Purification of Rat Liver and Mouse Ascites DNA-Dependent RNA Polymerase I†

Michael I. Goldberg,‡ Jean-Claude Perriard,¶ and William J. Rutter*

ABSTRACT: Three forms of RNA polymerase were assayed in nuclei and nucleoli isolated from rat liver and from Krebs II ascites cells. Assays of rat liver nuclei in the absence of exogenous DNA showed polymerase I accounted for 72% of the total activity, polymerase II for 17%, and polymerase III for 11%. The total activity in ascites nuclei was similar but the ratios of polymerase activities were different: polymerase I, 53%; polymerase II, 41%; and polymerase III, 6%. These values may reflect differences in the transcriptional activity of the nuclei. After isolation of nucleoli, both rat liver and ascites polymerase I accounted for 85% of enzyme activity. When exogenous calf-thymus DNA was added to nucleoli, there was a greater than 50% increase in activity suggesting that less than one-half of the polymerase I present was bound to endogenous template. Polymerase I was solubilized from either rat liver or ascites nucleoli by sonication at high ionic strength and subsequently purified by ion filtration, phosphocellulose, sucrose gradient centrifugation, and DNA-cellulose chromatography. The essentially homogenous ascites enzyme had a specific activity of 86 units/mg when assayed with native calf-thymus DNA and of 876 units/mg when assayed with poly(deoxycytidylic acid). Electrophoresis of the enzyme in sodium dodecyl sulfate indicated the presence of six subunits with molecular weights of 205 000, 125 000, 51 000, 44 000, 26 000 and 16 000. After the same purification procedure, the rat liver enzyme had a similar specific activity (98 units/mg) on native calf thymus and 362 units/mg on poly(deoxycytidylic acid).

Ribosomal RNA synthesis is localized in the nucleolus (Brown and Gurdon, 1964; Widnell and Tata, 1966). RNA polymerase I is selectively localized within the nucleolus and available evidence suggests it is involved in the transcription of ribosomal RNA (Roeder and Rutter, 1970; Blatti et al., 1970; Reeder and Roeder, 1972; Goldberg, Bell, and Rutter, unpublished results). Several groups have succeeded in obtaining milligram quantities of polymerase I, and have reported its subunit structure (Gissinger and Chambon, 1972; Buhler et al., 1974; Gornicki et al., 1974; Schwartz and Roeder, 1974). The activity and level of polymerase I in various organisms have also been compared (Schwartz et al., 1974).

We have compared the polymerase I in normal (rat liver) and neoplastic (mouse ascites) tissues. There are striking differences in the amount of enzyme present per unit weight of protein or DNA and in the way the two enzymes are associated with their respective endogenous (chromatin) templates. Parallel studies with polymerases II and III show similar results.

Polymerase I has been prepared from isolated nuclei by a procedure that is rapid and effective, yielding 0.5–0.7 mg of polymerase I per 100 g of ascites cells. A significant feature of this procedure is the use of ion-filtration chromatography, which is rapid, convenient, and avoids dilution of the sample. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the purified ascites enzyme shows six putative subunits present in stoichiometric proportions, all of which appear to be part of the native enzyme.

Materials and Methods

Biochemicals

The following chemicals were either purchased or obtained as gifts: ribohomopolymers and unlabeled nucleoside triphosphates from P-L Biochemicals; [^{3}H]UTP and [^{3}H]GTP from New England Nuclear; α-amanitin from Henley and Company; crystalline bovine serum albumin from Pentex; native calf-thymus DNA (nCT-DNA)[1] from Sigma; poly(deoxycytidylic acid) [poly(dC)] from Dr. Fred Bollum, University of Kentucky; electrophoresis grade acrylamide, bisacrylamide, ammonium persulfate, and sodium dodecyl sulfate from Bio-Rad; special enzyme grade sucrose and ammonium sulfate [$(NH_4)_2SO_4$] from Schwarz/Mann; tetramethylethylenediamine (TMED) from J. T. Baker; DNase from Worthington Biochemicals. All other chemicals were analytical or reagent grade.

Assays for DNA-Dependent RNA Polymerase

For assays of polymerase activity during purification, the reaction mixture contained the following in a total volume of 60 μL: 3 μmol of Tris-HCl (pH 7.9), 0.1 μmol of $MnCl_2$, 0.3 μmol of NaF, 0.15 μmol of mercaptoethanol, 30 nmol of GTR, ATP, and CTP, 0.5 nmol of UTP, 6×10^5 cpm of [^{3}H]UTP, 120 μg of bovine serum albumin, and 18 μg of calf-thymus DNA. When the specific activity of each fraction was determined, the UTP concentration was increased to 0.6 mM and the [^{3}H]UTP to 6×10^6 cpm. The salt concentration in the assay medium was 30–50 mM $(NH_4)_2SO_4$ for polymerase I and 70 mM for polymerase II.

When poly(deoxycytidylic acid) was the substrate, the reaction mixture contained in a total volume of 60 μL: 3 μmol of Tris-HCl (pH 7.9), 0.1 μmol of $MnCl_2$, 0.3 μmol of NaF,

† From the Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143. *Received June 30, 1976.* Supported by National Institutes of Health Grant No. GM 21830.

‡ Present address: Genetics Program, National Institutes of Health, Bethesda, Md. 20014.

¶ Present address: Institute for Cell Biology, Swiss Federal Institute of Technology, Honggerberg, 8093 Zurich, Switzerland.

[1] Abbreviations used are: nCT-DNA, native calf-thymus DNA; poly(dC), poly(deoxycytidylic acid); TMED, tetramethylethylenediamine; SSC, 0.15 M NaCl–0.015 M sodium citrate.

0.15 μmol of mercaptoethanol, 30 nmol of GTP, 2.4×10^6 cpm of [^{3}H]GTP, 120 μg of bovine serum albumin, 2.4 μg of poly(dC), and 9 μmol of $(NH_4)_2SO_4$.

All reactions were carried out for 10 min at 30 °C. The reaction was stopped by pipetting 50 μL of the reaction medium onto Whatman DE-81 paper disks. Unreacted [^{3}H]UTP or [^{3}H]GTP was removed by extensive washing in 5% sodium hydrogen phosphate before the disks were dried and counted in 5 mL of toluene–Omnifluor.

One unit of activity is defined as 1 nmol of [^{3}H]UMP or [^{3}H]GMP, incorporated in 10 min at 30 °C.

DNA, Protein, and Contaminating Enzyme Measurements

DNA was measured according to Burton (1956). Protein was measured according to a micromodification of Lowry et al. (1951) after the material was precipitated by 10% trichloroacetic acid in the presence of 250 μg/mL poly(adenylic acid). Bovine serum albumin ($E^{1\%} = 6.6$) was used as a standard.

The presence of DNase was determined by incubation of the enzyme solution with superhelical ^{32}P-labeled SV40 DNA and measurement of the extent of conversion of SV40 DNA from superhelical form I to closed circular form II (Weil and Vinograd, 1963).

DNA exonuclease activity was monitored by incubating ^{3}H-labeled HTC DNA (a gift of Graeme Bell of this laboratory) with either the enzyme or with ribonuclease-free DNase. The incubation mixture contained in a total volume of 60 μL: 3 μmol of Tris-HCl (pH 7.9), 0.3 μmol of $MgCl_2$, 0.6 nmol of EDTA, 8% (v/v) glycerol, 3 μmol of $(NH_4)_2SO_4$, 4.5×10^3 cpm of ^{3}H-labeled HTC DNA. The reaction was started by addition of enzyme and incubated for 1 h at 23 °C. It was stopped by addition of 30 μL of sodium dodecyl sulfate to a final concentration of 0.1%. A 75-μL aliquot from each reaction was spotted on DE-81 filters, washed, and counted in the usual manner.

