PURIFICATION OF RNA POLYMERASE B ACTIVITY FROM RAT LIVER

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Received 28 April 1971

1. Introduction

The discovery of multiple RNA polymerases in animal tissues (for references, see the preceding paper [1]) exhibiting template specificity [2, 3] suggested that these enzymes could play a fundamental role in the regulation of the expression of genetic information in eukaryotic cells. For example, at least some of the enzymes could be specific for a given tissue in order to transcribe the genes which are specifically expressed in that tissue. Such tissue-specificity of RNA polymerases could be achieved either through the existence of different enzymes in different tissues, or through the existence of non-specific enzymes acting in conjunction with tissue-specific factors analogous to the bacterial factors [4]. Alternatively both possibilities could occur.

As a first attempt to tackle these problems we have purified RNA polymerase B activity from rat liver in order to compare its components to those of calf thymus RNA polymerase B activity. The results so far obtained indicate that essentially the same components are present in both tissues, but do not exclude the presence of additional minor components in rat liver.

2. Material and methods

Unless specified all operations were carried out at 0–4° as fast as possible. Between each step the enzyme was stored in liquid nitrogen in the presence of 50% glycerol. The following buffers were used: 50 mM tris-HCl pH 7.9, 0.1 mM dithiothreitol, 0.1 mM EDTA, 10 mM EDTA, 10 mM thioglycerol (MS 0); MS 30 and MS 50 contained in addition 30% and 50% glycerol (v/v). P buffers were the same as MS 30, but potassium phosphate (pH 7.5) was used instead of tris-HCl. P 0.01, P 0.05 and P 0.12 were respectively 0.01, 0.05 and 0.12 M phosphate.

Nuclei were prepared from frozen Wistar rat livers using a modification of the method of Widnell and Tata [5] involving first a centrifugation at low speed in isotonic sucrose and then a centrifugation at high speed in 2.2 M sucrose.

DEAE-cellulose and phosphocellulose were DE-22 and P-11 from Whatman, respectively. Hydroxyapatite was prepared according to Levin [6]. Ammonium sulphate was enzyme grade (Mann).

3. Results

3.1. *Solubilization and separation of rat liver nuclear RNA polymerase activities A and B*

Solubilization and separation of RNA polymerase activities A and B from rat liver nuclei were carried out, with some slight modifications, as previously outlined for calf thymus enzymes [3].

Nuclei from 700 g of rat liver were suspended in 220 ml of MS 30; 50 ml of saturated (4°) ammonium sulphate (AS) were added. The viscous solution was sonicated at 0–5° with a Branson sonifier (model B-12, maximum power) until the viscosity had disappeared (usually 5–7 min). After centrifugation for 15 min at 48,000 *g*, ammonium sulphate was added to the supernatant up to 58% of saturation. The precipitates, collected by centrifugation for 75 min at 137,000 *g*, was dissolved in MS 50 (fraction P58).

Table 1
Purification of RNA polymerase B activity from rat liver.

Step*	Incubation conditions	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg protein)
Nuclear suspension	−AS	440	1,140	6,720	0.17
	+AS		2,970		0.44
P58	−amanitin	102	1,492	2,670	0.56
	+amanitin		700		
PS	−amanitin	460	1,470	1,650	0.88
	+amanitin		641		
P48	−amanitin	70	1,140	1,290	0.90
	+amanitin		450		
DEAE cellulose 0.12 M AS eluate	−amanitin	440	465	405	1.15
	+amanitin		435		
0.30 M AS eluate	−amanitin	160	510	58.5	8.72
	+amanitin		30		
HA eluate		26	390	8.1	48.1
Glycerol gradient	Fractions 4–11	9	240	1.5	160
	Fractions 6–9	4.5	180	0.9	200

Nuclear suspension was incubated at 37° for 10 min in a medium containing (final volume 0.25 ml): 100 mM tris-HCl pH 7.9, 4 mM Mn^{2+}, 1 mM each nucleoside triphosphate. Fractions P58, PS, P48 and DEAE eluates were incubated in the same medium but Mn^{2+} was 3 mM and 50 μg calf thymus DNA and 50 mM ammonium sulphate (AS) were added. Fractions HA and the glycerol gradient fractions were incubated in the same mixture except that AS was 96 mM. The incubations were processed as previously described [1]. One unit of activity corresponds to the incorporation of 1 nmole of $GM^{32}P$ in 10 min at 37°. When indicated ammonium sulphate (400 mM) or α-amanitin (1 μg) were added.

