

A Simplified Procedure for Large Scale Preparation of Purified Animal DNA-Dependent RNA Polymerase

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RNA polymerase, purification method

A procedure is described for obtaining DNA-dependent RNA polymerase form B from calf thymus in high purity. The procedure includes homogenization in low salt, sedimentation of chromatin at $50\,000 \times g$, adsorption to DEAE-cellulose in a batch procedure, DNA-agarose chromatography, and chromatography on DEAE-Sephadex. Analyses of the preparation by band sedimentation and gel-electrophoresis indicate a high purity of the enzyme.

Introduction

Current investigations of transcription in eucaryotic cells require the availability of sizable amounts of purified RNA polymerase from such cells for studies on the structure of this enzyme and for *in vitro* transcription systems.

A number of polymerases have been found to occur in eucaryotic cells (for review see¹). This paper describes a purification method for RNA polymerase B from calf thymus, the enzyme which is assumed to be responsible for the transcription of Hn- and m-RNA. This enzyme has been purified and characterized before^{2–4}, but the methods described are very laborious, including a comparatively large number of steps and exhibiting an accordingly low yield of the purified enzyme.

The method presented here avoids the previous preparation of nuclei, and the extraction of the enzyme by sonication in high salt, both of which steps complicate the handling of large amounts of tissue.

Furthermore, concentration of the enzyme by ammonium sulphate precipitation, which in our hands has always resulted in a loss of activity, is not used. Chromatography on phosphocellulose and/or hydroxyapatite give consistently low yields^{2–4}; these steps can be avoided. Finally, since the procedure described here does not include steps with limiting capacity such as density gradient centrifugation, a scaling up of the method is easily possible.

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Enzyme Preparation

Extraction of the tissue

Thymus from 12–16 weeks-old calves is roughly freed from fat and connective tissue, washed with 0.9% NaCl in distilled water, and stored at -70°C .

Portions of 250 g of the frozen thymus are homogenized together with 350 ml of homogenization buffer at room temperature for 2 min in a Waring blender at low setting. The homogenization buffer contains 10 mM Tris-HCl, pH 7.8; 1% Brij 58 (this improves the yield of extraction by a factor of 1.5); 0.1 mM EDTA; 1% mercaptoethanol. After addition of MnCl_2 to 2 mM and ammonium sulphate to 100 mM, another 2 min homogenization is performed at high setting. All following operations are performed at 4°C . To remove fat and cell debris, the homogenate is centrifuged for 5 min at $1700 \times g$, then the supernatant is mixed with one-half volume ice-cold glycerol (Merck) and centrifuged overnight in a Spinco rotor 19 at 19 000 rpm.

Batch adsorption to DEAE-cellulose

Ammonium sulphate is added to the clear supernatant to a concentration of 0.14 M. 1400 g of DEAE-cellulose (Whatman DE 52), equilibrated with TGMED buffer (50 mM Tris-HCl pH 7.8; 30% glycerol; 0.1 mM EDTA; 0.1 mM dithiothreitol; 5 mM 1-mercaptoglycerol) containing 0.14 M ammonium sulphate, is suspended in the extract from 1 kg of tissue. After stirring for 1 hour, the DEAE-cellulose is collected by filtration on a Büchner funnel and washed twice by resuspension and filtration using 6 l of TGMED containing 0.14 M ammonium sulphate. The material is resuspended in the same buffer, deaerated, transferred into two columns



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(5 × 50 cm), and packed at a flow rate of 180 ml/hour. For elution, the two columns are connected in tandem and a 2 l gradient of 0.3–0.6 M ammonium sulphate in TGMED buffer is applied. The steep continuous gradient following the step from 0.14 to 0.3 M ammonium sulphate was found to effectively concentrate the enzyme at the front of the gradient. An elution profile of the enzyme is shown in Fig. 1.