Protein kinase activity was determined in a 30-μL reaction mixture containing 0.15 μmol of $MgCl_2$, 1.5 μmol of Tris-HCl (pH 7.9), 6 μmol of NaCl, and 2×10^6 cpm of [α-^{32}P]ATP. When casein was used as a substrate, its concentration in the reaction mixture was 1.5 mg/ml. Reactions were carried out for 30 min at 30 °C and stopped by spotting 25 μL onto Whatman No. 3MM filter disks. Each disk was immediately suspended in 10% cold trichloroacetic acid (Cl_3CCOOH) containing a freshly prepared solution of 50 mM sodium pyrophosphate. After washing for 45 min, the solution was decanted and the filters were suspended in cold 5% Cl_3CCOOH containing 10 mM dibasic potassium phosphate and 10 mM sodium pyrophosphate. Filters were washed four times in the 5% Cl_3CCOOH solution, twice in 95% ethanol, and once in ether, dried, and counted in toluene–Omnifluor.

DNA Isolation and Solubilization

Nuclei or nucleoli were suspended in 10 vol of cold SSC (0.15 M NaCl–0.015 M sodium citrate) and then lysed by addition of sodium dodecyl sulfate to a final concentration of 1%. Following lysis, 7.5 M sodium perchlorate was added to a final concentration of 1 M and the DNA extracted according to Marmur (1961). Purified DNA was pooled and dissolved in 0.05 M Tris-HCl (pH 7.9) and 0.05 M KCl.

Calf-thymus DNA was dissolved by suspending in 0.05 M Tris-HCl (pH 7.9)–0.05 M KCl and allowing hydration to occur slowly over several days.

Preparation of Affinity Resins

Ribonucleotide homopolymers were dissolved in 0.2 M methylenesulfonic acid buffer (pH 6.0) at a concentration of 3.5 mg/mL (Wagner et al., 1971). Rat liver ribosomal RNA prepared by standard procedures was coupled to activated Sepharose in the same way. Denatured DNA-cellulose was prepared as described by Alberts and Herrick (1971). The amount of DNA bound was 0.5 mg/mL of bed volume.

Purification of Rat Liver Polymerase I

Preparation of Rat Liver Nucleoli. Frozen rat liver (480 g) (−90 °C, stored in liquid nitrogen) was pulverized and then thawed by addition of 1 L of 0.34 M sucrose containing 15 mM $MgCl_2$ and 0.25 mM spermine and maintained at 30 °C. Upon addition of the liver, the temperature rapidly dropped to 5 °C and the liver thawed in 3–5 min. Each 80 g of thawed liver was then added to 240 mL of 2.1 M sucrose containing 8 mM $MgCl_2$ and 0.12 mM spermine (Busch et al., 1967; Roeder and Rutter, 1970). The livers were homogenized by the Tekmar Dispex using a large probe set at maximum speed (Taylor et al., 1973). Homogenization took 4 min per batch. The homogenate was filtered through four layers of cheesecloth, and the filtrate poured into bottles, underlaid with 1/3 vol of 2.1 M sucrose, and spun at 37 000g for 90 min. The tightly packed, pink nuclei were taken up in 200 mL of 0.88 M sucrose–0.05 mM $MgCl_2$ (Higashinakagawa et al., 1972)–0.2% Triton X-100 and homogenized (10–12 strokes). The homogenate was then spun at 1900g for 15 min. The Triton wash was repeated (Blobel and Potter, 1966), and the nuclei were suspended in 200 mL of 0.34 M sucrose–0.05 mM $MgCl_2$ and homogenized as above. Twenty-five-milliter batches of homogenate were sonicated for 1–2 min using a Biosonik III with a medium probe set at 60% maximum intensity. The effectiveness of sonication was monitored with the light microscope. The sonicate was then layered over 0.5 vol of 0.88 M sucrose and centrifuged at 3000g for 20 min. The entire crude nucleolar pellet was taken up in 75 mL of 0.88 M sucrose–0.05 mM $MgCl_2$–0.2% Triton and 25-mL batches were sonicated for 15–30 s. The sonicate was then spun at 2000g for 30 min. The final nucleolar pellet is almost devoid of chromatin.

The nucleoli were taken up in 16 mL of TGED buffer [0.05 M Tris-HCl (pH 7.9), 25% glycerol, 0.1 mM EDTA, and 0.01 M dithiothreitol (DTT)] and thoroughly homogenized. $(NH_4)_2SO_4$ (4 M) was then added to (0.08 mL/mL of solution) to a final concentration of 0.3 M.

The slightly viscous, high salt lysate was then briefly sonicated (2×5 s with the Biosonik III medium probe set at 30% maximum intensity) and then centrifuged at 300 000g for 4 h.

Preparation of Nucleoli from Krebs Ascites Cells. Four-tenths milliliter of Krebs ascites cells was injected into the peritoneal cavity of each 25-g white mouse 8 days before the animal was sacrificed. The cell suspension from 600 mL of fluid (50 mice) was homogenized with a Potter-Elvehjem homogenizer and centrifuged at 1000g for 15 min, and the peritoneal fluid was removed by aspiration. The yield was 110 g (wet weight) of cells in the pellet. The cells were taken up in 500 mL of 0.34 M sucrose containing 0.015 M $MgCl_2$ and 0.25 mM spermine, and thoroughly homogenized. Cell lysis was accomplished in the Parr nitrogen bomb at 900 psi for 20 min (Hunter and Commerford, 1960). After release from the bomb, nuclei were collected by centrifugation at 100g for 10 min. More than 90% of the ascites cells and all the contaminating red blood cells were lysed in this manner.

To remove the outer nuclear membrane and adhering ribosomes, the nuclei were subsequently washed twice by homogenization in 5 vol (per initial wet weight of cells) of 0.88

TABLE I: Isolation of Nuclei and Nucleoli from Krebs II Ascites Cells (100 g Wet Weight) and Rat Liver (500 g Wet Weight).[a]

Step	DNA (mg)	Protein (mg)	Polymerase II (Units)	Polymerase I	
				Units	Units/mg
Ascites cells					
Homogenate	619	9 568	~455	~495	~0.05
Nuclei	658	5 874	455	493	0.08
Washed nuclei	540	3 165	292	564	0.18
Sonicated nuclei	540	384	177	623	0.18
Nucleoli	31.6	208	20	340	1.6
Rat liver					
Homogenate	986	60 600	1120	~988	~0.016
Nuclei	433	1 740	492	435	0.25
Washed nuclei	433	1 340	408	454	0.34
Sonicated nuclei	433	1 340	216	435	0.31
Nucleoli	33	98	15.5	249	2.54

[a] Nuclei and nucleoli from both ascites cells and rat liver were isolated as described in the text. Enzyme activities were determined at each step by the standard assay. Polymerase II activity was calculated from total enzyme activity in the presence and absence of 0.016 μg/mL α-amanitin. Polymerase I was defined as the activity remaining in the presence of 400 μg/mL α-amanitin. The $(NH_4)_2SO_4$ concentration for all assays was 0.07 M.

M sucrose–0.05 mM $MgCl_2$–0.2% Triton and centrifuged at 2500*g* for 15 min. The washed nuclei were suspended in 4 vol of 0.34 M sucrose–0.05 mM $MgCl_2$ and again placed in the Parr nitrogen bomb (1600 psi for 30 min). After release, the lysed nuclei were sonicated in 25-mL batches for 2 × 15 s with the Biosonik III (medium probe 150 W) to ensure complete breakage. Each 100 mL of sonicate was underlaid with 50 mL of 0.88 M sucrose–0.05 mM $MgCl_2$ and treated as described for rat liver nuclei.

Ion-Filtration Chromatography. Washed DEAE-Sephadex A-25 (250 mL) was poured into a 1.8 × 35 cm column and equilibrated with 2–3 column volumes of 0.1 M $(NH_4)_2SO_4$ in TGED. After equilibration, the column was drained until the top of the bed was just dry and the rat liver nucleolar lysate applied (0.5–1 mg of protein/mL of bed volume). The lysate was also allowed to drain into the bed. The column was then washed and the enzyme eluted with 0.3 M $(NH_4)_2SO_4$ in TGED. Fractions (3.5 mL) were collected at a flow rate of 100 mL/h. The same procedure was used for the ascites enzyme except the size of the column was 450 mL and 6.4 fractions were collected.

