PURIFICATION OF THE RAT LIVER FORM B DNA-DEPENDENT RNA POLYMERASES

C. James CHESTERTON
Department of Virology, Royal Postgraduate Medical School, Hammersmith Hospital, London, W12, England

and

Peter H.W. BUTTERWORTH
Department of Biochemistry, University College London, Gower Street, London, WC1E 6BT, England

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1. Introduction

Five differing mammalian DNA-dependent RNA polymerases have been reported [1–6]. Since the nomenclature of these enzymes is somewhat confused at present, we have adopted here that put forward by Chambon and coworkers [3].

If control of gene expression in animal cells is mediated by DNA-dependent RNA polymerases, the most interesting forms of this enzyme are probably those located in the nucleoplasm of animal cell nuclei. These are forms BI and BII (we can find only trace amounts of AIII) which may be responsible for mRNA and heterogeneous nuclear RNA synthesis. Below, a detailed procedure for the purification of these enzymes from rat liver nuclei is reported. The subunit pattern of the product, as demonstrated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, indicates that it is a mixture of the BI and BII enzymes recently separated by Kedinger, Nuret and Chambon [3].

2. Materials

Forty male Wistar rats of 300–400 g weight were used per preparation yielding about 600 g liver. Phosphocellulose (P11) and DEAE-cellulose (DE23), both from Whatman, were washed and stored as before [5]. DEAE-Sephadex (A25) from Pharmacia was stored in TGMED buffer (see below) containing 0.4 M $(NH_4)_2SO_4$ and was regenerated after use by washing with 4 M $(NH_4)_2SO_4$. Calcium phosphate gel was prepared by adding a 300 ml solution of $Na_3PO_4.12H_2O$ (46 g) dropwise to a 3200 ml solution of $CaCl_2.6H_2O$ (40 g) with vigorous stirring. After adjustment to pH 7.4 with acetic acid, the suspension was allowed to settle and the precipitate washed extensively by decantation with demineralised distilled water until completely chloride free. After ageing for at least one month suspended in a small volume of water at 4° in the dark, the concentration was determined by weighing dried samples. Stock solutions of $(NH_4)_2SO_4$ and EDTA were adjusted to pH 8. Other materials were as described before [2, 5, 6].

3. Methods

3.1. *Extraction of enzymes from nuclei*

All steps in the preparation of the form B polymerases were carried out at 0–4° unless stated otherwise. Rat liver nuclei were prepared as previously reported [5] except that KCl was omitted from the sucrose solutions. The extraction took place in 2 stages: first the selective removal of the form A polymerases [5, 6]; then extraction of the B enzymes [7]. The nuclear pellets were resuspended and homogenised [5] in 0.01 M tris-HCl, pH 8, 5 mM $MgCl_2$, 0.07 M KCl, 0.1 mM dithiothreitol (DTT), the volume being 0.5 times the initial total weight of liver used. After incubation of the suspension at 37° with shaking for 60 min, nuclei were spun off at 1000 *g*