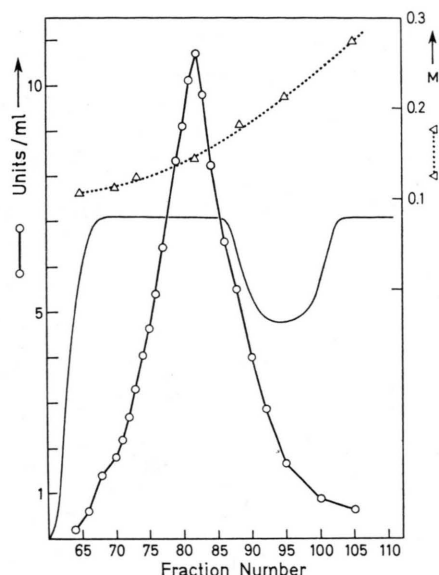


Fig. 1. Elution profile of the DEAE-cellulose column. Activity is assayed as follows: The assay mixture contains in 0.1 ml: ATP, CTP, GTP, UTP, 0.2 mM each; DNA (calf thymus, No V, Sigma), 0.2 mg/ml; ^3H -UTP 20 $\mu\text{Ci/ml}$; KCl 0.1 M; MnCl_2 1 mM; Tris-HCl 50 mM, pH 7.8; mercaptoethanol 5 mM. 10 μl of the enzyme are tested. After 10 min of incubation at 37 °C, the reaction is stopped by cooling to 4 °C and adding 100 μg RNA in 40 μl of saturated neutral sodium pyrophosphate. The samples are pipetted on 2 × 2 cm Whatman GF81 glass filters which are fixed on a stainless steel frame. The frame is placed for 5 min each in 6 successive washing baths containing: bath No. I, 4% TCA No. II–IV, 0.8% TCA; No. V–VI, methanol. The procedure effectively removes acid-soluble radioactive material, yielding a blank of less than 40 cpm. After drying (10 min at 80 °C), radioactivity on the filters is determined in a Packard Tri-Carb scintillation spectrometer in plastic vials containing 0.03% POPOP and 0.5% PPO in toluene. 1 activity unit of enzyme catalyzes the incorporation of 1 nmole UMP in 10 min at 37 °C. —○—, Enzyme activity in units/ml; —, A_{280} recording of LKB Uvicord; —△—, molarity of ammonium sulphate.

The active fractions are pooled, dialyzed over night against TGMED, and adjusted to an ammonium sulphate molarity of 0.05.

DNA-agarose chromatography

4% single-stranded DNA-agarose is prepared according to Schaller *et al.*⁵. DNA content, as determined after solubilization of DNA from the gel with DNase I, amounted in our preparations to 3 mg DNA per ml of packed agarose. A column of 1.6 × 12 cm is prepared in a Pharmacia K 16/20 tube using two flow adaptors. Care is taken not to apply too much pressure to the column during its preparation, so that a hydrostatic pressure of 30 cm results in a flow rate of 8 ml/hour. The column is run from the bottom to the top with this flow rate.

After loading the dialysate (about 300 ml) onto the column and washing with 125 ml of 0.07 M ammonium sulphate in TGMED, the enzyme is eluted with a step of 0.5 M ammonium sulphate in TGMED (Fig. 2).

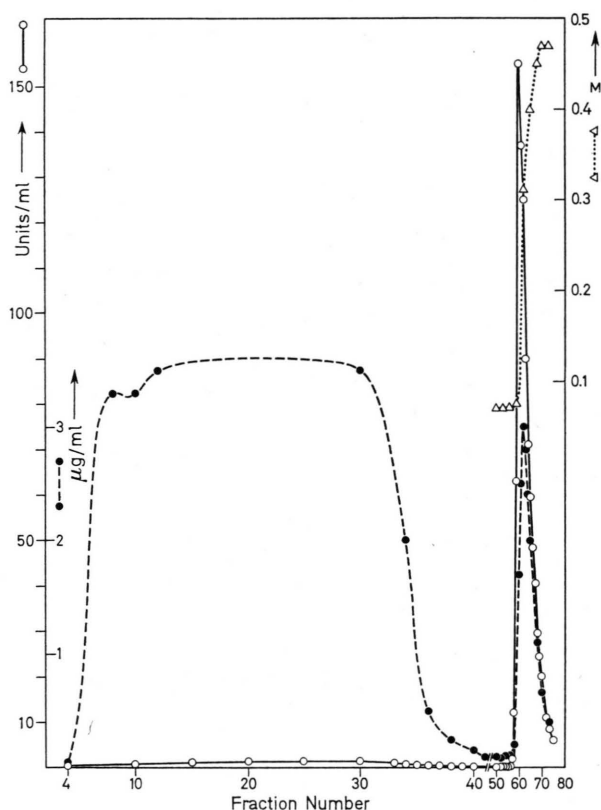


Fig. 2. Elution profile of DNA-agarose column; for assay conditions see Fig. 1. —○—, Enzyme activity in units/ml; —●—, protein concentration (Lowry method) in mg/ml; —△—, molarity of ammonium sulphate. Fractions 4–40 contained 8 ml, fractions 50–80 contained 1.2 ml effluent.

