THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 248, No. 11, Issue of June 10, pp. 3777-3788, 1973
Printed in U.S.A.

Mammalian Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerases

I. PURIFICATION AND PROPERTIES OF AN α-AMANITIN-SENSITIVE RIBONUCLEIC ACID POLYMERASE AND STIMULATORY FACTORS FROM HELA AND KB CELLS*

(Received for publication, December 4, 1972)

BILL SUGDEN AND WALTER KELLER

From the Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

SUMMARY

A procedure is described for the purification of DNA-dependent RNA polymerase II of the human tissue culture cell lines HeLa and KB. The enzyme has a molecular weight of approximately 500,000 and in sodium dodecyl sulfate polyacrylamide gels invariably shows 3 subunits with molecular weights of 220,000, 140,000, and 35,000. In addition there perhaps is a fourth subunit of about 25,000 daltons or less in mass and a subunit of 170,000 daltons occurring in variable amounts. RNA polymerase II has a strong preference for single-stranded over double-stranded DNA as template and is most active in the presence of 1.5 to 3 mM Mn^{++} as divalent cation.

We also describe the partial purification of two stimulatory factors (SF-A and SF-B) which specifically stimulate the activity of RNA polymerase II in the presence of double-stranded DNA as template.

The two basic requirements for the study of transcription *in vitro* are defined templates and purified DNA-dependent RNA polymerases. For mammalian systems the first requirement is readily fulfilled because DNA can be obtained in homogeneous form from some animal viruses. However, to purify RNA polymerase from the tissue culture host cells has proven more difficult because the supply of these cells is generally limited and mammalian RNA polymerases are notoriously unstable at low concentrations. Mammalian cells contain several forms of DNA-dependent polymerase (1), of which the major two can be separated by ion exchange chromatography. RNA polymerase I is localized in the nucleolus (2) and appears to be responsible for the synthesis of ribosomal RNA (3). RNA polymerase II is located in the nucleoplasm and can easily be identified by its characteristic sensitivity to the toxin α-amanitin (4, 5). There is evidence for the involvement of RNA polymerase II in the synthesis of heterogeneous nuclear RNA (6) and of virus-specific RNA in cells infected with adenovirus 2 or SV40 (7-9). By using kilogram quantities of animal tissues it was possible to purify extensively RNA polymerase II from rat liver (10) and calf thymus (10, 11). Here we report a procedure for purifying RNA polymerase II from the tissue culture cell lines, HeLa (12) and KB (13), where relatively small amounts of starting material are available. Using this procedure, partially purified RNA polymerase II can be obtained from as little as 1 g of cells while 10 g yield enough enzyme to allow its complete purification.

Fidelity of transcription *in vitro* may also require proteins that modify either the DNA template or the DNA-dependent RNA polymerase. We have partially purified two factors which specifically stimulate the *in vitro* transcription of native DNA by RNA polymerase II. Similar proteins have been isolated from calf thymus (14) and rat liver (15). Part of this work has been published in preliminary form (16, 17).

* This work was supported by Grant I P01 CA 13106-01 from the National Cancer Institute.

EXPERIMENTAL PROCEDURES

Cells—HeLa cells (S3) (12) and KB3 (13) cells were grown in suspension in Eagle's medium (18) (Gibco F-13) supplemented, respectively, with 5% calf serum (Gibco) and with 5% horse serum (Gibco). Six-liter batches of cells were grown in 12-liter florence flasks to a concentration of 4 to 5 × 10^5 cells per ml and diluted into new 6-liter batches for continued growth or collected. The cells were collected by allowing them to settle overnight at 4°, removing most of the supernatant by suction, and centrifuging the concentrated cell suspension at 1000 rpm in an International PR-J centrifuge for 10 min. The cell pellet was washed twice with phosphate-buffered NaCl solution and stored frozen at −70° or used directly.

The cells were free of mycoplasma contamination; no colonies were detected when the cells were plated on PPLO-agar (these tests were kindly performed for us by Professor Leonard Hayflick, Stanford University) and there was no incorporation of $[^3H]$uridine into 23 S or 16 S ribosomal RNA.

Nucleotides—Unlabeled ribonucleotides were purchased from Calbiochem. $[^3H]$UTP was purchased from New England Nuclear Corp. $[\gamma\text{-}^{32}P]$ATP was synthesized according to the methods of Penefsky *et al.* (19) using ^{32}P purchased from New England Nuclear Corp. and ADP purchased from Calbiochem.

DNA Templates—Poly(dAT) was purchased from Miles Laboratories. T_7 DNA was purified from CsCl-banded phage by the method of Goff and Minkley (20). φX174 single-stranded

DNA was purified from CsCl-banded Am-3 phage as described by Sinsheimer (21). Adenovirus 2 (Ad2) DNA was purified from CsCl-banded virions as described by Pettersson and Sambrook (22). SV40 component I DNA was purified from CsCl-banded virions as described by Trilling and Axelrod (23). Calf thymus DNA was purchased from Worthington.

Buffers—All buffers were made with deionized or deionized and distilled water; dithiothreitol was added immediately before use.

1. Buffer A contained 15% glycerol, 0.05 M Tris-HCl, pH 7.9, 0.006 M $MgCl_2$, 0.001 M EDTA, 0.001 M dithiothreitol, and varying concentrations of $(NH_4)_2SO_4$. The ammonium sulfate concentrations given throughout this paper were based on a saturated solution of ammonium sulfate at pH 7.6 (adjusted with NH_4OH) at 20° being 4.25 M in ammonium sulfate. (Twenty microliters of a 1:100 dilution of this saturated solution in 8 ml of H_2O at 0° have a conductivity of 15 μmhos per cm.)

2. Buffer B contained 20% glycerol, 0.05 M Tris-HCl, pH 7.9, 0.05 M NaCl, 0.001 M EDTA, and 0.001 M dithiothreitol. To make Buffer B with higher NaCl concentrations, the appropriate amount of a 5.0 M NaCl solution was added to the above buffer.

3. Buffer C contained 15% glycerol, 0.01 M potassium phosphate, pH 7.5, 0.1 mM EDTA, and 0.001 M dithiothreitol.

Conductivity Measurements—Conductivity measurements were made with a Serfass conductivity bridge (model RCM 15B1, A. H. Thomas Co.). Twenty microliters of a sample were diluted into 8 ml of deionized, distilled water at 0° and the conductivity determined. Buffers with known salt concentrations served as standards and the salt concentration in the sample was calculated by interpolation. Deionized, distilled H_2O had a conductivity of 1.0 ± 0.2 μmhos per cm, and 0.10 M NaCl solution had a conductivity of 15.5 ± 0.5 μmhos per cm.

Assay of RNA Polymerase Activity—The standard assay mixture contained in 0.125 ml: 0.05 μmole of ATP, GTP, and CTP; 0.5 nmole of UTP; 5 μCi of $[^3H]$UTP; 5 μmoles of Tris-HCl, pH 7.9; 5 μmoles of $(NH_4)_2SO_4$; 0.2 μmole of $MnCl_2$; 0.05 μmole of EDTA; 0.5 μmole of dithiothreitol; 5 μg of native or alkaline-denatured calf thymus DNA; 20% (v/v) glycerol; and 20 μl of enzyme. The concentration of UTP was limiting in this assay; although little RNA was synthesized a maximum of radioactive nucleotides was incorporated and measured counts per min were correspondingly high. These assay conditions constituted a UTP-limiting assay. For determining the amount of enzymatic activity present a UTP-nonlimiting assay mixture differing from the above only in containing 0.0125 μmole of UTP per assay was used. The enzyme was added last and either mixture incubated at 37° for 10 min. The incubation was terminated by adding 5 ml of 5% trichloroacetic acid in 0.01 M sodium pyrophosphate at 4°. The RNA was collected on Whatman GF/C glass fiber filters which were washed under suction with 50 ml of 2% trichloroacetic acid in 0.01 M sodium pyrophosphate. The filters were rinsed with 10 ml of 95% ethanol, air-dried, and counted in a toluene-based scintillation fluid.

One unit of enzyme activity is defined as the amount catalyzing the incorporation of 1 nmole of labeled ribonucleotide in 10 min under standard conditions. Specific activity is expressed as units per mg of protein.

Assay of Stimulatory Factors—Stimulatory factors were assayed in standard RNA polymerase assay mixtures containing 0.1 to 0.2 unit of KB RNA polymerase II (DNA cellulose fraction; see below) and 5 μg of native calf thymus DNA. Routine assays used UTP-limiting conditions, while for determining the amount of stimulatory activity present UTP-nonlimiting conditions were used.

Column Chromatography—DEAE-cellulose (Whatman DE-52) was washed first with 10 volumes of 0.5 M HCl, then with H_2O until its pH was 4, next with 10 volumes of 0.5 M NaOH, and finally with H_2O until its pH reached neutrality. The washed cellulose was equilibrated by stirring for 24 hours or longer in 10 volumes of 0.04 M $(NH_4)_2SO_4$ in Buffer A or Buffer B. DEAE-cellulose columns could be reused several times by washing the column after each elution with 5 volumes of 2 M $(NH_4)_2SO_4$ or 2 M NaCl. However, the binding capacity of the column decreased with each use so that after three to five runs the cellulose was removed, washed with acid and base, and re-equilibrated.

DEAE-Sephadex (A-25) was swollen in 0.05 M NaCl in Buffer B containing 0.05 M Tris-HCl, pH 7.2, and used directly. It was discarded after each use.

DNA-cellulose was prepared by the method of Alberts (24). Double-stranded T_4 or T_7 phage DNA, double-stranded HeLa or KB DNAs, and native and alkali-denatured calf thymus DNAs were used to prepare the DNA-cellulose. The packed columns could be reused many times over a period of 3 to 6 months so long as they were washed after each usage with 2 M NaCl in Buffer B and stored and run at 0–4°. The columns were loaded and run at a flow rate of not more than 0.5 column volume per hour to achieve optimal binding of enzyme and high resolution.

Carboxymethylcellulose (Whatman CM-52) was equilibrated with Buffer C without prior treatment.

