Ascites Tumor Ribonucleic Acid Polymerases. Isolation, Purification, and Factor Stimulation†

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ABSTRACT: RNA polymerase has been isolated from rat ascites tumor and resolved into three activities (Ia, Ib, and II) by phosphocellulose and DEAE-cellulose chromatography. Enzymological studies indicate that Ia and Ib are the nucleolar enzymes while II is nucleoplasmic. Ia and Ib have been purified further by sucrose density gradient centrifugation. Though Ia appears to be homogeneous by gel electrophoresis under nondenaturing conditions, its specific activity is quite low. Sodium dodecyl sulfate gel electrophoresis of both Ia and Ib indicates that each consists of several polypeptides, although the two enzymes do not appear to possess common polypeptides. One reason for the apparent low specific activity of Ia and Ib is the nonlinearity of activity with increasing enzyme concentration. Even in the presence of bovine serum albumin, the enzymes are inactive at low concentrations. This cooperative effect is alleviated by the addition of the runoff material from the DEAE-cellulose chromatography. Thus, this material greatly stimulates the activity of polymerases Ia and Ib at low enzyme concentrations.

The RNA polymerase of prokaryotic organisms has been implicated directly in the control of gene expression (see Burgess, 1971, for a recent review). Systems which exhibit temporal changes in RNA synthesis (T4, Weber and Goff, 1970; Schachner *et al.*, 1971; Schachner and Zillig, 1971; T7, Chamberlin *et al.*, 1970; phage infection, and *Bacillus subtilis* sporulation, Leighton *et al.*, 1971, for example) have proven to be examples of such control. After T4 infection, the physical structure of the host RNA polymerase becomes extensively modified. The extrapolation of similar mechanisms of gene control to eukaryotic systems may be possible but requires a thorough characterization of the eukaryotic RNA polymerases, both physically and enzymologically.

The existence of multiple RNA polymerase activities in eukaryotes is well established (Roeder and Rutter, 1969; Kedinger *et al.*, 1970, 1971; Mandel and Chambon, 1971; Chesterton and Butterworth, 1971a; Mondal *et al.*, 1972; see also *Cold Spring Harbor Symp. Quant. Biol.*, 1970). Factors which stimulate calf thymus (Stein and Hausen, 1970) and rat liver (Seifart, 1970) RNA polymerase II have been reported. These factors stimulate only form II and only with native DNA as a template. The active agent is probably a protein of mol wt ~70,000 (Seifart, 1970). The way in which multiple forms of RNA polymerase or these protein factors are involved in the control of gene expression is not yet understood, however. Earlier studies of gene control in eukaryotes with isolated chromatin have used bacterial RNA polymerase for *in vitro* transcription (Bonner *et al.*, 1968; Georgiev, 1969; Smith *et al.*, 1969). We do not know whether the heterologous system (bacterial RNA polymerase and mammalian chromatin) yields the same results which would be obtained with a homologous system. The study of chromatin transcription with mammalian polymerase is only beginning (Butterworth *et al.*, 1971) but the use of homologous RNA polymerase for chromatin transcription may, at least, clarify the problems involved.

Toward the solution of the problems discussed above, we have solubilized RNA polymerase from Novikoff ascites tumor nuclei and resolved it into three forms. Two of these forms (Ia and Ib) are the nucleolar enzymes while the other (II) is nucleoplasmic. Some evidence indicates that Ia is homogeneous, although the specific activity of both Ia and Ib is quite low. However, as we demonstrate here, the activity of both enzymes is greatly stimulated upon addition of proteins which do not bind to DEAE-cellulose during the enzyme purification. Thus, it appears that a stimulating factor, similar to that isolated from calf thymus and rat liver, is also present in ascites tumor and, contrary to other reports, stimulates the activity of the nucleolar enzymes.

Materials and Methods

Maintenance of Cell Line. The Novikoff ascites is a liver-derived tumor grown in the rat intraperitoneal cavity. Male Sprague-Dawley rats (150–250 g) were injected with 0.5 ml of fluid with an 18-gage needle and then killed by etherization after 6–7 days. The yield was approximately 20–25 ml of ascites fluid (4–5 ml of packed cells) per rat. Bacterial contamination was less than 100 cells/ml as judged by colony growth on media containing proteose peptone yeast extract and glucose.

Preparation of DNA. DNA was prepared from ascites tumor chromatin or calf thymus chromatin (Shih and Bonner, 1969) by the method of Marmur (1961) as modified by Dahmus and McConnell (1969).

RNA Polymerase Assay. The reaction mixture contained, in a total volume of 0.5 ml, 25 μmol of Tris-HCl (pH 7.9), 5 μmol of $MgCl_2$, 2.5 μmol of dithiothreitol, 0.5 μmol of ATP, UTP, and CTP (P-L Laboratories), 0.05 μmol of [^{3}H]GTP (100 μCi/μmol) (Schwarz/Mann), 50 μg of DNA, and enzyme. After incubation at 37°, usually for 10 min, the reaction was stopped by the addition of 1.0 ml of 0.1 M EDTA (pH 8), 1% sodium dodecyl sulfate, and 500 μg/ml of carrier RNA. Approximately 10 ml of cold trichloroacetic acid–pyrophosphate

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(100% trichloroacetic acid (w/v)–saturated sodium pyrophosphate (room temperature)–cold H_2O, 1:2:7, made up fresh each time) was added and the tubes allowed to stand for about 10 min. The precipitates were collected on Selectron membrane filters (Schleicher and Schuell, B-6, 25 mm, 0.45 μ). The tubes were rinsed three times with cold trichloroacetic acid–pyrophosphate and the filters were washed six–ten times with the same solution. The filters were then placed in glass scintillation vials, 0.5 ml of 5% trichloroacetic acid was added, and the capped vials were heated for 15 min at 95°. After cooling, 10 ml of Aquasol (New England Nuclear) was added and the samples counted in a Beckman LS-200B liquid scintillation counter. For time course experiments, the DEAE filter paper method of Litman (1968) was used. The specific radioactivity of the [^{3}H]CTP was increased tenfold to 1000 μCi/μmol and 50 μl was removed from the reaction mixture at the appropriate times. The aliquots were pipetted onto DEAE filter papers (Whatman, 2.3 cm) and after 15–30 sec the filters were submerged in 5% Na_2HPO_4 (5–10 ml/filter). The filters were then washed collectively six times with 5–10 ml/filter of 5% Na_2HPO_4 for 15–30 min each wash. After rinsing twice with deionized H_2O to remove excess Na_2HPO_4, the filters were washed twice with 95% ETOH and once with ether. After drying, the disks were hydrolyzed and counted as described.

