A., Ed., New York, N.Y., Academic Press.
Hsieh, H. S. (1974), Ph.D. Thesis, University of Wisconsin, Madison, Wis.
Massey, V., and Williams, C. H., Jr. (1965), *J. Biol. Chem. 240,* 4470.
Painter, E. P. (1941), *Chem. Rev. 28,* 179.
Palmer, I. S., Fischer, D. D., Halverson, A. W., and Olson, O. E. (1969), *Biochim. Biophys. Acta 177,* 336.
Rhead, W. J., and Schrauzer, G. N. (1974), *Bioinorg. Chem. 3,* 225.
Sandholm, M., and Sipponen, P. (1973), *Arch. Biochem. Biophys. 155,* 120.
Tietze, F. (1970a), *Arch. Biochem. Biophys. 138,* 177.
Tietze, F. (1970b), *Biochim. Biophys. Acta 220,* 449.

Partial Purification and Properties of Calf Thymus Deoxyribonucleic Acid Dependent RNA Polymerase III†

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ABSTRACT: DNA-dependent RNA polymerase III (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.-7.6) has been isolated and partially purified from calf thymus tissue. Significant amounts of enzyme III are present in this tissue (up to 15% of the total activity of thymus homogenates). This enzyme has been characterized with respect to its chromatographic properties, broad ammonium sulfate optimum (0.04–0.2 M), template requirements, divalent metal optima, and its unique α-amanitin sensitivity (50% inhibition of activity occurring at an α-amanitin concentration of 10 μg/ml).

The existence of multiple forms of DNA-dependent RNA polymerases in eukaryotic cells is well established (Roeder and Rutter 1969, 1970a). Three forms of RNA polymerase (I, II, and III) were originally described in yeast (Roeder and Rutter, 1969) and later in sea urchin (Roeder and Rutter, 1970a) and in rat liver (Roeder and Rutter, 1970b). RNA polymerase I is localized in the nucleolus (Roeder and Rutter, 1970a) and appears to be responsible for the transcription of rRNA (Blatti et al., 1970; Reeder and Roeder, 1972). Polymerase I is insensitive to inhibition by high levels of the bicyclic fungal toxin, α-amanitin (Lindell et al., 1970; Kedinger et al., 1970). However, the nucleoplasmic enzyme (Roeder and Rutter, 1970a), polymerase II, is inhibited by very low levels of α-amanitin. RNA polymerase II is responsible for the synthesis of heterogeneous nuclear RNA (Blatti et al., 1970; Zylber and Penman, 1971).

The third major class of RNA polymerases, form III, has been described in a number of lower eukaryotes (Roeder and Rutter, 1969; Adman et al., 1972; Ponta et al., 1972, Roeder 1974a,b) but until recently (Sergeant and Krsmanovic, 1973; Weil et al., 1974; Weinmann and Roeder, 1974a,b) RNA polymerase III has never been reproducibly isolated from mammalian cells. We report here the isolation, partial purification, and characterization of RNA polymerase III from calf thymus tissue.

Partially purified calf thymus RNA polymerase III exhibits characteristic elution properties from DEAE-Sephadex columns, ionic strength and divalent cation optima, and template specificities. But most noteworthy is the fact that calf thymus RNA polymerase III exhibits an intermediate sensitivity to α-amanitin, with 50% inhibition of activity occurring at a concentration of 10 μg/ml. This result is in agreement with the data of Weinmann and Roeder (1974a,b). The implications of these data are that the synthesis of any particular mammalian RNA species can be titrated with α-amanitin in vivo or in vitro, and the α-amanitin concentration required for 50% inhibition of its synthesis will be characteristic of the polymerase form responsible (polymerase I, no inhibition at α-amanitin levels >250 μg/ml; polymerase II, 50% inhibition at 0.01 μg/ml; polymerase III, 50% inhibition at 10 μg/ml). This approach has been used to titrate tRNA and 5S RNA synthesis in mouse myeloma nuclei (Weinmann and Roeder, 1974a,b) and HeLa cell nuclei (Weil et al., 1974; P. A. Weil and S. P. Blatti, manuscript submitted for publication) to show that RNA polymerase III is responsible for the transcription of the genes for these low molecular weight RNAs.

Materials and Methods

Biochemicals. All chemicals used were reagent grade. Tritium labeled UTP (specific activity >20 Ci/mmol) was obtained from New England Nuclear. α-Amanitin was purchased from Henley Co., New York, N.Y., calf thymus DNA (Grade I) was from Sigma Chemical Company, and poly[d(A-T)] was from Miles Laboratories. Crystalline bovine serum albumin was purchased from Pentex-Miles.

Calf Thymus. Fresh frozen calf thymus was obtained from Dubuque Pack, Dubuque, Iowa, and maintained at −70° until use.

Solutions. All buffers were prepared from distilled water and, when present, dithiothreitol was added immediately before use.

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Buffer A is 1.0 *M* sucrose, 0.01 *M* Tris-HCl (pH 7.9), 0.025 *M* KCl, 0.005 *M* $MgCl_2$ and 0.003 *M* $CaCl_2$. Buffer B contained 12% glycerol, 0.05 *M* Tris-HCl (pH 7.9), 0.005 *M* $MgCl_2$, 0.1 m*M* EDTA, and 0.5 m*M* dithiothreitol. Buffer C contained 33% glycerol, 0.05 *M* Tris-HCl (pH 7.9), 0.005 *M* $MgCl_2$, 0.1 m*M* EDTA, and 0.5 m*M* dithiothreitol. Buffer D is the same as buffer C except that $MgCl_2$ was not added. Buffer E is the same as buffer C except that the glycerol concentration was 25%.

