CHARACTERISTICS OF DNA-DEPENDENT RNA POLYMERASE ACTIVITY FROM ISOLATED YEAST NUCLEI

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1. Introduction

The biosynthesis of ribosomal RNA (rRNA) in the yeast *Saccharomyces carlsbergensis* has been extensively studied in our laboratory [1–3]. It is a complex process starting with the production of a high molecular weight precursor RNA with a sedimentation coefficient of about 42 S, which is converted in a number of steps into the mature rRNA components [2, 3]. How this process of rRNA formation is regulated is still largely unknown. However, the recent discovery of initiation factors needed for a specific transcription in prokaryotes [4, 5], and the demonstration of multiple forms of RNA polymerase activity in various eukaryotes [6–10], suggest that the DNA-dependent RNA polymerase might be an important factor in the regulation of rRNA synthesis. For this reason we decided to isolate and to characterize the RNA polymerase activity from yeast.

A DNA-dependent RNA polymerase has been isolated from a cell-free extract of the yeast *Saccharomyces cerevisiae* by Frederick et al. [11]. However, the enzyme isolated by these authors had a marked preference for a denatured template which suggests that this enzyme is incomplete and might have lost constituents which are required for binding to native DNA. More recently, the existence of multiple forms of RNA polymerase in yeast has been reported [12], but further particulars are still lacking.

In the present report we describe the isolation of 3 distinct RNA polymerase activities from yeast nuclei. The 3 enzymes, of which 2 have been purified to an apparent homogeneous state, differ in template requirement, in metal ion requirement, in sensitivity towards α-amanitin and in sedimentation behaviour.

2. Materials and methods

2.1. *Materials*

^{3}H-UTP was purchased from NEN Chemicals GmbH and DEAE-cellulose DE 32 from Whatman. Bovine pancreatic RNase (type IIIa), bovine pancreatic DNase (type I), calf thymus DNA (type I) and cycloheximide were obtained from Sigma Chemical Company. Yeast DNA was prepared as described previously [13]. α-Amanitin was a generous gift from Prof. Th. Wieland, Heidelberg, and rifampicin from Pharmaceutische Handelsafd. vh. Kerkhof and Co., Amsterdam.

2.2. *Preparation of yeast nuclei*

Nuclei from *Saccharomyces carlsbergensis* strain N.C.Y.C.74) were prepared in essentially the same way as described by Molenaar et al. [14].

2.3. *Assay for RNA polymerase activity*

The standard assay mixture contained in a total volume of 0.5 ml: 40 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 1.5 mM $MnCl_2$, 0.2 mM dithiothreitol, 150 mM KCl, 0.2 mM each of ATP, CTP and GTP, 0.04 mM ^{3}H-UTP (specific activity 0.1 Ci/mmole) 50 μg native calf thymus or yeast DNA, and enzyme preparation.

Incubation time was 30 min at 30°. The reaction was stopped by the addition of 1 ml cold 10% (w/v) trichloroacetic acid (TCA), containing 0.04 M $Na_4P_2O_7$. 100 μg bovine serum albumin was added as a carrier. After standing for 10 min at 0°, the acid-insoluble precipitates were collected on Whatman GF/C filters and washed 4 times with 10 ml cold 5%

(w/v) TCA, containing 0.04 M $Na_4P_2O_7$. The filters were dried, treated with 0.5 ml Nuclear Chicago Solubiliser and subsequently counted in a toluene scintillation liquid containing PPO (4 mg/ml) and POPOP (0.05 mg/ml)

One unit of enzyme activity corresponds to one pmole of ^{3}H-UMP incorporated in 30 min under the assay conditions.

