ROLE OF DNA–RNA HYBRIDS IN EUKARYOTS
1. PURIFICATION OF YEAST RNA POLYMERASE B

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1. Introduction

Multiple forms of RNA polymerase were described in eukaryots on the basis of differential elution on DEAE cellulose column, antibiotic sensitivity and catalytic properties [1, 2]. The 2 main species of nuclear RNA polymerases called A (or I) and B (or II) differ in template requirement since B enzyme is very active on denatured DNA but uses poorly native templates [3]. As the structure of DNA template in chromatin remains an open question it is not clear whether the low affinity of enzyme B for native DNA reflects the loss of some regulatory protein(s) during the course of the purification or is a real property of the native enzyme. To answer this question we thought that more should be known about RNA polymerase B. Resolution of multiple species of yeast RNA polymerase has already been mentioned [4, 5]. We report here on the rapid and large-scale purification of yeast RNA polymerase B and summarize some of its physical and biochemical properties. A fuller account will be given elsewhere [6]. Yeast RNA polymerase B obtained quasi-homogeneous on polyacrylamide gel electrophoresis has a molecular weight of about 440,000 daltons and is made up of the association of 2 large subunits in stoichiometric amounts with possibly 3 small polypeptide chains.

2. Materials

Yeast cells were either from commercial source (Fould Springer, baker's yeast) or haploid strain 4094 $B\alpha$ Ad_2 Ur_1 of *S. cerevisiae* grown in a medium containing 1% Bactopeptone, 1% yeast extract, 1.5% glucose, 0.002% adenine (w/v) to a density of 5.8×10^6 cells/ml. No difference was found as far as RNA polymerase B is concerned with both type of cells. *E. coli* core RNA polymerase and σ were purified as previously described [7]. T_7 and T_4 phage DNA were prepared by J.P. Dausse and J.M. Buhler in our laboratory. All DNA were denatured at Ph 12.5 for 10 min at room temp.

3. Results and discussion

3.1. *Resolution of yeast RNA polymerases A and B*

All operations were carried out at 0–4°. Yeast cells (300 g) were washed in buffer I (0.02 M Tris-HCl pH 8, 0.01 M β-mercaptoethanol, 0.001 M EDTA, 0.001 M $MgCl_2$, 0.3 M ammonium sulfate, 0.001 M phenylmethylsulfonyl fluoride, 10% glycerol). The cells suspended in 300 ml of buffer I were passed twice at 9,000 psi in a Manton Gaulin homogenizer, and the homogenate was adjusted to pH 8 with 1 M Tris. After 2 successive centrifugations at low speed (30 min at 8,000 rpm in a GSA rotor of the Sorvall RCII B) and high speed (1 hr at 100,000 *g*) the supernatant was diluted to an absorbance of 25 at 280 nm. 250 ml of 1% protamine sulfate was slowly added per liter of supernatant and after 30 min at 0°, the precipitate was discarded. Ammonium sulfate fractionation was carried out by adding successively 15.3 g and 17.5 g of solid salt per 100 ml of the respective supernatants. The second pellet was dissolved in a