Solutions containing ammonium sulfate were prepared by the addition of the appropriate amount of a concentrated solution of ammonium sulfate, 4 M, pH 7.9 (adjusted at 20° with NH_4OH).

Conductivity Measurements. The ammonium sulfate concentration of a given sample was determined by taking a 10-μl aliquot, mixing with 1 ml of distilled water, and measuring the conductivity at room temperature using a Radiometer CDM2b conductivity meter. The values obtained were compared with a standard curve previously constructed using ammonium sulfate solutions of known concentrations.

Enzyme Solubilization. RNA polymerase III was isolated using an extension of the method of Benson and Blatti (manuscript submitted for publication). Frozen calf thymus (1.5 kg) was thawed in 0.15 *M* KCl and homogenized in 2 volumes of buffer A using 40-sec bursts at low, medium, and high settings in a prechilled Waring Blendor. The mixture was strained through two layers of cheesecloth and β-mercaptoethanol (5 ml/l. of mixture) was added. Ammonium sulfate was added to 0.3 *M* and the resulting slurry was homogenized in a Waring Blendor by 20-sec bursts at low, medium, and high settings. The mixture was diluted 1:3 by the addition of buffer B and homogenized in the Waring Blendor for 60 sec at the high setting. The homogenate was subsequently centrigued for 30 min at 13,000*g* (9000 rpm GS3 Sorvall rotor). The resulting pellet was discarded and solid ammonium sulfate (0.234 g/ml of solution) was added to the supernatant. After the mixture was stirred at 0° for 90 min the resulting precipitate was collected by centrifugation (60 min, 13,000*g*) and resuspended in buffer C. This solution was centrifuged for 6 hr at 95,000*g*, in a Type 35 Spinco rotor. The resulting supernatant was filtered through four layers of glass wool and immediately dialyzed, utilizing a Bio-Rad hollow fiber dialysis device. To the resulting solution was added about 150 g of DEAE-Sephadex A-25. The slurry was stirred for 6 hr and then washed by vacuum filtration on a Buchner funnel with 2 l. of buffer D made 0.05 *M* in ammonium sulfate and 2.5 l. of buffer D made 0.08 *M* in ammonium sulfate. The Sephadex was not allowed to go dry during the filtration. The final slurry was suspended in 1.5 l. of buffer D, 0.08 *M* in ammonium sulfate, and poured into a column (5 × 60 cm) and polymerase activity was eluted with buffer D made 0.8 *M* in ammonium sulfate, utilizing reverse flow chromatography. The fractions containing polymerase activity were pooled, diluted if necessary, and then applied to a phosphocellulose column (2.5 × 20 cm). Polymerases were eluted with a linear ammonium sulfate gradient in buffer D containing bovine serum albumin at 0.2 mg/ml. The appropriate fractions were pooled, dialyzed, and rechromatographed on a second DEAE-Sephadex A-25 column. This column was eluted with a linear ammonium sulfate gradient in buffer C containing bovine serum albumin at a concentration of 0.2 mg/ml.

Assay of RNA Polymerase Activity. Assays were done as previously described (Blatti et al., 1970) but in a final volume of 60 μl. The following components were contained in each assay tube in a volume of 35 μl: 3 μmol of Tris-HCl (pH 7.9), 100 nmol of $MnCl_2$, 20 μg of DNA, 36 nmol each of GTP, CTP, and ATP, 600 pmol of unlabeled UTP, and 0.5 μCi of [^{3}H]UTP. This reaction mixture contained nonsaturating levels of UTP and was used in routine assays. However, when units of enzyme activity were recorded the UTP concentration was increased to a saturating level of 6 nmol/per assay; 15 μl of buffer C containing the appropriate amount of ammonium sulfate was then added to each tube. Unless otherwise noted polymerase I was assayed at 40 m*M* ammonium sulfate and polymerases II and III were assayed at 100 m*M* ammonium sulfate. When present α-amanitin was added to give the proper final concentration. The reaction was initiated by the addition of 10 μl of enzyme in either buffer C or D and incubated at 37° for 10 min; 50-μl aliquots were withdrawn from each tube and spotted on DEAE-cellulose paper discs (DE-81 filters 2.5-cm diameter). The filters were washed six times for 5 min each in 5% Na_2HPO_4. The filters were then washed two times in distilled water, twice in 95% ethanol, and twice in ether, and finally dried under an infrared lamp. Radioactivity was determined by placing the discs in 10 ml of toluene-based fluor containing 4 g/l. of Omnifluor (New England Nuclear). Tritium counting efficiency of UMP incorporated into RNA bound to filters was 21%; after NCS (Nuclear Chicago) solubilization of RNA, the efficiency was 32%.

Measured under saturating levels of UTP in the assay, one unit of enzyme activity represents the incorporation of 1 nmol of UMP into RNA in 10 min. One nmole of UMP incorporated is equal to 5×10^4 cpm.

Sucrose Gradient Centrifugation. Tubes containing polymerase III activity from a DEAE-Sephadex column were pooled and concentrated in an Amicon ultrafiltration cell (Model 8Mc) at 35 psi using a UM-10 filter. Buffer C was added and the sample was reconcentrated until the ammonium sulfate concentration was 50 m*M*. The concentrated enzyme samples were applied to the top of 5-ml, 5–20% sucrose gradients containing buffer E, 50 m*M* in ammonium sulfate, and the gradients were spun at 65,000 rpm in a Spinco SW65 rotor for 12 hr at 4°. Fractions were collected from the bottom of the tubes and assayed for polymerase activity immediately. Sedimentation coefficients were estimated by comparison with standards as previously described by Weaver et al. (1971).