DEAE-Sephadex chromatography

The active fractions from the DNA-agarose column are pooled and diluted with TGMED to 0.12 M ammonium sulphate as checked by conductivity.

About half of the diluted pool (containing 15.4 mg of protein in the example outlined in Table I) is loaded onto one 1.1 × 14 cm DEAE-Sephadex A 25 column equilibrated with 0.12 M ammonium sulphate in TGMED. A hydrostatic pressure of 30 cm is used to achieve a flow rate of 12 ml/hour. After washing the column with 30 ml of loading buffer the enzyme is eluted with a 30 ml linear gradient of 0.12–0.4 M ammonium sulphate in TGMED. A discrete protein peak paralleling enzyme activity was consistently seen in this chromatographic step (Fig. 3).

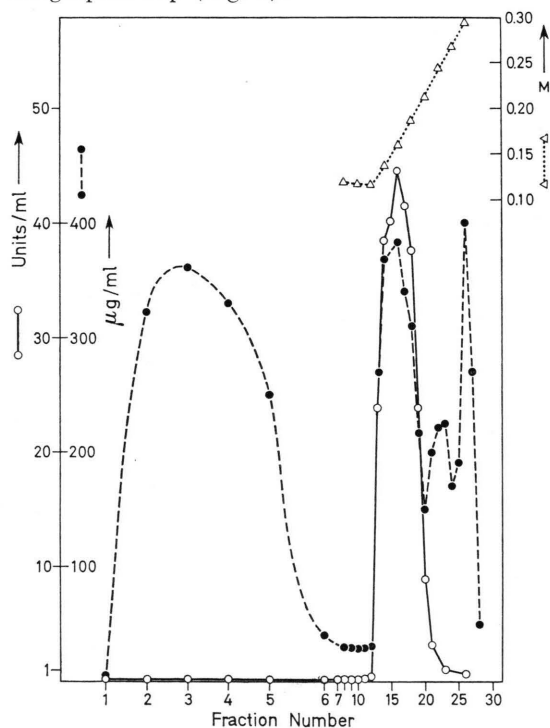


Fig. 3. Elution profile of DEAE-Sephadex column, for assay conditions see Fig. 1. —○—, Enzyme activity in units/ml; —●—, protein concentration (Lowry method) in µg/ml; —△—, molarity of ammonium sulphate. Fractions 1–5 contained 9 ml, fractions 7–30 contained 1.6 ml effluent.

Purity of the Enzyme*Sucrose-gradient-sedimentation*

Since the purification procedure includes no separation according to molecular size, the distribution

of protein and enzyme activity in a velocity sedimentation pattern should indicate the degree of enzyme purity. For such band sedimentation, 0.22 ml of the enzyme preparation was added to 0.08 ml H₂O and loaded onto a 5 ml 0–10% sucrose gradient in TGMED containing 0.1 M ammonium sulphate. After 16 hours centrifugation at 50 000 rpm, fractions are collected and assayed for protein concentration and enzyme activity. As can be seen from Fig. 4, about 85% of the protein distributes in the gradient in parallel with the enzyme activity.

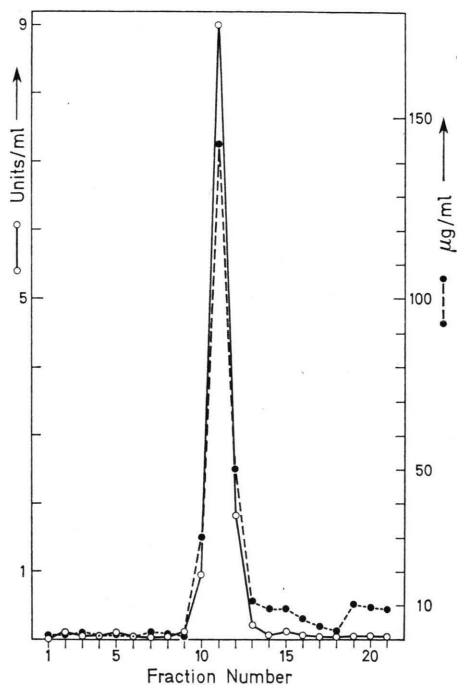


Fig. 4. Pattern of sucrose-gradient centrifugation; for assay conditions see Fig. 1. —○—, Enzyme activity in units/ml; —●—, protein concentration (Lowry method) in µg/ml.