Phosphocellulose Chromatography. Four milliliters (for 500 g of rat liver) or 12 mL (for 100 g of ascites cells) of washed phosphocellulose were packed into a small column and equilibrated with 8–10 column volumes of 0.1 M $(NH_4)_2SO_4$ in TGED.

The enzyme pool from the ion-filtration column (40–50 mL, 0.15 M $(NH_4)_2SO_4$ in TGED) was diluted with 0.5 vol of TGED buffer to a final salt concentration of 0.1 M $(NH_4)_2SO_4$. The solution was then loaded onto the column (2–3 mg of protein/mL of bed volume) at the maximum flow rate. After the column had been washed with 2–3 column volumes of 0.1 M $(NH_4)_2SO_4$ in TGED, a 4-column volume linear gradient of 0.1–0.5 M $(NH_4)_2SO_4$ in TGED was run. Fractions of 3% of the gradient volume were collected.

The enzyme pool had a final salt concentration of 0.15–0.17 M $(NH_4)_2SO_4$. Enzyme was precipitated from solution by careful addition of 0.4 g/mL solid $(NH_4)_2SO_4$ over a period of 5–6 h. After stirring, the pellet was collected by centrifugation at 280 000*g* for 1 h. The pellet was carefully washed and then taken up in TGED (10% glycerol).

Sucrose Gradient Centrifugation. Concentrated enzyme solution (0.25 mL) was layered over 5.2 mL of a 5–20% linear sucrose gradient containing either 0.05 M $(NH_4)_2SO_4$ or 0.1 M KCl, 0.05 M Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.003 M mercaptoethanol, and 35% glycerol and centrifuged at 400 000*g* for 28 h; 0.22-mL fractions were collected.

Poly(uridylic acid) [Poly(U)]-Sepharose, Ribosomal RNA-Sepharose, or DNA-Cellulose. Columns containing 1.5–3 mL of resin were equilibrated with 0.05 M $(NH_4)_2SO_4$ or 0.1 M KCl, 0.05 M Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.003 M mercaptoethanol, and 25% glycerol. Sucrose density gradient fractions having enzyme activity were pooled and loaded directly onto a column, and washed with four column volumes of equilibration buffer. All enzyme was assumed to be bound to the column since the effluent showed no activity when assayed with both nCT-DNA and poly(dC). The enzyme was then eluted with a four column volume linear gradient of either 0.05–0.5 M $(NH_4)_2SO_4$ or 0.1–0.6 M KCl in TGED. Fractions of 0.22 mL were collected and assayed. In some cases, particularly when the poly(U)- or ribosomal RNA-Sepharose columns were used, the enzyme was step eluted with 0.35 M $(NH_4)_2SO_4$ in TGED.

Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out according to the method of Laemmli (1970). Samples were prepared for electrophoresis by precipitation with 10% Cl_3CCOOH in the presence of 250 μg/mL poly(adenylic acid). The precipitate was washed twice with a mixture of cold absolute ethanol (50%) and ether (50%) and once with cold ether. The poly(adenylic acid) was then hydrolyzed by incubation at room temperature with 2–3 μL of 8 N NaOH for 30 min. The desired volume of sample buffer [10% glycerol, 0.5% mercaptoethanol, 3% sodium dodecyl sulfate, 0.0625 M Tris-HCl (pH 6.8)] was added and the solution heated at 100 °C for 2 min before loading on the gel.

The molecular weight of the individual components was estimated from a set of proteins of known molecular weight co-electrophoresed on the slab gel. The standards included myosin (210 000), β-galactosidase (135 000), phosphorylase (95 000), bovine serum albumin (69 000), and carbonic anhydrase (29 000). The mobility of the standards was deter-

TABLE II: Distribution of RNA Polymerase Activity in Nuclei and Nucleoli.[a]

Source	I			II			III		
	Exogenous	Endogenous	Total	Exogenous	Endogenous	Total	Exogenous	Endogenous	Total
Ascites nuclei	3.9	5.9	9.8	4.5	4.6	9.1	2.3	0.74	3.1
Ascites nucleoli	5.2	2.0	7.2	0.04	0.37	0.41	0.12		0.12
Rat nuclei	0.66	0.54	1.2	1.2	0.13	1.4	0.18	0.08	0.26
Rat nucleoli	0.36	0.37	0.73		0.04	0.04		0.02	0.02

[a] Values given in units/gram of Lowry protein. Assayed with nCT-DNA under standard conditions. Nuclei and nucleoli were isolated as described. Polymerase II activity was measured as the difference in enzyme level in the presence and absence of 0.016 μg/mL α-amanitin. Polymerase III activity was measured as the difference between α-amanitin insensitive activity at 0.016 μg/mL α-amanitin and α-amanitin sensitive activity at 400 μg/mL α-amanitin. Polymerase I activity was insensitive to the presence of 400 μg/mL α-amanitin. Endogenous activity was measured by incubating nuclei with cocktail in the absence of nCT-DNA. Exogenous activity was the difference between endogenous activity and total activity measured in the presence of nCT-DNA. The total units of enzyme activity in nuclei and nucleoli were determined for an initial wet weight of 100 g of cells. A similar determination was made for the number of grams of Lowry protein or DNA per 100 g wet weight of cells. The total units of activity were divided by the Lowry protein weight and then normalized.

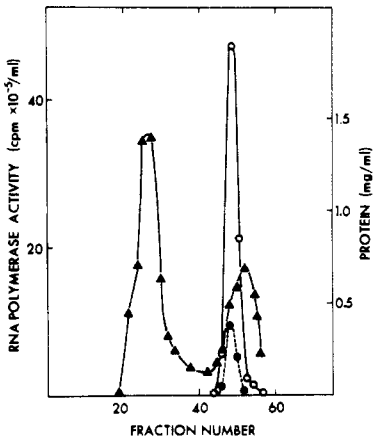

FIGURE 1: Ion-filtration chromatography of ascites polymerase I. Enzyme from 100 g of ascites cells was prepared and applied to the ion-filtration column as described in the text. Polymerase was eluted with 0.3 M $(NH_4)_2SO_4$-TGED at a flow rate of 150 mL/h and 6.5-mL fractions were collected. Every second fraction was assayed for both nCT-DNA and poly(dC) activity and the protein concentration was measured. Fractions 45–51 with a total volume of 47 mL were pooled; 180 mg of protein having 223 nCT-DNA units of polymerase I was applied to the column. Since the enzyme in the high salt nucleolar sonicate was still associated with endogenous template, the poly(dC) activity was not measured. Protein (19.4 mg) having 250.6 nCT-DNA and 965 poly(dC) units was recovered: (▲) protein; (○) nCT-DNA activity; (●) poly(dC) activity.

mined as described by Weber and Osborn (1969). Molar ratios were determined by scanning the gels at 590 nm with a Gilford spectrophotometer.

Native Polyacrylamide Gel Electrophoresis. Native gel electrophoresis was performed as described by Brown (1969). Gels were pre-run for 2 h at 10 mA/gel using 0.1 M potassium phosphate (pH 7.5)–12% glycerol before addition of sample.

One-hundred to two-hundred microliters of sample were dialyzed at 4 °C against sample buffer containing 0.05 M potassium phosphate (pH 7.0), 0.15 M mercaptoethanol, 0.1% Triton (v/v), 10% glycerol (v/v), and 0.001 M EDTA. Samples were then layered on the gels and run in 0.1 M potassium phosphate (pH 7.5), 0.05 M glycine, and 0.001 M EDTA for 10 h at 10 mA/gel at 4 °C. At the completion of the run, the gels were removed, frozen on dry ice, and then cut into 1-mm slices. Individual slices were washed 2–3 times with 0.5 mL of 0.05 M Tris-HCl (pH 7.9), 0.005 M $MgCl_2$, 25% glycerol, and 0.14 M mercaptoethanol to remove the phosphate and EDTA and then assayed with 60 μL of DNA or poly(dC) cocktail. The reaction was stopped by adding 60 μL of 5 mM EDTA and pipetting 100 μL onto DE-81 filters.