Chromatography. Phosphocellulose (P-11) was subjected to dilute acid and alkali washes before equilibration with buffer D containing 0.06 *M* ammonium sulfate. The sample previously adjusted by dilution with buffer D to 0.06 *M* ammonium sulfate was then loaded onto the column followed by 2 column volumes of buffer D containing 0.08 *M* ammonium sulfate and 0.2 mg/ml of bovine serum albumin. Polymerases were then eluted via a linear ammonium sulfate gradient of 5 column volumes (0.08–0.6 *M*) in buffer D plus 0.2 mg/ml of bovine serum albumin utilizing reversed flow.

DEAE-Sephadex (A-25) was equilibrated in buffer C containing 0.05 *M* ammonium sulfate. After pouring, all columns had a height to width ratio of at least 12:1. The samples were applied in low salt (<0.08 *M*) buffer C and, after loading, the column was washed with 2 column volumes of equilibration buffer. Polymerases were eluted via reversed flow and a linear ammonium sulfate gradient (0.1–0.8 *M*), containing bovine serum albumin of 4 column

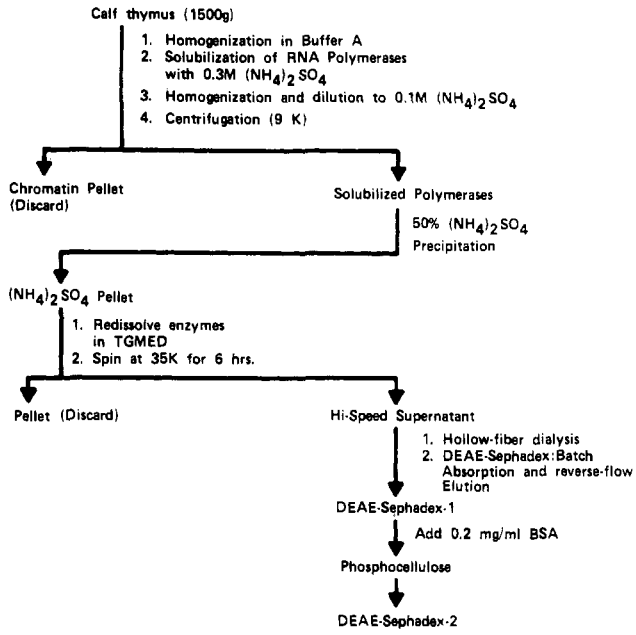

FIGURE 1: A flowsheet describing the major features in the purification scheme for calf thymus RNA polymerase III.

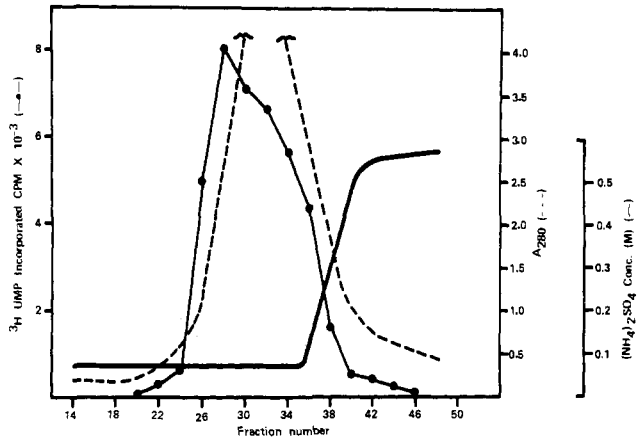

FIGURE 2: A typical DEAE-Sephadex column batch elution profile. The thymus extract from 1.5 kg of tissue after homogenization, solubilization, ammonium sulfate precipitation, and dialysis was adsorbed to 160 g of powdered DEAE-Sephadex A-25 and washed with buffer D as described under Materials and Methods. The resulting slurry was poured into a column, forming a bed of about 800 ml, 5 × 40 cm. The column was washed with buffer D, 0.08 *M* in ammonium sulfate, and step eluted utilizing reversed flow at a rate of 120 ml/hr with buffer D, 0.8 *M* in ammonium sulfate. Fractions (60) of 15 ml each were collected and aliquots were assayed for polymerase activity. (- - -) Absorbance at 280 nm, (●) polymerase activity, (—) ammonium sulfate concentration.

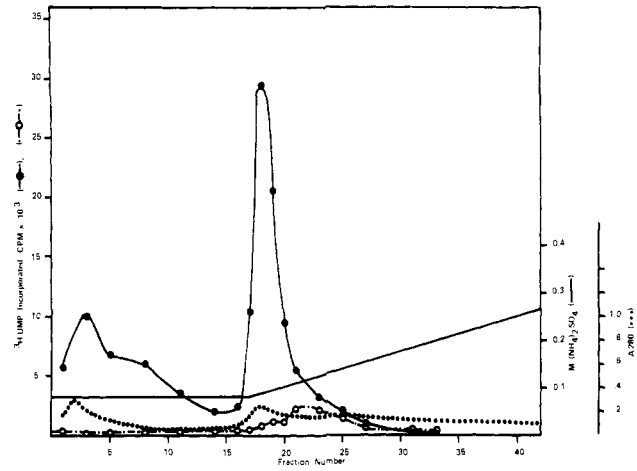

FIGURE 3: Phosphocellulose elution profile. The eluted polymerase activity from the DEAE-Sephadex batch column was diluted to 0.06 *M* ammonium sulfate with buffer D if necessary, and applied to a phosphocellulose column 2.5 × 15 cm. The column was washed with 2 column volumes of buffer D, 0.1 *M* in ammonium sulfate. Polymerase activity was eluted by a linear ammonium sulfate gradient 0.1–0.6 *M*, in buffer D containing bovine serum albumin (0.2 mg/ml). The column was run using reversed flow at a rate of 80 ml/hr and 10-ml fractions (60) were collected and assayed in the absence (●) and presence (○) of 0.5 μg/ml of α-amanitin, absorbance at 280 nm (····) and ammonium sulfate concentration (—).