Preparation of Nuclei for Enzyme Extraction. The procedure used is a modification of the method of Dahmus and McConnell (1969). Ascites fluid was diluted with an equal volume of cold TNKM[1] (0.05 M Tris (pH 6.7)–0.13 M NaCl–0.0025 M $MgCl_2$–0.025 M KCl) and centrifuged for 10 min at 1000*g* (2000 rpm in International No. 284 swinging bucket rotor). The cells were then resuspended in 5 vol of cold deionized H_2O and centrifuged at 1000*g* for 6 min. This preferentially lyses the red blood cells. TNKM was added and the cells recentrifuged as above. To lyse the ascites cells, the white pellets were suspended in 5 vol of 0.01 M Tris (pH 7.9)–0.01 M $MgCl_2$–0.025 M NaCl–0.33 M sucrose and the suspension was made 0.25% Triton X-100. After stirring in the cold for 15 min, the nuclei were pelleted by centrifugation at 1000*g* for 20 min.

Extraction of RNA Polymerase from Nuclei. RNA polymerase was solubilized by a modification of the method of Roeder and Rutter (1970). It is extremely important in this and all subsequent steps that the dithiothreitol is not oxidized since the enzyme is sensitive to air oxidation. Dithiothreitol is susceptible to autoxidation, particularly in a medium containing Mg^{2+} and at pH 8, and deteriorates over a period of a few days under these conditions. For best results the dithiothreitol should be added to the buffers immediately before use. The crude nuclear pellet was suspended in an equal volume of 0.02 M Tris (pH 7.9)–0.01 M $MgCl_2$–0.01 M dithiothreitol–2 M sucrose and then made 0.3 M $(NH_4)_2SO_4$ by the addition of $^1/_9$ vol of 3 M $(NH_4)_2SO_4$, pH 7.9. After thorough mixing, the viscous solution was sheared in 50-ml batches with a Polytron (Brinkman Instruments) twice for 45 sec each at a setting of 3, with cooling on ice and mixing between. The sheared solution was allowed to stir for 20–30 min to ensure maximum extraction. The salt concentration was then decreased to 0.1 M $(NH_4)_2SO_4$ by adding the extract to 2 vol of 0.05 M Tris (pH 7.9)–0.005 M $MgCl_2$–0.1 mM EDTA–5 mM dithiothreitol–25% glycerol (TGMED) while stirring with a glass rod. The precipitated "chromatin" was then removed by centrifugation at 78,100*g* for 4–5 hr (30,000 rpm in a Spinco 30 rotor). The supernatant, which contained 90% of the activity, was precipitated by the addition of 0.5 vol of saturated $(NH_4)_2SO_4$, pH 8 (saturated at 4°) with stirring. After diluting the precipitated solution with about 0.5 vol of 33% $(NH_4)_2SO_4$ to lower the density (otherwise the precipitate floats), the pellets were collected by centrifugation at 11,700*g* (12,000 rpm in a Sorvall GSA rotor) for 20 min and discarded. The supernatant was made 50% $(NH_4)_2SO_4$ by the addition of $^1/_3$ vol of saturated $(NH_4)_2SO_4$, pH 8, and allowed to stand overnight. The pellets were collected as above and dissolved in 0.05 M Tris (pH 7.9)–0.1 mM EDTA–5 mM dithiothreitol–25% glycerol (TGED) + 0.15 M KCl at a concentration of 5–7 mg/ml of protein. This crude extract was dialyzed against two changes of 10 vol of TGED + 0.15 M KCl for 6–8 hr each. Precipitated nucleohistone was then removed by centrifugation at 166,500*g* for 4 hr (50,000 rpm in a Ti50 rotor).

Phosphocellulose Chromatography. Whatman P-11 cellulose phosphate was precycled by suspension in 0.1 N HCl followed by washing with water and then with 0.1 N KOH. After excess KOH was removed by filtration and washing with H_2O, the resin was washed several times with TGED + 0.15 M KCl and the pH adjusted to 7.9. A 2.5 × 45 cm column was poured and equilibrated with TGED + 0.15 M KCl until the effluent was pH 7.9 and 0.15 M KCl (checked by conductivity). The sample (30–60 ml; <5 mg/ml of protein) was applied at a flow rate of 0.2 ml/min; the column was washed with one column volume of TGED + 0.15 M KCl at a flow rate of 0.5 ml/min and then developed with a gradient of 0.15–0.80 M KCl in a total volume of 600 ml of TGED. Approximately 100 9-ml fractions were collected, the A_{280} profile determined, and 100 μl of every other fraction assayed for RNA polymerase activity. The appropriate fractions were pooled, an equal volume of saturated $(NH_4)_2SO_4$, pH 8, was added, and the protein was allowed to precipitate overnight at 4°. The precipitates were collected by centrifugation at 12,500*g* for 20 min (12,000 rpm in a Sorvall SS-34 rotor) and redissolved in 3–5 ml of TGMED + 0.05 M $(NH_4)_2SO_4$. After dialysis against 50 vol of TGMED + 0.05 M $(NH_4)_2SO_4$ for 5–8 hr, the samples were used directly for further purification or stored at −70°.

