STRUCTURAL EVIDENCE FOR TWO α-AMANITIN SENSITIVE RNA POLYMERASES IN CALF THYMUS

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1. Introduction

We have previously reported that calf thymus contains two RNA polymerase activities: RNA polymerase A which is insensitive to amanitin, and RNA polymerase B which is inhibited by this compound [1]. Enzymes A and B are probably nucleolar and nucleoplasmic respectively. Structural studies of enzyme B indicated the presence of three large subunits B1, B2 and B3 with a MW of about 215,000, 185,000 and 150,000 respectively [2]. We now present results showing that RNA polymerase B is in fact a mixture of two enzymes BI and BII, containing the subunits B1 and B3, and B2 and B3, respectively.

2. Material and methods

Enzyme B was purified from calf thymus as previously described [2] except that the hydroxyapatite fraction was rechromatographed on a phosphocellulose column before the final glycerol gradient. All operations were carried out at 0–4°. Enzyme fractions were stored in liquid nitrogen.

The reaction mixture (0.25 ml) contained 100 mM tris-HCl, pH 7.9, 3 mM Mn^{2+}, 0.5 mM each nucleoside triphosphate, 4 mM thioglycerol, 96 mM ammonium sulphate and 50–60 μg calf thymus DNA. Incubations were processed as previously described [1]. One unit of activity corresponds to one nmole $GM^{32}P$ incorporated in 10 min at 37°. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecylsulphate (SDS) was carried out as described by Maizel [3] (5% gels). Prior to the run, the samples were incubated for 30 min at 37° in the presence of 1% 2-mercaptoethanol, 1% SDS, 0.01 M sodium phosphate pH 7.1 and 30% glycerol (final volume 100–250 μl). Incubations at 90° for 3 min in the presence of 3% 2-mercaptoethanol gave the same results. Electrophoresis was at 2.5 mA per tube for 5–6 hr. Polyacrylamide gel electrophoresis in non-denaturating conditions was carried out according to Krakow [4] (4% gels). In both cases, staining with Coomassie blue and destaining were as described by Burgess [5]. The standard buffer (MS 30) for DEAE chromatography contained: 50 mM tris-HCl pH 7.9, 10 mM thioglycerol, 0.1 mM dithiothreitol, 0.1 mM EDTA, 30% glycerol (v/v). DEAE cellulose was DE-52 from Whatman.

3. Results

As previously described [2], the last step of purification of enzyme B involved centrifugation through a glycerol gradient (fig. 1). The constant specific activity throughout the peak suggested that at this step the enzyme preparation was quite homogeneous. Aliquots of the fractions of the peak were subjected to polyacrylamide gel electrophoresis in SDS. Three major bands, B1, B2, and B3, were seen (fig. 2). As already reported [2], these bands are probably related to enzyme B since their intensity correlates well with the enzyme activity. In previous studies [2], densitometry of the gels has suggested a ratio of 1:1:2 between subunits B1, B2, B3. Subsequently we found that the ratio between B1 and B2 could change from one preparation to another, but that the sum of B1 + B2 was always close to B3. Rechromatography of this

