERIZATION OF THREE NUCLEAR ENZYME ACTIVITIES

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1. Introduction

Studies on DNA-dependent RNA polymerases from nuclei of animal cells had shown that these enzyme activities can be separated into two or three fractions on DEAE-Sephadex columns [1–3]. One of these fractions (enzyme B according to the nomenclature of Kedinger, Nuret and Chambon [4]), is inhibited by α-amanitin and thus contrasts to fraction A, which is of nucleolar origin and insensitive to the drug [3, 5]. More detailed characterization of the enzyme fractions A and B has provided evidence that both contain more than one enzyme protein differing in their subunit composition [4, 6–8].

We are interested in the DNA-dependent RNA polymerases from yeast for the following reasons: (1) yeast cells can be obtained in large quantities and thus provide a very suitable starting material for the purification and molecular characterization of RNA polymerases, (2) yeast is one of the simplest, non-differentiated eukaryotes and it is therefore of interest to compare its RNA polymerases with those of the more complicated, differentiated animal cells and (3) by variation of the growth conditions, yeast cells can be obtained in different physiological states and it would be interesting to investigate whether such changes have an effect on the distribution and activity of the RNA polymerases.

Multiple forms of DNA-dependent RNA polymerase from yeast have been reported recently [9, 10]; however a more detailed comparison of these enzymes with each other and with the respective polymerases from animal cells is still lacking.

We have obtained evidence for the occurrence in sonified yeast extracts of three DNA-dependent RNA polymerases of nuclear origin. The three enzymes differ in their template specificity and in their sensitivity towards α-amanitin.

2. Materials and methods

Two different strains of *Saccharomyces cerevisiae* were used in this work. One was a haploid, leucine-requiring mutant which was grown as described previously [11], the other one was a commercially obtained diploid wild type strain.

2.1. *Preparation of cellular extracts and chromatography on DEAE-Sephadex*

All operations were carried out at 0–4°. 5 g (wet weight) of cells were washed with water, suspended in 10 ml of 0.05 M Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithioerythrol, 15% glycerol containing a small amount of the protease inhibitor phenylmethylsulfonylfluoride and disrupted by shaking with glass beads for 40 sec in a Braun homogenizer. After decantation from the glass beads, the homogenate was treated for 20 sec with an MSE Model 3000 sonifier at maximum power. Ammonium sulfate was then added to a final concentration of 0.3 M and the sonication was repeated. From there on, the extract was processed further following closely the method described by Roeder and Rutter for preparations from animal nuclei [1, 3]. The homogenate was diluted twofold with the buffer

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solution described above and centrifuged for 20 min at 45,000 *g*. Ribosomes were removed from the supernatant by a 60 min centrifugation at 150,000 *g*. Proteins in the clear ribosome-free extract were precipitated by the slow addition of solid ammonium sulfate (0.42 g/ml solution). The precipitate was collected by centrifugation for 30 min at 45,000 *g* and dissolved in 3 ml of TGMED (0.05 M Tris-HCl pH 7.9, 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithioerythrol) containing 0.05 M ammonium sulfate and dialysed for 3 hr against the same buffer. If some material remained insoluble, it was removed by 60 min centrifugation at 150,000 *g*.

The chromatography was carried out on a column 2 × 17 cm of DEAE-Sephadex A-25 which was loaded with 40–45 mg of protein. The column was washed with 20 ml of TGMED containing 0.05 M ammonium sulfate followed by 20 ml of TGMED containing 0.1 M ammonium sulfate. Enzymes were finally eluted by 160 ml of a linear gradient ranging from 0.1–0.5 M ammonium sulfate in TGMED.

2.2. *Determination of RNA polymerase activity*

The standard assay mixture contained in a total volume of 0.125 ml: 0.05 M Tris-HCl (pH 7.9), 1.6 mM $MnCl_2$, 1 mM EDTA, 1 mM β-mercaptoethanol, 100 μM each of ATP, GTP and CTP, 25 μM UTP plus 0.25 μCi of ^{3}H-UTP (Radiochemical Center, Amersham, England, specific activities between 3–13 Ci/mmole), 12. 5 μg heat denatured calf thymus DNA and enzyme. After incubation for 15 min at 35°, samples were cooled in ice and 100 μl aliquots were applied onto Whatman GF/C filters (25 mm diameter) which were then washed according to the procedure described earlier [11].

