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Purification Using Polyethylenimine Precipitation and Low Molecular Weight Subunit Analyses of Calf Thymus and Wheat Germ DNA-Dependent RNA Polymerase II†

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ABSTRACT: DNA-dependent RNA polymerase II from calf thymus has been successfully purified using polyethylenimine precipitation. Thus, 5–6 mg of nearly homogeneous RNA polymerase II (>96% pure) can be prepared from 1 kg of calf thymus with three chromatography steps following extraction and precipitation of the enzyme from the polyethylenimine pellet. This procedure eliminates the high salt extraction of chromatin previously used in purification of this enzyme and makes possible the large scale preparation of mammalian RNA polymerase II. Calf thymus polymerase II prepared by this method is greater than 90% form IIb and consists of ten different subunits having the following molecular weights: 180 000; 145 000; 36 000; 25 000; 20 000; 18 500; 16 000; 15 000; 12 000; 11 500. The homologous enzyme isolated from wheat germ is greater than 90% form IIa and contains subunits of the following molecular weights: 206 000; 145 000; 44 000–47 000; 24 500; 21 000; 19 000; 17 000; 14 000; 13 500. The wheat germ and calf thymus enzymes exhibit similar subunit structures, but the molecular weights of individual subunits are clearly different between the enzymes. Wheat germ RNA polymerase II is 50% inhibited by 0.271 μg/mL of α-amanitin, a level 30-fold higher than that found for calf thymus RNA polymerase II. These enzymes are further distinguished by the absence of antigenic cross reactivity.

Detailed physical and chemical characterization of the eukaryotic tripartite transcriptive system has been impeded by the difficulty of obtaining large quantities of purified RNA polymerases I, II, and III. Although significant amounts of these polymerases can be isolated from yeast (Valenzuela et al., 1976a; Bucher et al., 1974), the subunit structure and catalytic properties of these enzymes are markedly different from their mammalian counterparts (Valenzuela et al., 1976b; Bucher et al., 1976; Biswas et al., 1975). Thus for in vitro studies of chromatin and viral DNA transcription it would be desirable to use mammalian RNA polymerases rather than the bacterial and plant polymerases that have been used previously. Recently Jendrisak and Burgess (1975) have successfully employed Polymin P, a polyethylenimine (Zillig et al., 1970), for the purification of milligram quantities of RNA polymerase II from wheat germ. This methodology avoids high salt sonication and ultracentrifugation and is conducive to large scale enzyme preparation. This paper describes the first application of polyethylenimine fractionation to the purification of RNA polymerase II from a mammalian tissue. This procedure results in high yields of this enzyme and is more suitable for scaleup than previously used methods (Weaver et al., 1971; Kedinger et al., 1972; Weil and Blatti, 1975). The subunit structure, antigenic determinants, and α-amanitin sensitivity of calf thymus RNA polymerase II are compared with those of wheat germ RNA polymerase II. In addition, using lower porosity gels, we report the presence of several smaller subunits or components of calf thymus RNA polymerase II not previously observed in this enzyme.

Materials and Methods

Materials. Wheat germ from General Mills was stored at 4 °C. Fresh frozen calf thymus was obtained from Dubuque Park, Dubuque, Iowa, and maintained at −70 °C until use.

All biochemicals were reagent grade. Tritium labeled UTP (specific activity, 16 Ci/mmol) was obtained from Schwarz/Mann, α-amanitin and dithiothreitol were purchased from Calbiochem, calf thymus DNA (grade I) was from Sigma Chemical Co., and bovine serum albumin was from Miles Laboratories. Polyethylenimine (30%) was obtained from Aldrich Chemical Co. Nonidet-P-40 was a gift of Shell Chemical Co.

Solutions. All buffers were prepared from reverse-osmosis purified deionized water. Dithiothreitol was prepared as a 0.1 M stock solution and was added immediately before use. Buffer

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A is 10 mM Tris[1]-HCl (pH 7.9, adjusted at 25 °C), 25 mM KCl, 5 mM $MgCl_2$, 0.1 mM Na_2EDTA, 12.5% glycerol, and 0.5% (v/v) 2-mercaptoethanol. Buffer B is 50 mM Tris-HCl (pH 7.9), 0.1 mM Na_2EDTA, and 0.5% (v/v) 2-mercaptoethanol. Buffer C is 50 mM Tris-HCl (pH 7.9), 10% glycerol, 0.1 mM Na_2EDTA, and 0.5 mM dithiothreitol. Buffer D is the same as buffer C, except that the glycerol concentration is 25%. Ten percent (v/v) stock solutions of polyethylenimine were prepared in buffer B containing no 2-mercaptoethanol and were stored at 5 °C. The pH of the polyethylenimine stock solution was pH 7.9, so additional neutralization was not required.

Conductivity Measurements. The ammonium sulfate concentration of a given sample was determined as described previously (Weil and Blatti, 1976) using a Radiometer CDM 2b conductivity meter.

Protein Determinations. Protein concentrations were measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Samples were precipitated in ice with trichloroacetic acid, washed with cold acetone, and resuspended in 0.1 N NaOH for Lowry determinations.

Preparation of Ion-Exchange Resins. DEAE-Sephadex A-25 was obtained from Pharmacia and was equilibrated with either 40, 150, or 200 mM ammonium sulfate as described previously (Weil and Blatti, 1976).

DE-52 was obtained from Whatman Inc. The cellulose was prepared by successively washing with 5 M NaCl, 0.5 M HCl, 0.5 M NaOH, and water until the pH of the effluent was pH 8.0. The washed resin was then suspended in 0.5 M Tris-HCl (pH 7.9) and allowed to equilibrate. The resin was resuspended three times in buffer D containing 150 mM ammonium sulfate, made 0.03% in toluene, and stored at 5 °C as a 2:1 (buffer-resin) slurry until use.

Phosphocellulose (P-11, Whatman Inc.) was prewashed in dilute acid and alkali before equilibration in buffer D, containing 50 mM ammonium sulfate and 0.2 mg/mL bovine serum albumin.

Gel Electrophoresis. Dodecyl sulfate-polyacrylamide gels were prepared and run as described by Laemmli and Favre (1973). Samples for electrophoresis were first precipitated in conical centrifuge tubes with cold 10% (w/v) trichloroacetic acid and centrifuged for 20 min at 2000g. The trichloroacetic acid supernatant was decanted and the pellets were washed with cold acetone. Residual acetone was removed by evaporation at room temperature. Immediately before application to the gel, the samples were dissolved in 60 μl of sample buffer (0.125 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50% 2-mercaptoethanol, and 0.001% bromophenol blue) and heated in a boiling water bath for 5 min.

Electrophoresis under nondenaturing conditions was carried out at 4 °C. Gels were prepared without the addition of dodecyl sulfate as described by Laemmli and Favre (1973), and were preelectrophoresed for 30 min prior to sample application.

At the conclusion of electrophoresis, the dye front was marked and the gels were fixed in hot 12% trichloroacetic acid–50% ethanol (v/v) prior to staining in 0.1% (w/v) Coomassie blue, 10% acetic acid (v/v), and 50% (v/v) ethanol (Schwartz and Roeder, 1974). Gels were initially destained in methanol–water–acetic acid (v/v, 90:90:25) until bands were visible; they were then allowed to destain in 10% acetic acid (v/v) until the background was clear. Gel scans were performed on an E-C 910 densitometer using a 570 nm filter.