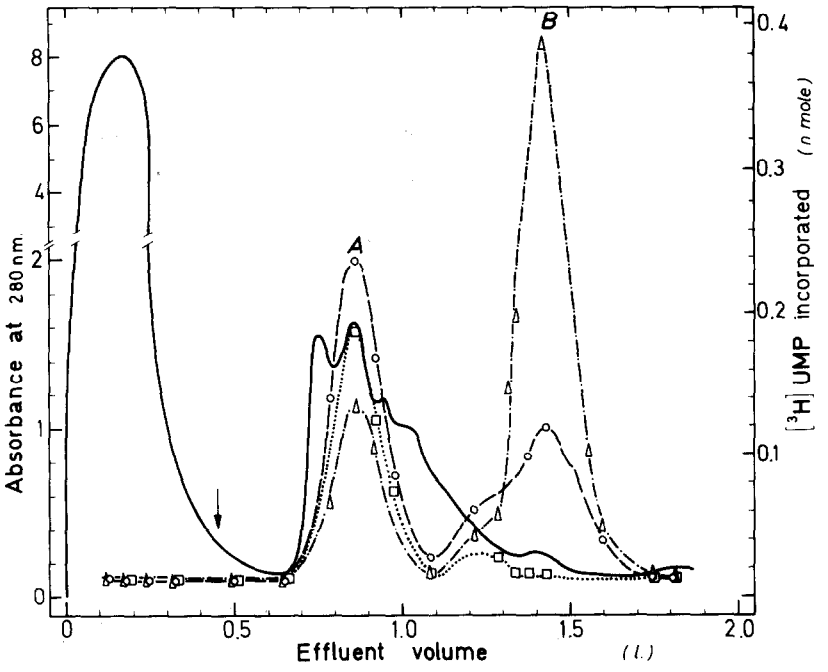


Fig. 1. Resolution of RNA polymerases A and B on DEAE-cellulose column. Chromatography was carried out as described in the text. Assay of RNA polymerase B: 20 μl of each fraction was added to reaction mixtures (0.25 ml) containing 70 mM Tris-HCl pH 8, 13 mM β-mercaptoethanol, 6 mM $MnCl_2$, 1 mM each of CTP, ATP, GTP, 0.2 mM ^{3}H-UTP (8,500 counts/min/nmole), 50 mM ammonium sulfate and 10 μg of denatured calf thymus DNA. After 15 min incubation at 37°, RNA was recovered by acid precipitation and radioactivity counted in a scintillation counter: (△—△—△). RNA polymerase A was assayed in the above conditions with 10 μg native calf thymus DNA, 10 mM ammonium sulfate and 4 mM $MgCl_2$ together with 6 mM $MnCl_2$: (○—○—○). α-amanitin (40 μg/0.25 ml) was added only in reactions mixtures giving optimal activity (□—□—□). Absorbance at 280 nm: (——).

small volume of buffer II (0.02 M Tris pH 8.4, 0.01 M β-mercaptoethanol, 0.001 M EDTA) and desalted rapidly on a Sephadex G-50 coarse column (5 × 75 cm) equilibrated in the same buffer. Protein containing fractions were made 0.1 M in ammonium sulfate and any precipitate was discarded by centrifugation. The supernatant (250 ml with an absorbance of 20 at 280 nm) was applied to a DEAE-cellulose column (16 cm^2 × 15 cm) equilibrated in buffer II with 0.1 M ammonium sulfate. Elution was carried out at 180 ml/hr with a linear gradient from 0.1–0.5 M ammonium sulfate in buffer II (total vol 1,500 ml). The procedure described allowed the resolution of enzymatic activity on DEAE-cellulose in 2 main fractions, A and B, eluted at 0.12 M and 0.28 M ammonium sulfate, respectively. Fraction B was more active on denatured DNA and totally sensitive to α-amanitin at 160 μg/ml, while fraction A was preferentially active on native calf thymus DNA and was only slightly inhibited by the toxic peptide at the same concentration (fig. 1). Fractions containing a high specific activity of α-amanitin sensitive RNA polymerase B were precipitated with solid ammonium sulfate (35 g per 100 ml). At this stage RNA polymerase B was purified 300-fold and its specific activity was about 500 U/mg, one unit corresponding to an incorporation of 1 nmole ^{3}H-UMP/hr under the conditions described in fig. 1. It should be noted that yeast RNA polymerase purified according to Frederick et al. [8] proved to be completely sensitive to α-amanitin [9] and thus corresponded to RNA polymerase B. An interesting observation was made at this stage: when cells were grown to stationary phase enzyme A activity was practically absent on DEAE-cellulose while enzyme B activity was normal (result not shown). This suggests that enzyme