3. Results and discussion

3.1. *Solubilization and separation of yeast nuclear RNA polymerase activities*

Nuclei obtained from about 2×10^{10} yeast protoplasts were lysed in 16 ml of 0.05 M Tris-HCl (pH 7.9), containing 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM glutathion, 0.1 mM dithiothreitol and 5% (v/v) glycerol (TMEG buffer) by gentle stirring for 15 min at 4° (fraction I in table 1). After centrifugation at 18,000 *g* for 10 min at 4°, the supernatant (I) was kept, while the pellet was resuspended in 10 ml of TMEG buffer, but now containing 0.5 M KCl. The suspension was sonicated at full power with a Branson medium probe for 15 sec, and centrifuged at 30,000 *g* for 30 min at 4°. The supernatant obtained was combined with supernatant I (fraction II in table 1).

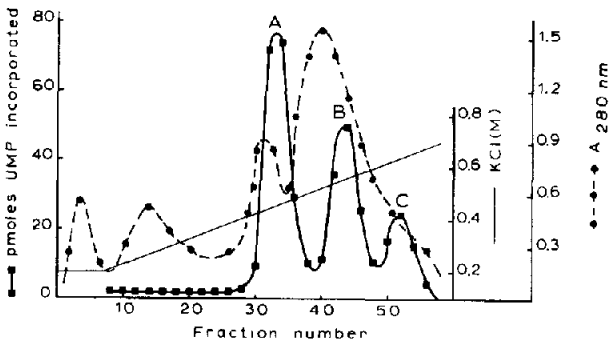

Fig. 1. DEAE-cellulose chromatography of RNA polymerase activity solubilized from yeast nuclei. Enzyme extracts from yeast nuclei were prepared as described in the text. The DEAE-cellulose column (2 × 18 cm) was loaded with 60–70 mg of protein, and a linear gradient of 0.2 M–0.7 M KCl in TMEG buffer (25%, v/v, glycerol) was applied. Fractions of 1.4 ml were collected at a flow rate of 10 ml/hr, and 0.1 ml aliquots of each fraction assayed for RNA polymerase activity using native calf thymus DNA as template (for the assay conditions see Materials and methods).

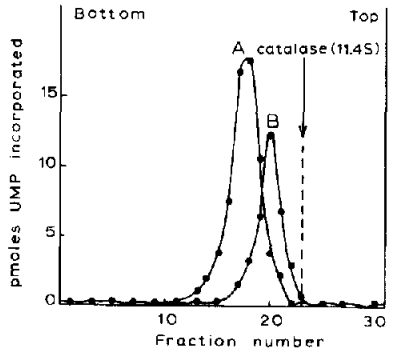

Fig. 2. Glycerol gradient centrifugation of RNA polymerases A and B. Polymerase A (about 900 units of fraction III A in table 1) and polymerase B (about 450 units of fraction III B in table 1) were separately centrifuged in a 15–30% glycerol gradient as described in sect. 3.2. In both cases catalase was used as a marker. For a better comparison the 2 sedimentation profiles are presented in one figure.

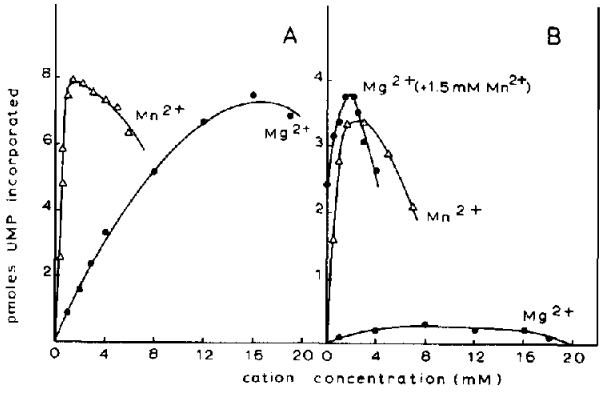

Fig. 3. Effect of magnesium and manganese ion concentration on the activity of RNA polymerases A and B. The enzymatic activities were assayed with native calf thymus DNA as a template as described in the Methods section, except for the metal ion concentration. (A) RNA polymerase A (8 units per assay). (B) RNA polymerase B (4 units per assay).