RNA Polymerase Assays. Assays were done as previously described in a final volume of 60 μL (Weil and Blatti, 1975). One unit of enzyme activity represents the incorporation of 1 nmol of UTP (under saturating conditions) in 10 min. To measure the relative levels of RNA polymerases I, II, and III, assays were performed at 50 mM ammonium sulfate. RNA polymerase I activity is defined as that activity resistant to an α-amanitin concentration of 107 μg/mL; polymerase III activity is that portion of the total activity which is sensitive to 107 μg/mL of α-amanitin, but resistant to 1.0 μg/mL of α-amanitin. Polymerase II activity represents the activity which is sensitive to 1 μg/mL α-amanitin. These levels of α-amanitin are taken from the titration curves of Weil and Blatti (1975).

Wheat Germ RNA Polymerase II Purification. RNA polymerase II was purified at 0–4 °C using methodology outlined by Jendrisak and Burgess (1975) with some modifications. One thousand grams of raw wheat germ was added to 3500 mL of buffer A, containing 0.5 mM dithiothreitol. The viscous mixture was allowed to sit in ice for 40 min with occasional stirring and then diluted with 4500 mL of buffer B, containing 0.5 mM dithiothreitol with stirring. The extract was centrifuged at 13 000*g* in the Sorvall GS-3 rotor for 10 min and the pellet discarded. RNA polymerase was precipitated from the yellow supernatant by the addition of 13 μL of a 10% polyethylenimine solution per mL of extract. After 40 min in ice the material was centrifuged at 13 000*g* as described above. The polyethylenimine pellet was resuspended in buffer D containing 100 mM ammonium sulfate and blended thoroughly using a Dounce homogenizer. This solution was centrifuged for 90 min at 95 000*g* in a Spinco type 35 rotor. The supernatant was filtered through glass wool and dialyzed against buffer D until the ammonium sulfate concentration reached 40 mM. This material was applied to a Sephadex A-25 column (1200 mL bed volume) previously equilibrated with buffer D containing 40 mM ammonium sulfate. RNA polymerase was eluted with a linear 40 to 500 mM ammonium sulfate gradient in buffer D. The appropriate fractions were pooled and bovine serum albumin was added to a final concentration of 0.2 mg/mL. This material was dialyzed for 4.5 h against 10 volumes of buffer D. The dialyzed material was then diluted with buffer D containing 0.2 mg/mL bovine serum albumin to 50 mM ammonium sulfate and applied to a phosphocellulose column (600 mL bed volume) equilibrated as described above. RNA polymerase II was eluted with a linear 50 to 500 mM ammonium sulfate gradient in buffer D containing 0.2 mg/mL bovine serum albumin.

The activity peak from the phosphocellulose column was pooled and dialyzed against buffer D until the ammonium sulfate concentration reached 50 mM. This material was applied to a Sephadex A-25 column (100 mL bed volume) and eluted with a 50 to 500 mM linear gradient of ammonium sulfate in buffer D. The active fractions from the second Sephadex A-25 column were concentrated by ammonium sulfate precipitation (0.39 g/mL) and dialyzed overnight against buffer D containing 100 mM ammonium sulfate.

Calf Thymus RNA Polymerase II Purification. One thousand grams of frozen calf thymus was thawed in 2 L of buffer A and homogenized in a prechilled Waring Blendor for 30 s each at low, medium, and high speeds. Two liters of buffer B was then added to the mixture and blended for an additional 30 s at low speed. The homogenate was centrifuged at 13 000*g* for 10 min in the Sorvall GS-3 rotor. The RNA polymerase

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethyleneaminotetraacetic acid; DEAE, diethylaminoethyl.

TABLE I: Purification of Calf Thymus RNA Polymerase.[a]

Purification step	Total act. (units)	Vol (mL)	Protein (mg)	Spec act. (units/mg of protein)	Polymerase act. (units) I	II	III
Crude supernatant (F_1)	4274	3680	42 170	0.101	1658	1879	706
200 mM ammonium sulfate extract (F_2)	5861	2075	15 580	0.276			
Dialyzed ammonium sulfate pellet (F_3)	2286	335	6 250	0.360	249	1744	290
DE-52 flow through	578	450	6 114		317	26	234
DE-52 peak	3100	184	135.6	22.8	0	3100	0
Phosphocellulose	1150	37	12.12	94.8	0	1150	0
Sephadex A-25-agarose A-1.5m	943	26	5.88	160	0	943	0

[a] The purification is based upon 1000 g of calf thymus.

activity in the crude supernatant (F_1) was precipitated by the dropwise addition of 4 μL of a 10% polyethylenimine solution per mL of extract. After 30 min of stirring the polyethylenimine precipitate was pelleted by centrifugation at 13 000*g* as described above.

The pellet was slowly blended with 1 L of buffer C containing sufficient ammonium sulfate to give a final concentration of at least 0.2 M. This material was centrifuged as described above and the supernatant fraction (F_2) was immediately precipitated by the addition of 0.24 g of ammonium sulfate per mL of extract. After 1 h of stirring the ammonium sulfate precipitate was resuspended in buffer D and dialyzed overnight to a concentration of 150 mM ammonium sulfate (F_3). The dialyzed material (335 mL) was loaded onto a 350 mL bed volume DE-52 column (5 × 18 cm) previously equilibrated with buffer D containing 150 mM ammonium sulfate. The flow through was collected and the column was washed with two column volumes of 150 mM ammonium sulfate-buffer D. The RNA polymerase II activity was eluted stepwise with buffer D containing 500 mM ammonium sulfate. The column was run at a rate of 300 mL/h and fractions of 23 mL each were collected. The active fractions (184 mL) were pooled and bovine serum albumin was added to a final concentration of 0.2 mg/mL. The DE-52 pool was diluted in aliquots to 50 mM ammonium sulfate with buffer D–0.2 mg/mL bovine serum albumin and applied to a 100-mL (5 × 12 cm) phosphocellulose column equilibrated as described above. After application of the sample the column was washed with 2.5 volumes of buffer D–50 mM ammonium sulfate, containing no bovine serum albumin. The RNA polymerase activity was eluted stepwise with 200 mM ammonium sulfate–buffer D. The column was run at 300 mL/h and 2.5-mL fractions were collected. The active fractions were pooled (37 mL), adjusted to 150 mM ammonium sulfate with buffer D, and applied to a 2.5 × 10-cm column of agarose A-1.5m capped with a 2.0-cm bed of Sephadex A-25 previously equilibrated with 150 mM ammonium sulfate–buffer D. After the sample was applied, the column was washed with 20 mL of the above buffer. The RNA polymerase activity was eluted stepwise with buffer D containing 500 mM ammonium sulfate. The column was run at 60 mL/h and 2.0-mL fractions were collected.

Density Gradient Centrifugation. The calf thymus polymerase II and wheat germ RNA enzymes used here were from the phosphocellulose step. They were each concentrated by adsorption to Sephadex A-25 in buffer D–200 mM ammonium sulfate followed by stepwise elution with buffer D containing 500 mM ammonium sulfate. Samples (0.3 mL) of wheat germ RNA polymerase II (specific activity, 180 units/mg) and calf thymus RNA polymerase II (specific activity, 104 units/mg) in buffer D, but with 12.5% glycerol, containing 100 mM ammonium sulfate were each layered onto 4.6-mL linear 17.5–35% glycerol gradients in 0.05 M Tris-HCl (pH 7.9)–0.1 mM Na_2EDTA–0.5 mM dithiothreitol–100 mM ammonium sulfate–0.2% Nonidet-P-40 detergent. The gradients were centrifuged for 8 h in a Spinco SW 65 rotor at 0 °C as described by Krebs and Chambon (1976). Thirteen drop fractions were collected from the bottom of the tube (19–21 fractions/gradient) and assayed immediately.