Results

Organelle Isolation. Isolated nucleoli from liver or ascites cells were lysed by addition of $(NH_4)_2SO_4$ to 0.3 M. The suspension was sonicated to release the enzyme from its chromatin template and centrifuged at 300 000g to pellet DNA and RNA. Examination of Table I shows that isolated rat nucleoli contain less than 0.2% of the protein and less than 4% of the DNA present in the rat liver cell homogenate. The removal of protein and DNA is not quite as dramatic in the isolation of ascites nucleoli (ca. 2% of the protein and 5% of the DNA remain), because of the different relationship between nuclear and cytoplasmic mass.

Determination of RNA Polymerase Activity in Nuclei and Nucleoli. RNA polymerase activity in isolated nuclei and nucleoli was assayed in the presence and absence of exogenous template. α-Amanitin was used to distinguish among the three forms of the enzyme (Schwartz et al., 1974; Weinmann and Roeder, 1974). Polymerase II was sensitive to 0.016 μg/mL α-amanitin and completely inhibited by 0.2 μg/mL toxin. Polymerase III was inhibited by 400 μg/mL while polymerase I was insensitive even at this high concentration.

Ascites cells contain 8–10 times more RNA polymerase than do liver cells, when activity is normalized to protein, or 2–3 times more polymerase when activity is normalized to DNA. The specific activities of ascites polymerases (units/gram of DNA) in nuclei are 150 for polymerase I, 139 for polymerase II, and 47 for polymerase III. The corresponding activities in rat nuclei are 74, 84, and 16, respectively.

The distribution of activity between endogenous and exogenous template is not only different for the two cell types but also changes for each enzyme during the course of the isolation procedure (Table II). Sonication affects the interaction of previously bound enzyme with template as shown by the change in the ratio of endogenous to exogenous polymerase I activity between nuclei and nucleoli (Table II). The finding that the poly(dC) activity is greater in ascites nucleoli than it is in rat liver nucleoli (Table I) demonstrates that the relationship between bound and unbound activity represents the difference between template-associated and free enzyme. In addition, sonication destroys 50% of the activity of rat liver polymerase II, suggesting that enzyme not associated with

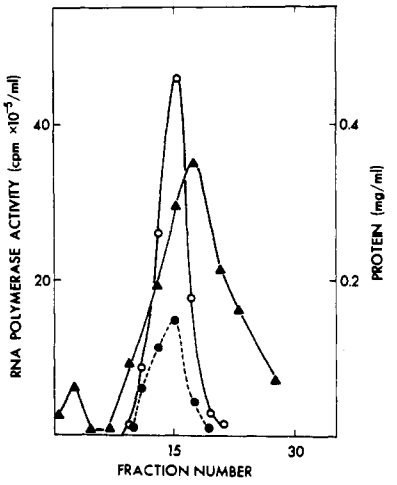

FIGURE 2: Phosphocellulose chromatography of ascites polymerase I. The pool from the ion-filtration column (47 mL) was diluted with 22 mL of TGED to a final concentration of 0.1 M $(NH_4)_2SO_4$. It was loaded onto a 12-mL (0.9 × 20 cm) phosphocellulose column equilibrated with 0.1 M $(NH_4)_2SO_4$-TGED and eluted as described in the text; 1.5-mL fractions were collected. Fractions 10–18 were pooled; 250 units of nCT-DNA and 965 units of poly(dC) activity were applied to the phosphocellulose column; 155 units of nCT-DNA and 1039 units of poly(dC) activity were recovered. Assays of DNA content and endogenous activity showed none above background; 2.8 mg of protein was recovered: (▲) protein; (○) nCT-DNA activity; (●) poly(dC) activity.

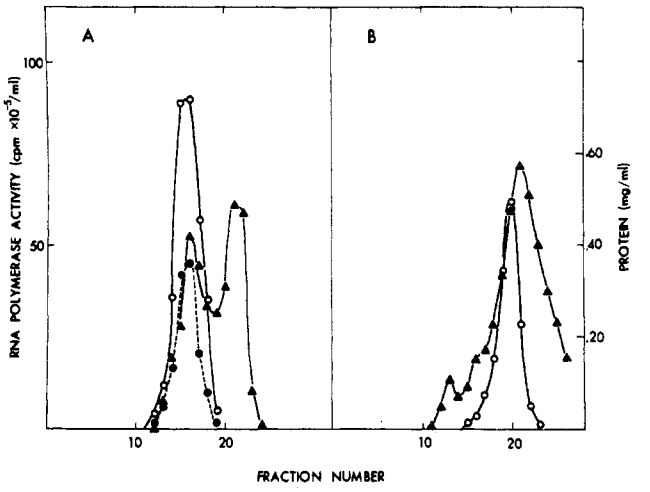

FIGURE 3: Sucrose gradient centrifugation of polymerase I. (A) Sucrose density gradient centrifugation of ascites polymerase I. The ammonium sulfate precipitate from the phosphocellulose column pool was pelleted by centrifugation. The protein pellet was taken up in 0.4 mL of TGED and layered in 0.25-mL aliquots onto 5–20% sucrose gradients in TGED (35% glycerol). The gradients (total volume 5.8 mL) were spun in the SW65 rotor for 24 h at 420 000*g*. After completion of the run, 0.2-mL fractions were collected and assayed for enzyme and protein activity. Fractions 13–18 were pooled for a total volume of 2.2 mL; 70.2 units of nCT-DNA and 575 units of poly(dC) activity were recovered. The protein concentration of the pool was 0.9 mg. (B) Sucrose density gradient centrifugation of rat liver polymerase I. Polymerase I from 1000 g of rat liver was purified according to the same protocol as described for the ascites enzyme, except that the sucrose gradient centrifugation was carried out at 270 000*g* for 24 h; 0.5-mL fractions were collected; 2.4 mg of protein containing 111 units of nCT-DNA activity and 696 units of poly(dC) activity was loaded onto two gradients; 0.82 mg of protein having 80 units of nCT-DNA activity and 296 units of poly(dC) activity was recovered: (▲) protein; (○) nCT-DNA activity.

endogenous template is particularly sensitive to such treatment.

Polymerase III activity represents from 18 (rat) to 24% (ascites) of the low α-amanitin insensitive activity (Table II). Although the greater proportion of polymerase III in ascites is reproducible, there is considerable variation. Polymerases II and III can be easily leached from the isolated nuclei because they are primarily not bound to template. This correlates with the observation that when nuclei are assayed after washing with 0.2% Triton, a procedure which removes the outer nuclear membrane, the polymerase II activity is reduced to between 70 and 80% of that of control (Table I).

Ion-Filtration Chromatography. Ion-filtration chromatography combines the techniques of gel filtration and ion-exchange chromatography (Kirkegaard et al., 1972; Kirkegaard and Agee, 1973). The enzyme in a solution less than 10% of the column volume and of high ionic strength (0.35 M $(NH_4)_2SO_4$) is applied to a column of DEAE-Sephadex equilibrated with a solution of low ionic strength. The equilibrating ionic strength solution is chosen so that the enzyme elutes after the excluded peak of basic proteins and in the sieving range of the gel. By adjusting the pH and ionic strength both ascites and rat liver polymerase I can be made to elute after the bulk of the protein (90%) and before the salt peak (Figure 1). Under these conditions, nucleic acids interact strongly with the ion exchanger and 90–95% of the residual DNA is separated from the enzyme. This is evident in the corresponding decrease in the amount of polymerase activity that is independent of exogenous DNA. Activity assays show that the enzyme at this stage can transcribe effectively all three templates, native calf-thymus DNA, ascites or liver nucleolar DNA, and poly(dC) (Table IV).