Ammonium Sulfate Precipitation—Fractions containing RNA polymerase activity were concentrated at early stages of purification by the addition of 0.38 g of solid ammonium sulfate per ml of solution, 0.1 ml of 1 M NaOH per 10 g of ammonium sulfate used, and 0.1 ml of 0.1 M dithiothreitol per 100 ml of solution. The suspension was stirred at 4° for 3 to 6 hours and the precipitate collected by centrifugation in a Sorvall SS-34 rotor at 18,000 rpm for 30 min. The pellet was resuspended in minimal volumes of either Buffer A or Buffer B.

Dialysis—Samples were dialyzed against 100 volumes of 0.04 M $(NH_4)_2SO_4$ in either Buffer A or Buffer B. The buffer was changed every 8 hours and the conductivity of the sample measured. Dialysis was stopped when the salt concentration of the sample was within 10% of that of dialysis buffer.

Assays for Contaminating Enzymes—DNAse activity was measured by incubating RNA polymerase samples in a standard RNA polymerase assay mixture with unlabeled UTP and labeled SV40 DNA component I (closed circular form) and determining the degree to which it was converted to the nicked form (component II) by alkaline sucrose velocity gradients (25). Reconstruction experiments with DNAse I showed that this method could detect as little as 10^{-5} μg per ml of pancreatic DNAse.

RNAse activity was assayed as follows: 10^5 dpm of ^{32}P-labeled 28 S ribosomal RNA (5×10^8 dpm from 5×10^7 KB cells) were incubated with an RNA polymerase sample in the normal RNA polymerase assay mix at 37° for 30 min. The incubation mixture was heated to 70° for 30 s, quickly cooled, and layered on a gradient containing 10 to 30% sucrose, 0.01 M Tris-HCl, pH 7.4, 0.1 M LiCl, 0.1% SDS,[1] and 0.001 M EDTA. It was centrifuged in a Spinco SW 56 rotor at 49,000 rpm for 2½ hours. Fractions were collected from the bottom of the tube, diluted to 1 ml with H_2O and counted directly by Cerenkov radiation in a liquid

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

scintillation counter. RNA sedimenting lighter than an untreated marker indicated the presence of RNAse activity. Reconstruction experiments showed that approximately 10^{-6} μg per ml of pancreatic RNAse could be detected by this assay.

Protein kinase activity was measured by the incorporation of [γ-^{32}P]ATP into material that was resistant to pancreatic RNAse but sensitive to Pronase. Either endogenous protein or commercial preparations of histones (Sigma) were used as phosphate acceptors. A standard RNA polymerase assay mixture containing 0.5 mM unlabeled UTP, no unlabeled ATP, 100 to 500 μCi of [γ-^{32}P]ATP (100 to 250 Ci per mM), with or without 10 μg of arginine-rich histones from calf thymus (Sigma) was incubated at 37° for 30 min. The mixture was then applied to a Sephadex G-50 column (1 × 20 cm) equilibrated in Buffer B without glycerol to separate the unreacted [γ-^{32}P]ATP from the heavier products. The flow-through material containing ^{32}P was pooled, brought to 0.01 M $MgCl_2$, and divided into three equal aliquots; one aliquot was adjusted to 100 μg per ml with a 2 mg per ml solution of Pronase (Calbiochem) the second aliquot was brought to 25 μg per ml with a 1 mg per ml solution of pancreatic RNase (Worthington), and the third was left as a standard. The three samples were incubated at 37° for 12 hours, precipitated with trichloroacetic acid, and counted as in the standard RNA polymerase assay. The presence of a protein kinase was indicated by the Pronase-treated acid-precipitable material being less (usually at least one order of magnitude) than the RNAse-treated precipitable material.

ATPase activity was measured by adding 5 × 10^5 dpm of [γ-^{32}P]ATP (100 Ci per mM) to an RNA polymerase sample in Buffer A with or without 0.01 μmole of ATP and incubating at 37° for 30 min. The reaction mixture was spotted on polyethyleneimine thin layer plates (Brinkmann Inc.) and developed in 1.5 M potassium phosphate buffer at pH 3.5 (26). The products and appropriate markers were identified by autoradiography.

Polynucleotide phosphorylase activity was assayed as described (27) except that glass fiber filters (Whatman GF/C, 24-mm diameter) were used and the radioactivity was determined by Cerenkov radiation.

DNA polymerase activity was assayed as described by Richardson (28) using [^{3}H]TTP with poly(dAT) as template.

Ribonuclease H activity was measured as described by Keller (29).

Isoelectric Focusing—Isoelectric focusing was performed as described by Pettersson *et al.* (30) in an LKB isoelectric focusing column with a 20 to 50% glycerol gradient in 0.005 M dithiothreitol. A gradient from pH 4 to 6 was established with "ampholines" purchased from LKB. The column was run at 0° for 48 hours and was focused initially with 400 volts which was increased to 1000 volts gradually so that the current remained less than 1 ma. Fractions were collected and assayed in the standard manner without removal of the ampholines. So long as each RNA polymerase had been purified through the DNA-cellulose chromatography stage, no precipitation of proteins occurred.

Polyacrylamide SDS Gel Electrophoresis—Polyacrylamide gel electrophoresis on discs containing a 4% acrylamide stacking gel and a 8.75% acrylamide running gel was run in the buffer system described by Laemmli (31).

Protein Determination—Protein concentrations were determined by precipitating an aliquot of the solution to be measured with 10% trichloroacetic acid at 0° for 60 min. The suspension was centrifuged in a Sorvall SS34 rotor at 18,000 rpm for 30 min; the pellet was resuspended in an equal volume of spectral grade acetone at −20° and recentrifuged in the same manner. The acetone was removed and the amount of protein in the centrifuge tube determined by the method of Lowry (32). For solutions containing less than 0.1 mg of protein per ml the sensitivity of the assay was increased by using equal volumes of the basic and the Cu_2SO_4 solutions instead of the usual 50:1 ratio.

RESULTS

Purification of α-Amanitin-sensitive RNA Polymerase

Extraction—All procedures were conducted between 0° and 4° unless specified. One volume (1 g to 200 g) of wet packed cells was swollen for 15 to 30 min in 4 volumes of 0.01 M Tris-HCl, pH 7.9, 0.001 M EDTA, and 0.005 M dithiothreitol. The cells were disrupted in a tight fitting Dounce homogenizer with 5 to 10 strokes. The volume of the homogenate was doubled and adjusted to a final concentration of 0.025 M Tris-HCl, pH 7.9, 0.3 mM EDTA, 3.5 mM dithiothreitol, 5 mM $MgCl_2$, 20% (v/v) glycerol, 10% (w/v) sucrose, and 10% of saturation with ammonium sulfate. The ammonium sulfate was added last and caused the nuclei to lyse; stirring was then stopped to prevent shearing of DNA. The lysate was heated to 35° for a period of 30 to 60 min. The heating was found to give a 2- to 3-fold higher recovery of RNA polymerase II and an unchanged recovery of the α-amanitin-resistant enzyme (RNA polymerase I). The lysate was cooled to 0° for 4 to 10 hours and centrifuged in a Spinco type 35 rotor at 35,000 rpm for 5 hours. The RNA polymerase activity was precipitated from the supernatant with ammonium sulfate as described above and the resulting pellet dissolved in Buffer A + 0.04 M $(NH_4)_2SO_4$ (1 to 2 ml per g of cells). This solution was dialyzed against Buffer A + 0.04 M $(NH_4)_2SO_4$ and 0.15 mg per ml of phenylmethylsulfonyl fluoride was added in order to inhibit proteases. The solution was clarified by centrifugation for 1 hour at 42,000 rpm in a Spinco type 42 rotor; it contained between 15 and 20 mg of protein per g of initial cells.

DEAE-cellulose Chromatography—The clarified solution was passed onto a DEAE-cellulose column (1 ml of packed DEAE-cellulose per g of cells). The column was washed with 4 to 5 column volumes of 0.04 M $(NH_4)_2SO_4$ in Buffer A and developed with a linear gradient of 6 to 8 column volumes from 0.04 to 0.4 M $(NH_4)_2SO_4$ in Buffer A. Fractions were collected and assayed for RNA polymerase activity (Fig. 1). Two activities were found; the α-amanitin-insensitive RNA polymerase I eluted around 0.07 M $(NH_4)_2SO_4$ while the α-amanitin-sensitive RNA polymerase II eluted in a broad peak around 0.2 M $(NH_4)_2SO_4$.

We have further purified RNA polymerase I by glycerol gradient centrifugation and DNA-cellulose chromatography. This purification will be reported in a forthcoming communication.

The fractions containing RNA polymerase II activity were pooled and precipitated with ammonium sulfate. The pellet was dissolved in Buffer B containing 15% glycerol (1 ml of buffer per 10 g of cells) and dialyzed against the same buffer. The dialyzed material was clarified by centrifugation in a Spinco type 40 rotor at 40,000 rpm for 1 hour. The supernatant contained approximately 0.7 mg of protein per g of initial cells.