* 1,500 g of rat liver were used.

Table 1 shows that under these conditions half of the maximum activity which could be measured in the initial nuclear suspension was solubilized. This activity is 90% DNA dependent and there was about the same amount of α-amanitin-resistant activity (A activity) and of α-amanitin-inhibited activity (B activity).

In order to eliminate the remaining nucleic acids, fraction P58 was brought to 0.2 M AS by dilution with MS 30 and protamine sulphate (1%) was slowly added (3 ml/100 ml of diluted P58). After centrifugation for 45 min at 137,000 *g* the supernatant (fraction PS), where RNA polymerase activity was completely DNA dependent, was precipitated with 48% saturated ammonium sulphate. The pellet (45 min, 137,000 *g*) was dissolved in MS 50.

Activities A and B were separated by stepwise elution after adsorption of the enzymes on DEAE-cellulose. The enzyme corresponding to 1,500 g of rat liver was diluted with MS 30 to decrease the concentration of AS down to 0.04 M. It was then adsorbed on a DE-22 cellulose column (20 × 4.6 cm). Stepwise elutions were performed with MS 30 containing 0.12, 0.14, 0.16 and 0.30 M AS successively. Most of the enzyme activity was present in two fractions eluted at 0.12 and 0.30 M. As shown in table 1, activities A and B were separated with very little cross-contaminations as judged by the effect of α-amanitin. The two active fractions were precipitated with 50% saturated AS, dissolved in MS 50 and stored in liquid nitrogen where they are stable for months.

3.2. *Further purification of RNA polymerase B*

The DEAE fraction of enzyme B was diluted with P 0.01 to a concentration of protein of about 1 mg/ml, and adsorbed on a 1.8 X 11 cm column of hydroxyapatite which was equilibrated with P 0.01. After washing with P 0.01 and P 0.05, the bulk of the activity was eluted with P 0.12 (fraction HA). After precipitation with ammonium sulphate (50% saturated) and centrifugation (90,000 *g* for 60 min) the pellet was dissolved in a minimal volume of MS 50.

The hydroxyapatite fraction corresponding to 1000 g of rat liver was diluted to a glycerol concentration of 10% (final volume 0.9 ml). Using the three buckets of SW-65 rotor, the enzyme was then centrifuged for 270 min (300,000 *g*) through a 15–30% glycerol density gradient (50 mM tris-HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, 10 mM thioglycerol, 2 mM $MnCl_2$, 50 mM ammonium sulphate). Fractions of 12 drops were collected from each of the 3 tubes. Fractions of each gradient were pooled and glycerol was added to bring the final glycerol concentration to about 50% (final volume 0.75 ml). From the sedimentation profiles of enzyme activity and proteins (fig. 1), it is obvious that there is a peak of protein corresponding to the peak of activity and that, within experimental error, the specific activity of the four major fractions is constant.