The combined supernatants (25 ml) were diluted with an equal volume of TMEG buffer containing 45% (v/v) glycerol, and then applied to a DEAE-cellulose column (2 × 18 cm), previously equilibrated with TMEG buffer containing 25% (v/v) glycerol. The column was washed with 10 ml of TMEG buffer (25%, v/v, glycerol) containing 0.2 M KCl, after which the polymerase activity was eluted by 70 ml of a linear

Table 1
Purification of the RNA polymerase activities from yeast nuclei.

Fraction	Activity (units)[a]	Protein (mg)[b]	Specific activity (units/mg protein)
I Nuclear lysate	3,755 (−amanitin)	126.1	29.8
	1,407 (+amanitin)		11.2
II Combined supernatants	4,508	74.7	60.3
III DEAE-cellulose chromatography			
RNA polymerase A	4,949	12.10	409
RNA polymerase B	3,329	12.04	276
RNA polymerase C	1,658	5.61	296
IV Glycerol gradient centrifugation			
RNA polymerase A	2,678	0.61	4390
RNA polymerase B	2,574	1.05	2451

[a] The activity in the nuclear lysate was measured in the absence and in the presence of α-amanitin (40 μg/ml) by incubation at 30° for 10 min in the reaction mixture described in Materials and methods, except that (for maximal activity) ammonium sulphate (200 mM) was added. All other fractions were assayed for activity as described in Materials and methods.
[b] Protein was determined according to Lowry et al. [15] using lysozyme as a standard.

gradient of 0.2–0.7 M KCl in TMEG buffer (25%, v/v, glycerol).

Fig. 1 shows that by DEAE-cellulose chromatography the RNA polymerase activity from yeast nuclei is resolved into 3 peaks which have been designated polymerases A, B and C. The first peak, polymerase A, is always the most prominent one if measured with a native template, and the yield of it is very reproducible. However, the relative amounts of polymerases B and C were found to vary with the nuclei preparation used for extraction, and sometimes polymerase C was even lacking. Since the yeast cells used for the preparation of nuclei were grown and harvested under carefully controlled standard conditions, we attribute this variability to a difference in quality of the nuclei preparations obtained. We tentatively assume that polymerases B and C are less firmly bound to the nuclear structure and may leak out in varying amounts during the isolation of the nuclei. The activity ratio of the polymerases A, B and C in the eluate, as measured with a native template, was at best 3:2:1 (table 1). The possibility that enzymes B and C are actually derived in varying amounts from enzyme A during the chromatography could be excluded. The chromatographic properties of each of the 3 polymerase activities appear to be maintained during concentration and subsequent rechromatography under identical conditions. This result makes it unlikely that the 3 activity peaks are dissociable complexes of a single RNA polymerase, or that an interconversion between the various peaks occurs.

It should be noted (see table 1) that the total polymerase activity found in the eluate after DEAE-cellulose chromatography (fraction III) is more than twice the activity present in the combined nuclear extracts. Apparently, the polymerases are separated from inhibiting factors by the chromatographic procedure. The nature of these inhibiting factors has not been elucidated yet.

3.2. *Further purification of RNA polymerases A and B*

The combined chromatographic fractions containing RNA polymerase A, and B, respectively, were diluted with TMEG buffer (25%, v/v, glycerol) to a KCl concentration of 0.1 M, and adsorbed on 1 × 4 cm columns of DEAE-cellulose, which were equilibrated with TMEG buffer (25%, v/v, glycerol). The polymerases A and B were eluted with TMEG buffer (25%, v/v, glycerol) containing 0.6 M KCl and 0.8 M KCl, respectively, in small volumes (about 1 ml). The enzyme solutions were dialyzed for several hr against 1000 ml of 0.05 M Tris-HCl (pH 7.9) containing 10% (v/v) glycerol, 0.1 mM EDTA, 0.5 mM glutathion, 0.5 mM dithiothreitol and 0.15 M KCl.

Table 2
The effect of the DNA template used on the activity of yeast RNA polymerases A, B and C.