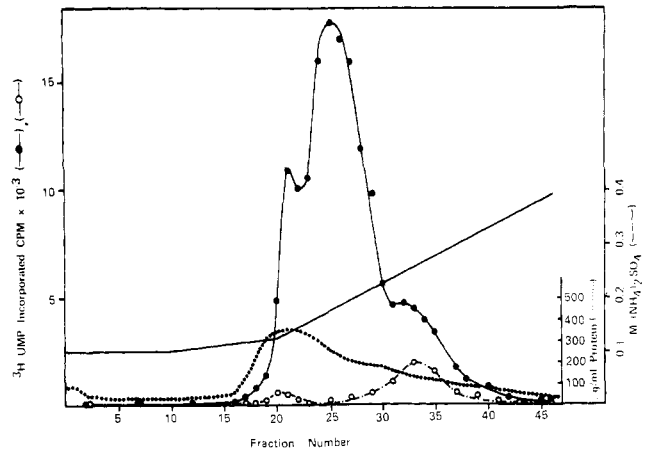

FIGURE 4: DEAE-Sephadex chromatography. The fractions from several phosphocellulose columns were pooled, dialyzed against buffer C to 0.03 *M* ammonium sulfate, and applied to a preequilibrated DEAE-Sephadex column, 1.5 × 20 cm, and the column was then washed with 2 column volumes of buffer C, 0.1 *M* in ammonium sulfate. RNA polymerases were eluted using reversed flow by a linear ammonium sulfate gradient, 0.1–0.8 *M*, and 1.5-ml fractions (60) were collected. Fractions were assayed in the absence, (●) or presence (○) of 1 μg/ml of α-amanitin; (···) absorbance at 280 nm; (—) ammonium sulfate concentration.

volumes. Fractions were collected and assayed immediately for polymerase activity.

Protein Determination. In cases where the protein content of dilute solutions were desired the Amido Schwarz method of Schaffner and Weissmann (1973) was used. In all other cases protein concentration was determined according to Lowry et al. (1951).

Sodium Dodecyl Sulfate Gel Electrophoresis. Five percent acrylamide–sodium dodecyl sulfate gels were run according to the methods of Weaver et al. (1971).

Results

Figure 1 summarizes the purification scheme for RNA polymerase III. Polymerase activity is solubilized by homogenization of the disrupted thymus tissue at high salt. Cellular debris and chromatin are removed by dilution to low salt and centrifugation. RNA polymerases are further purified and concentrated by ammonium sulfate precipitation, batch adsorption and elution from DEAE-Sephadex (Figure 2). Most of the polymerase I activity is selectively removed by the 0.08 *M* ammonium sulfate batch washes of the resin before the column is poured. Polymerase III activity although contaminated both by polymerase II and by small amounts of polymerase I is first observed during the phosphocellulose chromatography step as seen in Figure 3. Subsequently, form I activity is completely separated from

Table I: Purification of RNA Polymerase III from Calf Thymus.[a]

Purification Step	Total Units	Specific Activity (units/mg of Protein)	% Maximum Recovery
Cell homogenate	2440	0.012	
Solubilized enzyme	3000	0.016	100
After chromatin removal	2800	0.036	93
$(NH_4)_2SO_4$pellet	1360	0.038	45
High-speed supernatant	1220	0.055	41
After dialysis	720	0.034	24
DEAE-Sephadex-1	240	0.60	8
Phosphocellulose	160	9.1	5
DEAE-Sephadex-2	120	100–150[b]	4

[a] All fractions were assayed at low, medium, and high (40 m*M*, 100 m*M*, 200 m*M*) ammonium sulfate concentrations in the presence of 0, 0.5, and 133 μg/ml of α-amanitin in order to ascertain the true amount of polymerase III activity present. [b] This is the range of specific activities obtained from three preparations of RNA polymerase III. In the cases where bovine serum albumin was added during purification, the specific activity was based upon non-bovine serum albumin protein.

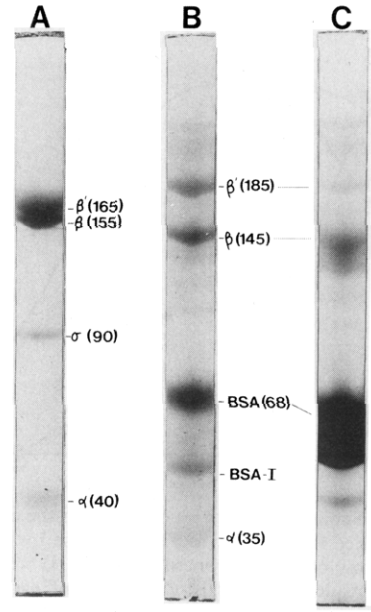

FIGURE 5: SDS polyacrylamide gel of an RNA polymerase III containing enzyme fraction. Fractions from a DEAE-Sephadex-2 column were pooled and concentrated by ultrafiltration as described in the text and electrophoresed on 8.5-cm, 5% acrylamide–sodium dodecyl sulfate gels for 3 hr and stained with Coomassie Blue. (A) *E. coli* RNA polymerase holoenzyme, (B) calf thymus RNA polymerase II, (C) calf thymus RNA polymerase III containing fraction. The direction of migration was from top to bottom. Numbers in parentheses represent approximate polypeptide molecular weights in thousands. Molecular weights were estimated by comparison with the following standards: spectrin (240,000 and 220,000), *E. coli* RNA polymerase β′ (165,000) and β (155,000) subunits, β-galactosidase (130,000), phosphorylase A (94,000), *E. coli* σ factor (90,000), bovine serum albumin (68,000), pyruvate kinase (57,000), ovalbumin (43,000), *E. coli* α subunit (39,000), and cytochrome *c* (12,500).