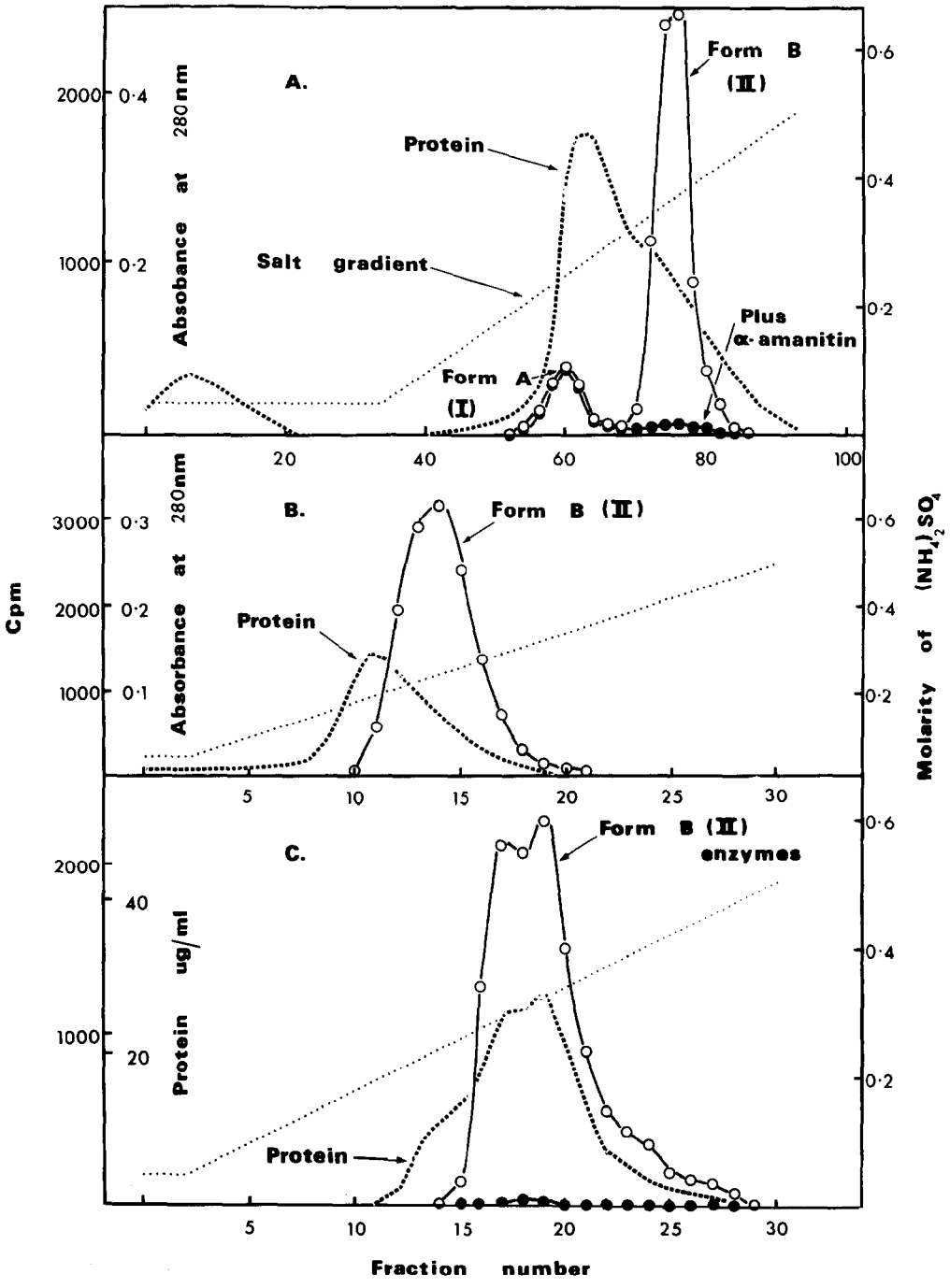

Fig. 1. Column chromatographic purification of the form B RNA polymerases. The enzyme nomenclature used previously [2, 5, 6] is shown in parentheses. The procedures involved are reported in the methods section. A) DEAE-Sephadex chromatography; B) phosphocellulose chromatography; C) DEAE-cellulose chromatography. Samples of 0.05 ml, 0.02 ml, and 0.02 ml were assayed from the eluate fractions of the three columns respectively. Closed circles show assays in the presence of 0.1 μg/ml α-amanitin. The protein profile in C) was derived from that of the absorbance at 280 nm.

for 10 min. Form A polymerases may be partially purified from this low salt extract as described elsewhere [5]. The pellet was resuspended in the same volume of 0.01 M tris-HCl, pH 8, 1.0 M sucrose, 5 mM $MgCl_2$, 1 mM DTT, and 11 ml portions were sonicated at full power with a Branson medium probe for 1 min in 10 sec bursts after the addition to each of 0.8 ml 4 M $(NH_4)_2SO_4$. Each was poured into 24 ml of 0.05 M tris-HCl, pH 8, 25% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT (TGMED) and cleared at 30,000 *g* for 30 min. The enzymes were precipitated by slowly adding 0.42 g $(NH_4)_2SO_4$ per ml with stirring under N_2 and pelleted at 30,000 *g* for 20 min. The pellet was dissolved in a small volume of 0.04 M $(NH_4)_2SO_4$ in TGMED buffer and dialysed against this buffer overnight. This dialysate was termed the high salt extract.