Preparation and Assay of Wheat Germ Polymerase II Antisera. Adult white Leghorn hens were immunized at 8 to 10 day intervals by both intramuscular and subcutaneous administration of 500 μg of wheat germ RNA polymerase II (specific activity, 188 units/mg) emulsified in a final volume of 1.0 mL of Freunds complete adjuvant (Ingles, 1973). Eight days after the fifth injection, the animals were bled from the jugular vein. The blood was allowed to clot for 1 h and centrifuged at 30 000*g* to remove clotted cells. The γ-globulin fraction was purified from the antisera as described by Linn et al. (1973) and dialyzed against buffer D containing 0.1 M NaCl; no dithiothreitol was present. Control γ-globulin was purified from serum obtained from the chickens prior to injection with wheat germ RNA polymerase II.

Results

Calf Thymus RNA Polymerase II Purification. The results of the calf thymus polymerase purification procedure detailed in the previous section are summarized in Table I. Significant quantities of RNA polymerase activity are solubilized by homogenization of the tissue in the absence of 0.3 M ammonium sulfate. Although only 43% of polymerase is initially extracted by this procedure compared with the previous report (Weil and Blatti, 1975) using the high salt extraction procedure, the overall yield of polymerase II per 1000 g of thymus is 1.6-fold greater at the phosphocellulose step.

The optimal conditions for precipitating total cellular RNA polymerase activity were determined from the data in Figure 1. When the pilot assays described in Figure 1 were scaled up, 4 μL of 10% polyethylenimine per mL of extract routinely precipitated 35% of the total soluble protein and 85% of the total polymerase activity present in the crude supernatant. The optimum level of polyethylenimine required for calf thymus polymerase precipitation is different from wheat germ polymerase precipitation. As for wheat germ (Jendrisak and Burgess, 1975), the required polyethylenimine concentration is strongly dependent upon the total protein, salt, and glycerol concentration of the initial homogenate. Selective precipitation of the cognate forms of RNA polymerase was not observed in these initial studies.

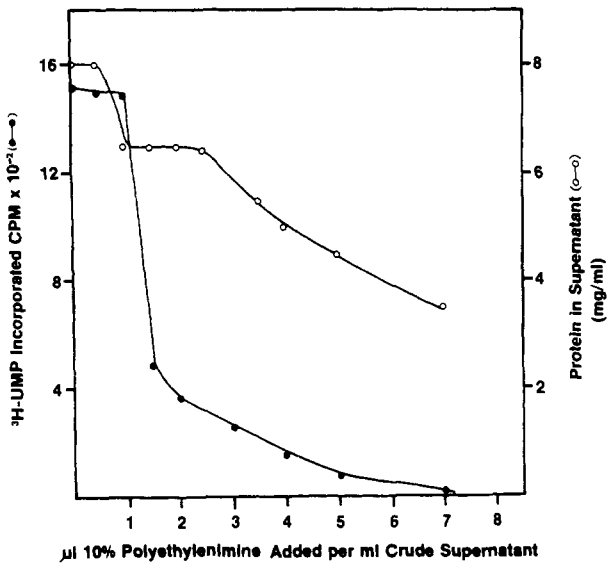

FIGURE 1: Precipitation of calf thymus RNA polymerase and protein with polyethylenimine. The appropriate volumes of 10% polyethylenimine were added to 7-mL aliquots of F_1 (crude supernatant) as described in Materials and Methods. The samples were mixed well and allowed to precipitate in ice for 30 min. After centrifugation at 10 000g for 15 min, the supernatants were assayed for RNA polymerase activity (●) and protein concentration (○).

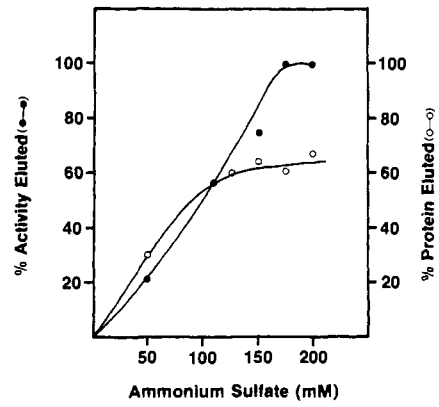

FIGURE 2: Elution of calf thymus RNA polymerase and protein from the polyethylenimine precipitate. Fraction F_1 (220 mL) was thoroughly mixed with 0.44 mL of 10% polyethylenimine and allowed to precipitate in ice for 30 min. This preparation was divided into six parts; each part was centrifuged 10 000g for 15 min and supernatants were assayed for RNA polymerase activity and protein. The RNA polymerase and protein content of the pellet was determined by subtraction of the supernatant values from that obtained for fraction F_1. The pellets were then dispersed in 7 mL of buffer C containing the indicated concentrations of ammonium sulfate. After centrifugation (10 000g for 15 min), the supernatants were assayed for protein (○) and polymerase activity (●) as described in Materials and Methods. The percent polymerase eluted is based on the maximum activity (100%) eluted at 200 mM; the maximum activity was actually 1.35× higher than the activity calculated to be present in the pellet. Percent protein represents that portion of the total protein eluted from the polyethylenimine pellet.

Appropriate conditions for the elution of RNA polymerase activity from the chalky polyethylenimine pellet were determined from the curves in Figure 2. Maximal recovery of activity was obtained at ammonium sulfate concentrations eluting 70% of the precipitated protein. At this step it is essential that the pellet be thoroughly blended with the eluting solution. Polyethylenimine precipitation does not appear to significantly stabilize calf thymus RNA polymerase since incubation in buffer for long periods prior to the elution step, i.e., 1 to 2 h, greatly reduces enzyme recovery.

The ammonium sulfate elution profile (see Figure 2) suggested that a preliminary low salt extraction of the polyethylenimine pellet would result in a substantial purification with little loss of polymerase activity. To test this, the polyethylenimine pellet was extracted with 50 mM ammonium sulfate prior to the 200 mM ammonium sulfate elution. The low salt elution removed 48% of the protein but only 18% of the total polymerase activity. It is noteworthy that polymerase I was 83% of the total polymerase present in this low salt extract (data not shown). Rapid precipitation of this extract with ammonium sulfate greatly increases the apparent overall yield of polymerase I. Thus, the inclusion of a 50 mM wash raises the apparent recovery of polymerase I to 45%, whereas the recovery of polymerase I is only 15% when extracted with 200 mM ammonium sulfate. The recoveries of polymerases II and III, however, do not appear to be substantially affected by the inclusion of a 50 mM wash. The 50 mM ammonium sulfate step is normally omitted when we want to purify either polymerase II or III.

The next step after the ammonium sulfate precipitation step in the purification of calf thymus polymerase II is chromatography on DE-52 cellulose. As shown in Table I, polymerase I and III are not retained on DE-52 when the sample is loaded at 150 mM ammonium sulfate. Moreover, when the DE-52 activity peak was assayed in the presence of α-amanitin (0.8 μg/mL), there was no detectable contamination of polymerase II by polymerases I or III. The enzyme can also be purified by batch adsorption at 150 mM ammonium sulfate. The results were essentially the same as in Table I, except that slightly more polymerase II was found in the flow through. Polymerases I and III found in the flow through were not purified further in these studies.