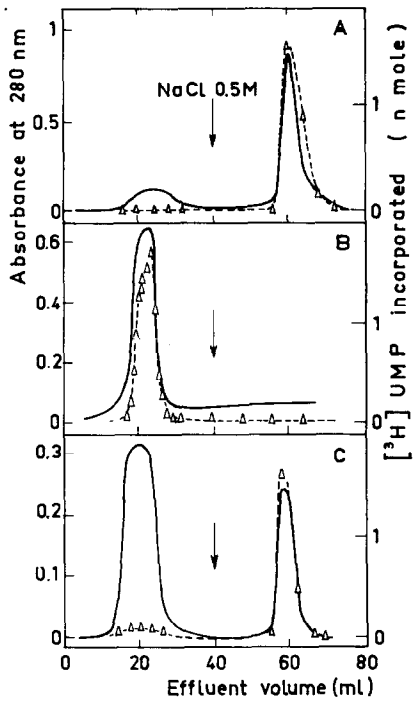


Fig. 2. DNA cellulose chromatography of RNA polymerase B. Calf thymus DNA cellulose column (0.9 × 12 cm) equilibrated with buffer II containing 0.05 M NaCl and 10% glycerol were prepared and run as described by Alberts et al. [10]. Elution was carried out at 15 ml/hr with 15 ml of the above buffer, then with buffer II containing 0.5 M NaCl and 10% glycerol. A) *E. coli* RNA polymerase holoenzyme (2 mg protein) was bound to a native DNA cellulose column and assayed as already described [7] with native calf thymus DNA as template. B) Yeast RNA polymerase B (3.6 mg of glycerol gradient enzyme) was loaded on native DNA cellulose. 5 μl aliquots of each fraction were assayed as in fig. 1 with 0.01 M dithiothreitol. C) Yeast RNA polymerase B (5 mg glycerol gradient preparation) was loaded on a denatured DNA cellulose column, eluted and assayed as above on 10 μl aliquots. Absorbance at 280 nm: (——); RNA synthesis: (△ - - △ - - △).

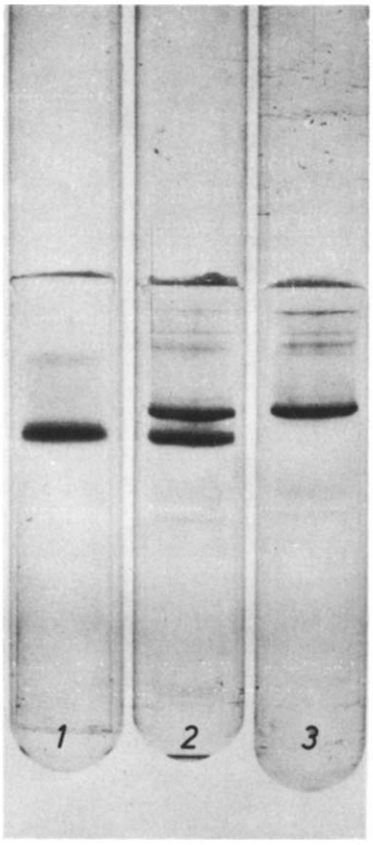
A activity, which is believed to be involved in ribosomal RNA synthesis, could be subject to regulatory control.

Further purification of yeast polymerase B was obtained by zone sedimentation at low ionic strength in a glycerol gradient. After this purification step 2 bands were still seen on polyacrylamide gel electrophoresis. Final purification was achieved by DNA cellulose chromatography. Fig. 2 shows that the enzyme was retained onto a denatured DNA cellulose column and could be eluted by 0.5 M NaCl. However, by contrast with *E. coli* RNA polymerase [10] yeast RNA polymerase B did not bind to native DNA cellulose. This result is in keeping with the observations of Frederick et al. [8]. Alternatively glycerol gradient enzyme was purified by phosphocellulose chromatography where a single peak of activity was observed [6]. The A_{280}/A_{260} ratio of the purified enzyme was 1.6–1.7 indicating a very low contamination by nucleic acids.