3. Results and discussion

Fig. 1. shows the elution profile obtained on chromatography of a cellular extract from yeast on DEAE-Sephadex A-25. Three peaks of RNA polymerase activity emerge, the second one being by

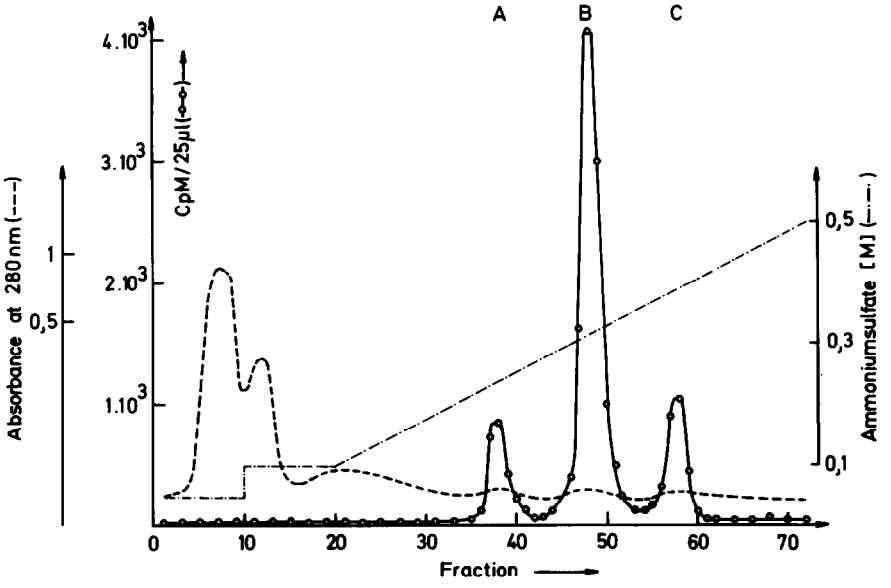

Fig. 1. Chromatographic separation of yeast RNA polymerases on DEAE-Sephadex A-25. Cellular extracts of yeast were prepared as described in Materials and methods. 45 mg of protein were applied onto a column 2 × 17 cm of DEAE-Sephadex and chromatographed according to the method of Roeder and Rutter [1] (see Materials and methods for details). Fractions of 2.5 ml were collected. The absorbance of the eluate was recorded automatically with an LKB UV-cord and 25 μl of each fraction used for the determination of RNA polymerase activity.

far the most prominent. The distribution of the peaks, the elution along the gradient and the relative amounts of activity eluted with each fraction is very reproducible. Thus in five different experiments peak B contained 63–68% and the peaks A and C between 15–20% of the total RNA polymerase activity. The same profile was obtained with both yeast strains used.

Some of the properties of the three enzyme activities are compared in table 1. Distinct differences exist between the enzymes. Enzyme B is the only one sensitive towards α-amanitin but at the same time shows the lowest activity with native calf thymus DNA and is almost inactive with native yeast DNA. With regard to the α-amanitin sensitivity, this enzyme corresponds to the B enzymes from animal cells and it also appears to be the yeast RNA polymerase that was purified earlier by Frederick, Maitra and Hurwitz [12]. Enzymes A and C both show considerable activity with native calf thymus DNA but differ in their activity when native yeast DNA is used as template. Only enzyme C exhibits high activity with this "natural" template. All three enzymes are insensitive to rifampicin.