3.2. *DEAE-Sephadex chromatography*

After clearing the dialysate at 30,000 *g* for 30 min, it was divided into 2 equal portions each of which was loaded onto a 40 × 2.5 cm column of DEAE-Sephadex which was subsequently washed with 0.4 M $(NH_4)_2SO_4$ in TGMED buffer until the absorbance at 280 nm of the eluate reached a plateau. A 180 ml linear gradient of 0.04 M to 0.5 M $(NH_4)_2SO_4$ in TGMED buffer was applied, followed by a further 100 ml of 0.5 M $(NH_4)_2SO_4$ in TGMED buffer. Fractions of 2.5 ml were collected. The flow rate was maintained at 80 ml/hr. Fractions were assayed for RNA polymerase [2, 5] as shown in fig. 1A. Those containing α-amanitin sensitive activity were combined, assayed for protein by the Lowry method [8] and stored at −70°.

3.3. *Calcium phosphate gel chromatography*

The combined fractions were diluted with an equal volume of TGMED buffer to reduce the $(NH_4)_2SO_4$ concentration and stirred gently as an amount of gel was added equal to 2.5 times the weight of protein present. After 10 min, the gel was pelleted at 20,000 *g* *g* for 5 min and washed with 10 ml of 0.15 M $(NH_4)_2SO_4$ TGMED buffer. The polymerases were eluted by washing the gel 3 times with 5 ml portions of 0.5 M $(NH_4)_2SO_4$ in TGMED buffer, allowing the gel to stand for 5 min after each resuspension.

3.4. *Phosphocellulose chromatography*

The eluate was dialysed in 1 cm width Visking tubing for 2 hr against two 1 litre changes of 0.05 M $(NH_4)_2SO_4$ in TGMED buffer containing 1 mM $MgCl_2$. The dialysate was adjusted to 40% glycerol and loaded onto a 6 × 1.5 cm column of phosphocellulose, previously washed for 1 hr with the above buffer containing 40% glycerol. After loading, the column was washed with the latter buffer until the absorbance at 280 nm of the eluate decreased to a constant value. A 30 ml linear gradient of 0.05 M to 0.5 M $(NH_4)_2SO_4$ in TGMED buffer containing 40% glycerol and 1 mM $MgCl_2$ was then applied, collecting 1 ml fractions. A flow rate of 40 ml/hr was used. Fractions were assayed for RNA polymerase (fig. 1B) which elutes at about 0.2 M $(NH_4)_2SO_4$. Active fractions were combined and may be stored at −70°. Rarely, a batch of enzyme will not bind to the resin. This step should then be repeated omitting $MgCl_2$ from the dialysis and column buffers. This, however, reduces the purification normally obtained at this stage.

3.5. *DEAE-cellulose chromatography*

The enzymes were dialysed against 0.05 M $(NH_4)_2SO_4$ in TGMED buffer as described in the previous section. After adjustment to 40% glycerol, the dialysate was loaded onto a 15 × 1 cm column of DEAE-cellulose, previously washed overnight with 0.05 M $(NH_4)_2SO_4$ in TGMED buffer and then for 1 hr with this buffer containing 40% glycerol. The column was washed with the latter buffer and a 30 ml linear gradient of 0.05 M to 0.5 M $(NH_4)_2SO_4$ in TGMED buffer containing 40% glycerol was applied using a flow rate of 40 ml/hr. The 1 ml fractions collected were assayed for enzyme (fig. 1C) and the active tubes were stored at −70°. The purification takes 3–4 days and yields about 100 μg of 4800-fold purified form B polymerases at 20–30 μg/ml with a specific activity of 25 nmoles UMP incorporated into RNA per min per mg protein. The preparation may be stored at −70° for several months without loss in activity.