Gel-electrophoresis

A high purity of the enzyme preparation after DEAE-Sephadex was confirmed by an SDS-acrylamide gel electrophoresis comparison of this material with protein from the peak fraction of the sucrose gradient illustrated in Fig. 4. Both profiles were found to be identical (Figs 5 a and b). In general agreement with the observations of other groups^{3, 4}, three bands in the region of 130 000–200 000 daltons and four additional bands in the range of 20 000–50 000 daltons are apparent.

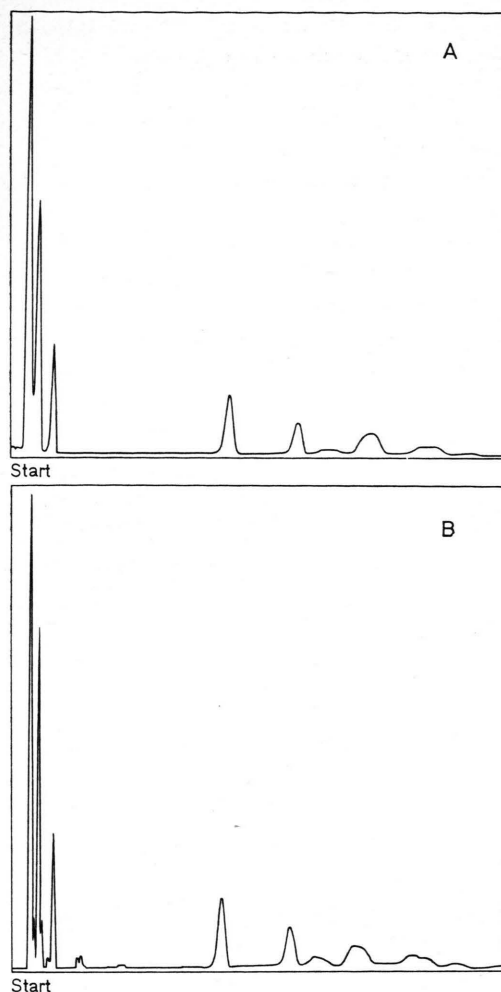


Fig. 5. Scan of SDS-gel-electrophoresis, in 10% polyacrylamide A, pool of DEAE-Sephadex column; B, peak fraction of sucrose-gradient centrifugation.

Fraction	Protein concentr. [mg/ml]	Enzyme activity [units/ml]	Vol. from 1 kg thymus [ml]	Spec. act. [units/mg pr.]	Yield [%]
homogenate	50	2 *	2400	0.04 *	100
Spinco supernatant	22.5	1.2 *	2500	0.054 *	62.5
DEAE-cellulose pool	4.5	6	280	1.34	35
DNA-agarose pool	2.5	70	14	29	20.3
DEAE-Sephadex pool	0.35	45	20	130	18.7

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³ C. Kedinger and P. Chambon, Eur. J. Biochem. **28**, 238 [1972].

Properties of the Enzyme

The efficiency of the whole purification procedure, applied to 1 kg of calf thymus, is summarized in Table I. As may be seen, a 3300-fold purification of the α -amanitin-sensitive enzyme is obtained; a total recovery of 18.5% of the activity yields 7 mg of enzyme per kg of thymus. The activity of the purified enzyme is entirely dependent on the presence of DNA, and is more than 98% sensitive to α -Amanitin. Of technical importance may be the observation that the enzyme activity is strongly dependent on the concentration of endogeneous protein in the assay: In the experiment summarized in Fig. 6, the enzyme

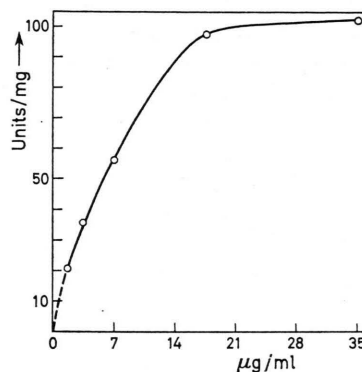


Fig. 6. Specific activity of enzyme at various concentrations of enzyme protein in the assay.

was diluted to different concentrations, and 10 μ l of the dilution added to the assay mixture. The observed decay of enzyme activity does not seem to be a general effect of protein concentration, however, since the addition of comparable amounts of bovine serum albumin is without detectable effect.

Table I. Efficiency of the purification.

Rössler for technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft.

⁴ R. F. Weaver, S. P. Blatti, and W. J. Rutter, Proc. nat. Acad. Sci. USA **68**, 2994 [1971].

⁵ H. Schaller, C. Nuesslein, F. J. Bonhoeffer, C. Kurz, and I. Nietschmann, Eur. J. Biochem. **26**, 472 [1972].