Glycerol Gradient Sedimentation—The supernatant (2.5-ml aliquots) from the previous step was layered on 36-ml gradients containing 0 to 15% sucrose, 20% glycerol, 0.1 M Tris-HCl, pH 7.9, 1 mM EDTA, 0.01 M $MgCl_2$, 0.05 M NaCl, 5 mM dithiothreitol, 0.1% β-mercaptoethanol, and 0.2 mg per ml of lysozyme

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

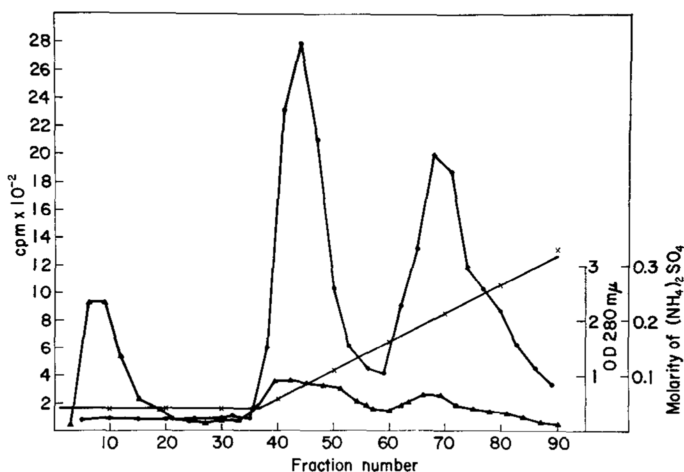

FIG. 1. DEAE-cellulose chromatography. The cell extract from 100 g of cells after high speed centrifugation, precipitation, and dialysis against 0.04 M $(NH_4)_2SO_4$ in Buffer A was passed onto a DEAE-cellulose column (3 × 15 cm) equilibrated with the dialysis buffer. The column was washed with 3 to 5 column volumes of the dialysis buffer and developed with a linear gradient of 4 to 8 column volumes of 0.04 M to 0.4 M $(NH_4)_2SO_4$ in Buffer A. Fractions of 20 ml were collected and 20-μl aliquots assayed as described under "Experimental Procedure." The first peak of activity to elute is the α-amanitin-insensitive RNA polymerase I, the second is α-amanitin-sensitive RNA polymerase II. ×— —×, molarity of $(NH_4)_2SO_4$; ▲— —▲, optical density measured at 280 nm; ●——●, RNA polymerase activity assayed with denatured calf thymus DNA.

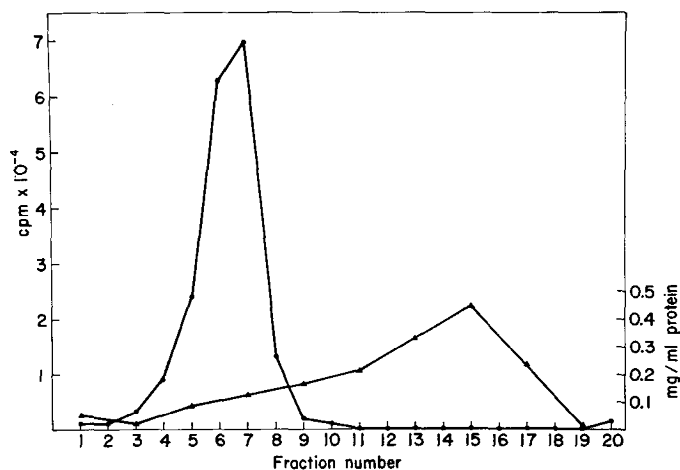

FIG. 2. Glycerol gradient centrifugation. Fractions containing RNA polymerase II activity from the DEAE-cellulose column were pooled, precipitated with solid $(NH_4)_2SO_4$, and dialyzed against Buffer B containing only 15% glycerol. After a high speed centrifugation to remove precipitated material, 2.5-ml aliquots were layered on 36-ml gradients (composition described in the text) and centrifuged at 26,500 rpm in a Spinco SW 27 rotor for 52 hours. Fractions of 2 ml were collected by puncturing the bottom of the tube and 20-μl aliquots were assayed. ▲——▲, protein concentration; ●——●, RNA polymerase activity.

(three times recrystallized, Calbiochem). The gradients were centrifuged in a Spinco SW 27 rotor at 26,500 rpm for 52 hours at 4°. Fractions (2 ml) were collected from the bottom of the tubes. Aliquots were assayed (Fig. 2) and those fractions containing RNA polymerase activity were pooled; 65 to 75% of the activity applied to the gradients was recovered. The pooled fractions contained (including the lysozyme) approximately 0.2 mg of protein per g of cells.

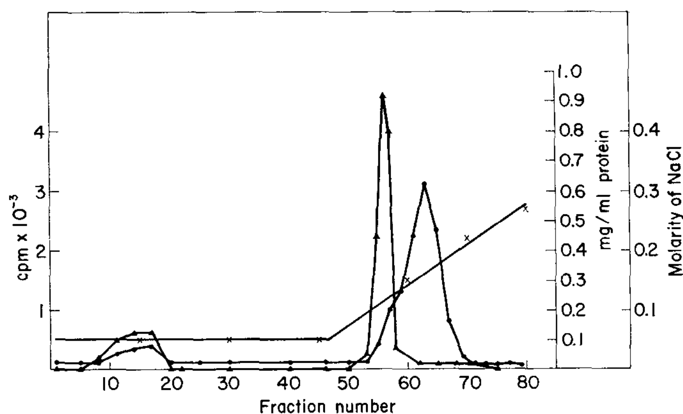

FIG. 3. Single-stranded calf thymus DNA-cellulose chromatography. Fractions of the glycerol gradients containing activity were pooled, dialyzed against Buffer B, and passed onto a DNA-cellulose column containing 20 mg of denatured calf thymus DNA and equilibrated with Buffer B. The column was washed with 4 to 5 column volumes of Buffer B and developed with a linear gradient containing 6 to 8 column volumes of 0.05 M to 0.5 M NaCl in Buffer B. Fractions of 2.5 ml were collected and 20-μl aliquots assayed. Symbols: × ——×, molarity of NaCl; ▲——▲, protein concentration; ●— —●, RNA polymerase activity.

DNA-cellulose Chromatography—The pooled gradient fractions were dialyzed against Buffer B and passed onto a column of single-stranded calf thymus DNA-cellulose. The column was washed with 4 to 5 column volumes of the onput buffer and developed with 6 to 8 column volumes of a linear gradient of 0.05 to 0.5 M NaCl in Buffer B. The RNA polymerase activity eluted in a broad peak around 0.175 M NaCl (Fig. 3). The fractions containing the activity were pooled; approximately 50% of the activity applied was recovered, with a protein concentration of 0.02 mg per g of cells.

DEAE-Sephadex Chromatography—The pooled active fractions from the DNA-cellulose column were dialyzed against Buffer B and passed onto a Sephadex A-25 column (0.2 ml of column volume per g of initial cells) equilibrated with Buffer B containing 0.05 M Tris-HCl, pH 7.2. The column was washed with 4 to 5 volumes of the equilibration buffer and developed with a 6 to 8 column volume gradient from 0.05 to 0.5 M NaCl in equilibration buffer. Twenty to 30% of the RNA polymerase activity applied to the column was recovered as a sharp peak (Fig. 4). The protein concentration in the final preparation was 0.001 and 0.003 mg per g of initial cells. The yields and specific activities of RNA polymerase II during the course of purification are summarized in Table I.

Storage and Stability of RNA Polymerase Preparations—Whenever possible enzyme preparations were stored at −80° in Buffer B. The most pure preparations were stable to at least five cycles of freezing and thawing. During the course of purification the enzyme activity became increasingly unstable. The addition of lysozyme as a protein carrier increased the recovery of activity during the glycerol gradient centrifugation but did not significantly affect recovery of activity during DNA-cellulose chromatography. In general the percentage of recovery of activity for each step was proportional to the amount added—the more activity applied, the greater the percentage of it recovered. Therefore, large preparations were used when the cells were available.

Contaminating Enzyme Activities—After the glycerol gradient the RNA polymerase preparation does not contain detectable DNase, ATPase, polynucleotide phosphorylase, DNA polymer-

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

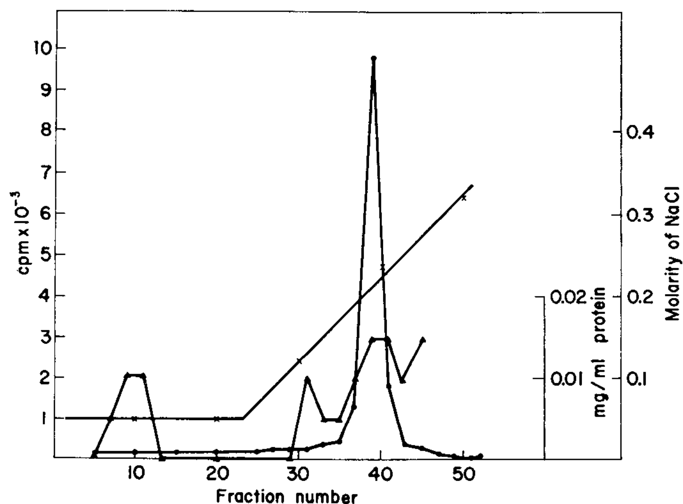

FIG. 4. DEAE-Sephadex chromatography. Fractions from the DNA-cellulose column containing RNA polymerase activity were pooled, dialyzed against Buffer B, and passed onto a DEAE-Sephadex column (1.5 × 10 cm) equilibrated with Buffer B containing 0.05 M Tris-HCl, pH 7.2. The column was washed with 4 to 5 column volumes of equilibration buffer and developed with a linear gradient containing 6 to 8 volumes of 0.05 M NaCl to 0.5 M NaCl in equilibration buffer. Fractions of 1.5 ml were collected and 20-μl aliquots assayed. ×——×, molarity of NaCl; ▲——▲, protein concentration; ●——●, RNA polymerase activity.

TABLE I

Purification of RNA polymerase II from 100 g of KB cells

Denatured phage T_7 DNA was used as template under UTP nonlimiting assay conditions. The samples containing RNA polymerase II activity for the last four purification steps were dialyzed against Buffer B before assaying so that final assay conditions would be consistent for all samples (0.0016 M $MnCl_2$, 0.04 M $(NH_4)_2SO_4$, 0.01 M NaCl).

Purification step	Total protein	Total activity	Specific activity	Specific activity of peak fractions	Total yield
	mg	*units*	*units/mg*	*units/mg*	%
High speed supernatant of crude extract	1500–2000	40[a]	0.02		
DEAE-cellulose chromatography	70	40	0.6	3	100
Glycerol gradient sedimentation	10	30	3.0	20	70
DNA-cellulose chromatography	2	20	10	50	50
DEAE-Sephadex chromatography	0.1–0.3	4–5	15–50	300	10

[a] This figure represents the sum of activities corresponding to RNA polymerase I + RNA polymerase II + stimulatory and inhibitory contaminants.

ase, or ribonuclease H activities. RNAse and protein kinase activities are detectable but are removed during DNA-cellulose chromatography. The amount of these activities that could be present but not detected by the assays used are given in Table II.