Phosphocellulose Chromatography. Enzyme is eluted with a linear gradient as shown in Figure 2. More than 90% of the protein and the residual DNA is removed by this procedure. The nCT-DNA activity and the ratio of nCT-DNA/poly(dC) activity are always shifted toward the high ionic strength side of the peak and increase with increasing fraction number. Careful examination of the yield, as determined using the three templates, shows that the recovery is less when assayed with nCT-DNA than when assayed with poly(dC) or nucleolar DNA (Table IV). These data suggest the possible loss of a protein cofactor that affects activity on nCT-DNA.

Sucrose Density Gradient Centrifugation. The distribution of enzyme activity and protein after sucrose gradient centrifugation is shown in Figure 3. Although the enzymes from both rat liver and ascites have the same specific activity prior to this step, there is little correspondence between the rat liver enzyme activity and protein distribution (Figure 3B). On the other hand, the activity peak of ascites enzyme is coincident with a protein peak (Figure 3A). As occurs on the phosphocellulose column, there is a distinct shift in the ratio of nCT-DNA/poly(dC) activity toward the rear of the gradient.

In Table III, the effect of a series of variables on the recovery of enzyme activity is compared in two separate experiments. As has been noted by other workers (Schwartz and Roeder, 1974; Sugden and Keller, 1973), a higher initial protein concentration leads to a greater recovery of activity (Table III, A–D). High glycerol concentrations (Table III, B and C) and shorter centrifugation times (Table III, C and D) also lead to better recovery. In contrast, a single enzyme concentration, a longer centrifugation time, or the presence of Mg^{2+} (Table III, E–H) leads to a lower recovery of activity.

Experiments (Goldberg et al., 1977) in which fractions from the lighter part of the gradient are added to those containing enzyme result in a stimulation of activity on nCT-DNA, but not when assayed with poly(dC). The extent of stimulation is

TABLE III: Recovery of Enzyme Activity after Sucrose Gradient Centrifugation.[a]

	Enzyme Load (mg/mL)	Glycerol (v/v %)	$MgCl_2$ (mM)	Time (h)	% Recovery of Act. (nCT-DNA)
A	1	35	5	14	105
B	1.2	35	5	24	65
C	1.2	25	5	8	66
D	1.4	25	5	16	11
E	1.0	25	5	12	57
F	1.0	25		12	98
G	1.0	25	5	16	24
H	1.0	25		16	48

[a] Rat liver polymerase I was employed for all experiments concerned with the effect of a series of variables on the recovery of enzyme activity after sucrose gradient centrifugation. The enzyme pool from phosphocellulose chromatography was concentrated by $(NH_4)_2SO_4$ precipitation as described in the text. The precipitate was collected by centrifugation and taken up in 0.5 mL of TGED. The protein concentration was 6.7 mg/mL and the ionic strength 0.1–0.15 M $(NH_4)_2SO_4$. Dilutions were made with 0.1 M $(NH_4)_2SO_4$ in TGED. Sucrose gradients (5–20%) were prepared in 0.1 M KCl in TGED containing either no extra cofactors, 0.005 M $MgCl_2$, or 35% glycerol. Centrifugation was for the indicated time at 400 000*g*. Assays with nCT-DNA have been described. From 3.7 to 7.5 units of nCT-DNA activity was applied to each gradient.

TABLE IV: Purification of Polymerase I from Krebs II Ascites Cells.[a]

Step	Total Protein (mg)	nCT-DNA			Nucleolar DNA			Poly(dC)			nCT-DNA/Poly(dC)	Nucleolar DNA/Poly(dC)
		Units	Yield	Sp Act. (Units/mg)	Units	Yield	Sp Act. (Units/mg)	Units	Yield	Sp Act. (Units/mg)		
1. Ion-elution chromatography	24	247	72.5	10.3	120.8	92.3	5.0	1456	121	60.7	0.17	0.08
2. Phosphocellulose chromatography	3.6	166	49	46.1	110.7	84.6	30.8	1085	90.5	301	0.15	0.10
3. Sucrose density gradient chromatography	0.99	73.9	21.8	74.6	66	50.5	66.7	640	53.3	646	0.12	0.10
4A. Poly(U)-Sepharose chromatography	0.23	9.0	2.6	39.3	18.6	14.2	80.8	454	37.8	1973	0.02	0.04
4B. DNA-cellulose chromatography	0.37	31.9	9.4	86.2	34.6	26.4	93.5	325	27.1	876	0.10	0.11

[a] 100 g of cells. The number of units of enzyme activity (as assayed with each template) and of protein concentration is an average value calculated from 12 separate preparations. Ascites cells (100 g, wet weight) were used as the starting material. The details of the purification procedure and the assays are described in the text.

proportional to the concentration of enzyme loaded onto the gradient.

Poly(uridylic acid)-Sepharose. Poly(U)-Sepharose was chosen because it can function either as a cation exchanger or as an affinity column, and therefore provides a binding environment different than phosphocellulose. In addition, the use of a homopolymer minimizes the number of different sites of varying affinity to which polymerase can bind.

The fractions from the sucrose gradient containing enzyme activity were pooled, applied directly to a poly(U)-Sepharose column, and then eluted with a linear salt gradient. The extent of purification and the distribution of activity are seen in Figure 4A and Table IV. Approximately 70% of the protein is removed, leading to a three-fold increase in specific activity when the enzyme is assayed with poly(dC). However, only 12% of the nCT-DNA activity is recovered, so that no specific activity increase is observed with this template. It is significant that the ratio of nCT-DNA to poly(dC) activity, which was 0.10–0.11 prior to chromatography, has undergone a fivefold decrease to 0.02. Once again, there is a shift in template activity across the enzyme peak, although the purification is so extensive that both activities now coincide with the peak of protein. Sodium dodecyl sulfate–polyacrylamide gels of individual fractions show a change in subunit stoichiometry that appears to correlate with the change in activity ratio (see below).

Ribosomal RNA-Sepharose. A loading and elution procedure similar to that for poly(U)-Sepharose was employed for ribosomal RNA-Sepharose columns. In Figure 4D and Table V, the distribution of activity and protein for polymerase I from rat liver is shown. As has been noted for the chromatography of ascites enzyme on poly(U)-Sepharose, the ratio of nCT-DNA/poly(dC) activity has changed from that of the load (from 0.27 to 0.04) and the peak of nCT-DNA activity is shifted toward the region of higher ionic strength. Although there is general coincidence of protein and enzyme activity, the specific activity varies considerably across the peak.

DNA-Cellulose. The enzyme bound at low ionic strength to DNA-cellulose and then eluted with a linear gradient of $(NH_4)_2SO_4$. Although there was not a large change in specific activity, 30–40% of the protein present in the load was removed (Table IV). The nCT-DNA/poly(dC) ratio did not change and is reflected in the juxtaposition of the two peaks of enzyme activity (Figure 4B). The coincidence of both enzyme activities with the protein peak suggests the presence of few impurities. A sodium dodecyl sulfate gel of the enzyme pool shows six

TABLE V: Purification of Polymerase I from Rat Liver.[a]

Step	Total Protein (mg)	nCT-DNA			Poly(dC)			nCT-DNA/Poly(dC)
		Units	Yield	Sp Act. (Units/mg)	Units	Yield	Sp Act. (Units/mg)	
1. Ion-elution chromatography	10.6	133	59	12.5	955	100	90	0.13
2. Phosphocellulose chromatography	1.4	67	27	47.8	445	47	317	0.15
3. Sucrose density gradient centrifugation	0.82	80	32	98	297	31	362	0.27
4. Ribosomal RNA-Sepharose chromatography	0.2	3.2	1.2	16	89	0.3	445	0.04

[a] The values given in the table for steps 1-3 are an average calculated from 6 preparations. The starting material was 480 g of frozen rat liver. The livers were thawed and the enzyme purified as described in the text. Ribosomal RNA-Sepharose chromatography was performed on a single preparation.