DEAE-Cellulose Chromatography. The procedure used is a modification of the method of Roeder and Rutter (1970). DEAE-cellulose (type 20, Schleicher and Schuell) was precycled by suspension in 0.25 N NaOH; excess base was removed by washing with water on a Buchner funnel and then resuspended in 0.25 N HCl. After removal of excess acid, the resin was suspended in TGMED + 0.05 M $(NH_4)_2SO_4$ and the pH adjusted to 8. A 1.5 × 30 cm column was poured and washed with TGMED + 0.05 M $(NH_4)_2SO_4$ until the eluate was pH 7.9 and 0.05 M $(NH_4)_2SO_4$. The sample (3–7 ml at 1 mg/ml of protein) was applied at a flow rate of 0.2–0.3 ml/min and allowed to stand for about 30 min. After washing with one column volume of TGMED + 0.05 M $(NH_4)_2SO_4$, a gradient of 0.05–0.5 M $(NH_4)_2SO_4$ in a total volume of 200 ml of TGMED was applied at a flow rate of 0.6 ml/min and 3-ml fractions were collected. The A_{280} profile was determined and 100-μl aliquots of every second fraction were assayed for activity. The appropriate fractions were combined, an equal volume of saturated $(NH_4)_2SO_4$, pH 8, was added, and the protein was allowed to precipitate overnight at 4°.

Sucrose Density Gradient Centrifugation. The precipitated protein from the DEAE-cellulose column was collected by centrifugation at 64,700*g* for 1 hr (25,000 rpm in a SW 25.1

[1] Abbreviations used are: TGED, 0.05 M Tris (pH 7.9)–0.1 mM EDTA–5 mM dithiothreitol–25% glycerol; TGMED, 0.05 M Tris (pH 7.9)–0.005 M $MgCl_2$–0.1 mM EDTA–4 mM dithiothreitol–25% glycerol; TNKM, 0.05 M Tris (pH 6.7)–0.13 M NaCl–0.0025 M $MgCl_2$–0.025 M KCl; PC-A and PC-B, ascites RNA polymerases purified by phosphocellulose chromatography.

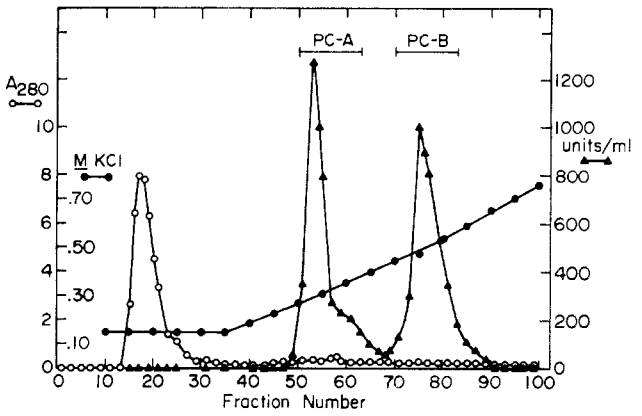

FIGURE 1: Phosphocellulose chromatography of the ammonium sulfate fraction. A crude extract (25 ml) containing 170 mg of protein and 291,000 units of RNA polymerase was applied to the column and processed as described under Materials and Methods.

rotor) and the tubes wiped free of excess $(NH_4)_2SO_4$ solution. The pellet was dissolved in 100–200 μl of 0.05 M Tris (pH7.9)–0.005 M $MgCl_2$–0.1 mM EDTA–10 mM dithiothreitol–5% glycerol. The final $(NH_4)_2SO_4$ concentration (due to that remaining in the pellet) was determined by conductivity (10 μl diluted into 6 ml of H_2O and compared to a standard curve). The volume of the sample was adjusted so that the $(NH_4)_2SO_4$ concentration was less than 0.6 M. The sample was then layered on a 5-ml 5–20% sucrose gradient (containing 0.05 M Tris, pH 7.9, 0.005 M $MgCl_2$, 0.1 mM EDTA, 10 mM dithiothreitol, 0.2 M $(NH_4)_2SO_4$, and 10% glycerol), overlayered with paraffin oil and centrifuged for 10 hr at 300,000g (65,000 rpm in a SW 65 rotor). Approximately 25 fractions (10 drops) were collected by puncturing the bottom of the tube and 20-μl aliquots assayed for activity. Protein concentrations were determined on 20-μl aliquots of each fraction by the method of Bramhall *et al.* (1969).

Factor Preparation (RO_{Ia-II} and RO_{Ib}). The run-off fractions of the DEAE-cellulose chromatography of PC-A and PC-B enzymes are used as the source of the factor. The fractions are pooled and precipitated by the addition of 1.5 vol of saturated $(NH_4)_2SO_4$, pH 7.9 (4°). After precipitation overnight at 4°, the protein is collected by centrifugation, dissolved in approximately 5 ml of TGMED + 0.05 M $(NH_4)_2SO_4$, and dialyzed against 100 vol of the same buffer for 5–6 hr. The sample is then subjected to another pass through the

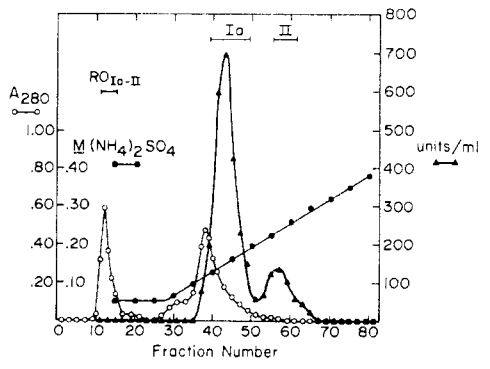

FIGURE 2: DEAE-cellulose chromatography of PC-A. PC-A (4.1 ml) containing 9.7 mg of protein and 46,400 units of RNA polymerase was applied to the column and processed as described under Materials and Methods.