polymerase III by rechromatography on another DEAE-Sephadex column. As Figure 4 shows, there is a significant amount of polymerase II trailing through the peak of form III activity. This polymerase II contamination of enzyme III could not be removed using DEAE-Sephadex chromatography. However, this partially purified enzyme fraction proved very suitable for the determination of the properties of calf thymus RNA polymerase III as long as α-amanitin was present in all assays at a concentration of 0.5 μg/ml.

The steps in the isolation scheme resulting in the greatest purifications are the various columns (Table I). After the second DEAE-Sephadex column polymerase III activity has been purified approximately 10,000-fold relative to the thymus homogenate, to an estimated purity of 10–20%. These values represent rough estimates on fold purification and percent purity, taking into account the addition of bovine serum albumin to the enzyme fractions in the later stages of purification. Figure 5 shows a sodium dodecyl sulfate polyacrylamide gel of a concentrated polymerase III containing fraction and illustrates that the major contaminants are bovine serum albumin and form II enzyme. Enzyme at this stage of purity stored in buffer C at −80° was stable for at least 6 months.

Table II shows the relative amounts of the cognate forms of RNA polymerase present in calf thymus tissue. It is readily apparent that there are significant amounts of RNA polymerase III present in this tissue.

The kinetics of the RNA polymerase III catalyzed reaction is a nonlinear function of time. The reaction rate is linear to 5 min, but gradually drops to about 80% of the expected rate at 20 min. This nonlinearity cannot be corrected even if the reaction is conducted in the presence of 1 mg/ml of bovine serum albumin.

The dependence of polymerase III activity on enzyme concentration is also nonlinear, even in the presence of 1 mg/ml of bovine serum albumin. At very low enzyme concentrations activity is depressed somewhat. The reasons for this phenomenon are unknown, but it could possibly be that the polymerase is selectively inactivated at low protein concentrations or that certain enzyme cofactors are absent.

Figure 6 illustrates the ammonium sulfate titration

Table II: Relative Amounts of the Multiple Forms of RNA Polymerases in Calf Thymus.[a]

Fraction	Polymerase I[b] (%)	Polymerase II (%)	Polymerase III (%)
Homogenate	32	52	16
Phosphocellulose		85	15
DEAE-Sephadex-2		90	10

[a] All values represent the averages of at least three experiments. [b] Polymerase I has been selectively removed before the phosphocellulose and DEAE-Sephadex-2 steps.

curves of the three cognate RNA polymerase forms from calf thymus. Polymerases I and II exhibit salt optima of 40 and 100 m*M*, respectively, but most notable is the very broad salt optima exhibited by calf thymus RNA polymerase III. Form III enzyme is at least 80% active over a salt range of 40–200 m*M* ammonium sulfate. This result is in agreement with that obtained from lower eukaryotes (Roeder and Rutter, 1969; Adman et al., 1972) and mammalian cells (Sergeant and Krsmanovic, 1973).

Figure 7 depicts the divalent cation titration curve of polymerase III. Optimal activity in the presence of manganese ion is observed at a concentration of 1.6 m*M* Mn^{2+}, while for magnesium ion the optimal concentration is about 4 m*M* Mg^{2+}, resulting in a Mn/Mg activity ratio of 2.0.

The template specificity of enzyme III is represented in Figure 8. RNA polymerase III clearly exhibits optimal activity when utilizing native calf thymus DNA as template.

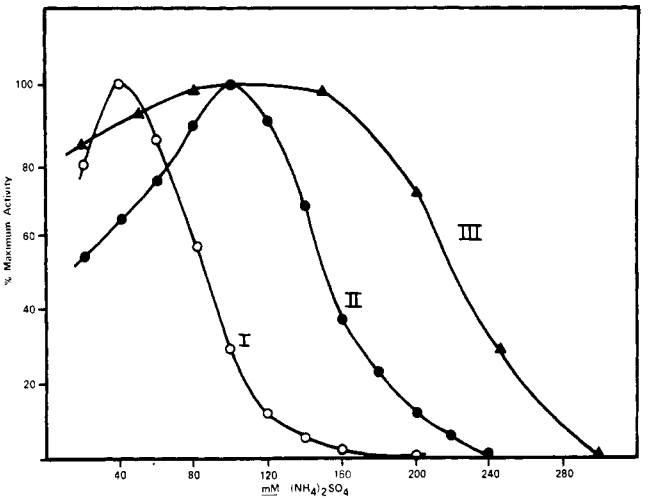

FIGURE 6: Ammonium sulfate titration curves of the cognate polymerase forms obtained from calf thymus. Polymerase I was obtained from a phosphocellulose column, polymerases II and III were from the second DEAE-Sephadex column; 10-μl aliquots of each enzyme form were assayed at the indicated ammonium sulfate concentration. Polymerase III was assayed in the presence of 0.5 μg/ml of α-amanitin. 100% acitivity for enzymes I, II, and III were respectively; 64, 550, and 26 pmol of UMP incorporated/10 min; (O) polymerase I, (●) polymerase II, (▲) polymerase III.