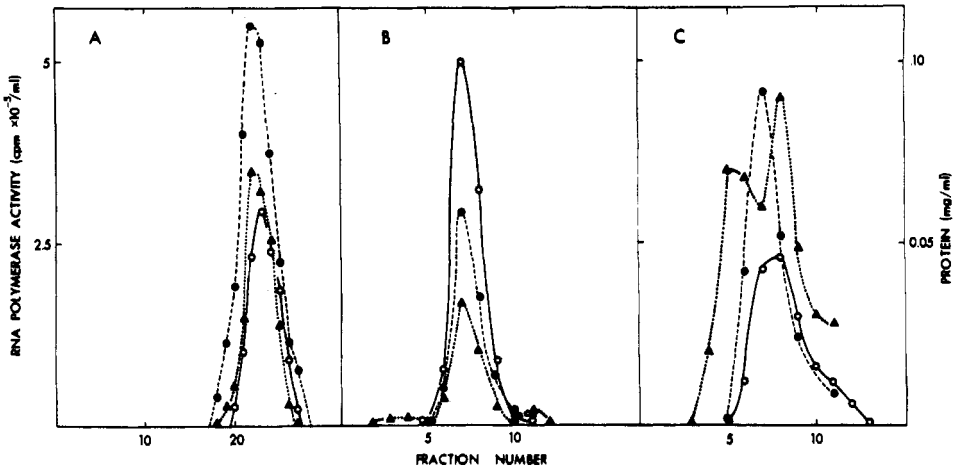

FIGURE 4: Affinity column chromatography of polymerase I. (A) Poly(uridylic acid)-Sepharose chromatography of polymerase I. The active fractions from a sucrose density gradient were pooled, diluted with 0.05 M Tris (pH 7.9) to lower the glycerol concentration to 25%, and applied to a 2-mL poly(uridylic acid)-Sepharose column equilibrated with 0.05 M $(NH_4)_2SO_4$-TGED. The column was washed with the equilibration buffer and the enzyme eluted with a 4 column volume linear gradient of 0.05-1.0 M $(NH_4)_2SO_4$-TGED; 0.4-mL fractions were collected at a flow rate of 10 ml/h; 0.45 mg of protein containing 35 units of nCT-DNA and 288 units of poly(dC) activity was applied to the column. The pool contained 4.8 units of nCT-DNA activity, 186 units of poly(dC) activity, and 0.1 mg of protein. (B) DNA-cellulose chromatography of ascites polymerase I. A 1.7-mL column of calf-thymus denatured DNA-cellulose was prepared and equilibrated as described in the text. The enzyme pool (1.1 mL) from a sucrose gradient was diluted with 0.05 M Tris (pH 7.9) to reduce the glycerol concentration to 25% and then applied to the column. The column was washed with the equilibration buffer. After 2 column volumes of buffer, the enzyme was eluted with a 5 column volume linear gradient of 0.05-1.0 M $(NH_4)_2SO_4$-TGED; 0.22-mL fractions were collected; 0.47 mg of protein was applied containing 36.7 units of nCT-DNA and 315 units of poly(dC) activity. The eluate contained 0.30 mg of protein, 22.3 units of nCT-DNA, and 251 units of poly(dC) activity in a total volume of 1.0 mL. The RNA polymerase activity units and protein concentration should be multiplied by a factor of 20 to reflect the actual values in a typical experiment. (C) rRNA-Sepharose chromatography of rat liver polymerase I. A 1.5-mL column of rRNA-Sepharose was packed and equilibrated with 0.05 M $(NH_4)_2SO_4$-TGED. The enzyme pool from a sucrose density gradient was loaded onto the column and the column washed with the equilibration buffer. The enzyme was step eluted with 0.35 M $(NH_4)_2SO_4$ and collected in 0.2-mL fractions. The load contained 0.4 mg of protein, 19.7 units of nCT-DNA, and 74 units of poly(dC) activity. The enzyme pool contained 1.6 units of nCT-DNA, 44.5 units of poly(dC) activity, and 0.1 mg of protein in a total volume of 1.2 mL. The RNA polymerase activity should be divided by a factor of two, and the protein concentration multiplied by a factor of two to reflect the actual values in a typical experiment: (▲) protein; (○) nCT-DNA activity; (●) poly(dC) activity.

protein bands (see below). Their stoichiometry is relatively constant across the peak.

Sodium Dodecyl Sulfate Gel Electrophoresis. Enzyme purification and subunit structure were monitored by sodium dodecyl sulfate gel electrophoresis. The gel patterns show that the high molecular weight subunits are visible when the ascites enzyme has been purified through the ion-elution column step. The low molecular weight subunits do not become distinguishable until either sucrose density (Figures 5a and 6A) or DNA-cellulose chromatography (Figures 5b and 6B) has been completed. A scan of the enzyme pool from a DNA-cellulose column shows six protein bands (Figure 5, c-h). Their molecular weights and stoichiometry are given in Table VI, part A. In Table VI, part B, are the molecular weights and stoichiometry for an enzyme pool from a poly(U)-Sepharose column (Figure 6C). All six protein bands are still present, but the molar ratio of the 51 000 molecular weight band has decreased from 1.0 to 0.66. There is also a decrease in the ratio of the 44 000 band from 1.0 to 0.85. A scan across the poly(U) column (Table VI, part B) reveals a marked change in the amount of the band at 52 000 that correlates with the increase in activity when nCT-DNA is used as a template. Although the agreement is not quantitative (the increase for the protein is twofold, while that for activity is fourfold), it certainly

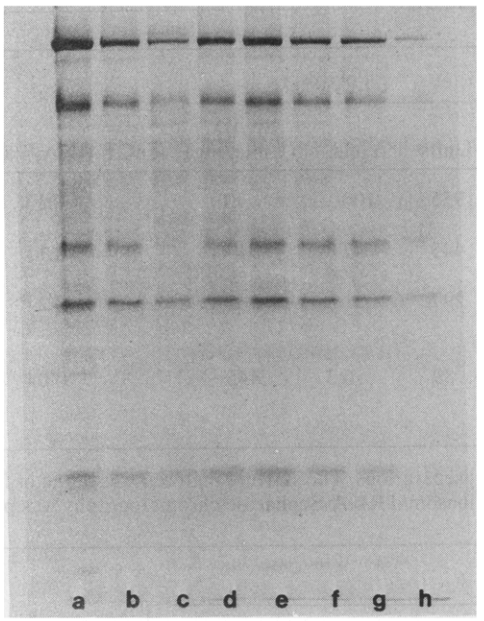

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ascites polymerase I. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ascites polymerase I was performed in the Hofer gel apparatus. The ascites enzyme was purified as described in the text. The enzyme from the pool of the sucrose gradient centrifugation is shown in a and the pool enzyme from the DNA-cellulose column in b; c–h are the shoulders and peak fractions of a typical DNA-cellulose column. The enzyme pool applied to the column contained 29 units of nCT-DNA and 306 units of poly(dC) activity. The eluate contained 11.2 units of nCT-DNA and 222 units of poly(dC) activity.

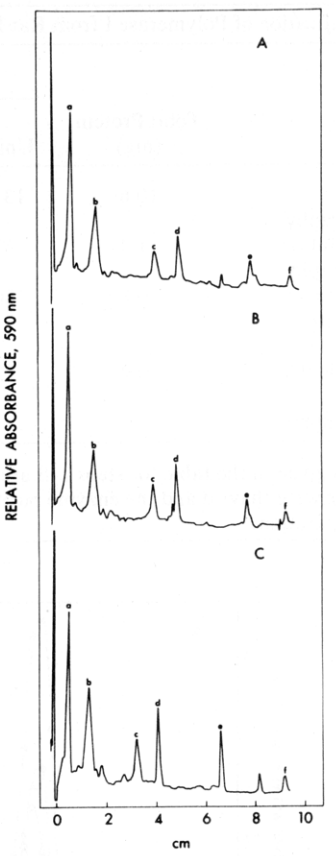

FIGURE 6: Gel scans of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of polymerase I. (A) Ascites polymerase I from the pool of the sucrose density gradient was prepared for sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described in the text. After completion of the run and staining, the strip containing the sucrose gradient enzyme was excised and then scanned at 590 nm using the linear transporter of the Gilford spectrophotometer. Full scale optical density was 1 OD unit. Subunits are labeled a–f in order of decreasing molecular weight. (B) Ascites polymerase I from the pool of a DNA-cellulose column. Full scale was 0.5 OD unit. (C) Ascites polymerase I from the pool of a poly(U)-Sepharose column. Full scale was 0.4 OD unit.

suggests that the presence of this subunit affects the activity of the enzyme. This can be clearly seen if the nCT-DNA/poly(dC) activity ratio is examined as a function of the distribution of enzyme across the column.