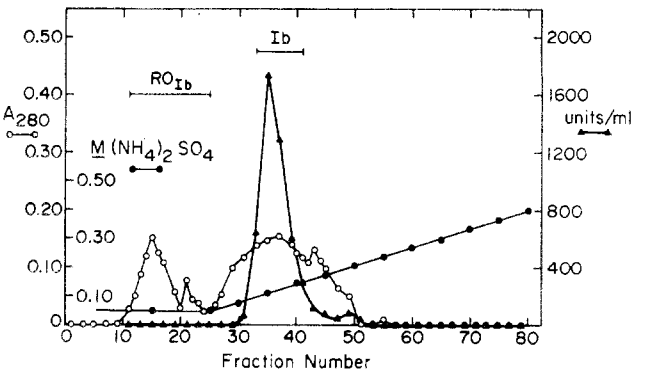

FIGURE 3: DEAE-cellulose chromatography of PC-B. PC-B (6 ml) containing 6 mg of protein and 30,750 units of RNA polymerase was applied to the column and processed as described under Materials and Methods.

DEAE-cellulose column to ensure that all RNA polymerase activity has been removed. The run-off fractions are again precipitated with $(NH_4)_2SO_4$, redissolved, dialyzed as described above, and stored at −90° in TGMED + 0.05 M $(NH_4)_2SO_4$.

Polyacrylamide Gel Electrophoresis. For gel electrophoresis under nondenaturing conditions, the method of Davis (1964) was used (5% acrylamide, pH 8.7; with pH 6.9 stacking gel; Tris–glycine reservoir buffer, pH 8.3). Approximately 10 μg of protein in 100 μl of TGMED + 0.05 M $(NH_4)_2SO_4$ was applied to a 0.5 × 8 cm gel and electrophoresed for 2 hr at 3 mA/gel. Sodium dodecyl sulfate gel electrophoresis was performed according to the method of Shapiro *et al.* (1967). Samples were dialyzed against 0.01 M sodium phosphate, pH 7.1, 0.1% sodium dodecyl sulfate, 0.1% β-mercaptoethanol, and 10% glycerol, and up to 100 μl was applied to a 0.5 × 6 cm gel. For the analysis of the sucrose gradient fractions, 100 μl of each fraction was diluted with 100 μl of dialysis buffer and then dialyzed overnight against 500 ml of the same buffer. Samples (50 μl) were applied to each gel. Electrophoresis was for 2.5 hr at 6 mA/gel. Bovine serum albumin and *Escherichia coli* RNA polymerase were used as molecular weight standards. To determine the molecular weights of unknowns, bovine serum albumin was electrophoresed on the same gel and the mobilities of the unknowns relative to bovine serum albumin were determined. The molecular weights were then determined from a standard curve of bovine serum albumin and the subunits of *E. coli* RNA polymerase (β' = 165,000, β = 155,000, σ = 90,000, α = 40,000). Both the native and sodium dodecyl sulfate gels were stained with Coomassie Blue and destained according to Elgin and Bonner (1970).

Preparation of Poly(dT)-[^{3}H]Poly(rA) Hybrid. To hybridize poly(dT) and poly(A), 0.2 ml of poly(dT) (5 A_{260} units/ml in 0.01 M Tris, pH 7.9; mol wt 1–5 × 10^6; General Biochemicals, Chagrin Falls, Ohio) plus 0.05 ml of [^{3}H]poly(A) (10 μCi/ml in 0.12 M phosphate buffer, pH 6.8 [equimolar quantities of mono- and dibasic $NaPO_4$]; 51.0 mCi/mmol of polynucleotide phosphorus, mol wt 0.24 × 10^6; Schwarz/Mann) were diluted to 1 ml with 0.12 M phosphate buffer, pH 6.8, and heated at 95° for 3 min. The solution was then placed at 60° for 2 hr to allow reannealing. With trichloroacetic acid, 98% of the ^{3}H counts were precipitable.

Results

Phosphocellulose chromatography is used as the initial step in the purification because of its high protein capacity and be-

TABLE I: Summary of Purification Scheme.[a]

Purification Step	Protein (mg)		Act. (Total Units)		Sp Act.		Yield of Act. (%)
	Ia	Ib	Ia	Ib	Ia	Ib	
$(NH_4)_2SO_4$ precipitation	274		271,000		0.99		100
Phosphocellulose chromatography	31.0	19.2	117,000	85,500	3.75	4.50	75
DEAE-cellulose chromatography	9.8	1.12	35,100	28,200	3.60	25.2	23
Sucrose gradient centrifugation	0.21	0.19	8,100	2,610	3.40	13.6	4

[a] One unit of enzyme incorporates 1 pmol of GMP in 20 min at 37°. Specific activity is defined as units per microgram of protein. The calculation of units of enzyme and amount of protein recovered at each step includes the concentration by $(NH_4)_2SO_4$ precipitation from the previous step (see Methods), except for the phosphocellulose chromatography.

cause large amounts of inactive protein are removed. Also, the resolution of the activity into two forms (PC-A and PC-B) is accomplished (Figure 1). Chromatography of these two fractions separately on DEAE-cellulose resolves PC-A into two more forms (Ia and II) (Figure 2) and achieves some purification of Ib (Figure 3). The final purification of Ia and Ib is accomplished by sucrose density gradient centrifugation in high ionic strength (Figures 4 and 5). A summary of the purification procedure is shown in Table I.

The results presented in the purification scheme (Table I) indicate that no increase in specific activity of Ia is achieved with the DEAE-cellulose chromatography or sucrose gradient centrifugation. Also, the specific activity of Ib decreases in the final purification step. However, substantial amounts of inactive protein are removed at each step. Further investigations indicated that the nonlinearity of enzyme activity with protein concentration (see below) drastically affected the apparent specific activity and yield of both Ia and Ib. Thus, it is likely that the specific activities of these enzymes do not reliably indicate their purities.