Physical Properties of RNA Polymerase II

Sedimentation Properties—In buffer conditions in which DNA-dependent RNA polymerase from *E. coli* sediments as a monomer (33) with a sedimentation constant of 14.9 S, RNA polymerase II has a sedimentation constant of 16.5 S (Fig. 5). The mammalian enzyme does not appreciably change its sedimentation constant in glycerol gradients containing concentrations between 0.01 and 0.5 M NaCl. In contrast *E. coli* RNA polymerase has

TABLE II

Maximum levels of some possible contaminating enzyme activities in RNA polymerase II preparations

Assay conditions are described under "Experimental Procedures."

Contaminating enzyme activity assayed	Purification step where assayed	Maximum possible activity present as determined by the sensitivity of the assay
DNAase (endo)	Glycerol gradient	$<2.5 \times 10^{-3}$ μg of DNAse I eq per mg of protein
RNAase	DNA cellulose	$<2.5 \times 10^{-3}$ μg of RNAse A eq per mg of protein
Protein kinase	DNA cellulose	<2.5 pmoles of [γ-^{32}P]ATP rendered trichloroacetic acid-soluble by Pronase digestion per mg of protein
ATPase	Glycerol gradient	<5 pmoles of [γ-^{32}P]ATP converted to $^{32}P_i$ per mg of protein
Polynucleotide phosphorylase	Glycerol gradient	<2.5 μmoles of $^{32}P_i$ incorporated into nucleoside diphosphate per mg of protein
DNA polymerase	Glycerol gradient	<2.5 pmoles of thymidine triphosphate rendered acid-precipitable per mg of protein
Ribonuclease H	Glycerol gradient	<10 pmoles of [^{3}H]poly(rA)·poly(dT) degraded to acid-soluble products per mg of protein

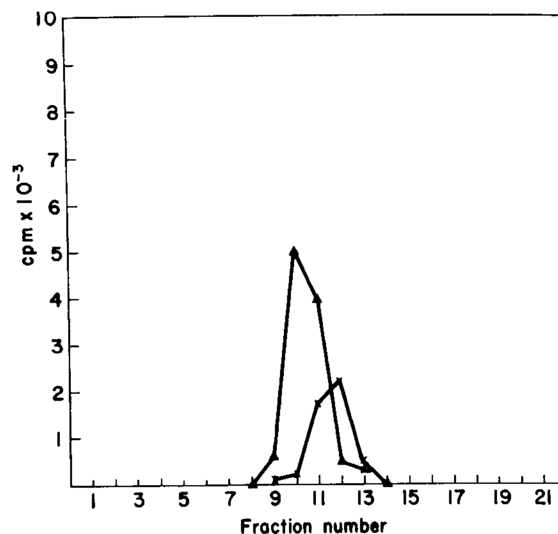

FIG. 5. Co-sedimentation of *Escherichia coli* RNA polymerase with KB polymerase II. The sample was layered on 11-ml gradients (composition described in the text) and centrifuged in a Spinco SW 41 rotor at 41,000 rpm for 32 hours. Fractions of 0.5 ml were collected by puncturing the tube. ▲——▲, RNA polymerase activity assayed with denatured calf thymus DNA and with 15 μg of rifampicin included; ×——×, the same assay but with 15 μg of α-amanitin included.

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

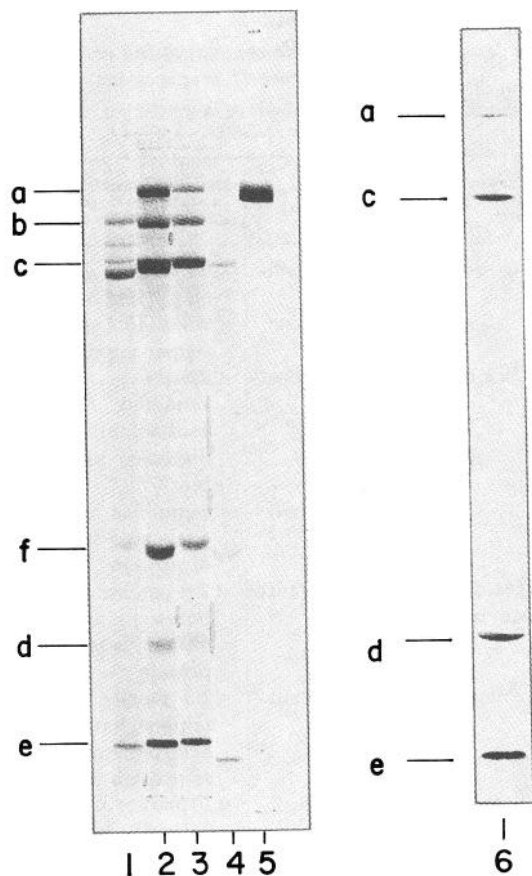

Fig. 6. Sodium dodecyl sulfate polyacrylamide gels. Molecular weights were determined by the interpolative method of Weber and Osborn (43). *Gels 1* to *4* contain samples from the shoulders and peak fractions (38 to 41 of Fig. 4) of activity from a final DEAE-Sephadex column. *Gel 5* contains a 20-μg sample of myosin. *Gel 6* contains a sample of the peak of activity from an isoelectric focusing column. The individual bands and their molecular weights are described in the text.

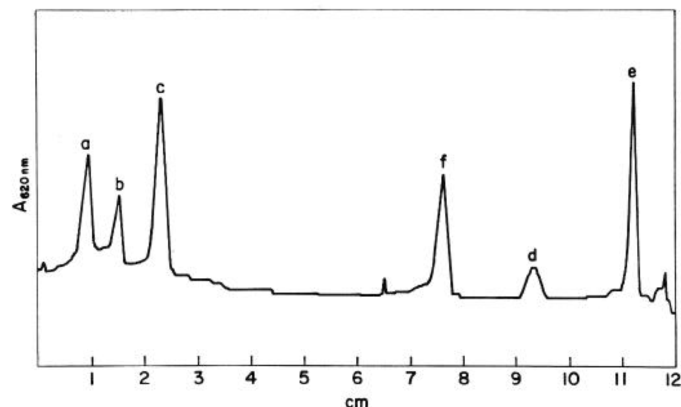

Fig. 7. A tracing of Gel 2 of Fig. 5 taken with a Joyce Loebl chromoscan using a 620 nm wave length filter. A "d" cam was used to expand the vertical axis. The horizontal axis begins with the stacking-running gel interface.

a tendency to form aggregates in low ionic strength conditions (33). Without a more detailed study, the molecular weight of the mammalian enzyme can only be estimated to be in the range of *E. coli* RNA polymerase, approximately 500,000.

Subunit Composition—Acrylamide gel electrophoresis in SDS suggests that the subunit structure of RNA polymerase II isolated from KB and HeLa cells is similar to that described for the enzymes from rat liver (10) and calf thymus (10, 11). To aid in identifying the polypeptides associated with the RNA polymerase activity, the enzyme was purified via two different routes. The first was our standard procedure (see above) while the second used DEAE-cellulose chromatography, glycerol gradient centrifugation, phosphocellulose chromatography (in potassium phosphate buffer, pH 8.0, 20% glycerol, 0.001 M EDTA, and 0.001 M dithiothreitol), DEAE-Sephadex chromatography at pH 7.2, and isoelectric focusing. In our hands, the yields of RNA polymerase II in the chromatography on phosphocellulose and during isoelectric focusing are extremely low. These steps were therefore not suitable for the routine purification procedure. For each of the two differently purified samples acrylamide SDS gels (Fig. 6) showed four bands (*a*, *c*, *d*, and *e* in Fig. 6) whose concentration in the gel appeared to correlate with the activity applied to the gel. Myosin (mol wt = 200 to 220,000), *E. coli* RNA polymerase β and β' subunits (mol wt = 160,000 and 150,000), and human γ-globulin (mol wt = 55,000 and 23,000) were used as markers to determine the approximate molecular weights of polypeptides *a*, *c*, and *d*. In 8.75% *Gel a* has a molecular weight of 220,000, *c* of 140,000, and *d* of 35,000. The polypeptide corresponding to Band *b* is present in varying amounts in different preparations of RNA polymerase II. Weaver *et al.* (10) suggest that this 170,000 dalton polypeptide is a proteolytic product of the heavier subunit, *a*. We find that though we always added the protease inhibitor phenylmethylsulfonyl fluoride to our preparations, the amount of Band *b* still varies in different preparations. The 8.75% gels do not include material having molecular weights of 25,000 or less; therefore, the molecular weight of Subunit *e* could not be determined accurately. Band *f*, which is seen in enzyme preparations purified by the standard procedure, appears to be a contaminant since it is absent in preparations carried through the isoelectric focusing step. The heavily loaded Gels 2, 3, and a third from a different preparation have been scanned using a Joyce Loebl chromoscan densitometer reading in the transmission mode at a wave length of 620 nm. A tracing of Gel 2 is shown in Fig. 7. The three gels have been scanned with a 1:3 expansion, peaks corresponding to Bands *a*, *b*, *c*, and *d*, where possible, have been cut out and weighed (the weight being proportional to the area). In order to assay the molar ratios of polypeptides in Bands *a*, *b*, *c*, and *d* the weights of the peak traces must be divided by the molecular weights of their corresponding polypeptides because the amount of Coomassie blue bound is approximately proportional to the number of peptide bonds present. The molar ratios of Bands *a*, *b*, *c*, and *d* (arbitrarily setting that of Band *c* equal to unity) are given in Table III. Because these values depend on the assigned molecular weights which certainly have at least a ±10% error, they can be taken only as approximate values. The data in Table III are consistent with molar ratios of $a + b = 1$, $c = 1$, and $d = 1$ as suggested by Weaver *et al.* (10); however, there is enough error in the measurements for them to be also consistent with molar ratios of $a + b = 1$, c 2, and $d = 1$.

Isoelectric Point—RNA polymerase II focuses as a single peak of activity in a pH gradient established either over a wide (pH 4 to 10) or a narrow (pH 4 to 6) range of pH. The activity peaks at about pH 4.7 to 4.8 (Fig. 8).