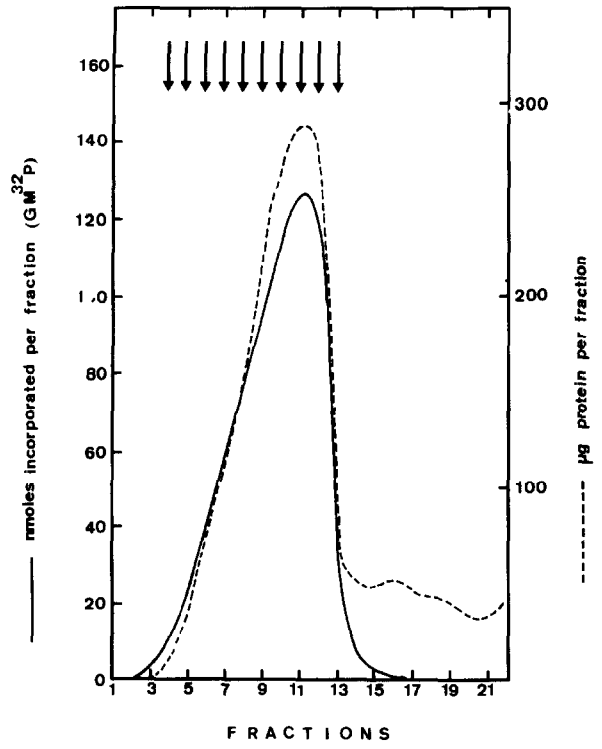

Fig. 1. Sedimentation or purified enzyme B in a glycerol density gradient. 1100 units of enzyme B purified as described in Material and methods (hydroxyapatite fraction rechromatographed on phosphocellulose) were layered on 4.3 ml of a 15 to 30% glycerol gradient containing 50 mM tris-HCl pH 7.9, 80 mM ammonium sulphate, 0.1 mM EDTA, 10 mM thioglycerol and 0.1 mM dithiothreitol. The gradient was centrifuged at 65,000 rpm (300,000 g) in a Spinco SW-65 rotor for 240 min at 0°. Fractions, 0.2 ml each, were collected through a hole in the bottom of the tube. 0.05 ml glycerol was added to each fraction. The bottom of the tube is at the left. Proteins were measured by the method of Lowry. Enzyme recovery was 68%.

material on phosphocellulose did not reveal any heterogeneity, but rechromatography on DEAE cellulose showed clearly that enzyme B was heterogeneous and probably consisted of at least two fractions exhibiting a slight difference in charge (fig. 3). When samples of the column eluate were electrophoresed in the presence of SDS (fig. 4a), it was apparent that, although subunit B3 was always associated with the enzyme activity, subunit B1 was predominantly associated with the first part of the enzyme activity, while subunit B2 was found in the second part. Separate rechromatography on DEAE cellulose of these two parts resulted in more homogeneous peaks of activity (figs. 5, 6). Analysis of the fractions by polyacrylamide gel electrophoresis in the presence of SDS showed that in one case, the enzymatic activity at the top of the peak was associated with bands B1 and B3 (fig. 4b), while in the second case (fig. 4c) most of the enzyme activity was associated with the presence of B2 and B3 bands. These results indicate that enzyme B is actually composed of two enzymes, BI and BII, containing the subunits B1 and B3, and B2 and B3, respectively. The presence of a band migrating like B2 in the very first fractions during rechromatography of enzyme BI (fig. 4b, fraction 32) was unexpected. This band could belong either to some BII enzyme modified during the purification, for instance by the loss of a small acidic subunit, or to a third B enzyme. Further studies are required to elucidate this point.

The existence of two B enzymes, BI and BII, was further supported by results of polyacrylamide gel electrophoresis of enzyme BI and BII in non denaturating conditions. Electrophoresis of enzyme BI and BII resulted in two separate bands (fig. 7, gels 1 and 3). As expected from the more acidic behavior of BII on DEAE cellulose, the band corresponding to this enzyme migrated more rapidly. When the enzymes were mixed with ^{14}C-methyl γ-amanitin before electrophoresis, the label was found only in the regions corresponding to the two bands (C. Kedinger, see the following paper). This observation supported the identification of the two bands as enzymes BI and BII. This was confirmed by the fact that when the two bands were cut out, denatured with SDS and run on polyacrylamide-SDS gels (fig. 7), the band corresponding to enzyme BI contained the two subunits B1 and B3 (gel 4), while the band corresponding to enzyme BII contained B2 and B3 subunit (gel 6).