The three activities resolved by this purification procedure have been characterized according to their enzymological properties. The following information is consistent with the idea that Ia and Ib are nucleolar while II is located in the nucleoplasm, as shown by Roeder and Rutter (1969) and Chesterton and Butterworth (1971b) for rat liver RNA polymerase. (1) Ia and Ib elute from the DEAE-cellulose column at 0.11–0.15 M $(NH_4)_2SO_4$ while II is released only above 0.2 M. (2) Ia and Ib show optimum activity at low ionic strength while II exhibits maximal incorporation at 0.1 M $(NH_4)_2SO_4$. (3) II prefers Mn^{2+} as the divalent cation while Ia and Ib work best with Mg^{2+}. (4) II is extremely sensitive to α-amanitin while Ia and Ib are inhibited only at extremely high concentrations of this material.

Thus, these enzymes appear to have the enzymological properties which are typical of most mammalian RNA polymerases which have been studied (see *Cold Spring Harbor Symp. Quant. Biol.*, 1970). Since relatively small amounts of polymerase II are isolatable from ascites cells, the physical studies of these enzymes have been restricted to Ia and Ib.

Examination of the sucrose gradient profiles reveals coincidence of the protein and RNA polymerase peaks, particularly so in the case of Ia, indicating a relatively high state of purity of the enzymes, although the presence of contaminating protein of similar sedimentation characteristics cannot be eliminated. Aggregation of the proteins seems unlikely since the gradients were performed at high ionic strength (0.2 M $(NH_4)_2SO_4$). Aliquots of each fraction were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the results are shown in Figures 4 and 5. In the profile of Ia, six polypeptides of mol wt 170,000, 125,000, 69,000, 49,000, 44,000, and 37,000 are present in the fractions which contain enzyme activity. The amounts of these six bands are highest in fraction 8 (the one with the highest enzyme activity) and decrease on either side of the peak with approximate corres-

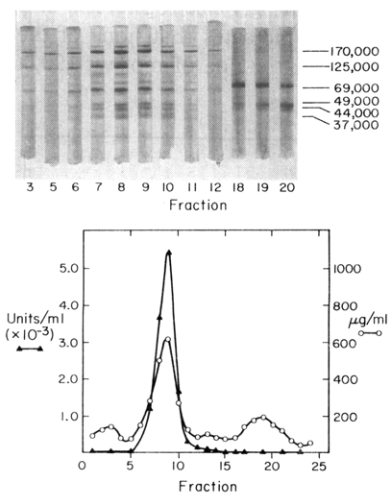

FIGURE 4: Sucrose density gradient centrifugation of Ia. The procedure was as described under Materials and Methods.

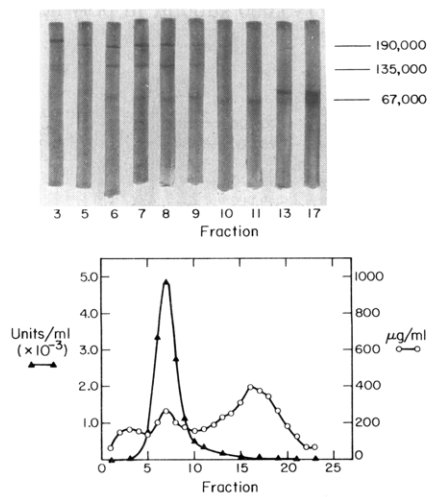

FIGURE 5: Sucrose density gradient centrifugation of Ib. The procedure was as described under Materials and Methods except the centrifugation was for 10.5 hr at 300,000*g*.

TABLE II: Molar Ratios of the Subunits of Polymerase Ia.[a]

Subunit Mol Wt	Molar Ratio: Fraction 7	Fraction 8	Fraction 9	Fraction 10	Av ± Std Dev
170,000	1.00	1.00	1.00	1.00	1.00 ± 0
125,000	0.91	0.93	0.73	0.94	0.88 ± 0.10
69,000	1.56	1.44	1.32	1.40	1.43 ± 0.10
49,000	0.83	0.92	0.87	0.86	0.87 ± 0.12
44,000	1.25	1.26	1.12	0.92	1.14 ± 0.16
37,000	1.49	1.53	1.38	1.63	1.51 ± 0.10

[a] The fractions refer to the sucrose density gradient fractions of Ia (Figure 4). The molar ratios were calculated from spectrophotometric scans of the sodium dodecyl sulfate–polyacrylamide gels. Gaussian curves were fitted to the peaks and the areas determined by a computer program.

pondence to the activity, as would be expected if these polypeptides constitute the enzyme. Fractions 18 and 19, which do not contain activity, show an entirely different spectrum of polypeptides, except that a protein of mol wt ~70,000 is present. It is not possible to determine whether this is identical with that present in fractions 6–11.

If these polypeptides are all part of RNA polymerase, the relative molar ratios should be integral numbers. These ratios were determined and the results are shown in Table II. Within a reasonable margin of error, the polypeptides are all present in equimolar ratios, with the exception of the mol wt 69,000 and 37,000 polypeptides. These latter two have calculated molar ratios of about 1.5. It is possible that these polypeptides have abnormal affinities for the dye used to stain the gels. They may also be simply contaminating proteins. It is important that the molar ratios for all six polypeptides remain constant (within the margin of error) across the peak of RNA polymerase activity, as would be expected if they sedimented as one complex (Table II).