4. Results and discussion

Details of the recovery and purification at all stages of the preparation are shown in table 1. Exami-

Table 1
Purification of the form B RNA polymerases from rat liver. One enzyme unit incorporates 1 pmole UMP/min into RNA. All enzyme assays were carried out at 0.13 M $(NH_4)_2SO_4$ ± α-amanitin and the sensitive activity is shown.

Fraction	Enzyme activity (Units)	Recovery (%)	Protein (mg)	Specific activity (Units/mg protein)	Purification (fold)
Nuclei	9,520	100	1,840	5.2	1
High salt extract	3,820	40	385	9.9	2
DEAE-Sephadex eluate	8,170	86	15.6	524	100
$CaPO_4$ gel eluate	5,910	62	1.9	3,130	600
Phosphocellulose eluate	3,840	40	0.405	9,490	1,820
DEAE-cellulose eluate	2,650	28	0.105	25,310	4,870

nation of the subunit pattern of the preparation by SDS polyacrylamide gel electrophoresis on 5% gels [9] showed the presence of 3 large subunits (fig. 2). Using the nomenclature of Chambon and coworkers [3], these are termed B1, B2, and B3, and have approximate molecular weights of 200,000, 180,000, and 160,000 respectively. The similarity of this pattern to that reported for the calf thymus enzyme [10] now shown to be a mixture of two enzymes called BI and BII [3] indicates that the rat liver B polymerase may also be a mixture of these forms. If this is the case, the relative intensity of the stained protein bands in the gel (fig. 2) suggests that form BI predominates in this preparation. Similar results for the rat liver form B enzyme are reported by Mandel and Chambon [4].

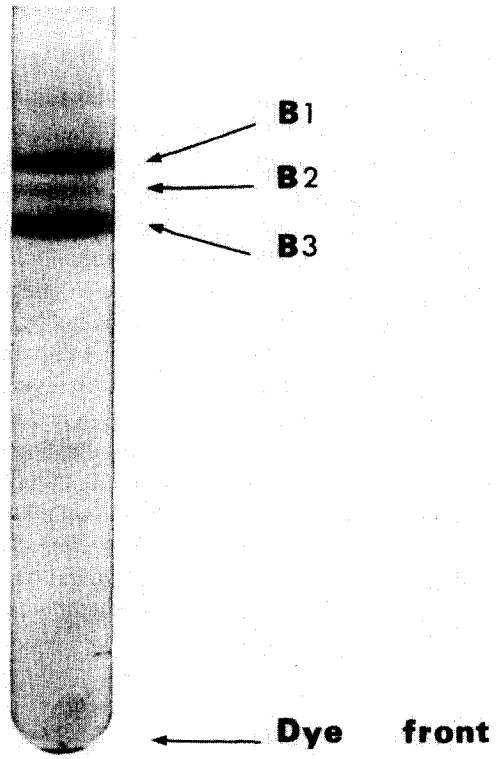

Fig. 2. Analysis of the form B RNA polymerases, purified 4800-fold, by sodium dodecyl sulphate polyacrylamide gel electrophoresis [9].

Comparison of the subunit pattern of this 4800-fold purified preparation with that we reported earlier for a 900-fold purified preparation of form B [2] shows that inclusion of the calcium phosphate gel and DEAE-cellulose steps has virtually removed the components of 100,000 and 60,000 molecular weight. The minor band of 180,000 molecular weight, which was previously regarded as an impurity with a low relative molar ratio to the other large subunits, is retained and is probably the B2 subunit of the BII enzyme. Low molecular weight components, which are not removed by G-100 Sephadex filtration, are still present in the preparation and their relationship to the large subunits is under investigation.

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