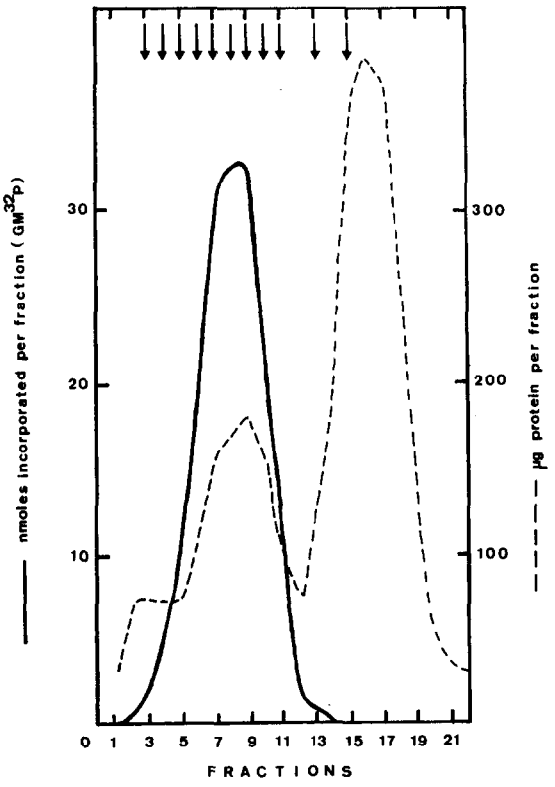

Fig. 1. Glycerol density gradient of hydroxyapatite fraction of rat liver RNA polymerase B activity. The bottom of the tube is at the left. The centrifugation was performed as described in section 3.2.

In some experiments the enzyme after glycerol gradient centrifugation was further purified by phosphocellulose chromatography as described for calf thymus RNA polymerase B activity [3], without any loss of activity when using native calf thymus as a template. This preparation will be referred to as fraction PC.

3.3. *Structural components of RNA polymerase B activity of rat liver*

Results presented in fig. 2 suggest that at least two large polypeptide chains B1 and B3 are related to the enzyme since there is a strong correlation between the intensity of their staining and the enzymatic activity throughout the gradient. These two bands are also found in the PC fraction which is free of the contaminants which are seen at the edges of the peak of activity in the glycerol gradient. Two other bands (B0 and B2) seem also related to the enzyme activity within the glycerol gradient and are also found in the PC fraction. The presence of components B1, B2 and B3 in purified rat liver form B RNA polymerase is also reported by Chesterton and Butterworth [13]. Comparison of the SDS gel patterns (fig. 3) obtained for the rat liver B activity and the calf thymus enzymes BI and BII, indicates that, within experimental error, the molecular weight of the rat liver components B1, B2 and B3 are identical to those of the subunits B1, B2 and B3 of calf thymus enzymes BI and BII and different from those of calf thymus A enzyme (A1 and A2). This observation suggests that the rat liver RNA polymerase B activity contains two enzymes analogous to enzymes BI and BII of calf thymus [1]. Indeed, rat liver enzyme B activity run in non denaturating gels gave three bands, two of which migrated like calf thymus BI and BII enzymes (fig. 4a). It is unlikely that phosphocellulose column chromatography of the rat liver B activity results in the loss of some component of the enzymes since their migration

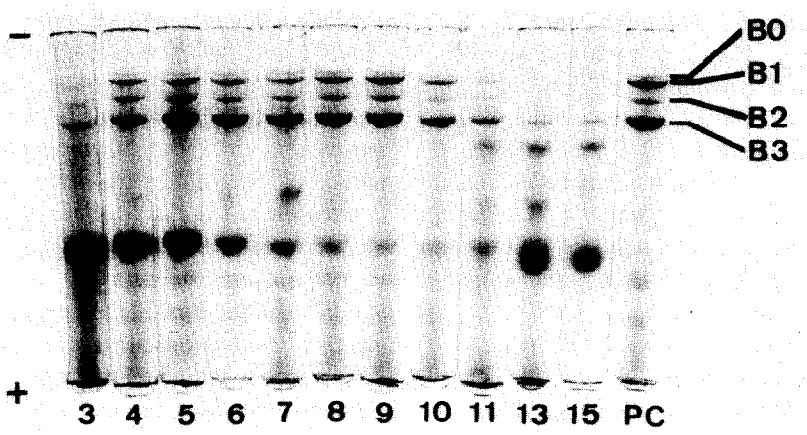

Fig. 2. SDS polyacrylamide gel electrophoresis of glycerol gradient fractions. Aliquots of fractions 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15 (fig. 1) were treated and run on SDS gels as described previously [1]. The protein content of each aliquot was about 6 μg. One gel corresponding to fraction PC (PC, 0.70 unit of activity) is also shown.