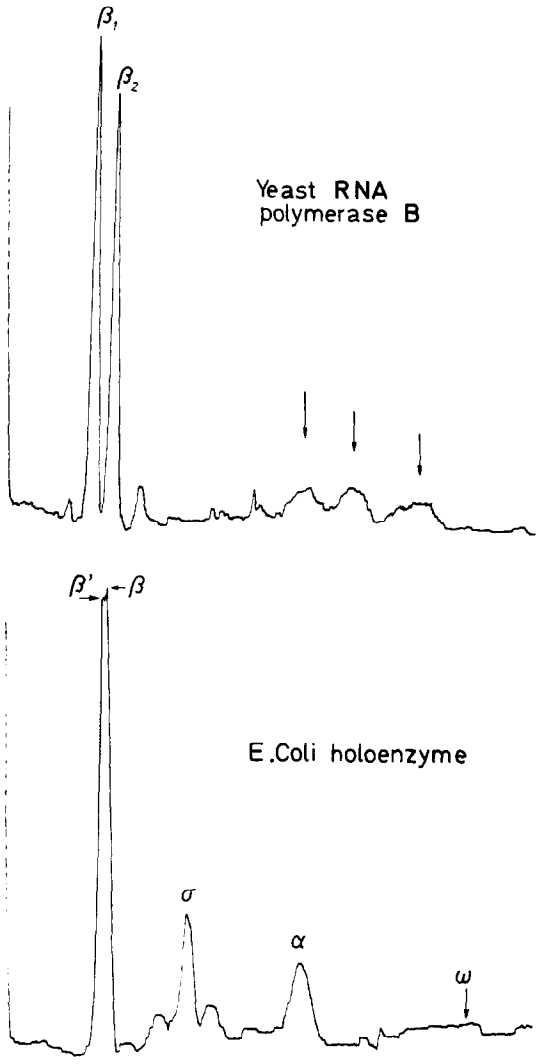
3.2. *Properties of RNA polymerase B*

At this stage of purification enzymatic activities of 7000 U/mg were obtained using denatured calf thymus DNA as template; native calf thymus DNA was also active but much less so. While the enzyme transcribed efficiently denatured T_7 and T_4 phage DNA it was 10 times less active on the native templates. No significant difference could be observed between the glycerol gradient and the DNA cellulose enzyme. Both enzymatic preparations had a similar behaviour on T_4 DNA progressively denatured at increasing temperatures since in both cases total denaturation of the DNA was required for transcription. A drop in template activity of T_4 DNA when heated above 90° was constantly observed with our best enzyme preparations. When template activities of synthetic polydeoxyribonucleotides were compared, poly (dC) proved to be a 5-fold better template than poly (dT) while poly (dA) and poly (dG) were almost inactive. This observation should be related to the preferential initiation of RNA chains by GTP [8, 9]. RNA polymerase B may then normally initiate transcription on unpaired (dC) clusters.

Purified RNA polymerase B migrated as a main protein band on 5% polyacrylamide gel electrophoresis (fig. 3A) and was estimated more than 90% pure. By cutting the gel and testing the different fractions, enzymatic activity was found at the level of the band. The enzyme migrated faster than *E. coli* holoenzyme and the 2 enzymes did not interact detectably during electrophoresis. Analysis of the enzyme using SDS acrylamide gels revealed 2 large polypeptide chains of 180,000 and 150,000 M.W. (fig. 3B). There were also 3 lower molecular weight proteins in current enzyme preparations which might be part of the enzyme. Using *E. coli* RNA polymerase subunits as



A



B

Fig. 3. Analysis of purified RNA polymerase B using 5% polyacrylamide gel electrophoresis. A) Native yeast RNA polymerase B (3.2 μg) was analysed either with or without *E. coli* holoenzyme (5.5 μg) as previously described [7]: 1, yeast enzyme; 2, yeast enzyme + *E. coli* holoenzyme; 3, *E. coli* holoenzyme. B) Analysis of yeast RNA polymerase B in 0.1% SDS gels [11]: yeast RNA polymerase (6.4 μg); *E. coli* holoenzyme (7 μg). Scanning of the gel was carried out at 550 nm with the Joyce and Loebl Chromoscan. Corresponding migration of ω not visible here was indicated in the figure.

standard their molecular weight was estimated as 39,000, 28,000 and 19,000 (± 10%). Scanning of the gel showed that the 2 large subunits (called β_1 and β_2 by analogy with the bacterial enzyme) were in a 1:1 ratio (fig. 3B); the mean tentative number of small components, estimated from several experiments, relative to β_1 or β_2 was 1 (39,000 M.W.), 2 (28,000 M.W.) and 2 (19,000 M.W.). However, the precision of these figures was low due to band diffusion and uncertainty in molecular weight determination. The other proteic bands, much less abundant, could well be minor contaminants.

Using electrophoresis on polyacrylamide gels of graded porosity the molecular weight of *E. coli*