Enzymatic Properties

Cation Salt Requirements—Purified RNA polymerase II from KB and HeLa cells is most active when manganese is present as

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

TABLE III

Molar ratios of Bands a, b, c, and d as determined by Joyce Loebl chromoscan tracings of Gels 2 and 3 of Fig. 6, and a gel from a different preparation

The molar ratio of *c* is set arbitrarily as 1. Errors in these values arise from a ±10% error in assigning the molecular weights of the polypeptides *a*, *b*, *c*, and *d*, from nonlinear response of the densitometer at low adsorption levels, and in a necessary arbitrariness in determining the base-line for each peak in measuring its area. These values certainly can have errors of greater than ±10%.

Band	Molar ratios of polypeptides determined from stained gel bands		
	Gel 2	Gel 3	Peak gel from another preparation
a	0.4	0.2	0.6
b	0.4	0.4	0.4
c	1.0	1.0	1.0
d	0.7	Could not be determined	Could not be determined

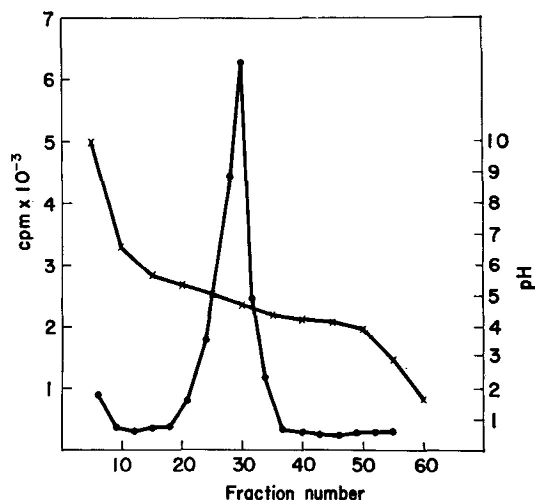

FIG. 8. Isoelectric focusing. RNA polymerase II was focused in pH 4 to 6 "ampholines" and assayed in the presence of the ampholines as described in the text. ×——×, pH; ●——●, RNA polymerase activity.

a divalent cation and the $(NH_4)_2SO_4$ concentration is between 0.05 and 0.1 M. It utilizes magnesium less efficiently than manganese as a cation (Fig. 9). These cation and salt requirements are similar to those of other α-amanitin-sensitive RNA polymerases (1) isolated from mammalian cells.

α-Amanitin Sensitivity—α-Amanitin specifically inhibits RNA polymerase II (4, 5). When aliquots of 3×10^{-3} units of RNA polymerase are incubated under standard assay conditions in the presence of 10^{-6} M α-amanitin RNA synthesis is inhibited by 98%. At a concentration of 10^{-9} M α-amanitin inhibits the enzyme by approximately 50%.

Template Specificity—Throughout the course of its purification RNA polymerase II utilizes single-stranded DNA more efficiently than double-stranded DNA as a template. However, the relative efficiency of synthesis on the two kinds of template changes drastically during the purification. Prior to glycerol gradient centrifugation there is still RNA polymerase activity without added DNA. However, addition of native T_7 or adenovirus 2 DNA stimulates RNA synthesis 1- to 2-fold while alkali-denatured T_7 or Ad2 DNA stimulates RNA synthesis 100-fold or more. After glycerol gradient centrifugation RNA polymerase II preparations have little or no detectable activity without added template and alkali-denatured DNAs are now utilized only 2 to 10 times more efficiently than native DNAs as added templates (Table IV). During the glycerol gradient centrifugation not more than 30 to 40% of the activity of RNA polymerase II as measured with poly(dAT) or denatured T_7 DNA is lost; the 10-fold or greater change in its efficiency of utilization of native *versus* denatured DNA as template therefore reflects an increase in the ability of the enzyme to synthesize RNA from native DNA. This effect could be due to the removal during gradient centrifugation of a substance which inhibits transcription of double-stranded DNA. However, addition of aliquots from glycerol gradient fractions not containing RNA polymerase II activity to those containing the activity does not restore the inhibition of synthesis with native DNAs.

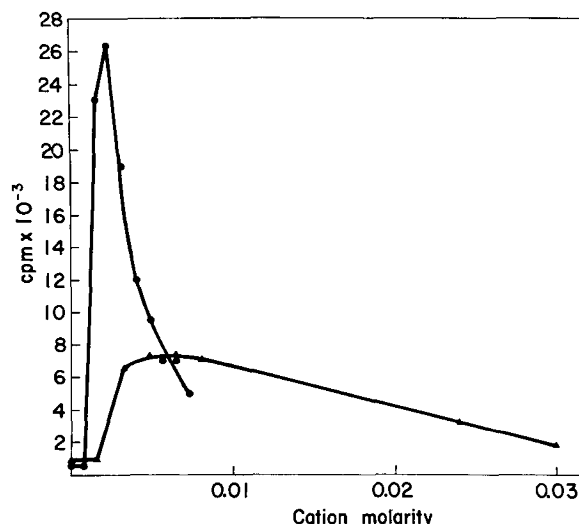

FIG. 9. Cation optima. RNA polymerase II (0.1 unit) was assayed using φX174 single-stranded DNA as a template with 0.06 M $(NH_4)_2SO_4$ as the salt in varying concentrations of $MgCl_2$ (▲——▲) or $MnCl_2$ (●——●).

TABLE IV

RNA synthesis with different templates and RNA polymerase II at different stages of purification

Assays were performed under standard UTP nonlimiting conditions with 5 μg of DNA per assay. Denatured DNA templates were prepared by incubating of the DNA with 0.1 M NaOH for 5 min at room temperature. RNA polymerase activity after the DEAE-Sephadex chromatography is more dilute than after the DEAE-cellulose chromatography; consequently only relative activities can be compared.

RNA polymerase II fraction	DNA template	UMP incorporated
		pmoles
DEAE-cellulose	None	0.2
	T7	0.36
	Denatured T7	15.3
	Ad2	0.2
	Denatured Ad2	11.5
Final DEAE-Sephadex	None	0.0
	T7	1.5
	Denatured T7	2.5
	Ad2	1.0
	Denatured Ad2	3.1

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

Product Formation on ϕX174 Single-stranded DNA—Because RNA polymerase II uses single-stranded more efficiently than native DNAs as templates, it was of interest to determine the nature of the product synthesized on a defined single-stranded DNA. When ϕX174 single-stranded DNA is used as a template (synthesis conditions and product isolation described in legend to Fig. 10) present in excess and the product synthesized by RNA polymerase II on it is centrifuged to equilibrium in cesium sulfate, the product bands as a single peak at a density (ρ = 1.51 to 1.55) typical of an RNA-DNA hybrid (Fig. 10*a*). If the ϕX174 template is present in limiting concentrations the product bands in cesium sulfate as both an RNA-DNA hybrid and as free RNA (ρ = 1.65) (Fig. 10*b*) suggesting that RNA polymerase II can displace the RNA from an RNA-DNA hybrid. Chamberlin and Berg (34) have shown that *E. coli* RNA polymerase similarly synthesizes a RNA-DNA hybrid with ϕX174 DNA as a template and with sufficient synthesis can displace the RNA from that RNA-DNA template. It has not yet been determined whether the free RNA synthesized by mammalian RNA polymerase II is complementary only to the DNA; the most simple notion suggests that it is, arising via semi-conservative synthesis as in the case of *E. coli* RNA polymerase's product (34).

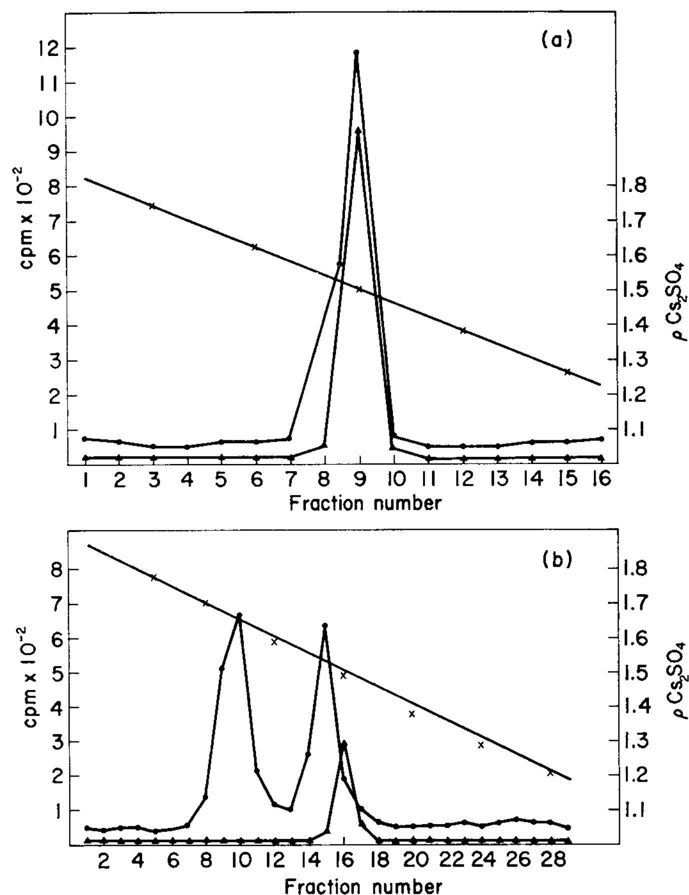

FIG. 10. Cs_2SO_4 banding of RNA polymerase II products synthesized on ϕX174 single-stranded DNA. RNA was synthesized under standard assay conditions ("Experimental Procedure") in a 100-μl volume. The reactions were quenched with 400 μl of 0.1 M EDTA containing boiled [^{32}P]adenovirus 2 DNA as a marker. A solution of Cs_2SO_4 was added and the density adjusted to 1.55 g per ml. The samples were centrifuged at 37,000 rpm at 20° for 72 hours in a Spinco SW 56 rotor. Fractions were collected by puncturing the bottom of the tubes and were precipitated and counted as described for standard assay mixtures ("Experimental Procedure"). *a*, 0.025 unit of RNA polymerase II was incubated with 0.25 μg of ϕX174 DNA for 10 min at 37°; *b*, 0.25 unit of RNA polymerase II was incubated with 0.25 μg of ϕX174 DNA for 30 min at 37° with one-tenth of the standard concentration of [^{3}H]-UTP. ▲——▲, denatured [^{32}P]adenovirus 2 DNA; ●——●, [^{3}H]RNA product; ×——×, density.