The DE-52 chromatography step also results in an overall increase in the total units of RNA polymerase II activity (Table I). This increase is most likely due to the removal of low molecular weight inhibitors, i.e., RNA or protein, rather than the concentration of an activator in the peak of activity. Thus, when enzyme prior to DEAE-Sephadex (Weaver et al., 1971) is sedimented on a sucrose gradient, there is again a two to threefold increase in activity; this activity is inhibited by component(s) found at the top of the gradient (S. P. Blatti, unpublished observation).

The pool of polymerase II from DE-52 was next chromatographed on phosphocellulose (P-11). The major loss in polymerase activity occurred during this step in the purification procedure. The equilibration of the resin with bovine serum albumin was essential to prevent complete loss of activity. Recovery of activity after phosphocellulose chromatography is generally 30 to 50%. Dodecyl sulfate gel analysis of the DE-52 enzyme and the phosphocellulose purified material indicates that the major protein contaminating these fractions has a molecular weight of 44 000. Furthermore, polymerase II obtained from either the DE-52 or the phosphocellulose stages is predominantly of the 11B form.

The polymerase II from phosphocellulose was further purified by Sephadex A-25-agarose A-1.5m chromatography (Figure 3). As shown in Table I, this step resulted in excellent recovery (82%) and a significant increase (1.7-fold) in specific activity of the enzyme. The behavior of polymerase II on this column is somewhat unusual since the enzyme elutes just ahead of the salt front, suggesting that it is totally included in the agarose. This phenomenon apparently results from a tight interaction between the enzyme and agarose at 150 mM ammonium sulfate (H. G. Hodo, unpublished observation).

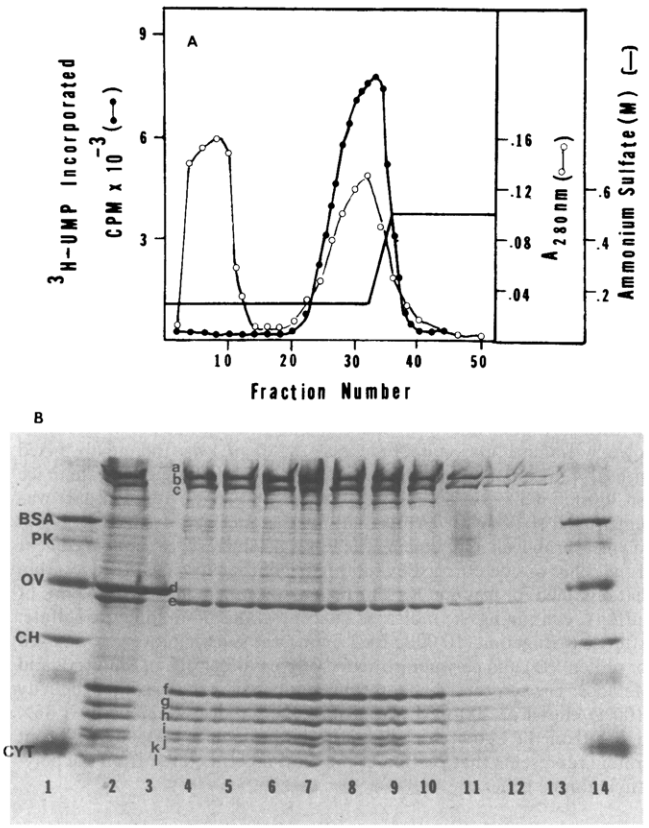

FIGURE 3: Sephadex A-25-agarose A-1.5m chromatography of calf thymus RNA polymerase II. (A) Active fractions from phosphocellulose chromatography were pooled and applied to a 2.5 × 10 cm column of A-1.5m agarose capped with Sephadex A-25 as described in Materials and Methods. The column was washed with 20 mL of 150 mM ammonium sulfate, buffer D. RNA polymerase activity was eluted stepwise with buffer D, 500 mM ammonium sulfate. The column was run at 60 mL/h and 2.0-mL fractions were collected and assayed for RNA polymerase (●) and protein (○). (B) Sodium dodecyl sulfate gel analysis of Sephadex A-25-agarose A1.5m column fractions. Aliquots (0.2 mL) from the phosphocellulose pool and the Sephadex A-25-agarose A-1.5m column fractions were precipitated with 2 mL of 10% trichloroacetic acid and analyzed on 12.5% dodecyl sulfate gels as described in Materials and Methods. Gels 1 and 14 contain protein markers bovine serum albumin, pyruvate kinase, ovalbumin, chymotrypsinogen, and cytochrome *c*. Gel 2 represents phosphocellulose purified enzyme; gels 3–13 represent fractions 8, 26, 30–34, 36, 38, and 40, respectively.

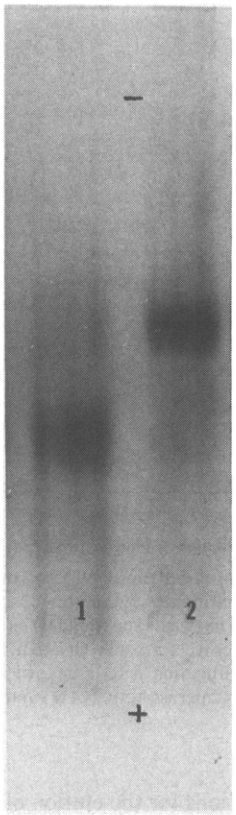

FIGURE 4: Electrophoresis of calf thymus and wheat germ RNA polymerase II under nondenaturing conditions. Seventeen micrograms of Sephadex A-25-agarose A-1.5m purified calf thymus polymerase II and glycerol gradient purified wheat germ polymerase II each were electrophoresed under nondenaturing conditions at 5 °C as described in Materials and Methods. Gel 1 contains calf thymus RNA polymerase II. Gel 2 contains wheat germ RNA polymerase II.

However, the major contaminant (component d, Figure 3B) is not retained under these conditions. Thus this step results in the purification of RNA polymerase II to near homogeneity, i.e., >96% pure, based upon density scan of gels 3–13, shown in Figure 3B. In addition, when this enzyme was subjected to electrophoresis under nondenaturing conditions, only one band was observed (Figure 4, gel 1).

Purification and Properties of Wheat Germ RNA Polymerase II. Table II summarizes the purification of polymerase II from wheat germ. The recovery of enzymatic activity at the phosphocellulose step is similar to that reported by Jendrisak and Burgess (1975).

The α-amanitin titration profiles of the pooled fractions from both the first Sephadex A-25 column (specific activity, 24.3) and the second Sephadex A-25 column were identical (see Figure 5). The enzyme was 50% inactivated at 0.271 μg/mL of α-amanitin. Wheat germ RNA polymerase II is considerably more resistant to α-amanitin than the calf thymus enzyme, i.e., 50% inhibition at 0.01 mg/mL α-amanitin (Weil and Blatti, 1975). RNA polymerase II isolated from other nonanimal sources is also more resistant than the mammalian enzyme. Polymerase II from yeast is 50% inhibited by 0.8–1.0 μg/mL α-amanitin (Valenzuela et al., 1976b; Schultz and Hall, 1976); 0.1 μg/mL α-amanitin inhibits maize polymerase II by 58%; and polymerase II from *Drosophilia melanogaster* is 50% inactivated by 0.03 μg/mL α-amanitin (Strain et al., 1971; Greenleaf and Bautz, 1975).