A similar series of gels has been used to examine the purification of the enzyme from rat liver. The two high molecular weight bands are easily discernible; it is not easy to detect bands of low molecular weight, even after DNA-cellulose chromatography. Since the specific activity of the rat enzyme at comparable stages in the purification is similar to that of the ascites polymerase (see Tables IV and V), the lower degree of purity of the rat polymerase implies a more active enzyme. One explanation of such high activity is the presence of a protein or other cofactor in the rat preparation that is capable of activating the enzyme. Preliminary experiments in which the rat and ascites enzymes were mixed suggest that some component in the rat preparation is capable of stimulating the activity of the ascites enzyme when native calf-thymus DNA is used as a template.

Native Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was also used as a means of investigating enzyme heterogeneity. Assay of either the rat liver or ascites polymerase gels showed a single peak of enzyme activity (Figure 7A). Both the nCT-DNA and poly(dC) activities were coincident with each other. Their co-electrophoresis strongly implies that they represent different polymerizing functions of the same protein molecule. In addition, a poly(dC) activity has been found to be associated with polymerase II and is inhibited to the same extent as the nCT-DNA activity by α-amanitin and the eucaryotic polymerase inhibitor, rifampicin AF/013 (Meilhac et al., 1972).

Rat liver polymerase II was also electrophoresed under the same conditions. Assay of the gel revealed the existence of two peaks of activity (Figure 7B). Changing the time of electrophoresis and the porosity of the gel shifted the mobility but did not affect the ratio of the two activities.

Assay for Contaminating Enzyme Activities. Assay of the DNA-cellulose fraction of ascites polymerase I shows that there is no detectable DNA endonuclease above background and less than 0.1% contamination of enzyme protein. Similar experiments indicate very low degrees of contamination with RNA endonucleases (equivalent to less than 0.1 ng of RNase A/μg of polymerase).

Assays for autophosphorylation and transfer of phosphate to casein were determined as a function of purification. The data suggest that the enzyme has a low level of protein kinase activity that remains constant throughout purification. Although the endogenous substrate is removed by the sucrose gradient step so that the specific activity for autophosphorylation decreases tenfold, the specific activity for casein phosphorylation remains the same. Whether the protein kinase is an integral part of the enzyme and/or affects enzyme activity remains to be determined.

Discussion

The discovery by Roeder and Rutter (1970) that RNA polymerase I was localized in the nucleolus of rat liver sug-

TABLE VI: Subunit Structure and Stoichiometry of Ascites Polymerase I.[a]

Subunit (Mol. Wt.)	DNA-Cellulose Chromatography					Sucrose Gradient
	11	12	13	14	Pool	Pool
A.						
a. 205 000	1	1	1	1	1	1
b. 125 000	0.8	0.9	1.0	0.9	0.8	1.2
c. 51 000	0.8	0.7	1.0	1.1	0.85	0.8
d. 44 000	1.3	1.4	1.6	1	1.0	1.0
e. 26 000	1.2	1.4	1.4	.4	0.8	0.8
f. 16 000	0.3	0.8	0.7	1.2	0.7	0.8
nCT-DNA/ poly(dC)	0.064	0.073	0.060	0.062		

Subunit (Mol. Wt.)	Poly(uridylic acid)-Sepharose Chromatography			
	No. 9	No. 10	No. 11	Pool
B.				
a. 205 000	1.0	1.0	1.0	1.0
b. 125 000	0.92	1.2	1.2	1.15
c. 51 000	0.44	0.89	1.6	0.63
d. 44 000	1.0	1.0	1.3	0.84
e. 26 000	0.64	0.71	0.64	0.96
f. 16 000				1.9
nCT-DNA/ poly(dC)	0.044	0.075	0.095	

[a] Individual fractions and pools from a DNA-cellulose and poly(U)-Sepharose column and from a sucrose density gradient were prepared for sodium dodecyl sulfate gel electrophoresis as described. Lanes were scanned at 590 nm. The area under the peak of the highest molecular weight component (205 000) was set at a stoichiometry of one, and the molar ratios of all the other subunits were normalized to it. The nCT-DNA/poly(dC) ratios were calculated from the total units of nCT-DNA and poly(dC) activity assayed in each fraction.

gested to us that prior isolation of nuclei and nucleoli might facilitate purification of the enzyme. Such a procedure would eliminate both nonnucleolar protein and the bulk of the cellular DNA and RNA. Accordingly, their method for the isolation of rat liver nuclei and nucleoli was adopted with minor but significant modifications. By use of the Tekmar Dispex, minced rat liver could be homogenized directly in hypertonic sucrose at high cation concentrations. The isolated nuclei retained their structure, including the inner and outer nuclear membranes. The yield of nuclei, as measured by recovery of DNA, was 40–60% and compared favorably with other fairly large scale procedures.

Neither hypertonic solutions nor homogenization could be used successfully with the Krebs ascites cells. Homogenization by Potter-Elvehjem or by Tekmar Dispex was ineffective at rupturing the cell membrane. Furthermore, ascites nuclei prepared in hypertonic sucrose (1.7 M) failed to sediment through a 2.1 M sucrose pad. However, after removal of lipids by flotation, cell breakage could be effectively accomplished by the use of a Parr nitrogen bomb (Hunter and Commerford, 1960). Two passes through the bomb were sufficient to rupture both the ascites cell membrane and the erythrocytes, but not the nuclei.

Polymerase Levels in Nuclei and Nucleoli. Assays performed in nuclei and nucleoli confirm older findings (Roeder and Rutter, 1969, 1970; Schwartz et al., 1974) that, while polymerase II and III are located in the nucleoplasm, polymerase I is found specifically in the nucleolus. The low levels of polymerases II and III in the nucleolus are probably a consequence of chromatin contamination, since the activity is due to bound enzyme and is not increased by exogenous DNA.

The ratio of exogenous to endogenous activity varies for the three enzymes and depends upon the isolation procedure. This has important implications for the conclusions drawn by Yu and Feigelson (1971, 1973) and by Yu (1974, 1975) from their studies in isolated nuclei and nucleoli. Their claim that the activity of polymerase I is mediated by a rapidly turning over subunit is based on assays carried out with poly(dC) (an exogenous template) in the presence of actinomycin. Obviously the activity and class of enzyme (i.e., I or III) will depend on the way in which the organelles were isolated.

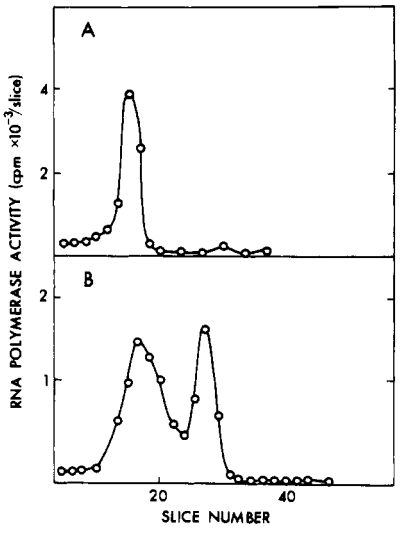

FIGURE 7: Native gel electrophoresis of rat liver polymerase. (A) Rat liver polymerase I. Rat liver polymerase I was purified as described in the text. (B) Rat liver polymerase II. Rat liver polymerase II was purified through the sucrose gradient step as described by Weaver et al. (1971). The enzyme was dialyzed against the low salt dialysis buffer and then electrophoresed as described in the text; (O) nCT-DNA activity.