Purification of Stimulatory Factors

DEAE-cellulose Chromatography—The protein which did not bind to DEAE-cellulose during the purification of RNA polymerase was precipitated with ammonium sulfate, collected by centrifugation, and dialyzed against Buffer A not containing any ammonium sulfate. A precipitate which formed during dialysis was removed by low speed centrifugation and the clarified supernatant was applied to a DEAE-cellulose column (0.5 ml of packed DEAE-cellulose per g of cells) equilibrated with Buffer A not containing any $(NH_4)_2SO_4$. The column was washed with Buffer A and the material not binding to it concentrated by ammonium sulfate precipitation and dialyzed against Buffer C.

CM-cellulose Chromatography—The dialyzed material was passed onto a column of CM-cellulose (0.2 ml of packed CM-cellulose per g of cells), the column washed with 5 volumes of Buffer C, and developed with a linear gradient of 8 column volumes containing 0 to 0.4 M KCl in Buffer C. Fractions were collected and assayed for stimulatory activity. Two peaks of stimulatory activity were usually found and called SF-A and SF-B (Fig. 11). SF-B varied greatly in amount from preparation to preparation and has not been further purified.

Gel Filtration—The fractions containing SF-A from the CM-cellulose column were concentrated by ammonium sulfate precipitation and dialyzed against Buffer C. The dialyzed material was loaded on a Sephadex G-150 column (2 × 80 cm) equilibrated with Buffer C free of glycerol but containing 0.2 M KCl. Elution of the column was carried out at a flow rate of 5 ml per hour. Fractions were collected and assayed for stimulatory activity (Fig. 12).

DNA-cellulose Chromatography—Fractions from the Sephadex

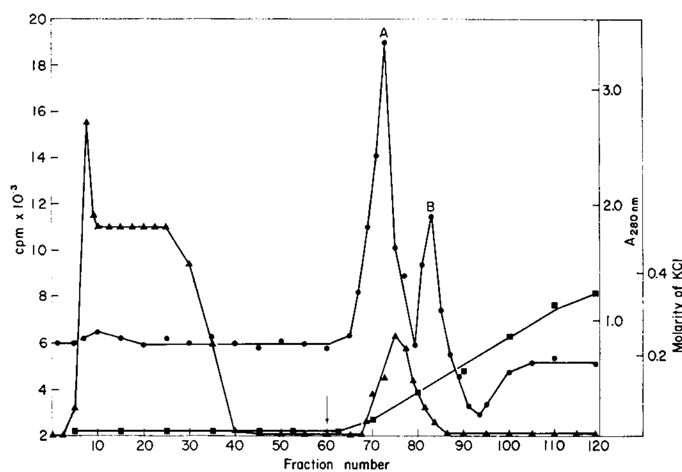

FIG. 11. Chromatography of stimulatory factors on CM-cellulose. Flow-through material from the second DEAE-cellulose column (see text) of a 100-g KB cell preparation was precipitated with $(NH_4)_2SO_4$ and dialyzed against Buffer C. The dialyzed material was applied to a column (2 × 9 cm) of CM52 equilibrated with Buffer C. After extensive washing with Buffer C, the column was developed with a linear gradient of 400 ml of Buffer C containing 0 to 0.4 M KCl. Fractions of 6 ml were collected and the stimulatory activity assayed with 20-μl aliquots as described under "Experimental Procedure." ▲——▲, absorbance at 280 nm; ●——●, incorporation of [^{3}H]UMP; ■– –■, concentration of KCl; the *arrow* indicates the start of the gradient elution.

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

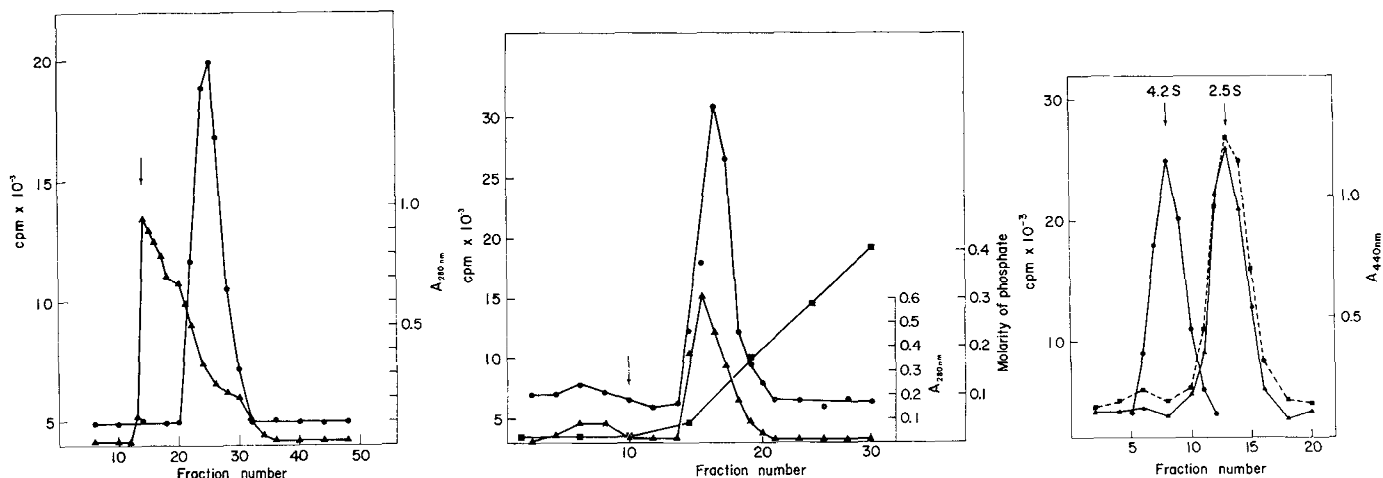

FIG. 12 (*left*). Gel filtration of SF-A on Sephadex G-150. Peak A of stimulatory factor from the CM-cellulose column (Fig. 11) was precipitated with $(NH_4)_2SO_4$ and briefly dialyzed against Buffer C containing 0.2 M KCl and no glycerol. The material (5 ml) was applied to a column (2 × 62 cm) of Sephadex G-150 and eluted with a flow rate of 6 ml per hour. Fractions of 6 ml were collected and stimulatory activity assayed with 20-μl aliquots. ▲——▲, absorbance at 280 nm; ●——●, incorporation of [³H]UMP; the *arrow* indicates the void volume of the column.

FIG. 13 (*center*). Chromatography of SF-A on DNA-cellulose. The fractions containing stimulatory activity from the gel filtration step (Fig. 12) were combined, dialyzed against Buffer C, and applied to a column (1 × 6 cm) of double-stranded T4 DNA-cellulose equilibrated with Buffer C. After washing with Buffer C, the column was developed with a 60-ml linear gradient from 0 to 0.5 M potassium phosphate in Buffer C. Fractions of 2 ml were collected and 20-μl aliquots assayed for stimulatory activity. ▲——▲, absorbance at 280 nm; ●——●, incorporation of [³H]UMP; ■——■, concentration of potassium phosphate; the *arrow* indicates the start of the gradient elution.

FIG. 14 (*right*). Zonal centrifugation of SF-A and SF-B. Fractions of CM-cellulose Peak A and Peak B (Fig. 11) were concentrated by $(NH_4)_2SO_4$ precipitation and dialyzed against Buffer C. Aliquots of 0.15 ml were mixed with 0.05 ml of hemoglobin and layered on 3.6-ml gradients containing 5 to 20% sucrose; 0.05 M Tris-HCl, pH 7.9; 0.05 M NaCl; 1 mM EDTA; 1 mM dithiothreitol; and 20% (v/v) glycerol. The gradients were centrifuged for 48 hours at 55,000 rpm and 5° in a Spinco SW 56 rotor. Fractions were collected from the bottom of the tubes and assayed for stimulatory activity. Symbols: ■- - -■, SF-A; ▲——▲, SF-B; ●——●, absorbance at 440 nm (hemoglobin).

G-150 column containing stimulatory activity were combined, dialyzed against Buffer C, and then passed onto a column of native T4 DNA-cellulose (0.05 ml of packed DNA-cellulose per g of cells). After washing with several column volumes of Buffer C, proteins were eluted with a linear gradient of 3 column volumes containing 0 to 0.5 M potassium phosphate in Buffer C. Fractions were collected and assayed (Fig. 13). The stimulaory factor was concentrated by ammonium sulfate precipitation and subsequently dialyzed against Buffer C.

Properties of Stimulatory Factors

Purity—Neither of the stimulatory factors have been purified to homogeneity and therefore show a number of bands after polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. They appear to be proteins since heating to 70° for 5 min completely destroys their activity. Both factors are free of the contaminating enzymes described under "Experimental Procedures." The levels of contaminating enzyme activities possibly present but not detected by the assays are approximately the same as in the case of RNA polymerase II (see Table II). It is important to note that there is less than 2.5×10^{-2} μg of DNAse I eq per mg of protein in the most purified SF-A preparations. There is, however, a DNA exonuclease activity found in these preparations of SF-A. It has in addition to its ability to degrade double-stranded DNA, the ability to degrade the RNA of a RNA-DNA hybrid. This activity is distinct from RNAse H in that it can degrade DNA and that on degrading RNA-DNA hybrids, it yields a large proportion of ribomononucleotides while RNAse H generates larger oligonucleotides with the mononucleotides as minor species (35). The amount of this exonuclease contamination is small. SF-A preparations purified through the DNA-cellulose stage will render less than 10^{-2} μg of ³²P-labeled Ad2 DNA acid-soluble per mg of protein per 10 min at 37.