Figure 6 shows that potent antiserum was obtained after administration of wheat germ RNA polymerase II to chickens. When equal amounts of calf thymus and wheat germ polymerase II were each assayed with varying amounts of anti-wheat germ polymerase II γ-globulin, no significant inhibition of the calf thymus enzyme was observed. Preincubation of the enzyme and γ-globulin at 0 °C for 45 min (Ingles, 1973) instead of 37 °C for 15 min gave results identical with those shown in Figure 6. Similarly, performing the preincubation in 30 mM potassium phosphate buffer, pH 7.0, failed to demonstrate any inhibition of the calf thymus enzyme. Thus, there does not appear to be any detectable antigenic similarity between polymerase II from calf thymus and wheat germ.

Subunit Composition of Glycerol Gradient Purified Enzymes. To compare the subunit structures of calf thymus and wheat germ polymerase II, both enzymes were purified through phosphocellulose, concentrated with Sephadex A-25, and subjected to glycerol gradient centrifugation. The peak of activity obtained in the glycerol gradient was in each instance coincident with the peak of protein (Figures 7 and 8). The recoveries of calf thymus enzyme activity were variable, with an average recovery of 60%. The variability in recovery was found to be due, in part, to the fact that the enzyme rapidly

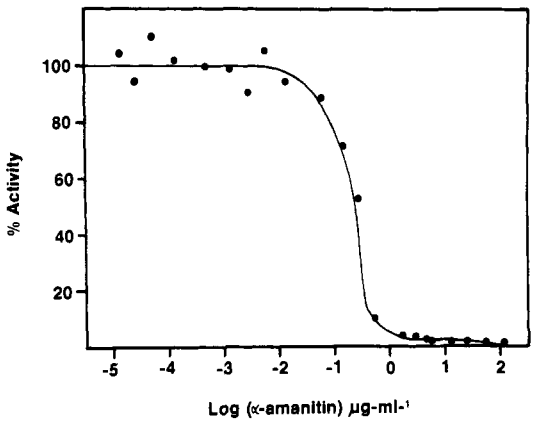

FIGURE 5: α-Amanitin titration of wheat germ RNA polymerase II. Pooled enzyme from Sephadex A-25 was assayed at 100 mM ammonium sulfate in the presence of different concentrations of α-amanitin. One hundred percent activity represents the incorporation of 0.45 μmol of UMP/10 min.

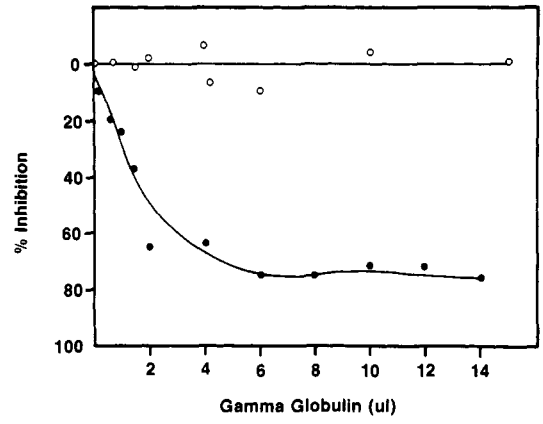

FIGURE 6: Effect of anti-wheat germ RNA polymerase II γ-globulin on wheat germ and calf thymus RNA polymerase II. Wheat germ and calf thymus RNA polymerase II (0.13 unit) were each incubated for 15 min at 37 °C in the presence of varying amounts of γ-globulin. After 15 min, nucleoside triphosphates, Mn^{2+}, and DNA were added and the respective RNA polymerase activities were determined as described in Materials and Methods. Percent inhibition is calculated with respect to control assays containing equivalent amounts of control γ-globulin. Wheat germ RNA polymerase II activity (●); calf thymus RNA polymerase II activity (○).

TABLE II: Purification of Wheat Germ RNA Polymerase II.[a]

Purification Step	Total act. (units)	Protein (mg)	Spec act. (units/mg of protein)
Crude supernatant	9631	110300	0.087
100 mM ammonium sulfate extract	10110	8810	1.14
Sephadex A-25	11193	461	24.26
Phosphocellulose	5228		
Sephadex A-25	7332	42.8	171
Ammonium sulfate concentrate	4644	24.7	188

[a] All fractions were assayed at 100 mM ammonium sulfate. The purification is based upon 1000 g of wheat germ.

loses activity after the fractions are collected. However, the peak tube exhibits a specific activity which is comparable to that obtained from Sephadex A-25–agarose A-1.5m chromatography (160 units/mg). Inclusion of bovine serum albumin (0.2 mg/mL) in gradients did not affect the variable recovery of this enzyme. Furthermore, attempts to prepare preparative quantities of polymerase II using glycerol gradients (Kedinger and Chambon, 1972) were unsuccessful. Polymerase II from wheat germ, however, is much more stable to purification by glycerol gradient centrifugation with recoveries between 90 and 100%. Wheat germ polymerase II had a specific activity of 306 units/mg and gave a single band (Figure 4, gel 2) upon gel electrophoresis under native conditions.

Dodecyl sulfate gels of the glycerol gradient purified enzymes (Figures 7 and 8) show as expected that polymerase II from both sources is composed of multiple subunits. The wheat germ enzyme is composed of predominantly form IIA, while the calf thymus enzyme is predominantly form IIB. The molecular weight of the large subunits in the wheat germ enzyme was obtained by dodecyl sulfate electrophoresis in 5% polyacrylamide gels with the following marker proteins: spectrin = 240 000 and 220 000; *E. coli* RNA polymerase β = 165 000, β = 155 000, and σ = 95 000. Although not shown, the calf thymus enzyme was analyzed on 5% polyacrylamide–dodecyl sulfate gels and the molecular weights of the large subunits are in agreement with those previously published (Blatti et al., 1970; Weaver et al., 1971; Kedinger and Chambon, 1972; Kedinger et al., 1974). To determine the molecular weight of the small subunits, glycerol gradient fractions of wheat germ and calf thymus polymerase II were each electrophoresed under denaturing conditions on 12.5% polyacrylamide gels with the following standards: bovine serum albumin (67 500), pyruvate kinase (58 000), ovalbumin (46 000), chymotrypsinogen (25 700), and cytochrome *c* (12 400). The estimated subunit molecular weights for wheat germ polymerase II (Figure 6) are as follows: *a* = 206 000, *b* = 177 000, *c* = 145 000, *d* = 47 000, *e* = 44 000, *f* = 24 500, *g* = 21 000, *h* = 19 000, *i* = 17 000, *j* = 14 000, *k* = 13 500, *l* = 12 000. The subunit analysis (Table III) for this enzyme is quite complex. These data confirm the previous studies (Jendrisak and Becker, 1974) in which the molar ratio of $(a + b)/c$ is about 1.0. This ratio suggests that polymerase II in wheat germ exists in two forms and is consistent with the idea that subunit b arises from subunit a by proteolytic cleavage (Weaver et al., 1971). Recently the proteolytic conversion of yeast polymerase IIA to IIB has been demonstrated in vitro (Dezelee et al., 1976). All of the peptides less than 50 000 daltons cosedimented with the polymerase II activity, suggesting that they are integral components of the enzyme. The only small peptides present in less than molar quantities are peptides d and e. Interestingly, the sum of their molar ratios in all scans was about 1.0. This result is consistent with the presence of either subunit d or e as part of each enzyme molecule. On the other hand, subunit d might be modified, perhaps by proteolysis, to form subunit e. The molar ratios for peptides a–k did not exhibit any substantial variation across the peak of enzymatic activity. However, peptide l (12 000) was quite variable in its molar ratio (4–11). This range of variation indicates that it may not be a subunit, but a peptide nonspecifically bound to the enzyme. The most probable subunit structure then for wheat germ polymerase IIA is the following: $206\,000_1$; 145 000; 44 000–$47\,000_1$; $24\,500_1$; $21\,000_3$; $19\,000_1$; $17\,000_2$; $14\,000_5$; $13\,500_2$. This structure would have a molecular weight of approximately 630 000. This value agrees well with the molecular weight of polymerase IIA from calf thymus and rat liver (600 000) as determined by sedimentation analysis (Kedinger