DNA used	Activity (%) Polymerase A	Polymerase B	Polymerase C
Native calf thymus DNA	100	100	100
No DNA added	6	5	5
Denatured calf thymus DNA	67	450	160
Native yeast DNA	45	40	65
Denatured yeast DNA	29	192	110

The enzymatic activity was assayed as described in Materials and methods. Results are expressed as percent of the activity obtained with native calf thymus DNA, being 7 units for polymerase A, 4 units for polymerase B and 2 units for polymerase C. Denatured DNA was obtained by heating DNA at 100° for 10 min, followed by rapid cooling in ice.

The dialyzed enzyme solutions were layered on 30 ml of 15–30% (v/v) glycerol gradients in 0.05 M Tris-HCl (pH 7.9) containing 0.1 mM EDTA, 0.5 mM glutathion, 0.5 mM dithiothreitol and 0.15 M KCl. In some runs 0.1 ml of a catalase solution (1 mg/ml) in the same buffer but with 10% glycerol was also layered on the gradient. The gradients were centrifuged at 25,000 rpm in a Spinco SW-25.1 rotor for 20 hr at 4°. Fractions of 1.0 ml were collected of which 0.1 ml was used for the assay of RNA polymerase activity.

RNA polymerase A appeared to have a distinct higher sedimentation rate than RNA polymerase B (fig. 2). Using catalase (11.4 S) as a marker the sedimentation value of polymerases A and B were found to be 18 S and 16 S, respectively. The constant specific activity found throughout the 2 peaks suggested that at this stage the 2 enzyme preparations are quite homogeneous. This was also indicated by preliminary electrophoretic analysis on 5% polyacrylamide gels at pH 8.9. Compared with the initial activity of the 2 enzymes in the nuclear lysate (see table 1), the whole purification procedure resulted in a roughly 400-fold purified preparation of enzyme A and a roughly 200-fold purified preparation of enzyme B. Both purified enzymes were rather labile; the activities were completely lost within a week, even if the enzymes were stored in a 50% (v/v) glycerol solution at 0°. Polymerase C (fraction III C in table 1) was still more labile, and has not yet been purified further.

3.3. *Characteristics of the yeast RNA polymerases A, B and C*

For a study of the enzymatic properties of the 3 polymerases, the enzyme preparations IV A, IV B and III C, respectively, have been used (cf. table 1). The 3 RNA polymerases required the presence of all 4 ribonucleoside triphosphates for activity; the ^{3}H-UMP incorporation was linear with time for 30 min at 30° in all cases.

As shown in table 2, the activity of all polymerases is highly dependent on added DNA. Native DNA was found to be a better template for RNA polymerase A than heat-denatured DNA. On the contrary, the polymerases B and C exhibit the highest activity with a denatured template. With both kinds of denatured DNA the activities of polymerases B and C are about 4.5 and 1.6 times, respectively, higher than with native DNA. It is noteworthy that all 3 enzymes show a lower activity with the homologous template than with the heterologous calf thymus DNA. This might be due to a larger number of false initiation sites present on the heterologous template.

Fig. 3 shows the requirements for divalent cations of the polymerases A and B. Both enzymes are fully dependent on the presence of Mn^{2+} ions, with an optimal concentration of about 1.5 mM Mn^{2+} for polymerase A, and about 2.5 mM Mn^{2+} for polymerase B. Only in the case of RNA polymerase A could Mg^{2+} – but at a much higher concentration (16 mM) – be a substitute for Mn^{2+} to obtain a comparable activity level. However, in the presence of 1.5 mM Mn^{2+} addi-

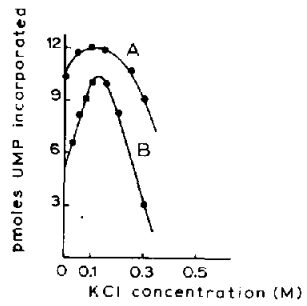

Fig. 4. Effect of KCl concentration on the activity of RNA polymerases A and B. The activities were assayed with native calf thymus DNA as described in the Materials and methods section except that the KCl concentration present was varied. Curve A: polymerase A (12 units per assay); Curve B: polymerase B (10 units per assay).