Some preparations of Ia, including the one shown here, contain a polypeptide of mol wt 190,000 which comigrates with the active complex. The molar ratio of this subunit varies considerably from one preparation to another, although it is never greater than about 0.3. Weaver *et al.* (1971) reported the presence of a mol wt 190,000 subunit in polymerase II from

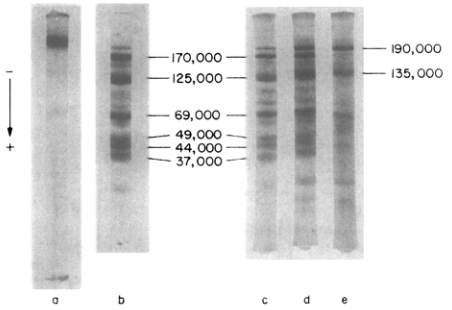

FIGURE 6: Native and sodium dodecyl sulfate gel electrophoresis of Ia and Ib: (a) native gel of Ia (13 μg); (b) sodium dodecyl sulfate gel of Ia (13 μg), same preparation as a; (c) sodium dodecyl sulfate gel of Ia (10 μg), different preparation from a and b; (d) sodium dodecyl sulfate gel of Ia (10 μg) + Ib (5 μg); (e) sodium dodecyl sulfate gel of Ib (5 μg), same preparation as d. Procedures for electrophoresis are described under Materials and Methods; c, d, and e were electrophoresed simultaneously.

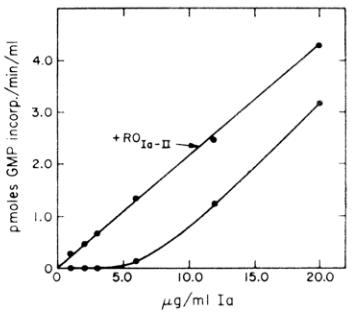

FIGURE 7: Effect of RO_{Ia-II} on the activity of Ia at different enzyme concentrations. Reaction mixtures (0.25 ml) contained 10 mM $MgCl_2$, 200 μg/ml of rat DNA, Ia at the indicated concentrations, plus RO_{Ia-II} (140 μg/ml) where indicated. Bovine serum albumin (80 μg/ml) was included if RO_{Ia-II} was not present. Rates of synthesis were determined by removing 50-μl aliquots at 0, 15, 30, and 45 min for analysis by the DEAE filter paper method.

rat liver and calf thymus which appears to be converted to mol wt 170,000 by a protease present in the crude extract. It seems possible that a similar situation may exist with polymerase Ia, although experiments designed to inhibit the presumptive protease with phenylmethylsulfonyl fluoride were not definitive (Froehner, S. C., unpublished results). The minor bands which appear on the sodium dodecyl sulfate gels and which have been ignored to this point, namely those of mol wt ~100,000, ~80,000, and ~55,000, are thought to be contaminants on the basis of their low molar ratios.

The most convincing evidence for the high state of purity of Ia is obtained by analytical gel electrophoresis in the absence of denaturants. Ia electrophoresis as a single major component with a very minor amount of contamination (Figure 6a). It is assumed that the major component is the RNA polymerase, since attempts to elute the enzyme from the gel for assay were unsuccessful. Also, the possibility that aggregation occurs during electrophoresis cannot be eliminated, although essentially all of the protein migrated into the gel.

The sucrose gradient centrifugation profile of Ib also shows some coincidence of the activity and protein peaks (Figure 5). Sodium dodecyl sulfate gel electrophoresis indicates the presence of polypeptides of mol wt 190,000, 135,000, and 67,000 in the active fractions, although the mol wt 67,000 polypeptide is probably contamination from the slower sedimenting material since it is also present in fractions 13 and 17. The two large polypeptides are present in molar ratios of approximately 1.0. Sodium dodecyl sulfate gel electrophoresis of Ia and Ib separately and together indicates that none of the polypeptides comigrate, except for the one of mol wt 190,000 (Figure 6).

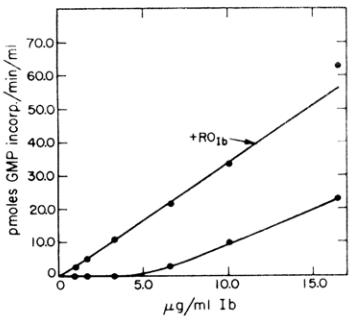

FIGURE 8: Effect of RO_{Ib} on the activity of Ib at different enzyme concentrations. The procedure was identical with that of Figure 6. Where indicated, RO_{Ib} was included at 129 μg/ml.

TABLE III: Heat Lability of RO_{Ia-II}.[a]

Additions	Rate of RNA Synthesis (pmol of GMP incorp/min per ml)
Ia	0
RO_{Ia-II}	0
Ia + RO_{Ia-II}	3.34
Ia + RO_{Ia-II} (heated)	0.33

[a] Reaction mixtures contained 2 μg/ml of Ia and 69 μg/ml of RO_{Ia-II}. Where indicated, RO_{Ia-II} was heated for 5 min at 85°, cooled, and centrifuged to remove precipitated protein.

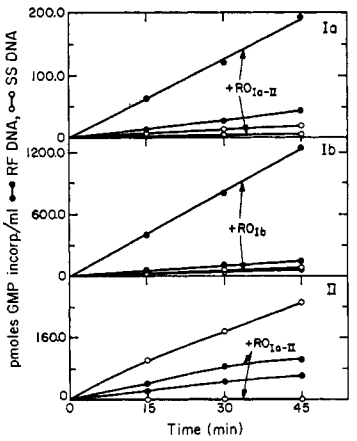

FIGURE 9: Stimulation of Ia, Ib, and II by RO_{Ia-II} or RO_{Ib} on φX174 DNA. Reaction mixtures contained 10 mM $MgCl_2$ for Ia (10 μg/ml) or Ib (6.6 μg/ml). II (1 μg/ml) was incubated at 2 mM $MnSO_4$ + 0.10 M $(NH_4)_2SO_4$. The DNA concentration (for both RF and SS) was 60 μg/ml. The φX174 RF-DNA was at least 80% form II (nicked circles). RO_{Ia-II} (140 μg/ml) and RO_{Ib} (129 μg/ml) were included where indicated.