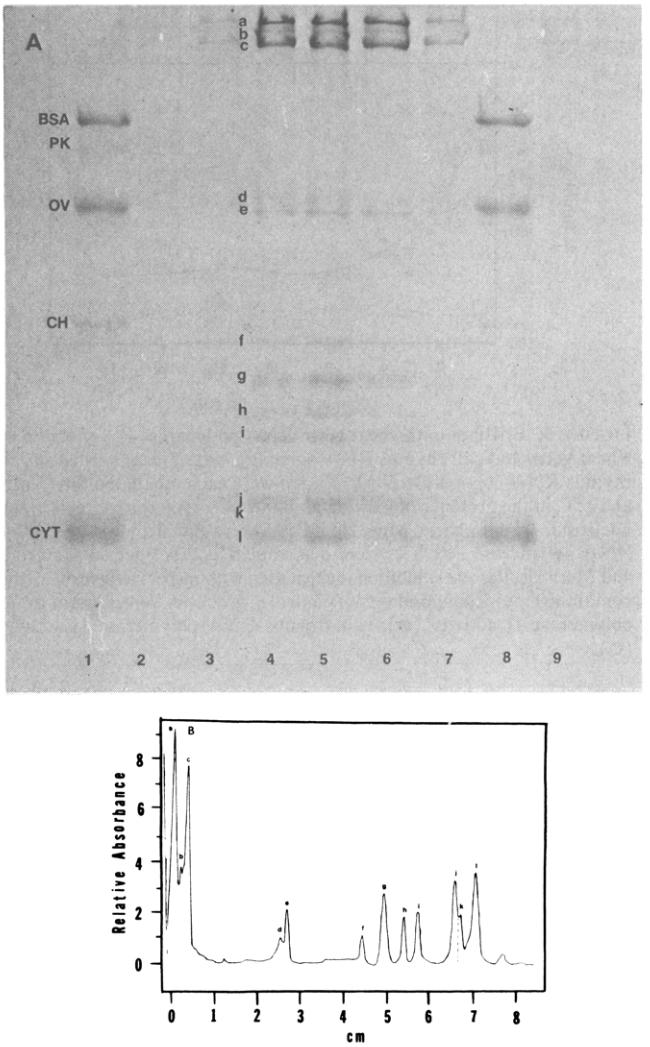

FIGURE 7: Subunit structure of wheat germ RNA polymerase II. (A) Fractions (0.15 mL) obtained from a glycerol gradient of wheat germ RNA polymerase II were each precipitated with 2 mL of 10% trichloroacetic acid and analyzed on 12.5% dodecyl sulfate gels as described in Materials and Methods. Gel 1, protein markers: BSA, bovine serum albumin; PK, pyruvate kinase; CH, chymotrypsinogen; CYT, cytochrome *c*. Gels 2–7 represent glycerol gradient fractions 6–11, respectively; gel 8, protein markers; and gel 9, glycerol gradient fraction 13. The activities of the gradient fractions in units/10 μL were as follows: fraction 6, <0.005; fraction 7, 0.04; fraction 8, 0.314; fraction 9, 0.543; fraction 10, 0.286; fraction 11, 0.08; and fraction 13, 0.02. (B) Densitometer tracing of gel 5 (glycerol gradient fraction 9).

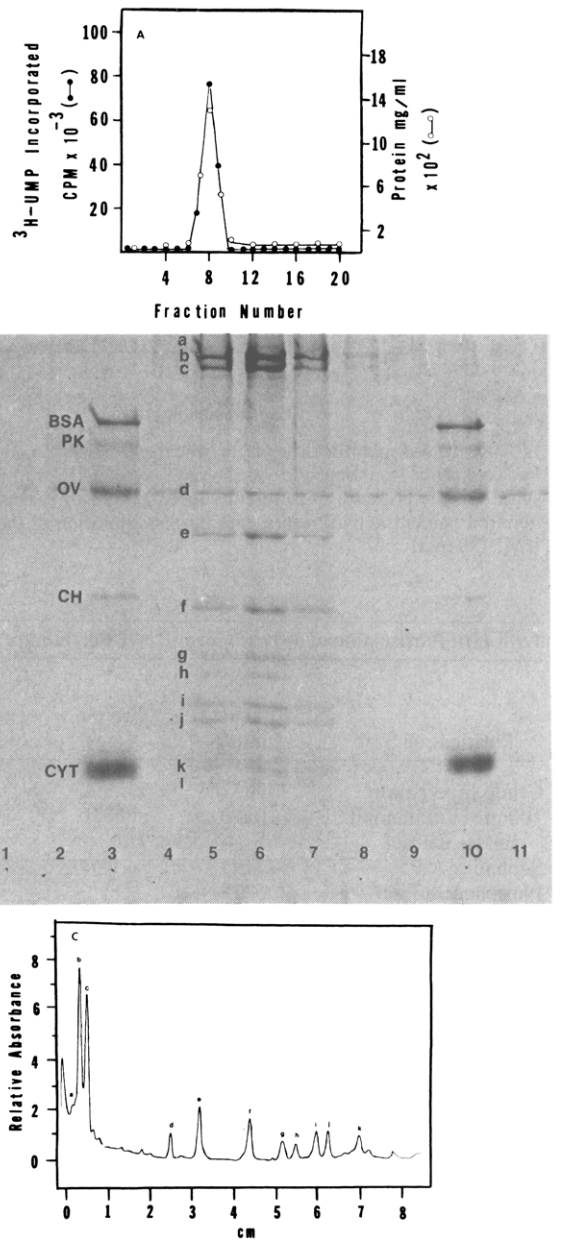

FIGURE 8: Subunit structure of glycerol gradient purified calf thymus RNA polymerase II. (A) Glycerol gradient of RNA polymerase II activity. Samples of calf thymus RNA polymerase II were layered onto 17.5 to 35% glycerol gradients (see Materials and Methods) and centrifuged for 8 h in a Spinco SW 65 rotor at 0 °C. Fractions (0.23 mL) were collected from the bottom of the tube and assayed for RNA polymerase activity (●) and protein (○). (B) Fractions (0.15 mL) obtained from a glycerol gradient of calf thymus RNA polymerase II were each precipitated with 2 mL of 10% trichloroacetic acid and analyzed on 12.5% dodecyl sulfate gels as described in Materials and Methods. Gel 1 represents fraction 3; gel 2 contains fraction 5; gels 3 and 10 contain protein markers bovine serum albumin, pyruvate kinase, ovalbumin, chymotrypsinogen, and cytochrome *c*; and gels 4–9 and 11 represent fractions 6–11 and 13, respectively, from the glycerol gradient. (C) Densitometer tracing of gel 6 (glycerol gradient fraction 8).

et al., 1974). Recently published studies of wheat germ polymerase IIA (Jendrisak et al., 1976) suggest a native molecular weight of 550 000–600 000 for this enzyme.