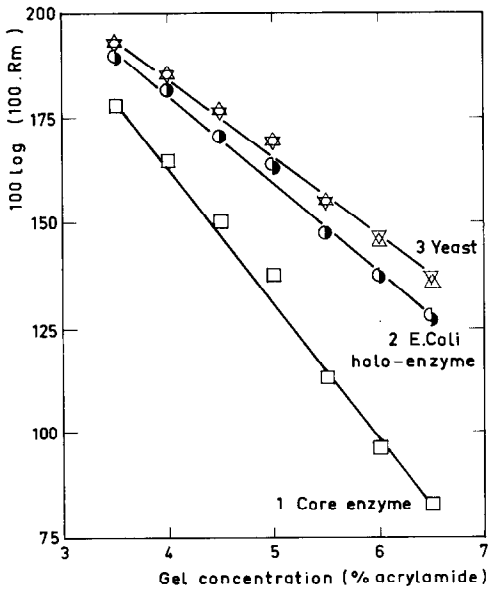


Fig. 4. Electrophoretic mobility of yeast and *E. coli* RNA polymerase. The relative migration of the proteins at different gel concentration was determined using bromophenol blue as tracking dye [13]. Yeast RNA polymerase alone (3.2 μg): △, or with *E. coli* RNA polymerase (5.5 μg): ▽, *E. coli* RNA polymerase alone: ●, or with yeast RNA polymerase: ◐. Dimeric *E. coli* core enzyme: □.

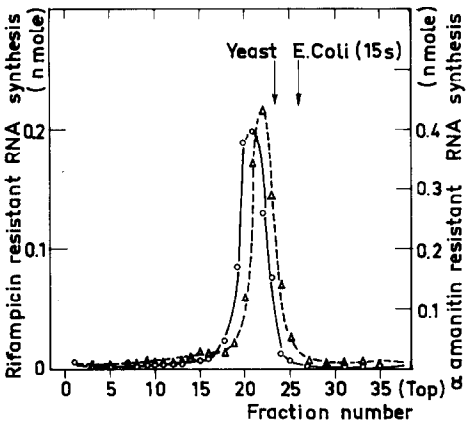


Fig. 5. Sedimentation analysis of a mixture of yeast and *E. coli* RNA polymerase. A mixture (0.1 ml) of 36 μg of purified yeast RNA polymerase and 27 μg of *E. coli* core enzyme saturated with purified σ (on the basis of maximum enzymatic activity on T_4 phage DNA) was layered on a 4.6 ml 5–20% sucrose gradient in buffer II containing 20 mM ammonium sulfate. After centrifugation for 4 hr at 50,000 rpm in a SW 65 Spinco rotor at 3°, 38 fractions of about 125 μl were collected and assayed on 50 μl aliquots, under 2 different conditions: for RNA polymerase B activity as indicated in fig. 1, in the presence of rifampicin (3 μg/ml) and with 2.5 μg of denatured T_4 DNA (○—○—○); for *E. coli* RNA polymerase activity as previously described, in the presence of α-amanitin (30 μg/ml) and with 2.5 μg of native T_4 DNA (△—△—△). The arrows indicate the sedimentation of *E. coli* and yeast RNA polymerase in a parallel gradient containing 0.2 M ammonium sulfate.

RNA polymerase was estimated as 480,000 ± 30,000 [12]. Fig. 4 shows the relative mobility of yeast and *E. coli* RNA polymerase at different gel concentration. Compared to *E. coli* holoenzyme the migration of yeast RNA polymerase was a little less hindered in concentrated gels. This indicated that yeast RNA polymerase B had a slightly smaller molecular weight than bacterial RNA polymerase. Identical results were obtained whether yeast enzyme migrated with or without *E. coli* RNA polymerase. Using this technique the molecular weight of yeast RNA polymerase B was estimated as 440,000 daltons. Thus very likely the enzyme migrated as a protomer. As the 2 large subunits weighted 330,000 daltons, the low molecular weight proteins found on SDS gels probably represented additional subunits. However, when yeast RNA polymerase B was sedimented through a sucrose gradient containing 0.2 M ammonium sulfate together with *E. coli* RNA polymerase holoenzyme the yeast enzyme sedimented faster than the bacterial enzyme in a position corresponding to a sedimentation coefficient of about 17 S (fig. 5). The sedimentation coefficient of yeast RNA polymerase increased to 21 S in 0.02 M ammonium sulfate (fig. 5). Thus, like the bacterial enzyme [14], RNA polymerase B protomers may aggregate at low ionic strength.

Some degree of similarity in physical properties was found between bacterial and yeast RNA polymerase B. Both enzymes were also found to be inhibited by the same rifampicin derivative AF/013 [6]. However, they differed drastically on the basis of rifampicin and α-amanitin sensitivity and template requirement; moreover, antibodies against bacterial core enzyme did not inhibit yeast enzyme B. On the other hand the above results indicated that yeast RNA polymerase B and mammalian RNA poly-

merase B shared similar catalytic properties, antibiotic sensitivity and probably subunit composition [15, 16].

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