4. Discussion

The present results demonstrate that it is possible to fractionate RNA polymerase type B from calf thymus into two separate enzymes BI and BII, containing the subunits B1 and B3, and B2 and B3, respectively. Assuming that the ratios of B1:B3 and B2:B3 are one, as suggested by densitometry of the gels, these subunits account for a weight

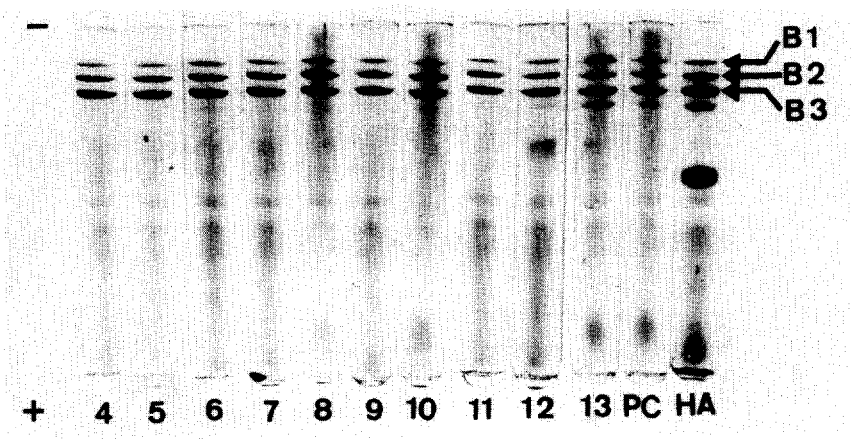

Fig. 2. Polyacrylamide gel electrophoresis of glycerol gradient fractions of enzyme B in the presence of SDS. Aliquots of fractions 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 (fig. 1) were treated and run on SDS gels as described in Material and methods. The corresponding activities applied were 0.46, 0.48, 0.51, 0.50, 0.50, 0.48, 0.50, 0.49, 0.49 and 0.44 unit. Two gels corresponding to the hydroxyapatite step of the purification (HA, 1.0 unit) and to the rechromatography of this fraction on phosphocellulose (PC, 0.50 unit) are also shown.

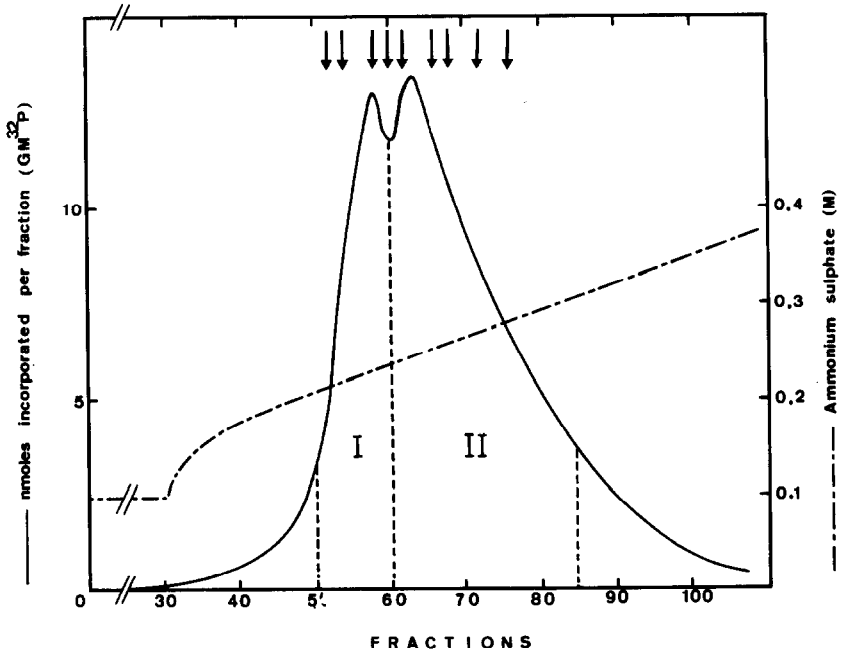

Fig. 3. DEAE cellulose column chromatography of enzyme B. Fractions 4 to 13 of the glycerol density gradient (fig. 1, 550 units of activity) were pooled, diluted to 0.06 M ammonium sulphate (AS) and adsorbed on a 9 × 1 cm DE-52 column. The enzyme was eluted with 20 ml of a linear gradient of AS (0.14 M to 0.35 M). The flow rate was 0.05 ml/min. 0.07 ml of concentrated glycerol was added to each fraction (0.24 ml). Enzyme recovery was 62%.