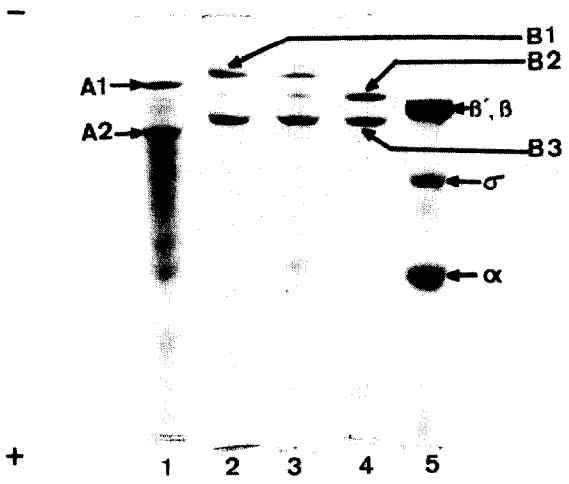

Fig. 3. Comparison by SDS polyacrylamide gel electrophoresis of the components of calf thymus RNA polymerase A (gel 1), BI (gel 2) and BII (gel 4), *E. coli* RNA polymerase (gel 5) and rat liver RNA polymerase B activity (gel 3). Conditions for SDS gel electrophoresis were as described in the legend of fig. 2. Calf thymus enzyme A and *E. coli* RNA polymerase were purified as previously reported [3]. Calf thymus enzyme BI and BII were the DEAE-fractions obtained as described in the preceding paper. The molecular weight of the various components was established by the method of Shapiro et al. [11] taking the subunits of *E. coli* RNA polymerase as markers [12] (β' = 165,000; β = 155,000; σ = 95,000; and α = 39,000). Under these conditions the values were 200,000 and 135,000 for components A1 and A2, 230,000, 215,000, 185,000 and 150,000 for components B0, B1, B2 and B3.

during electrophoresis in non-denaturating gels is not modified by this chromatography. The fact that in rat liver the band corresponding to enzyme BI is much stronger than the band corresponding to enzyme BII fits very well with the hypothesis of two rat liver enzymes analogous to calf thymus BI and BII. Enzyme BI should contain the subunit B1 which, compared to subunit B2, is indeed predominant in rat liver (figs. 2 and 3). The identity of the two components with enzymes BI and BII is confirmed by the migration of labeled amanitin with the two bands (fig. 4b). Moreover when comparing rat liver and calf thymus enzymes, there is a good correlation between the ratios of intensity of staining of the bands and the ratios of label associated with these bands.

Further studies are required to investigate if component B0 (fig. 2) is a contaminant or belongs to a new RNA polymerase which would not exist in activity B purified from calf thymus. In this respect it is interesting to observe that there is some labeled amanitin (fig. 4) associated with the third band which follows enzyme BI during electrophoresis in non-denaturating conditions; this band could correspond to an additional RNA polymerase B activity containing the B0 component.