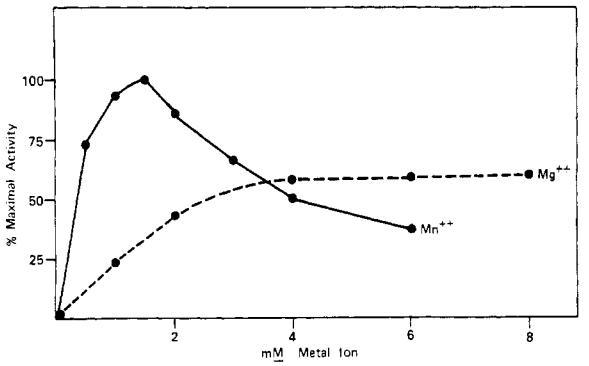

FIGURE 7: The effects of divalent cations on polymerase III activity; 10 μl of the enzyme from the second DEAE-Sephadex column was assayed at the depicted metal ion concentrations. α-Amanitin was present in all assays at 0.5 μg/ml. Activity assayed in the presence of $MgCl_2$ (●- - -●); activity measured with $MnCl_2$ in the assay (●—●). Maximal activity represents the incorporation of 27 pmol of UMP in 10 min.

In this respect calf thymus polymerase III resembles lower eukaryotic RNA polymerase III from *Xenopus leavis*, which also is more active on native DNA. The ratio of native DNA template activity to denatured DNA template activity is approximately 1.2.

Figure 9 shows the α-amanitin sensitivities of the calf thymus enzymes I, II, and III. Enzyme I is not inhibited significantly by the amatoxin even at concentrations as high as 133 μg/ml. Form II enzyme exhibits the classical α-amanitin sensitivity curve of mammalian RNA polymerase II species (Lindell et al., 1970; Kedinger et al., 1970; Cochet-Meilhac and Chambon, 1974) with 50% inhibition of activity occurring at a concentration of 0.01 μg/ml of α-amanitin. The α-amanitin titration curve for a mixture of calf thymus polymerases III and II (obtained from DEAE-Sephadex chromatography, see Figure 4) is also plotted in Figure 9. The dashed line represents the proposed titration curve of polymerase III if the sample were not contaminated with polymerase II. The exact shape of the solid line depicting the α-amanitin sensitivity of the III + II mixture varies with the amount of enzyme II contaminating the form III enzyme. In these cases the plateau region of the curve is either raised or lowered, depending upon the amount of polymerase II activity present in the sample. That this curve is indeed representative of polymerase III is also substantiated by the fact that the salt titration curve of the partially purified polymerase III is that expected from a III + II mixture when assayed in the presence or absence of α-amanitin and at different salt concentrations (data depicted in Table III). Thus, it can be clearly seen (Figure 9) that calf thymus RNA polymerase III exhibits an intermediate sensitivity to α-amanitin, with 50% inhibition of enzymatic activity occurring at a concentration of 10 μg/ml of α-amanitin.

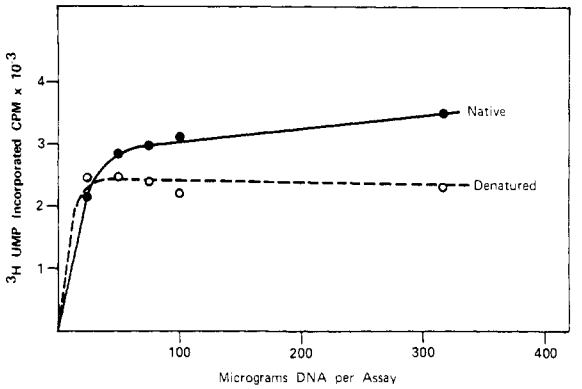

FIGURE 8: The titration of polymerase II with native and denatured DNA templates; 10-μl fractions of DEAE-Sephadex-2 enzyme were assayed in the presence of 0.5 μg/ml of α-amanitin utilizing increasing amounts of native (●—●) or denatured (O- - -O) calf thymus DNA as template.

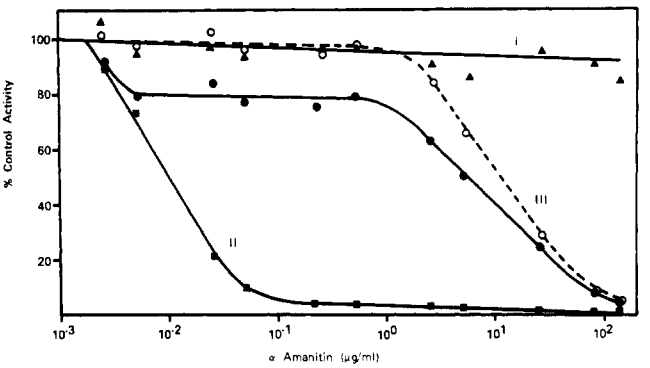

FIGURE 9: The α-amanitin sensitivities of calf thymus RNA polymerases. The source of enzymes was the same as in Figure 6; 10-μl aliquots of polymerases I, II, and III were assayed with increasing amounts of α-amanitin. (▲—▲) form I, (●—●) form II, (O—O) form III, (O- - -O) proposed RNA polymerase III inhibition curve when there is no contaminating polymerase II present in the sample. All three enzyme forms were assayed at their respective salt optima; 100% activity represents 64, 253, and 26 pmol of UMP incorporated/10 min, respectively, for polymerases I, II, and III.

Table IV describes the reaction properties of the form III enzyme. Enzyme III is DNA dependent, requires all four ribonucleotides for the synthesis of RNA, and is inhibited by actinomycin D. Using the copolymer poly[d(A-T)] as template results in a twofold increase in activity. Thus calf thymus RNA polymerase III exhibits all the properties expected from a bona fide DNA-dependent RNA polymerase.