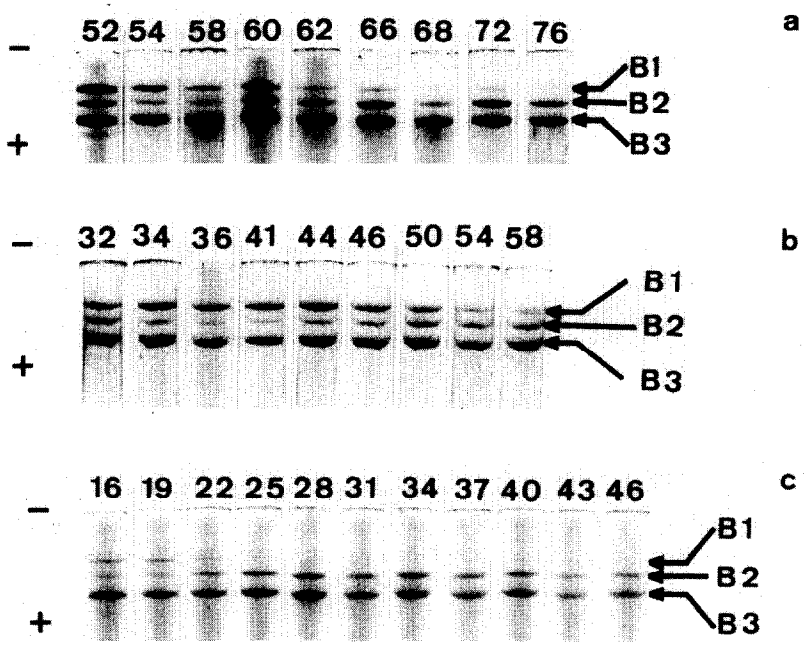

Fig. 4. Polyacrylamide gel electrophoresis of aliquots of DEAE cellulose fractions. Only the upper part of gels is shown. Aliquots of fractions were treated and run on SDS gels as described in Material and methods. (a) Aliquots of fractions 52, 54, 58, 60, 62, 66, 68, 72 and 76 (fig. 3). The corresponding activities applied were 0.64, 0.58, 0.50, 0.66, 0.52, 0.59, 0.52, 0.57 and 0.47 unit. (b) Aliquots of fractions 32, 34, 36, 41, 44, 46, 50, 54 and 58 (fig. 5). The corresponding activities applied were 0.50, 0.41, 0.32, 0.35, 0.38, 0.44, 0.37, 0.35 and 0.40 unit. (c) Aliquots of fractions 16, 19, 22, 25, 28, 31, 34, 37, 43, 46 (fig. 6). The corresponding activities applied were 0.60, 0.60, 0.60, 0.73, 0.73, 0.60, 0.68, 0.65, 0.67, 0.53 and 0.60 unit.

of 365,000 for enzyme BI and 335,000 for enzyme BII. Since the sedimentation of a mixture of B enzymes in glycerol gradient was slightly faster than that of *E. coli* RNA polymerase [2], the MW of the enzymes BI and BII should be around 500,000. This value was also suggested by results of binding studies of amanitin to a mixture of B enzymes [2]. The difference between the MW of the enzymes, and the sum of the weights of the large subunits could be explained by the existence of 3 small subunits of MWs around 20,000, 30,000 and 40,000 respectively (C. Kedinger, unpublished results). Studies are in progress to ascertain the exact MW and stoichiometry of these small subunits.

It is interesting to note that all of the high molecular weight RNA polymerases so far described contain two large subunits, which are β' and β for *E. coli* RNA polymerase [5], A1 and A2 for animal enzyme A [2] and those presently described for animal BI and BII enzymes. Whether the functions of the large subunits of animal enzymes are similar to those ascribed to the β' and β subunits of *E. coli* RNA polymerase [6] is still unknown. Since polyacrylamide gel electrophoresis suggests [2] that both the large subunits A1 and A2 of enzyme A are different from the large subunits B1, B2 and B3 of enzymes B, a ready interconversion between enzymes B and enzyme A as recently proposed by Chesterton and Butterworth [7] appears unlikely.