For technical reasons we have used extracts of whole cells rather than isolated nuclei for these studies; nuclei being destroyed when yeast cells are opened by shaking with glass beads. Since it is known that a DNA-dependent RNA polymerase is present also in mitochondria, we have done a number of experiments in order to prove that the three RNA polymerases described are in fact of nuclear origin. These experiments are summarized in fig. 2. We can immediately exclude enzyme B as being the mitochondrial RNA polymerase. Enzyme B is the major species of yeast RNA polymerases while the mitochondrial enzyme is expected to be a minor fraction. Furthermore, enzyme B is inhibited by α-amanitin in contrast to the mitochondrial RNA polymerase which is known to be resistant to the drug [13–15]. Enzyme B is present in the 150,000 *g* supernatant and is readily solubilized without sonication (fig. 2, panel b). This, together with the α-amanitin sensitivity suggests that enzyme B originates from the nucleoplasm. If the ribosomal supernatant is sonified prior to chromatography (fig. 2, panel c), enzyme C is found in addition to enzyme B. We interpret the fact that sonication is required to solubilize enzyme C as indicating that in untreated extracts this enzyme is strongly bound to some nuclear structure, probably DNA, which is adsorbed irreversibly on the DEAE-Sephadex column. Immunological experiments to be described below (table 2) make it unlikely that enzyme C is derived from enzyme B through sonication. It can

Table 1

Conditions	RNA polymerase A	B	C
Standard assay mixture	100	100	100
plus 2 μg α-amanitin	94	22	98
minus denatured DNA plus native calf thymus DNA	43	10	61
minus denatured calf thymus DNA plus native yeast DNA	11	3	51

Properties of the RNA polymerase activies A, B and C from the DEAE-Sephadex column. 25 μl of the combined fractions of each peak were used for activity measurements. Results are expressed as percent of the activity obtained with heat denatured calf thymus DNA. (100% equals, in pmoles UMP incorporated/15 min at 35°/25 μl sample; 6.7 for enzyme A, 34 for enzyme B, 8.2 for enzyme C).

Table 2

Enzyme	(pmoles UMP incorporated) minus antiserum	plus antiserum	Inhibition (%)
A	20.1	19.2	5
B	35.1	12.1	66
C	27.2	27.6	0

Influence of antiserum against purified polymerase B on the activity of the three RNA polymerases. Enzymes (7–12 μg protein) were preincubated in the absence of triphosphates and DNA with 10 μl of rabbit antiserum prepared against purified polymerase B in the buffer solution used for the assay of RNA polymerase. After 5 min at 35° triphosphates and DNA were added and RNA polymerase activity measured as described in Materials and methods. Controls were treated identically except that control serum was added in place of antiserum. Control serum did not inhibit the activity of any one of the three enzymes.