Figure 10 shows the results of sucrose gradient centrifugation of a polymerase III + II sample. As can be seen,

Table III: Salt Titration of a Polymerase II and III Mixture in the Presence and Absence of α-Amanitin.[a]

$(NH_4)_2SO_4$ (m*M*)	Total Activity [^{3}H]UMP Incorporated (cpm)	α-Amanitin Resistant Activity (% Maximal Activity) (cpm)	α-Amanitin Sensitive Activity (% Maximal Activity) (cpm)
20	3500	1680 (90%)	1820 (32%)
60	5100	1800 (96%)	3300 (58%)
100	7500	1860 (100%)	5640 (100%)
150	3800	1880 (100%)	1920 (34%)
200	2400	1500 (80%)	900 (16%)

[a] When present α-amanitin was at a concentration of 0.5 μg/ml. Source of enzyme was a DEAE-Sephadex-2 column.

Table IV: Properties of the RNA Polymerase III Catalyzed Reaction.[a]

Reaction Conditions	[^{3}H]UMP Incorporated (cpm)
+ DNA + enzyme	2500
+ DNA − enzyme	
+ DNA + enzyme + actinomycin D	50
− DNA − enzyme	
− DNA + enzyme	140
− DNA + enzyme + actinomycin D	
+ DNA + enzyme − XTP'S	
+ Poly[d(A − T)] + enzyme	5920

[a] Poly[d(A − T)] when present in the assay was at a concentration of 100 μg/ml. All assays were conducted in the presence of 0.5 μg/ml α-amanitin. When present, actinomycin D was at a concentration of 50 μg/ml.

polymerase III exhibits approximately the same sedimentation coefficient as calf thymus polymerase II, which is about 15 S, and assuming a globular shape for the molecule, corresponds to a molecular weight around 400,000–500,000.

Discussion

In this paper we have described methods for the partial purification of DNA-dependent RNA polymerase III from calf thymus. We have shown that there are significant amounts of the enzyme present in this tissue. The enzyme can be readily solubilized and exhibits all the properties expected of true DNA-dependent RNA polymerases such as a requirement for a DNA template and all four ribonucleotide triphosphates for RNA synthesis. The exact reasons why this form of RNA polymerase has never been observed in calf thymus by other investigators are unknown, but it could be due to the fact that they chromatographed their enzyme preparations on DEAE-cellulose columns instead of on DEAE-Sephadex. On DEAE-cellulose, form III chromatographs with form I (Seifart et al., 1972, Sergeant and Krsmanovic, 1973) and therefore would not be detected unless the peak of α-amanitin resistant activity were titrated with α-amanitin or subjected to rechromatography on a DEAE-Sephadex column.

Enzyme II appears to be more stable than polymerase III as can be seen from the relative increase in the amount of form II during the purification procedure (Table II). However, the isolation procedure was originally designed for the isolation of polymerase II and modifications of the procedure to increase the yield of polymerase III are currently under study in our laboratory. Table V summarizes the properties of the three calf thymus enzymes. As can be seen

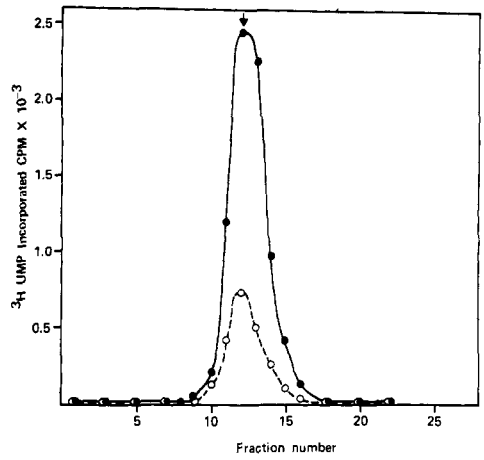

FIGURE 10: Sucrose gradient centrifugation of a calf thymus RNA polymerase III + II mixture; 5-ml, 5–20% sucrose gradients in buffer E, 50 m*M* in ammonium sulfate, were prepared and 100-μl samples were applied before centrifugation for 12 hr in a Spinco SW65 rotor at 4°. Fractions were collected from the bottom of the tubes and assayed in the absence (●) or presence (○) of 0.5 μg/ml of α-amanitin. The arrow represents the position of the peak of calf thymus RNA polymerase II when it is sedimented on a parallel 5–20% sucrose gradient. Sedimentation was from right to left.

Table V: A Comparison of the Properties Which Distinguish the Various Forms of Calf Thymus RNA Polymerase.

Property	Form I	Form II	Form III
Salt Elution from DEAE-Sephadex (*M*)	0.08	0.18	0.26
$(NH_4)_2SO_4$ optima (*M*)	0.04	0.10	0.04–0.20
Mn/Mg Ratio	2.0	10	2.0
α-Amanitin sensitivity	−	+	+
Concn required for 50% inhibition (μg/ml)		0.01	10
Native/denatured DNA activity	1.2	0.5	1.2

from the data in this table, all three enzymes exhibit distinctive properties.