TABLE V

Specificity of stimulatory factors for mammalian RNA polymerase II

Standard reaction mixtures contained 5 μg of native calf thymus DNA, 0.2 unit of KB RNA polymerases I or II, 0.6 unit of *E. coli* RNA polymerase (glycerol gradient fraction (42)), or 0.1 unit of α-amanitin-sensitive RNA polymerase (DEAE-cellulose fraction) isolated from yeast by a procedure similar to the one described in this paper for mammalian RNA polymerases.[a] Numbers represent picomoles of [³H]UMP incorporated.

Enzyme	Control	+ SF-A	+ SF-B
KB RNA polymerase II	18	198	204
KB RNA polymerase I	210	160	190
E. coli RNA polymerase	570	610	590
Yeast RNA polymerase	72	76	69

[a] W. Keller, unpublished work.

Molecular Weight—As illustrated in Fig. 14, both SF-A and SF-B sediment at 2.5 S which corresponds to a molecular weight of 20,000 to 30,000.

Specificity—Both SF-A and SF-B preparations exhibit a 2-fold specificity. As shown in Table V, the factors exclusively stimulate RNA polymerase II and only in the presence of double-stranded DNA as template (Table VI). The activity of RNA polymerase I or *E. coli* RNA polymerase is not affected by addition of the factors. *In vitro* transcription of single-stranded DNA by RNA polymerase II is inhibited in the presence of the factors possibly due to the DNA exonuclease activity found in

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

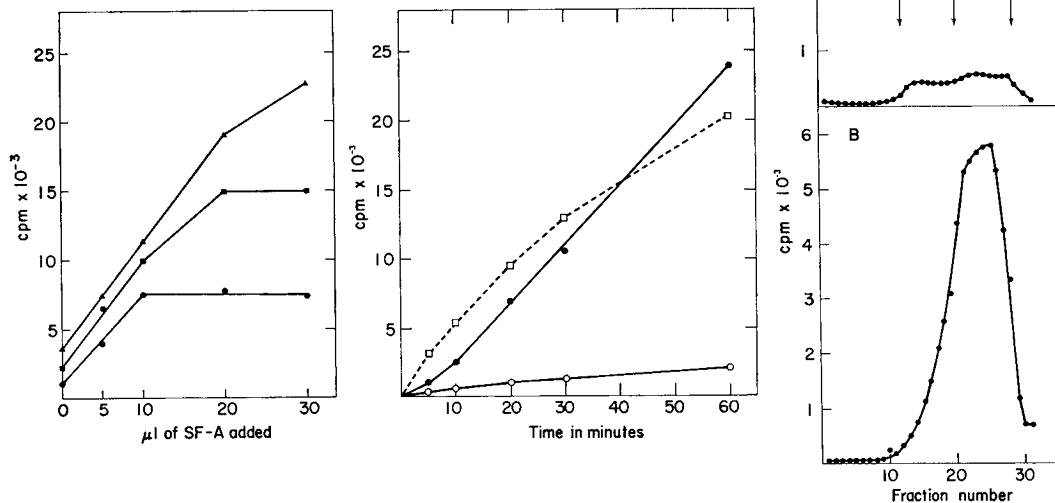

FIG. 15 (*left*). Saturation of KB RNA polymerase II with SF-A. Standard reaction mixtures containing 5 g of native calf thymus DNA and 0.5 unit (●——●), 1 unit (■——■), and 1.5 units (▲——▲) of KB RNA polymerase II (DNA-cellulose fraction), and increasing amounts of SF-A (G-150 fraction; 0.3 mg of protein per ml) as indicated.

FIG. 16 (*center*). Time course of RNA synthesis by KB RNA polymerase II on native and denatured adenovirus 2 DNA in the presence and absence of SF-A. Standard reaction mixtures contained 3 µg of native (○——○) or alkali-denatured (□- - -□) adenovirus 2 DNA, 0.5 unit of KB RNA polymerase II (DNA-cellulose fraction), and 10 µl of SF-A (0.3 mg per ml; G-150 fraction; (●——●).

FIG. 17 (*right*). Size distribution of RNA synthesized on native adenovirus 2 DNA in the presence or absence of SF-A. Standard reaction mixtures, containing 0.5 unit of KB RNA polymerase II, 3 µg of native adenovirus 2 DNA, were incubated with or without 10 µl of SF-A (G-150 fraction, 0.3 mg of protein per ml) for 90 min. The reactions were stopped by the addition of 0.4 ml of 0.1% sodium dodecyl sulfate and extracted with phenol, saturated with 0.1 M Tris-HCl, pH 7.4-0.1% SDS. The material from the aqueous phases was precipitated with 2.5 volumes of cold ethanol, collected by centrifugation, and dissolved in 0.075 ml of 0.03 M LiCl, 0.03% sodium dodecyl sulfate, 1 mM Tris-HCl, pH 7.3, 0.5 mM EDTA. The samples were mixed with a 10-µl aliquot of ^{32}P-labeled ribosomal RNA from HeLa cells as molecular weight marker, heated for 1 min at 80°, and layered onto 3.6-ml gradients containing 10 to 30% sucrose, 0.1 M LiCl, 0.1% sodium dodecyl sulfate, 0.01 M Tris-HCl, pH 7.3, 1 mM EDTA. After centrifugation for 2½ hours at 49,000 rpm in a Spinco SW 56 rotor at 20°, fractions were collected from the bottom of the tubes, mixed with 50 µg of yeast RNA as carrier, and precipitated with 2 ml of 5% trichloroacetic acid. The precipitates were collected on Whatman GF/C filters, washed with cold trichloroacetic acid and 95% ethanol, air-dried, and counted in a toluene-based scintillation fluid.

TABLE VI

Specificity of SF-A and SF-B for double-stranded DNA

Standard reaction mixtures contained 0.2 unit of KB RNA polymerase II (DNA-cellulose fraction), 5 µg of the appropriate DNA, and 20 µg of SF-A (G-150 fraction), or 10 µg of SF-B (CM-cellulose fraction). Numbers represent picomoles of [^{3}H]UMP incorporated.

	Control	+ SF-A	+ SF-B
Native calf thymus DNA	31	142	138
Denatured calf thymus DNA	222	24	17
Native Ad2 DNA	20	239	223
Denatured Ad2 DNA	184	14	16
SV40 DNA (component I)	45	220	192
poly(dAT)	620	990	690

the factor preparations. The DNA exonuclease activity is not responsible for factor stimulation on double-stranded DNA because closed, circular SV40 DNA is unaffected by the SF-A preparations which do stimulate RNA synthesis when SV40 DNA is used as a template. When the amount of factor needed for maximal stimulation is titrated in the presence of an excess of DNA and varying amounts of RNA polymerase, we find that higher levels of RNA polymerase require an increased amount of stimulatory factors (Fig. 15). Therefore, the amount of factor required for maximal stimulation depends on the amount of RNA polymerase in the reaction and not on the concentration of DNA. Fig. 16 shows the time course of RNA synthesis using RNA polymerase II in the presence of SF-A and native adenovirus 2 DNA. After an initial lag of about 5 min, the reaction in the presence of SF-A proceeds at a linear rate for 60 min. This figure also shows that in the presence of SF-A native DNA is transcribed by RNA polymerase II with equal efficiency as denatured DNA. In the absence of factor there is very little transcription of native DNA. The size of RNA synthesized by RNA polymerase II on native adenovirus 2 DNA *in vitro* is very heterogeneous ranging from 4 S to about 29 S (Fig. 17, Panel *A*). RNA synthesized in the presence of SF-A has a size distribution with a peak between 4 S and 18 S (Fig. 17, Panel *B*). The same size distribution was obtained with SF-B (results not shown).

In an attempt to determine which step of the RNA polymerase reaction is stimulated by the factors, the experiments summarized in Table VII were performed. Incubation of factors with RNA polymerase in the presence of the rifamycin derivative AF/05 resulted in a complete inhibition of RNA synthesis. AF/05 has been shown to inhibit specifically the initiation step of RNA synthesis (36).[2] When factor was added 5 min after the start of the reaction, it could still exert its stimulatory effect. However, when AF/05 and factor were added simultaneously, no stimulation occurred. It appears, therefore, that the factors stimulate the frequency of initiations and have no effect on the rate of

[2] B. Sugden and W. Keller, unpublished results.

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

TABLE VII

Stimulation of initiation of RNA synthesis by SF-A and SF-B and its inhibition by rifamycin SV-AF/05

Standard reaction mixtures contained 3 μg of adenovirus 2 DNA, 0.25 unit of KB RNA polymerase II (DNA-cellulose fraction), and when indicated, 20 μg of SF-A (G-150 fraction) or 10 μg of SF-B (CM-cellulose fraction), and 20 μg of the rifamycin derivative AF/05.

Addition at $t = 0$				Addition at $t = 5$ min			Counts per min at $t = 20$ min
RNA polymerase II	SF-A	SF-B	AF/05	SF-A	SF-B	AF/05	
+							470
+			+				12
+	+						4175
+		+					3306
+	+		+				319
+		+	+				420
+						+	242
+				+			3349
+					+		2351
+				+		+	567
+					+	+	721

elongation. Proof of this mechanism requires the direct measurement of RNA chain initiation by determining the incorporation of γ-labeled ribonucleoside triphosphates into RNA. Due to the limited amounts of purified RNA polymerase available, this experiment has not been performed yet.

DISCUSSION

We have described a procedure for the solubilization and purification of RNA polymerase II from tissue culture cells with an over-all yield of 10%. In contrast to the commonly used solubilization methods (1, 4, 37), the procedure does not include a sonication step which in our experience causes a loss of activity of between 20 to 50%. There are several aspects peculiar to this protocol that are important for high recovery of activity. Firstly, high concentrations of dithiothreitol in the cell swelling buffer, heating of the cell lysate to 35,° and the described over-all composition of the glycerol gradients are essential for recovery of RNA polymerase II during the early stages of purification. Secondly, the use of a single rather than double-stranded DNA-cellulose chromatography and of DEAE-Sephadex at pH 7.2 rather than 7.9 are required for the success of the later stages of this procedure. Several different DNAs in DNA-cellulose columns have been used. Bacteriophage DNAs bind more reproducibly to cellulose perhaps because they are generally of greater average length (24). However, because they reanneal rapidly it is difficult to make single-stranded bacteriophage DNA cellulose. Single-stranded eukaryotic DNA-cellulose is readily made, even with commercial calf thymus DNA and single-stranded DNA-cellulose binds RNA polymerase II more effectively than native DNA-cellulose. RNA polymerase II activity purified through the DNA-cellulose chromatography step is sufficiently free of contaminating enzymes to be useful for most *in vitro* transcription studies and can be obtained in 30 to 40% yields by these methods. The purification can be carried out with as little as 1 g and as much as 200 g of packed cells. In addition to human cell lines the solubilization and initial steps of purification have been used successfully with tissue culture cell lines of monkey, mouse, and hamster origin. These methods also serve well with KB cells infected with adenovirus 2.