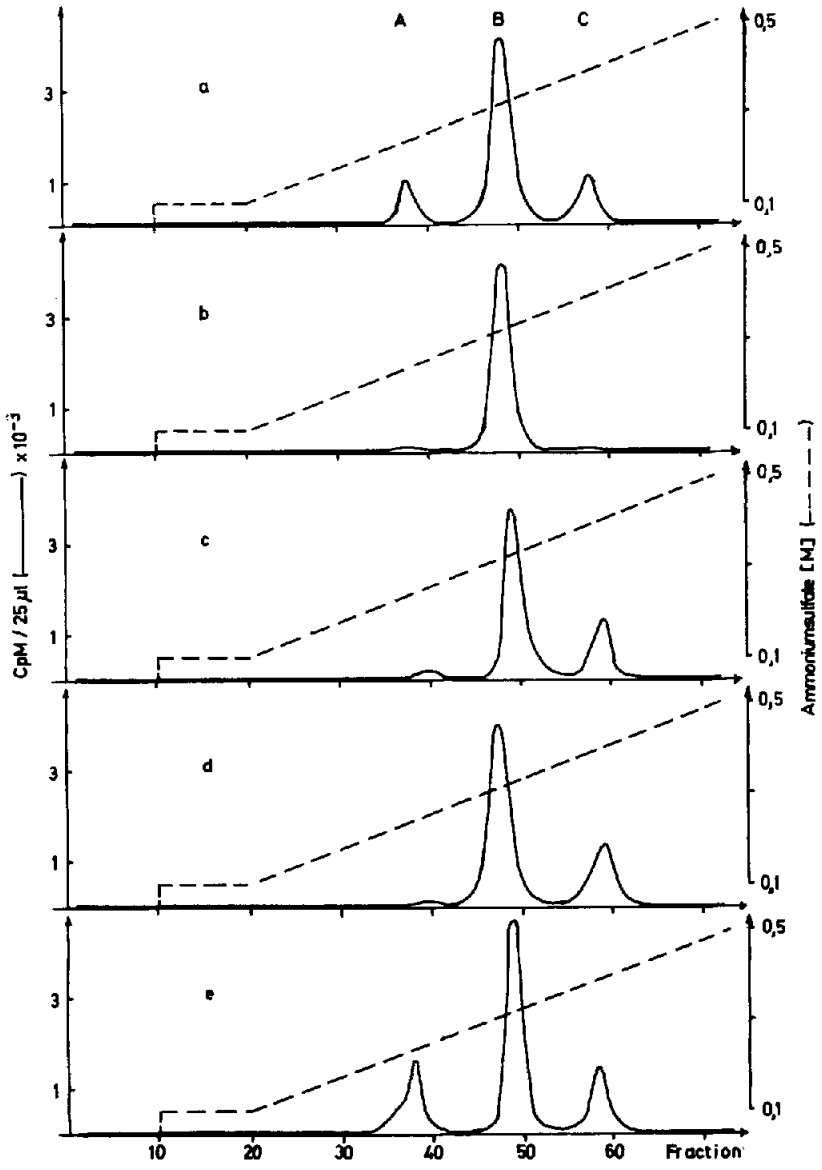

Fig. 2. Chromatography of RNA polymerases from different fractions of the cell homogenate. All experiments were done according to the general scheme described in Materials and methods starting from 5 g of yeast. The modifications are indicated below. Only the profiles of RNA polymerase activity are shown for clarity. Panel a: Control experiment identical to the one shown in fig. 1. Panel b: Sonication was totally omitted. Panel c: Sonication of the homogenate was omitted, the ribosome-free extract was prepared and sonified. Panel d: A ribosome-free extract was prepared by omitting sonication of the homogenate. To this was added a suspension of mitochondria isolated from 15 g of yeast cells and the mixture was sonified in the usual manner. Insoluble material was then removed by centrifugation for 60 min at 150,000 *g*. Panel e: A homogenate of yeast cells was prepared without sonication. This was first centrifuged for 10 min at 1500 *g* to remove nuclear fragments followed by 20 min centrifugation at 45,000 *g* to remove mitochondria. After centrifugation for 60 min at 150,000 *g* a ribosome-free extract was obtained back to which the first pellet containing the nuclear fragments was added. This mixture was then sonified and the insoluble material removed by another centrifugation at 150,000 *g*.

be excluded that enzyme C is of mitochondrial origin because not only is this enzyme present in a mitochondria-free extract, but addition of a large excess of mitochondria to the ribosomal supernatant prior to sonication does not increase the amount of enzyme C (fig. 2, panel d). From this experiment it is furthermore clear that addition of mitochondria does not result in the appearance of enzyme A.

Enzyme A is present in the crude fraction which is obtained by centrifuging the cell homogenate (without prior sonication) at 1500 *g*. This fraction contains nuclear fragments and membranes. If it is collected and added to an extract from which mitochondria and ribosomes have been removed, sonication releases all three enzymes in soluble form (fig. 2, panel e). Removal of mitochondria therefore does not result in the loss of any one of the enzymes. This, together with the results of fig. 2, panel d, conclusively eliminates the possibility that one of the RNA polymerases described here originates from mitochondria. Earlier work had already indicated that mitochondrial RNA polymerase from yeast is difficult to extract and appears to be quite labile [13, 14, 16] which is in agreement with the present results.

Although enzyme B is by far the largest fraction of yeast RNA polymerases, it has the surprising and unexpected property of being stimulated only by denatured DNA which suggests that this enzyme is incomplete and may lack one or more subunits which are required for binding to native DNA. It is therefore possible that enzyme B is actually derived from either enzyme A or enzyme C. We have carried out immunological experiments to test this possibility and have found (table 2) that rabbit antiserum prepared against pure enzyme B [17] has no effect on the activity of either enzyme A or enzyme C although it inhibits enzyme B considerably. This experiment therefore does not support the idea of a structural relationship of enzyme B with the enzymes A and C. A structural relationship between enzyme A and enzyme C can of course not be excluded.

We have purified all three RNA polymerases from yeast in order to characterize these enzymes more fully and to determine their subunit structure [17]. These studies also point to a structural difference between enzyme B and enzymes A and C.

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