Calf thymus polymerase III has properties similar to those previously described for the lower eukaryotic enzymes, such as a broad salt optimum, Mn^{2+}/Mg^{2+} ratio of about 2, elution after polymerase II on DEAE-Sephadex columns, and a preference for double-stranded DNA. However, the α-amanitin sensitivity is markedly different than that reported for the yeast form III enzyme. With 50% inhibition of activity occurring at an α-amanitin concentration of 10 μg/ml, calf thymus form III enzyme exhibits an intermediate sensitivity to this fungal toxin. Enzymes with similar α-amanitin sensitivities have been observed in rat liver cytosol (Seifart et al., 1972), in *Xenopus laevis* oocytes (Wilhelm et al., 1974), and recently in mouse myeloma cells (Weinmann and Roeder, 1974a,b). All three of the above described enzymes have properties similar to calf thymus polymerase III, and taken with the data of Sergeant and Krsmanovic (1973) describing KB cell polymerase III, seem to indicate that RNA polymerase III is present in all eukaryotic cells. This seems quite likely in view of the fact that polymerase III has recently been shown to be responsible for the synthesis of pretransfer RNA and 5S rRNA in isolated mouse myeloma nuclei (Weinmann and Roeder, 1974a,b) and in HeLa cell nuclei (Weil et al., 1974, and manuscript submitted for publication).

The molecular weight of calf thymus polymerase III, as

here determined (see Figure 10) to be approximately the same as homologous form II enzyme, is in agreement with the data of Seifart et al. (1972). They show that rat liver enzymes II and C (III) exhibit similar sedimentation rates. Sergeant and Krsmanovic (1973), however, report that KB cell RNA polymerase III sediments faster than KB cell polymerase II. The reason for the discrepancy in molecular weights in unknown, but it could be that there is a difference in the subunit composition of the enzymes or that there may be small amounts of DNA contaminating their enzyme III preparation. However, our data do not rule out the possibility that the α-amanitin resistant activity (polymerase III) here described is a small polymerase II associated polypeptide. The data from the sedimentation experiments (Figure 10) tend to rule this idea out though, because a polypeptide small enough not to affect the sedimentation rate of enzyme II (mol wt <30,000), with RNA synthesizing activity and similar properties, has never been described in the literature. Also the fact that the polymerase III activity exhibits the expected salt titration curve in the presence of polymerase II (Table III) further reinforces the concept of distinct molecular entities. Final resolution of this problem awaits purification of the form III enzyme to homogeneity and subsequent subunit structural analysis.

It is important to ascertain the subunit structure of calf thymus polymerase III since thymus is the only mammalian tissue for which the subunit structures of both polymerases I and II are currently known (Weaver et al., 1971; Kedinger and Chambon, 1972; Kedinger et al., 1974). Therefore it would be interesting to see if enzymes II and III share any subunit(s) in common and, if so, correlate this information with the α-amanitin sensitivity of these two enzymes. Also it has been reported by Kedinger et al. (1974) that enzymes I(A) and II(B) from calf thymus each contain subunits with polypeptide molecular weights of 25,000 and 16,500. It is possible that form III also contains similar small subunits and that all three major forms of calf thymus RNA polymerase share a common pool of these low molecular weight subunits.

Preliminary data also indicate that calf thymus polymerase III activity is not increased by a calf thymus factor (Stein and Hausen, 1970; Benson, Spindler and Blatti, manuscript submitted for publication) which specifically stimulates homologous RNA polymerase II activity. These data are in agreement with that of Seifart et al. (1972) who also show that rat liver enzyme C(III) activity is not stimulated by a similar factor.

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References

Adman, R., Schultz, L. D., and Hall, B. D. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1702–1706.

Blatti, S. P., Ingles, C. J., Lindell, T. J., Morris, P. W., Weaver, R. F., Weinberg, F., and Rutter, W. J. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 649–657.

Cochet-Meilhac, M., and Chambon, P. (1974), *Biochim. Biophys. Acta 353,* 160–184.

Kedinger, C., Gniazdowski, M., Mandel, J. L., Gissinger, F. and Chambon, P. (1970), *Biochem. Biophys. Res. Commun. 38,* 165–171.

Kedinger, C., and Chambon, P. (1972), *Eur. J. Biochem. 28,* 283–290.

Kedinger, C., Gissinger, F., and Chambon, P. (1974), *Eur. J. Biochem. 44,* 421–436.

Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., and Rutter, W. J. (1970), *Science 170,* 447–449.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

Ponta, H., Ponta, V., and Wintersberger, E. (1972), *Eur. J. Biochem. 29,* 110–118.

Reeder, R. H., and Roeder, R. G. (1972), *J. Mol. Biol. 67,* 433–441.

Roeder, R. G. (1974a), *J. Biol. Chem. 249,* 241–248.

Roeder, R. G. (1974b), *J. Biol. Chem. 249,* 249–256.

Roeder, R. G., and Rutter, W. J. (1969), *Nature (London) 224,* 234–237.

Roeder, R. G., and Rutter, W. J. (1970a), *Biochemistry 9,* 2543–2553.

Roeder, R. G., and Rutter, W. J. (1970b), *Proc. Natl. Acad. Sci. U.S.A. 65,* 675–682

Seifart, K. H., Benecke, B. J., and Juhasz, P. P. (1972), *Arch. Biochem. Biophys. 151,* 519–532.

Sergeant, A., and Krsmanovic, V. (1973), *FEBS Lett. 35,* 331–335.

Schaffner, W., and Weissman, C. (1973), *Anal. Biochem. 56,* 502–514.

Stein, H., and Hausen, P. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35,* 709–717.

Weaver, R. F., Blatti, S. P., and Rutter, W. J. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2994–2999.

Weil, P. A., Benson, R. H., and Blatti, S. P. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1350.

Weinmann, R., and Roeder, R. G. (1974a), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1349.

Weinmann, R., and Roeder, R. G. (1974b), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1790–1794.

Wilhelm, J., Dina, D., and Crippa, M. (1974), *Biochemistry 13,* 1200–1208.

Zylber, E. A. and Penman, S. (1971), *Proc. Natl. Acad. Aci. U.S.A. 68,* 2861–2865.