A similar point may be made concerning the activity of polymerase II. In rat liver, an organ that has ceased rapid growth, polymerase II is not template bound (<10%) and is perhaps physiologically inactive, whereas in ascites cells, 50% of the enzyme is bound to its template. The redistribution of a fixed amount of enzyme could easily account for the apparent increase in polymerase II activity in nuclei as a function of steroid hormone administration or of regeneration following partial hepatectomy (Blatti et al., 1970; Glasser et al., 1972; Novello and Stirpe, 1970). It would also provide an explanation for the finding by Benecke et al. (1973) that although the activities of polymerases I and II were affected by such short-term physiological transitions, the amounts (or levels) of the solubilized enzymes were unchanged.

On the other hand, there is a difference in enzyme levels between rat liver and a rapidly growing source such as ascites cells (Table II). When the total amount of each enzyme is considered on a protein basis, there are 5–10 times more polymerases I and III present in ascites as compared to liver cells. The results are similar to previous findings (Schwartz et al., 1974; Schwartz and Roeder, 1974; Jaehning et al., 1975).

Purification. The purification procedure described above is rapid and effective. It requires no new or unusual methodology and takes less than 4 days to complete. It has a significant advantage in that it minimizes dilution, a phenomenon that Schwartz and Roeder (1974) have shown, and our data support, severely restricts recovery.

The enzyme pool from the ion-elution column is assayed with several templates. These have been chosen so that differences in enzyme activity during the course of isolation might provide clues to the existence of protein cofactors. The first differences in the ratio and distribution of the three activities occur during phosphocellulose chromatography (Figure 2). Native calf-thymus DNA/poly(dC) activity ratios increase toward higher ionic strength. The displacement of the poly(dC) activity and the nCT-DNA activity peaks suggests a partial dissociation of a basic protein that binds to phosphocellulose.

A similar shift in activity and yield occurs after sucrose density gradient centrifugation, so that the dissociation of enzyme and putative factor must be a consequence of mass action as well. Table III and Figure 3 bear this out. The possibility exists that the increase in the ratio of nCT-DNA/poly(dC) activity might be due to the relative increase in enzyme concentration rather than to a change in the molar ratio of one of the subunits. However, the data in the accompanying paper (Goldberg et al., 1977) indicate that such a response is a function of the stage of purification. Prior to sucrose gradient centrifugation or poly(U)-Sepharose chromatography, the response of activity with protein concentration is linear.

Although only the ascites polymerase I has been purified to homogeneity, the specific activities of both enzymes after DNA-cellulose chromatography (Table IV) are comparable with those found in the literature (Schwartz and Roeder, 1974; Buhler et al., 1974; Gissinger and Chambon, 1972). The fact that the specific activity of the rat enzyme is greater than 100 units/mg while the enzyme preparation is still obviously heterogeneous could be due to the presence of an activating molecule. As noted above, experiments in which both enzymes were mixed show that the ascites enzyme is stimulated by some factor in the rat preparation.

It might also be noted that the yield of enzyme is comparable to those reported previously (Schwartz and Roeder, 1974; Gissinger and Chambon, 1972). The recovery of enzyme activity can be measured as a function of two template activities. When total nCT-DNA activity is determined, the yield from nuclei or nucleoli to ion-elution pool is 50–60%. Similarly, when poly(dC) activity is measured, the yield from ion-elution pool to DNA-cellulose or poly(U)-Sepharose is 30%. The overall recovery is therefore 15–18%.

Subunit Structure of Polymerase I. The molecular weight and stoichiometry of the subunits of ascites and rat liver polymerases were examined by the use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Since the determination of stoichiometry was strongly dependent upon the staining intensity of the bands, slab gels were employed and the protein was stained for 24 h at room temperature. Six protein bands were present in the DNA-cellulose column pool of the ascites enzyme (Figures 5 and 6B). Their molecular weights and stoichiometry were 205 000 (1), 126 000 (1), 51 000 (1), 44 000 (1), 27 000 (1), 16 000 (1–2) (Table V, part A).

The number of subunits and their molecular weight and stoichiometry are remarkably analogous to the two best-characterized preparations from calf-thymus (Gissinger and Chambon, 1972) and myeloma cells (Schwartz and Roeder, 1974). Other sources from which polymerase I has been purified have similar although not identical structures (Buhler et al., 1974; Gornicki et al., 1974; Young and Whiteley, 1975; Froehner and Bonner, 1973).

The enzyme from rat liver has not yet been purified to homogeneity. Two high molecular weight bands (205 000 and 126 000) are present after ion-elution chromatography. However, even after the completion of DNA-cellulose chromatography, enzyme protein accounts for no more than 50% of the total protein. When the enzyme is electrophoresed under nondenaturing conditions, there is a single broad diffuse band of protein that is roughly coincident with enzyme activity. Electrophoresis of the ascites enzyme under the same conditions yields a more sharply defined band. This suggests there may be a complex of proteins associated with rat liver polymerase I which is involved in the regulation of activity. Such regulatory proteins might mediate the activity of enzyme in stable, differentiated tissue in a manner distinct from that in rapidly proliferating or neoplastic cells.

Acknowledgments

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Restriction Endonuclease Map of *Euglena gracilis* Chloroplast DNA†

Patrick W. Gray and Richard B. Hallick*

ABSTRACT: A physical map of the *Euglena gracilis* chloroplast genome has been constructed, based on cleavage sites of *Euglena gracilis* chloroplast DNA treated with bacterial restriction endonucleases. Covalently closed, circular chloroplast DNA is cleaved by restriction endonuclease SalI into three fragments and by restriction endonuclease BamHI into six fragments. These nine cleavage sites have been ordered by fragment molecular weight analysis, double digestions, partial digestions, and by digestion studies of isolated DNA fragments. A fragment pattern of the products of EcoRI restriction endonuclease digestion of *Euglena* chloroplast DNA is also described. One of these fragments has been located on the cleavage site map.

Chloroplasts of the unicellular alga *Euglena gracilis* contain double-stranded, covalently closed circular DNA of molecular weight 92 × 10^6 (Manning and Richards, 1972), corresponding to 140 kbp.[1] Depending on the stage of chloroplast development and the conditions of cell culture, there are 500–2000 copies of chloroplast DNA per *Euglena* cell (Rawson and Boerma, 1976a; Chelm et al., 1977). The chloroplast genome is extensively transcribed in vivo, both in light grown and in dark adapted cells. Estimates of the extent of genome transcription of from 12 to 23% (Chelm and Hallick, 1976) and from 23 to 26% (Rawson and Boerma, 1976b) for various stages of light-induced chloroplast development have been reported. Two classes of RNA transcripts are known to be of chloroplastic origin. The most abundant chloroplast transcripts are the 16S and 23S rRNAs (Scott and Smillie, 1967; Stutz and Rawson, 1970), accounting for as much as 26% of *Euglena* cellular RNA (Chelm et al., 1977; Cohen, 1973; Cohen and Schiff, 1976). Chloroplast DNA also contains genes for approximately 25 tRNAs (Schwartzbach et al.,

† From the Department of Chemistry, University of Colorado, Boulder, Colorado 80309. *Received November 2, 1976.* This work was supported by Grant GM21351 from the National Institutes of Health and Grant IN-103A to the University of Colorado from the American Cancer Society.

[1] Abbreviations used: kbp, kilobasepair; ct DNA, chloroplast DNA; SalI, endonuclease isolated from *Streptomyces albus G*; BamHI, endonuclease isolated from *Bacillus amyloliquefaciens H*; EcoRI, restriction endonuclease isolated from *E. coli RY13*; PstI, endonuclease isolated from *Providencia stuartii*; BS, DNA fragments resulting from a BamHI, SalI double digestion of ct DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.