Although these two enzymes have been extensively purified, their specific activities are still quite low. It is likely that inactive RNA polymerase molecules are present in the preparation since both enzymes are quite unstable, particularly after DEAE-cellulose chromatography. Another possibility is that some cofactor or protein factor necessary for optimal activity has been removed during the purification process. To investigate the reasons for the low activity of the preparations, the rate of RNA synthesis as a function of enzyme concentration was determined. As shown in Figures 7 and 8, the results are not as expected for a typical enzyme: the rate of synthesis does not increase linearly with enzyme concentration but shows a very pronounced concave shape. In fact, at low enzyme concentrations (less than 4 μg/ml for Ia and 3 μg/ml for Ib), no detectable synthesis of RNA occurs. The lack of activity of Ia and Ib is not due to denaturation of the enzyme at very low concentration since the presence of bovine serum albumin (final concentration of 80 μg/ml) has no effect on the curve. In contrast, polymerase II exhibits an almost linear activity *vs.* concentration curve, with only a slight deviation from linearity at low concentration of enzyme (less than 0.5 μg/ml) (Froehner, S. C., unpublished results). Thus the effect is probably not due to lack of sensitivity or some artifactual problem with the assay.

If the run-off protein from the DEAE-cellulose chromatography (the run-off from the chromatography of PC-A will be designated RO_{Ia-II} while that of PC-B will be referred to as RO_{Ib}) is added to the reaction mixtures (at a constant amount), the activity *vs.* enzyme concentration curves are then linear (Figures 7 and 8), at least over the enzyme concentration ranges tested. Thus, at low enzyme concentrations, RO_{Ia-II} and RO_{Ib} greatly increase the rates of RNA synthesis by Ia and Ib, respectively. In fact, at very low concentrations of enzyme, Ia and Ib show an absolute requirement for the factor for activity. As the enzyme concentration is increased, the stimulation decreases until at 20 μg/ml of Ia, the stimulation is only 30%.

It has been reported that the stimulating factor from calf thymus is stable to heating (5 min at 80° has little effect on the activity while 15 min at 100° results in less than 50% decrease in the activity) (Stein and Hausen, 1970). If RO_{Ia-II} from ascites tumor is subjected to 85° for 5 min, 90% of the activity is lost (Table III). (Note also that RO_{Ia-II} alone has no RNA polymerase activity.)

The activity of *E. coli* core RNA polymerase can be increased by alterations in the DNA. Introduction of single-stranded nicks into the template, for instance, produces artifactual initiation sites for the polymerase, thus increasing the RNA-synthesizing activity (Vogt, 1969). Therefore, one explanation for the stimulation of Ia by RO_{Ia-II} might be that the latter alters the template to make it more available for transcription. To investigate this possibility, two reaction mixtures were prepared, one containing Ia and the other Ia plus RO_{Ia-II}. After incubation as usual, the DNA from these reactions was isolated by phenol extraction and then used again in identical reaction mixtures. The DNA was not permanently "activated" by RO_{Ia-II} for transcription (data not shown). In fact, the amount of stimulation of Ia by RO_{Ia-II} was the same on either of the reisolated DNA preparations or on DNA which has not been subjected to preincubation and reisolation.

The factors isolated from calf thymus (Stein and Hausen, 1970) and from rat liver (Seifart, 1970) stimulate the activity of polymerase II on native DNA only. This property has also been investigated for RO_{Ia-II} and RO_{Ib} from ascites tumor. Since mammalian DNA contains highly repetitive sequences which reanneal almost instantaneously (Britten and Smith, 1969), rat RNA is not a suitable template for this investigation. Any stimulation on "denatured" DNA could be due to the selective utilization of renatured, double-stranded regions of template. Therefore, φX174 RF- (double-stranded) and SS- (single-stranded) DNA were used (Figure 9). It is clear that Ia and Ib prefer native DNA, while II uses denatured DNA; Ia and Ib both show a higher rate of RNA synthesis with φX174 RF-DNA while II prefers φX174 SS-DNA. RO_{Ia-II} stimulates synthesis by Ia and II only on RF-DNA, as does RO_{Ib} with Ib. (Essentially the same results are obtained with native and denatured ascites DNA.) Not only does RO_{Ia-II} not stimulate synthesis on SS-DNA, the activity of Ia and II alone on SS-DNA is completely inhibited by the addition of RO_{Ia-II}. RO_{Ib} slightly inhibits the activity of Ib on SS-DNA. This suggests that the factor may limit initiation by the enzymes to certain sites.

However, another explanation is possible. If RNase H (Hausen and Stein, 1970) is contaminating RO_{Ia-II}, then any synthesis which occurs on SS-DNA will remain as a DNA–RNA hybrid and be degraded by the hybridase. RNase H activity of RO_{Ia-II} and RO_{Ib} was assayed and the results are shown in Table IV. Both RO_{Ia-II} and RO_{Ib} contain significant amounts of RNase H as demonstrated by their ability to

TABLE IV: RNase Activity of Ia, Ib, and II.[a]

Additions	[^{3}H]Poly(A) (cpm)	Poly (dT)–[^{3}H]Poly(A) (cpm)	[^{3}H]RNA (cpm)
None	3050	3170	12,507
Ia	3065	3089	12,491
Ib	3043	3143	13,070
II	3048	3028	11,731
RO_{Ia-II}	2700	379	
RO_{Ib}	2928	1009	

[a] [^{3}H]Poly(A) and the poly(dT)–[^{3}H]poly(A) hybrid were prepared as described under Materials and Methods. [^{3}H]-RNA (~80,000 cpm/μg) was synthesized *in vitro* with *E. coli* RNA polymerase on native rat liver DNA and subsequently purified (gift of Maurice Dupras). Fifty microliters yielded 12,208 cpm (precipitable with trichloroacetic acid). Ten microliters of [^{3}H]poly(A) or poly(dT)–[^{3}H]poly(A) hybrid, or 50 μl of [^{3}H]RNA was incubated with Ia (20 μg/ml), Ib (5 μg/ml), II (1 μg/ml), RO_{Ia-II} (35 μg/ml), or RO_{Ib} (65 μg/ml), in the standard assay mix ([^{3}H]GTP was replaced with unlabeled nucleotide) for 30 min at 37°. The samples were then processed as described under Materials and Methods for RNA synthesis assays.

degrade [^{3}H]poly(rA) in the form of a hybrid with poly(dT). Stein and Hausen (1970) and Seifart (1970) did not report the RNase H activity of their factor preparations.