The estimated molecular weights for the small subunits of calf thymus polymerase IIb are e = 36 000, f = 25 000, g = 20 000, h = 18 500, i = 16 000, j = 15 000, k = 12 000, and l = 11 500. Peptide d (44 000) is most likely an impurity since (i) it is found throughout the gradient, (ii) exhibits a molar ratio substantially below unity, and (iii) can be removed by Sephadex A-25–agarose A-1.5m chromatography (see Figure 3B). The molar ratios for the individual peptides (Table III) as determined from densitometer tracings are consistent with the following structure for calf thymus polymerase IIB: 180 000$_1$; 145 000$_1$; 36 000$_1$; 25 000$_2$; 20 000$_1$; 18 500$_1$; 16 000$_2$; 15 000$_2$; 12 000; 11 500. The molar ratios of the two lowest molecular weight subunits varied between preparations and, therefore, are not listed. This consist of subunits would give a native molecular weight of approximately 550 000 which is consistent with the sedimentation analyses for this enzyme (Kedinger et al., 1974).

The subunit and molar ratios of RNA polymerase II purified through both phosphoscellulose and Sephadex A-25–agarose A-1.5m were similar to those obtained from glycerol gradient purified enzyme. Two major differences were noted: (i) the intensity of staining for peptides k and l (Figures 3B and 8B)

TABLE III: Subunit Composition of RNA Polymerase II From Wheat Germ and Calf Thymus.

	Wheat germ			Calf thymus	
Subunit	Mol wt	Molar ratio (±SE)[a]	Subunit[b]	Mol wt	Molar ratio (±SE)
a	206 000	0.95 ± 0.15 = 1.0	a	210 000	
b	177 000	0.10 ± 0.01 = 0.1	b	180 000	1
c	145 000	1	c	145 000	1
d	47 000	0.37 ± 0.06 = 1.0	d	44 000	0.50 ± 0.07
e	44 000	0.70 ± 0.06	e	36 000	1.24 ± 0.08 = 1
f	24 500	0.75 ± 0.07 = 1.0	f	25 000	1.67 ± 0.09 = 2
g	21 000	3.12 ± 0.43 = 3.0	g	20 000	0.99 ± 0.06 = 1
h	19 000	1.36 ± 0.28 = 1.0	h	18 500	0.72 ± 0.09 = 1
i	17 000	2.10 ± 0.31 = 2.0	i	16 000	1.87 ± 0.19 = 2
j	14 000	4.75 ± 0.39 = 5.0	j	15 000	1.78 ± 0.17 = 2
k	13 500	2.17 ± 0.12 = 2.0	k	12 000	
l	12 000		l	11 500	

[a] SE = standard error of the mean. [b] Subunit d, impurity.

were reversed in the agarose purified enzyme; (ii) the 25 000 molecular weight subunit (f) displays abnormal behavior when large quantities of enzyme are put on the gel (Figure 3B). Under these conditions (Figure 3B), this polypeptide moves faster with an apparent molecular weight of 20 000. However, when the same fractions are analyzed on sodium dodecyl sulfate gels, but, at lower enzyme concentrations, polypeptide f again migrates as a 25 000 molecular weight protein (data not shown) which is identical with that found in the glycerol gradient studies. Furthermore, polypeptides g and h (20 000 and 18 500) occasionally migrate as a single 19 000 polypeptide. When this occurs, the molar ratio is the sum of polypeptides g and h. The reasons for this anomalous behavior are at present unclear; however, the Tris–glycine–sodium dodecyl sulfate gel system is known to sometimes separate polypeptides with identical molecular weights but with different charge densities. Therefore, the molecular weights and molar ratios of these low molecular subunits must be interpreted with some caution.

Discussion

The results presented in this paper clearly demonstrate that large quantities of homogeneous RNA polymerase II can be purified from calf thymus using polyethylenimine precipitation. The major advantage of this method over other procedures (Weaver et al., 1971; Kedinger et al., 1972; Weil and Blatti, 1975) is that it avoids the extraction with ammonium sulfate, and the large dilutions required for the condensation and precipitation of the extracted chromatin. Thus, initial volumes are kept to a minimum which allows the processing of much larger quantities of material. Furthermore, the absence of glycerol in the early stages eliminates the need for extensive high speed centrifugation. This procedure yields 5–6 mg of homogeneous enzyme from 1 kg of tissue as compared with 3.5 mg using other methods (Kedinger and Chambon, 1972). This represents an overall yield of at least 30% (see Table I) vs. 8.6% (Kedinger and Chambon, 1972). Furthermore, the polyethylenimine procedure involves only 6 steps including one ammonium sulfate precipitation and no ultracentrifugation. In contrast, the procedure of Kedinger and Chambon (1972) achieves homogeneous polymerase II after 12 steps which includes 5 ammonium sulfate precipitation and ultracentrifugation steps. The amount of polymerase II solubilized using this procedure is quite different in wheat germ and calf thymus (see Tables I and II). Roughly three times more polymerase II per kilogram of tissue is extracted from wheat germ than from calf thymus using this procedure. Since this difference is due in part to the dehydrated nature of wheat germ, the initial polymerase II specific activities differ by a factor of two, i.e., 0.044 and 0.087 for calf thymus and wheat germ, respectively.

Polyethylenimine does not appear to selectively precipitate the cognate forms of calf thymus RNA polymerase. However, the three polymerases are not bound to polyethylenimine with identical affinity since polymerase I is preferentially eluted at 50 mM ammonium sulfate. The data demonstrate that 60% of the recoverable polymerase I activity is eluted from the polyethylenimine pellet with little contamination by polymerases II and III. Hence, this procedure is potentially useful for quickly preparing quantities of polymerase I of low specific activity without using column chromatography.

Little or no RNA polymerase II activity is apparently lost during purification until the phosphocellulose step (Table I). Despite equilibration with buffers containing bovine serum albumin and stepwise elution of the enzyme, polymerase II recovery from this column is generally no better than 35–50%. This loss of activity is apparently due to enzyme denaturation on the column since phosphocellulose columns which are run without pretreatment with bovine serum albumin result in total loss of activity. The peak of activity when assayed in the presence of the flow through or other inactive fractions is not enhanced above the original observed activity. Thus, the loss of activity does not appear to result from the removal of a loosely associated stimulatory factor. However, the removal of stability or stimulatory factors which bind tightly to phosphocellulose cannot be precluded.

Dodecyl sulfate gel analysis of Sephadex A-25–agarose A-1.5m or glycerol gradient enzyme shows that RNA polymerase II purified using these methods is almost entirely form IIB (Figures 3, 4, and 8). It is unclear whether the predominance of polymerase IIB throughout this procedure is a result of proteolysis (Weaver et al., 1971; Dezelee et al., 1976) or a stronger affinity of polymerase IIA for chromatin. An argument against the latter possibility is that, when wheat germ polymerase II (Figure 7) is purified in a similar manner, about 90% of the activity is form IIA. On the other hand, wheat germ extracts are relatively free of protease activity (Jendrisak et al., 1976) and this would favor the purification of form IIA.

The subunit analysis of calf thymus RNA polymerase IIB (Figures 3B and 8B and Table III) demonstrates the presence of multiple small subunits. The subunits smaller than 16 000

have not been observed previously in this tissue (Blatti et al., 1970; Weaver et al., 1971; Kedinger et al., 1974). These earlier studies may not have revealed these small subunits because the enzyme preparations were not examined on low porosity acrylamide gels. Kedinger et al. (1974) examined calf thymus RNA polymerase II structure using discontinuous acrylamide gels (5% at the top and 10% at the bottom). We have found that the small subunits (<16 000) are not resolved in 10% dodecyl sulfate gels and run with the tracking dye. A mixture of calf thymus RNA polymerases IIa and IIb purified using conventional procedures (Weil and Blatti, 1975; Blatti et al., 1970) exhibits the same subunit profile as observed in the enzyme purified utilizing the polyethylenimine procedure. Thus the presence of multiple small subunits in the calf thymus enzyme is not an artifact introduced by this procedure. The subunit pattern was essentially identical, except for the intensity of bands k and l, for enzyme isolated from the agarose column, glycerol gradient, or enzyme eluted from gels run under nondenaturing conditions, suggesting that the polypeptides listed in Table III are part of the enzyme. The 44 000 molecular weight band is most likely not a subunit of RNA polymerase II since it can be removed by agarose chromatography (Figure 3B).