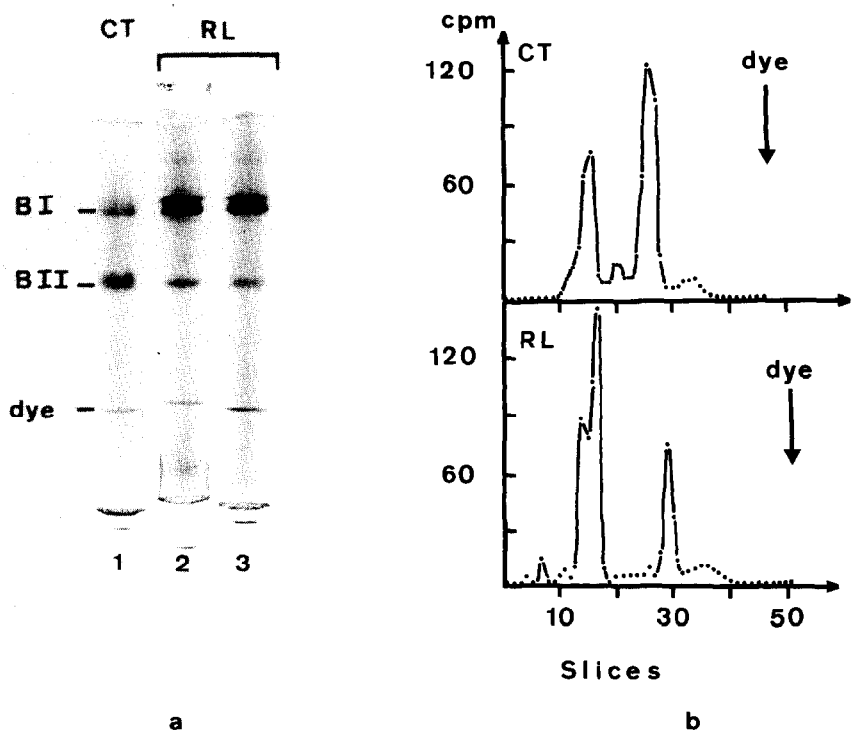

Fig. 4. Comparison by non-denaturating polyacrylamide gel electrophoresis of the components of rat liver RNA polymerase B activity (RL) and RNA polymerases BI and BII of calf thymus (CT). Rat liver enzyme was fraction 9 of the glycerol gradient (gel 2, 1 unit of activity) or PC fraction (gel 3, 1 unit of activity). The mixture of calf thymus enzymes (gel 1) was obtained as described in the preceding paper (fig. 7). Conditions for electrophoresis were as described in the preceding paper. (b) In parallel electrophoresis RL and CT enzyme B activities were run after preincubation in the presence of ^{14}C-methyl γ-amanitin. Two units of enzyme and 2×10^{-2} μg of ^{14}C-methyl γ-amanitin (about 3,000 dpm) were mixed in 0.1 ml MS 30 for 10 min at 0°. The gels were sliced immediately after the electrophoresis and the slices (1 mm width) were counted on a scintillation counter.

4. Discussion

Our purification procedure for rat liver RNA polymerase activity B has three main advantages over the method so far described [7–9]. First, while avoiding the use of a phosphocellulose step which could separate some σ-like factor [10], it nevertheless produces a highly purified enzyme which has a specific activity in the range of the purest animal RNA polymerases so far obtained [1, 3, 8, 9]. Secondly, the yield of enzyme is higher than that previously reported [8]. Finally the purification was specifically designed for the processing of kilograms of tissue in order to obtain the milligrams of RNA polymerase B activity which are required for structural studies.

Structural studies suggest very strongly that RNA polymerase B activity from rat liver results from at least two enzymes, BI and BII, with structures very similar to those of enzymes BI and BII purified from calf thymus [1].

At the present time, although quantitative differences are not excluded, it seems that most of the differences which are observed when comparing the components of enzyme B activity from calf thymus and rat liver are quantitative: the ratio of enzyme BI/enzyme BII is much higher in rat liver than in calf thymus. Whether this difference is a characteristic of the animals (or tissues) or results from selective losses of enzymes during the purification is presently unknown.

Acknowledgements

We are greatly indebted to Prof. T. Wieland for gifts of α-amanitin and ^{14}C-methyl γ-amanitin. We

wish to thank C. Kedinger who carried out the electrophoretical studies in non-denaturating conditions. The technical assistance of Mrs. C. Hauss is gratefully acknowledged. This investigation was supported by grants from the Ligue Française Contre le Cancer and the Délégation Générale à la Recherche Scientifique et Technique.

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