Although subunits B1 and B2 are different, our results suggest that BI and BII enzymes share a common subunit B3, or at least that they have one subunit of similar MW polyacrylamide gel electrophoresis in the presence of urea will be necessary to ascertain whether the charge is also identical. Since enzymes BI and BII have the same affinity for amanitin (M. Meilhac, unpublished results) it is tempting to speculate that amanitin inhibits the formation of the phosphodiester bond by binding to subunit B3.

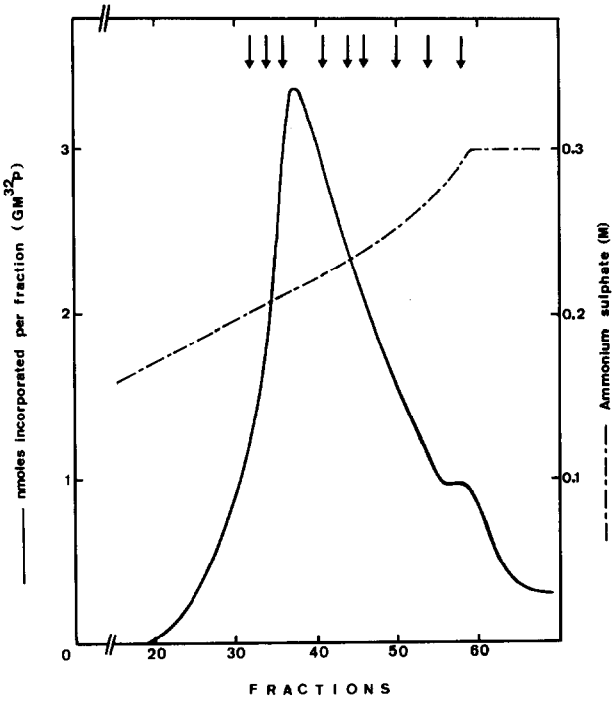

Fig. 5. DEAE cellulose column rechromatography of part I of the peak of fig. 3. Fractions 50–60 (fig. 3, 85 units of activity) were pooled, diluted to 0.08 M ammonium sulphate (AS) and adsorbed on a 8.5 × 0.6 cm DE-52 column. The enzyme was eluted with 11.5 ml of a linear gradient of AS (0.14 to 0.30 M). The flow rate was 0.03 ml/min. 0.05 ml of glycerol was added to each fraction (0.17 ml). Enzyme recovery was 74%.

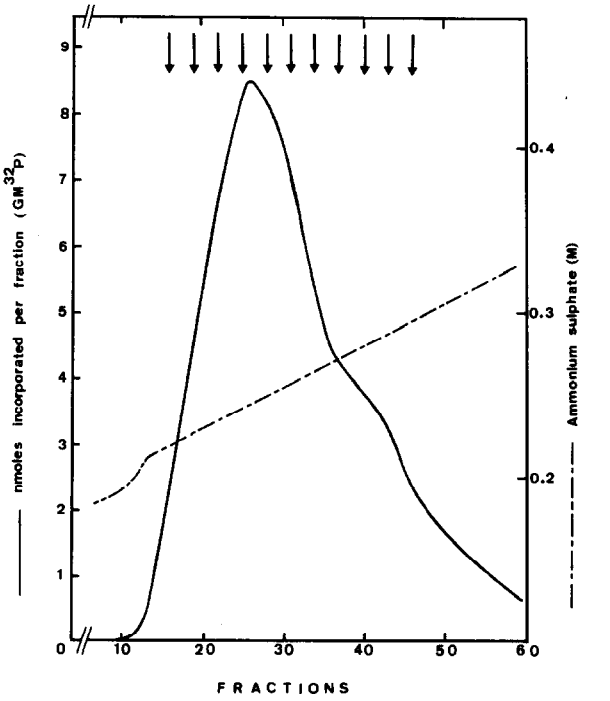

Fig. 6. DEAE-cellulose column rechromatography of part II of the peak of fig. 3. Fractions 61–85 (fig. 3, 200 units of activity) were pooled, diluted to 0.12 M ammonium sulphate (AS) and adsorbed on a 8.5 × 0.6 cm DE-52 column. The enzyme was eluted with 13 ml of a linear gradient of AS (0.18 to 0.35 M). The flow rate was 0.03 ml/min. 0.05 ml of glycerol was added to each fraction (0.17 ml). Enzyme recovery was 88%.