The calf thymus enzyme does not display any cross-reactivity with anti-wheat germ polymerase II γ-globulin. This is somewhat surprising since calf thymus enzyme II has previously been reported to react with antiserum prepared against other nonmammalian polymerase II, and vice versa (Greenleaf and Bautz, 1975; Ingles, 1973). This apparent lack of cross-reactivity of catalysis inhibition may be due to structural differences between the active sites of these enzymes.

Wheat germ polymerase II is considerably more resistant to α-amanitin than is the calf thymus enzyme. Wheat germ polymerase II is inhibited by 50% at 0.271 μg/mL of α-amanitin; the calf thymus enzyme is similarly inhibited at concentrations of 0.01 μg/mL (Weil and Blatti, 1975). This difference in sensitivity to α-amanitin probably reflects structural or conformational differences between subunit c (145 000) since this subunit is the site of amatoxin interaction (Broder and Wieland, 1976).

The subunit structure reported here for wheat germ polymerase II (Table III) is similar to the structure recently published by Jendrisak et al. (1976) for this enzyme. Although both studies show 8–9 polypeptides less than 50 000 molecular weight, our preparation exhibits polypeptides smaller than 16 000 molecular weight. The reason for this discrepancy is unclear since the same gel system and acrylamide concentration (12.5%) were employed in both cases. The small differences in molecular weight could result from the use of different standards since the marker proteins used by Jendrisak et al. (1976) were not specified.

When the subunit structure of the wheat germ enzyme is compared with the calf thymus enzyme (Table III), several differences are noted. The wheat germ enzyme preparation is composed of predominately (90%) form IIA while the calf thymus enzyme is predominately form IIB. Wheat germ polymerase II contains two subunits exhibiting molecular weights of 44 000 and 47 000. Since the sum of their molar ratios is near 1, the 44 000 subunit might be derived from the 47 000 subunit. As mentioned earlier, the calf thymus enzyme does not appear to contain a subunit in this molecular weight class. On the other hand, the calf thymus polymerase II contains a 36 000 molecular weight subunit which is not present in the wheat germ enzyme. Both enzymes contain a polypeptide (f) with a molecular weight of about 25 000. Both enzymes contain a polypeptide (g) with a molecular weight of approximately 20 000. The calf thymus polypeptides g and h sometimes run as one diffuse band in this region and exhibit a molar ratio as the sum of g and h (data not shown). Whether the wheat germ band (g) which is also diffuse and exhibits a high molar ratio actually contains two polypeptides with similar molecular weight but with different charge densities awaits further experimentation. It should be mentioned that the molecular weight values for the various subunits can be directly compared between the calf thymus and wheat germ enzyme since they were run together and separately in the same slab gel, together with marker proteins.

To ascertain which subunits are an integral part of polymerase II activity, accessory control proteins, or proteins adventitiously bound to polymerase must await reconstitution studies from individual subunits. The purification scheme described here should facilitate the isolation of much larger quantities of nearly homogenous RNA polymerase II for the reconstitution studies.

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Effect of Anisomycin on the Cellular Level of Native Ribosomal Subunits†

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ABSTRACT: Treatment of Ehrlich ascites cells with anisomycin induces an almost threefold increase in the level of native 60S ribosomal subunits. This increase is not the result of an increase in rate of synthesis or transport of these subunits but is caused by a defect in the joining of the 60S subunit to the smaller initiation complex to form an 80S complex. Experimental evidence for such a blocking of the "joining reaction" could be found in the formation of "half-mer"-type oligosomes and by the release of extra 40S subunits when these oligosomes were treated with ribonuclease. Cycloheximide, an inhibitor of the translocation reaction, and inhibitors of the initiation prevent the increase of native 60S subunits induced by anisomycin. Our results imply that the increase of 60S subunits induced by anisomycin may be helpful in estimating the amount of initiating mRNAs in the cell.

It has been described by several authors that in cultured cells large pools of native ribosomal subunits are present.[1] These subunits may have an important function in the control of initiation of protein synthesis because they serve as a reservoir of initiation factors (Ayuso-Parilla et al., 1973; Freienstein and Blobel, 1975; Sundkvist and Staehelin, 1975; van Venrooij and Janssen, 1976). Under normal growth conditions the level of native subunits is fairly constant. It has been found that limitation of protein synthesis (with NaF, cycloheximide, and deprival of amino acids or glucose) does not influence the total amount of native ribosomal subunits significantly (Hirsch et al., 1973). In this paper we report the effect of anisomycin, an antibiotic isolated from cultures of various streptomyces (Sobin and Tanner, 1954), on the level of native ribosomal subunits. Anisomycin (2-*p*-methoxyphenylmethyl-3-acetoxy-4-hydroxylpyrrolidine; Beereboom et al., 1965) inhibits protein synthesis in mammalian cells but not in bacteria (Pestka, 1971). It has been shown that the transpeptidation step is reversibly inhibited by the binding of anisomycin to the peptidyltransferase center of the large ribosomal subunit (Barbacid and Vazquez, 1975). In addition to these data we now present evidence that treatment of Ehrlich ascites tumor cells with anisomycin also leads to increased levels of native 60S subunits. This effect is explained by experimental evidence that binding of native 60S subunits to the Met-tRNA$_f$–mRNA–40S initiation complex is inhibited by the antibiotic.

Materials and Methods

Materials. Tissue culture media and new-born calf serum were bought from Flow Lab Ltd. All chemicals used were of analytical grade. [5,6-^{3}H]Uridine (sp. act. 51 Ci mmol^{-1}) was bought from the Radiochemical Centre, Amersham. Anisomycin (a generous gift from Pfizer Inc.) was added to the medium as a 1 mM solution in water. All buffers were standardized to the desired pH with concentrated HCl at 4 °C, and, unless otherwise stated, all operations were carried out at 0–4 °C. Centrifugation gravity values are quoted as the average *g* values generated at particular speeds of rotation.

Cell Culture Conditions. The Ehrlich ascites tumor cells were grown in spinner culture as described earlier (van Venrooij et al., 1970) using MEM-S autopow neutralized with $NaHCO_3$ and complemented with 0.15% lactalbumin hydrolysate, 2 mM glutamine, and 10% new-born calf serum. Penicillin (100 IU mL^{-1}) and streptomycin (50 μg mL^{-1}) were routinely used. Three hours before the start of the experiment the cells were replenished with fresh medium (van Venrooij et al., 1970) and incubated at a density of 2×10^6 cells mL^{-1}.

Cell Fractionation. The cell fractionation procedure has been described in detail (van Venrooij et al., 1975). In short, cells were washed and, after swelling in a hypotonic buffer, homogenized by 5–10 strokes of a tight-fitting Dounce ho-

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[1] Native subunits are defined here as those subunits that appear as free subunits in cell extracts, in contrast to the subunits which are present in ribosomes. We refer to the smaller and larger mammalian subunits as the 40S and 60S subunits, respectively. These designations are for the purpose of identification only and do not imply accurate measurements of sedimentation coefficients.