We have not detected a third RNA polymerase activity (1). If, however, distinct RNA polymerases were to co-elute with either of the activities we observed and were labile, they might readily be lost during the subsequent purification procedures. Besides RNA polymerases I and II, a number of other enzymes can be obtained from the same cell extract. DNA polymerases I (38, 39) and II (38, 40) as well as ribonuclease H (41) and two stimulatory factors of RNA polymerase II (SF-A and SF-B) are present in the flow-through material of the first DEAE-cellulose chromatography. DNA polymerase I appears in the flow-through material of the second DEAE-cellulose step together with the stimulatory factors. DNA polymerase II and ribonuclease H bind to the second DEAE-cellulose column and can be separated and purified further by procedures to be described elsewhere. DNA polymerase I binds to CM-cellulose and co-elutes with SF-A. It separates from the stimulatory factor in the gel filtration step where it elutes ahead of the stimulatory protein.

The subunit structure of RNA polymerase II as revealed by SDS-polyacrylamide electrophoresis is similar to that reported for the enzyme from rat liver and calf thymus (10, 11). If the variable band with a molecular weight of 170,000 (Band *b* in Fig. 6) is an integral part of a modified RNA polymerase as has been suggested (10), then the final purity of the KB cell enzyme is greater than 70%. However, only reconstitution of enzyme from its dissociated polypeptides can identify the subunits necessary for its activity. Densitometer tracings of SDS-polyacrylamide gels very approximately suggest molar ratios of polypeptides $a + b$, c, and d as being 1:1:1 or 1:2:1, respectively. The errors in these measurements might permit other ratios, too. The suggested molar ratios would generate molecular weights of 400,000 and 540,000 (excluding contributions from polypeptides of 25,000 daltons or less, Band *e*) which are each close enough to the active enzyme's molecular weight of 500,000 to be possible. The specific activity of the purified KB or HeLa cell RNA polymerase II is similar to that of the *E. coli* enzyme (42) and other mammalian RNA polymerases (10, 11). Correcting for loss during purification we can estimate that there are 1 to 3 μg of RNA polymerase II per g of packed cells. One gram of wet KB or HeLa cells corresponds to about 2×10^8 cells, therefore the total number of RNA polymerase II molecules per cell is approximately 10,000.

A prominent feature of RNA polymerase II is its preference for single-stranded DNA as template which is in contrast to RNA polymerase I.[2] At present we do not know whether this predilection reflects an artifact due to a modification of the enzyme during the purification or whether this is an intrinsic property of RNA polymerase II. We must of course remember that eukaryotic DNA is largely associated with proteins within the cell, which might influence its "template activity." The use of free DNA as a template *in vitro* therefore may not adequately reflect physiological conditions.

Similar uncertainties prevail in the discussion of a physiological role for the two stimulatory factors described in this paper. Only after an extensive investigation of their influence on the *in vitro* transcription of defined templates may we be able to assess their possible significance. Experiments described above have indicated that the effect of the stimulatory factors is abolished when rifamycin-AF/05 is added simultaneously 5 min after the start of the reaction. This result could be explained in three ways. (*a*) AF/05 might bind to the stimulatory

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

factors and prevent their action, independently from its effect on RNA polymerase. (*b*) Stimulatory factors might act by increasing the frequency of chain initiations. AF/05 will prevent this via its regular effect on RNA polymerase. (*c*) Stimulatory factors might act as "release factors" for polymerase molecules which are bound to the template and do not synthesize RNA. After being released by stimulatory factors, these RNA polymerase molecules would be able to reinitiate, a process that is blocked by the simultaneous addition of AF/05. The finding that the stimulatory factors are required in a constant ratio with RNA polymerase indicates that they somehow interact with the enzyme. Therefore, they do not seem to be DNA unwinding proteins. In accord with this interpretation is our observation that the factors do not bind strongly to either single or double-stranded DNA.[2] DNA unwinding proteins generally bind very strongly to single-stranded DNA (24). However, attempts to demonstrate specific binding of the factors to RNA polymerase II by co-sedimentation in sucrose gradients have been unsuccessful. In contrast, a factor isolated from calf thymus tissue has been shown to co-sediment with RNA polymerase (14). The calf thymus stimulatory factor differs also from the HeLa or KB cell factors in its marked heat stability (14). To determine the relationship of the stimulatory factors isolated from calf thymus and rat liver (15) to those found in tissue culture cells will be possible only after a more extensive purification and characterization of these proteins.

Acknowledgments—We thank Ms. Nabi Sullivan for helping with the tissue culture work and Gruppo Lepetit for generously providing the rifamycin derivatives, PR/19 and AF/05. We are indebted to Dr. J. D. Watson for his continuing advice and encouragement and to Joe Sambrook for his helpful suggestions and guiding criticisms.

REFERENCES

1. Roeder, R. G., and Rutter, W. J. (1969) *Nature* **224,** 234
2. Roeder, R. G., and Rutter, W. J. (1970) *Proc. Nat. Acad. Sci. U. S. A.* **65,** 675
3. Reeder, R. H., and Roeder, R. G. (1972) *J. Mol. Biol.* **67,** 433
4. Kedinger, C., Gniazdowski, M., Mandel, J. C., Gissinger, G., and Chambon, P. (1970) *Biochem. Biophys. Res. Commun.* **38,** 165
5. Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., and Rutter, W. J. (1970) *Science* **170,** 447
6. Zylber, E. A., and Penman, S. (1971) *Proc. Nat. Acad. Sci. U. S. A.* **68,** 2861
7. Price, R., and Penman, S. (1972) *J. Virol.* **9,** 621
8. Wallace, R. D., and Kates, J. (1972) *J. Virol.* **9,** 627
9. Jackson, A., and Sugden, W. (1972) *J. Virol.* **10,** 1086
10. Weaver, R. F., Blatti, S. P., and Rutter, W. J. (1971) *Proc. Nat. Acad. Sci. U. S. A.* **68,** 2994
11. Kedinger, C., and Chambon, P. (1972) *Eur. J. Biochem.* **28,** 283
12. Gey, G. O., Coffman, W. D., and Kubicek, M. T. (1952) *Cancer Res.* **12,** 264
13. Eagle, H. (1955) *Proc. Soc. Exp. Biol. Med.* **89,** 362
14. Stein, H., and Hausen, P. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35,** 709
15. Seifart, K. H. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35,** 719
16. Sugden, W., and Sambrook, J. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35,** 663
17. Keller, W., and Goor, R. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35,** 671
18. Eagle, H. (1959) *Science* **130,** 432
19. Penefsky, H. S., Pullman, M. E., Datta, A., and Racker, E. (1960) *J. Biol. Chem.* **235,** 3330
20. Goff, C. G., and Minkley, E. G. (1970) in *Lepetit Colloquy on RNA Polymerase and Transcription* (Silvestri, L., ed) p. 124, North-Holland Publishing Co., Amsterdam
21. Sinsheimer, R. L. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., and Davies, D. R., eds) p. 569, Harper and Row, New York
22. Pettersson, U., and Sambrook, J. (1973) *J. Mol. Biol.* **75,** 125
23. Trilling, D. M., and Axelrod, D. (1970) *Science* **168,** 268
24. Alberts, B., and Herrick, G. (1971) *Methods Enzymol.* **21,** 198
25. Weil, R., and Vinograd, J. (1963) *Proc. Nat. Acad. Sci. U. S. A.* **50,** 730
26. Randerath, K., and Randerath, E. (1967) *Methods Enzymol.* **12A,** 323
27. Singer, M. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., and Davies, D. R., eds) p. 245, Harper and Row, New York
28. Richardson, C. C. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., and Davies, D. R., eds) p. 263, Harper and Row, New York
29. Keller, W. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69,** 1564
30. Pettersson, U., Philipson, L., and Hogland, S. (1968) *Virology* **10,** 204
31. Laemmli, U. K. (1970) *Nature* **227,** 680
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265
33. Berg, D., and Chamberlin, M. (1970) *Biochemistry* **9,** 5055
34. Chamberlin, M., and Berg, P. (1963) *Cold Spring Harbor Symp. Quant. Biol.* **28,** 67
35. Keller, W., and Crouch, R. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69,** 3360
36. Meilhac, M., Tysper, Z., and Chambon, P. (1972) *Eur. J. Biochem.* **28,** 291
37. Ishihama, A. (1967) *Biochim. Biophys. Acta* **145,** 272
38. Weissbach, A., Schlabach, A., Fridlender, B., and Bolden, A. (1971) *Nature New Biol.* **231,** 167
39. Chang, L. M. S., and Bollum, F. J. (1971) *J. Biol. Chem.* **246,** 5835
40. Yoneda, M., and Bollum, F. J. (1965) *J. Biol. Chem.* **240,** 3385
41. Stein, H., and Hausen, P. (1969) *Science* **166,** 393
42. Burgess, R. R. (1969) *J. Biol. Chem.* **244,** 6160
43. Weber, K., and Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017

Mammalian Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerases: I. PURIFICATION AND PROPERTIES OF AN α-AMANITIN-SENSITIVE RIBONUCLEIC ACID POLYMERASE AND STIMULATORY FACTORS FROM HELA AND KB CELLS
Bill Sugden and Walter Keller

J. Biol. Chem. 1973, 248:3777-3788.

Access the most updated version of this article at http://www.jbc.org/content/248/11/3777

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/248/11/3777.full.html#ref-list-1

Downloaded from http://www.jbc.org/ at Universitaetsbibliothek Medizin on November 7, 2017