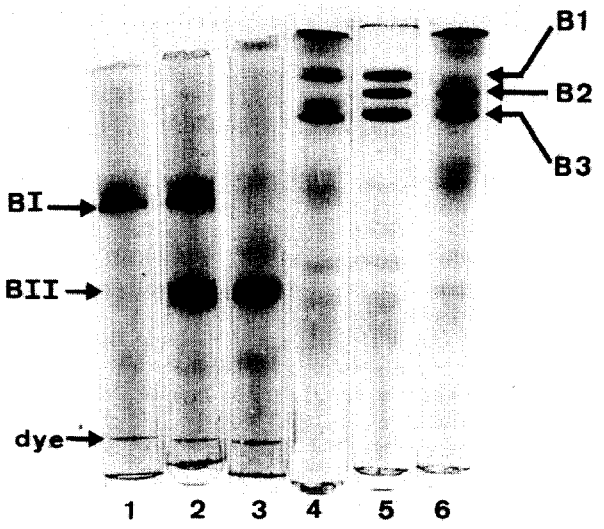

Fig. 7. Polyacrylamide gel electrophoresis of purified RNA polymerases BI and BII. Aliquots of fraction 39 (fig. 5, 0.51 unit) and 29 (fig. 6, 0.71 unit) were run separately (gels 1 and 3 respectively) or together (gel 2) in non-denaturating conditions as described in Material and methods. On parallel gels, the regions corresponding to the bands were cut out, denatured with SDS and then run on polyacrylamide SDS gels. Gels 4, 5 and 6 correspond to gels 1, 2 and 3, respectively.

Table 1
Proposed terminology for the various animal RNA polymerases.

Class of enzyme	Proposed terminology	Large subunits	Localization	Previous terminology
A	Enzyme AI	A1, A2 [2]	nucleolar [7, 8, 11, 13]	Enzyme Ia } [7, 8]
(insensitive to amanitin)	Enzyme AII	?	nucleolar	Enzyme Ib }
	Enzyme AIII	?	nucleoplasmic [13]	Enzyme III [8]
B	Enzyme BI	B1, B3	nucleoplasmic [11, 13]	Enzyme II } [8]
(sensitive to amanitin)	Enzyme BII	B2, B3	nucleoplasmic	Enzyme II }

The relation between enzymes BI and BII and RNA polymerase type II which was identified by Roeder and Rutter in rat liver [8] and also purified from calf thymus [9] is somewhat obscure. The fact that Blatti et al. [9] found only the bands corresponding to subunits B2 and B3 in their calf thymus preparation suggests that enzyme BI was lost during the purification, and that their calf thymus enzyme II is mainly enzyme BII. On the other hand, we have found (see the following paper) that RNA polymerase type II (as defined by Roeder and Rutter [8]), when purified from rat liver, corresponds mainly to enzyme BI. It is obvious that it is becoming more and more difficult to use the initial terminology of Roeder and Rutter which was based on the order of elution from DEAE cellulose, especially since the enzyme activity corresponding to their RNA polymerase I (our enzyme A) was recently resolved on phosphocellulose into two fractions [7]. Since the intranuclear localization of these enzymes could be difficult to assess, we propose to base the terminology of animal nuclear RNA polymerases both on the inhibitory effect of amanitin and on the substructure of the enzymes. Two classes of enzymes can then be defined, those which are insensitive to amanitin (class A) and those which are inhibited by amanitin (class B). The proposed terminology is summarized in table 1.

The physiological functions of RNA polymerases BI and BII are unknown. Both should be nucleoplasmic since all the aminitin sensitive RNA polymerase activity seems to be extra-nucleolar [10, 11]. Penman et al. [12] have recently suggested that messenger RNA and nuclear heterogeneous RNA could be transcribed by two enzymes with different in vivo sensitivities to cordycepin. A possible function for RNA polymerases BI and BII would be to catalyze the synthesis of these two classes of RNA.

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