tion of 1.0 mM Mg^{2+} caused an increase of 15% in the activity of polymerase B (fig. 3B). Such an effect was not found for RNA polymerase A. Therefore, the combination of Mn^{2+} (1.5 mM) plus Mg^{2+} (1.0 mM) was routinely used in the enzymatic assays. The Mn^{2+}/Mg^{2+} activity ratios (at the optimal concentration of each ion) for RNA polymerases A and B are approximately 1 and 13, respectively. These results are analogous to the results of Roeder and Rutter [16] obtained with the RNA polymerases I and II from rat liver and from sea urchin.

The activities of polymerases A and B are both markedly influenced by the ionic strength. Fig. 4 shows the effect of various concentrations of KCl on the activity of the 2 enzymes with native DNA as a template. For both enzymes the optimal KCl concentration is about 0.15 M.

As shown in table 3, polymerase B is the only one sensitive towards α-amanitin, resulting in an inhibition of the activity for nearly 90%. None of the enzymes is affected by the antibiotics rifampicin and cycloheximide at concentrations up to 5 μg/ml and 200 μg/ml, respectively. The effect of cycloheximide on the polymerase activities was investigated, because it has been reported [17] that this antibiotic specifically inhibits the RNA polymerase I (A) from *Blastocladiella emersonii*, and is without effect on RNA polymerases II (B) and III (C). In the case of the yeast polymerases, however, cycloheximide does not have any effect on the activity.

The properties of RNA polymerase B show a striking resemblance with the enzyme isolated from yeast cell homogenate by Frederick et al. [11], as for instance the pronounced preference for denatured DNA as a template, and the high Mn^{2+}/Mg^{2+} activity ratio. In addition it has been reported [18] that the yeast RNA polymerase prepared by the method of Frederick et al. is also strongly inhibited (about 95%) by α-amanitin (20 μg/ml). Whether RNA polymerase B is a deficient enzyme which has lost one or more essential constituents during the isolation, or is only *in vitro* unable to transcribe double stranded templates efficiently, is difficult to say. Anyway, our results indicate that polymerase B is distinctly different from

Table 3
Influence of nucleases and antibiotics on the activity of yeast RNA polymerases A, B and C.

DNA template	Additions to reaction system	Activity (%) Polymerase A	Polymerase B	Polymerase C
Native yeast DNA	–	100	100	100
Native yeast DNA	+ DNase (20 μg/ml)	1	2	–
Native yeast DNA	+ RNase (20 μg/ml)	15	–	–
Native yeast DNA	+ Rifampicin (5 μg/ml)	98	112	104
Native yeast DNA	+ α-Amanitin (40 μg/ml)	99	12	98
Native yeast DNA	+ Cycloheximide (200 μg/ml)	111	113	108
Denatured yeast DNA	–	65	470	–
Denatured yeast DNA	+ α-Amanitin (40 μg/ml)	65	71	–

The enzymatic activity was assayed as described in Materials and methods. Results are expressed as percent of the activity obtained with native yeast DNA, being 6 units for polymerase A, 4 units for polymerase B and 3 units for polymerase C. Denatured DNA was obtained by heating DNA at 100° for 10 min, followed by rapid cooling in ice.

the other two polymerases, and it is unlikely that enzyme B is derived from one of the two other enzymes.

Since yeast RNA polymerase A has many features in common with the RNA polymerase activity found in the nucleolus of other eukaryotes, such as its chromatographic behaviour on DEAE-cellulose [16], its response to Mn^{2+} and Mg^{2+} [16] and its insensitivity to α-amanitin [9, 19], we tentatively assume that RNA polymerase A is concerned in the synthesis of rRNA. It was recently shown by Chesterton and Butterworth [20] that RNA polymerase A from rat liver can be separated into 2 different species. Whether the same holds true for yeast RNA polymerase A is now under study as well as the subunit structure of the enzymes isolated.

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