It is clear that the run-off fractions of the DEAE-cellulose chromatography of both PC-A and PC-B contain a factor(s) which stimulates the activity of Ia and Ib, respectively. It was of interest to determine if RO_{Ia-II} is specific for Ia or if it also stimulates Ib and if RO_{Ib} stimulates Ia. At saturating factor concentrations, RO_{Ia-II} and RO_{Ib} stimulate Ia and Ib equally well, and independently of which fraction is used with a given enzyme.

Other steps in the enzyme purification procedure removed large amounts of protein which contain no RNA polymerase, particularly the phosphocellulose chromatography and sucrose density gradient centrifugation. These nonactive fractions have also been tested for stimulation of RNA polymerase activity. Neither the run-off of the phosphocellulose column nor the slowly sedimenting protein in the sucrose gradient of Ia have any effect on the rate of RNA synthesis by I (Froehner, 1972). Thus it appears that the factor chromatographs on phosphocellulose together with the enzyme and is lost only when applied to DEAE-cellulose.

Discussion

Some of the data presented here suggest that RNA polymerase Ia, as purified by this procedure, is homogeneous, particularly the native polyacrylamide gel electrophoresis. If so, then the subunit structure, as indicated by sodium dodecyl sulfate gel electrophoresis, is quite complex. However, the low specific activity of Ia, as compared to other RNA polymerases, is an important point, and permits only a tentative conclusion as to the structure of Ia. Ib also has a quite low specific activity, even though the presence of several polypeptides is indicated by sodium dodecyl sulfate gel electrophoresis. However, it seems likely that Ia and Ib are physically distinct enzymes since, even at this state of purity, none of the polypeptides comigrate in sodium dodecyl sulfate gels.

It seems highly unlikely that the low specific activity of Ia and Ib is due entirely to contaminating protein. As previously mentioned, inactive polymerase molecules may be present. Also, it is noteworthy that the activity is doubled when the concentration of GTP is increased from 0.1 to 1.0 mM. At any rate, accurate determination of the specific activities of Ia and Ib is very difficult because the specific activity varies depending on the enzyme concentration used. Both Ia and Ib show very strong cooperative effects, *i.e.*, the activity increases nonlinearly with enzyme concentration. No activity is observed at low enzyme concentrations. One interpretation of this phenomenon is that at low enzyme concentrations, one of the subunits dissociates from the enzyme, yielding an inactive enzyme. At higher concentrations, the equilibrium is shifted toward association of the subunit and the rest of the complex, resulting in an active enzyme. Certain trivial explanations have been ruled out. Small amounts of RNase present in the assay reagents could destroy small amounts of product synthesized at low enzyme concentrations. At higher enzyme concentrations, more RNA is synthesized and the RNase is not able to destroy it completely during the time course of the experiment, resulting in net synthesis. This is probably not the case since neither the reagents nor Ia nor Ib show any trace of RNase activity (Table IV). It is also possible that the enzymes become inactive at low concentration simply due to dilution of the protein. However, bovine serum albumin has been included to avoid this. It is possible that earlier reports that the run-off factor does not stimulate polymerase I (Stein and Hausen, 1970; Seifart, 1970) can be explained by the fact that stimulation is dependent on enzyme concentration. No significant stimulation would be observed at high enzyme concentrations, at least with poymerase Ia.

It is impossible to determine at present if the stimulation is due to one or more factors, since RO_{Ia-II} and RO_{Ib} are not homogeneous preparations. However, the active agent is probably a protein since it is heat sensitive and is destroyed by treatment with proteolytic enzymes (Dahmus, M. E., personal communication). Also, preliminary experiments indicate that RO_{Ia-II} can be purified by chromatography on carboxymethylcellulose, although the purified factor is unstable (Froehner, S. C., unpublished results).

At present, the mechanism of action of the ascites factor is unknown. The experiments involving reisolation of the DNA suggest that permanent physical alteration of the template is not involved. Any proposed mechanism involving recognition of native RNA must be viewed with caution since stimulation by the factor on denatured DNA cannot be ruled out because of contaminating RNase H. Several lines of evidence, however, suggest that the factor, whatever its function, is a subunit of RNA polymerase. First, the factor chromatographs with the enzyme on phosphocellulose, though this may be due either to its association with the enzyme or with the phosphocellulose or both. It is noteworthy that RO_{Ia-II} and RO_{Ib} appear to chromatography with PC-A and PC-B, respectively, which are well resolved on phosphocellulose, suggesting that they do in fact remain associated with the enzyme during this purification step. Secondly, the cooperative effect of Ia and Ib is abolished by the addition of RO_{Ia-II} and RO_{Ib}, respectively, yielding a linear dose–response curve. If the cooperativity is due to the dissociation of a subunit from the complex, the addition of this subunit should shift the equilibrium toward association, and thus toward active enzyme. At high enzyme concentrations, little dissociation occurs and

addition of the subunit would have little effect. The reduction of stimulation at high enzyme concentrations (especially with Ia) is consistent with this proposal. Finally, Seifart (1970) has reported a mol wt of 70,000 for the rat liver factor. It is interesting that polymerase Ia preparations from ascites tumor contain a polypeptide of mol wt 69,000, present in a molar ratio of about 1.5 when compared to the other subunits. Whether RO_{Ia-II} is identical with this subunit awaits purification of the factor.

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