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Purification of a Factor from Ehrlich Ascites Tumor Cells Specifically Stimulating RNA Polymerase II†

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ABSTRACT: A factor stimulating RNA polymerase II from Ehrlich ascites tumor cells was purified. The final preparation appeared almost homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a molecular weight of 38 000. The endonuclease activity of about 10 μg of purified factor, if any, was well below the 10^{-5} μg equivalent of pancreatic deoxyribonuclease, indicating that the stimulation of RNA synthesis by this factor was not due to contaminating endonuclease. This factor specifically stimulated RNA polymerase II on native DNA as template and did not affect RNA polymerase I at all. The molecular size of RNA synthesized in the presence of this factor increased markedly compared with that synthesized by RNA polymerase II alone.

Multiple forms of DNA-dependent RNA polymerase exist in eukaryotic cells (Roeder and Rutter, 1969). Among these, RNA polymerase II (type B), which is completely inhibited by a low concentration of α-amanitin, is thought to be responsible for the synthesis of messenger RNA in the nucleoplasm (Roeder and Rutter, 1970). It is generally believed that many thousands of genes are actively transcribed in dividing cells by this enzyme. Thus the questions arise of how the enzyme selects the genes required and regulates the synthesis of messenger RNA quantitatively and qualitatively. There are probably many species of regulatory proteins controlling the expressions of specific genes. So the first step in elucidating their effects in regulation of RNA synthesis is to find proteins which affect transcription in vitro. In studies along these lines, many protein factors which stimulate or inhibit mammalian RNA polymerase II have been found (Stein and Hausen, 1970; Seifart et al., 1973; Natori, 1972; Lee and Dahmus, 1973; Sugden and Keller, 1973; Shea and Kleinsmith, 1973; Natori et al., 1974; Kostraba et al., 1975; Kostraba and Wang, 1975; Chuang and Chuang, 1975). However, most of the factors isolated were impure and the possibility was not excluded that their stimulatory or inhibitory effects on RNA synthesis may have been due to contaminatory materials, such as nucleases. Moreover, there have been no reports of quantitative studies on the purification of these factors. Recently we reported two factors which stimulate RNA polymerase II of Ehrlich ascites tumor cells (Natori et al., 1973a). To elucidate the precise mechanism of stimulation of RNA synthesis by these factors, we tried to purify one of them, named S-II. This paper describes a method to purify this factor. We were able to determine the yield and specific activity of this factor at each step of purification by measuring stimulation quantitatively. The amount of endonuclease contaminating the final preparation was negligible, so that stimulation of RNA synthesis by this factor was not due to introduction of random nicks in template DNA by contaminating nucleases.

Materials and Methods

Cells. Male ddY mice, weighing 25 to 30 g, were each inoculated with 3×10^6 Ehrlich ascites tumor cells. Ascites fluid was harvested from the abdominal cavity at 8 days after inoculation. Cells were collected by centrifugation for 10 min at 150*g*. The cells harvested from 5 mice were combined and rapidly suspended in 27 ml of deionized water to lyse erythrocytes. Then 3 ml of 10 × PBS[1](−) was added and the mixture was centrifuged. The resulting white pellet of cells was washed three times with PBS(−) and stored at −80 °C.

Preparation and Assay of RNA Polymerase II. DNA-dependent RNA polymerase II was isolated from Ehrlich ascites tumor cells by the procedure described previously (Natori et al., 1973b) involving the following steps: extraction of RNA polymerase by sonication in solution of high salt concentration, ammonium sulfate precipitation, DEAE-cellulose chromatography, and glycerol gradient centrifugation. The specific activity of the RNA polymerase II used usually 1000–2000 units per mg of protein. One unit of enzyme activity was defined as the amount catalyzing incorporation of 1 pmol of UMP into the acid-insoluble fraction under the standard conditions. The standard assay medium contained, in a total volume of 0.25 ml: 10 μmol of Tris-HCl, pH 7.9, 0.75 μmol of

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[1] Abbreviations used: DEAE, diethlaminoethyl; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl.

$MnCl_2$, 1.15 μmol of $MgCl_2$, 12.5 μmol of $(NH_4)_2SO_4$, 0.017 μmol of EDTA, 1 μmol of β-mercaptoethanol, 0.0625 μmol each of ATP, GTP, and CTP, 0.00625 μmol of UTP, 0.5 μCi of [^{3}H]UTP (20 Ci/mmol), 5 μg of purified Ehrlich ascites tumor DNA, and 10–20 units of RNA polymerase II. After incubation for 60 min at 37 °C, samples were chilled in ice and 0.25 ml of cold 10% Cl_3CCOOH solution was added. The volume was increased by adding 1 ml of cold 5% Cl_3CCOOH solution, and after 15 min the sample was applied to a Whatman GF-C glas fiber filter. The filter was washed successively with 60 ml of cold 5% Cl_3CCOOH containing 0.01 M sodium pyrophosphate and 1 ml of 95% ethanol and dried under an infrared lamp. The radioactivity on the filter was counted in a Packard liquid scintillation spectrometer.

Assay of Endonuclease. Endonuclease activity was assayed in the standard RNA polymerase assay mixture (omitting nucleoside triphosphates, radioactive UTP and DNA) with labeled SV40 DNA, and the degree of conversion of cyclic coiled DNA (component I) to single-stranded circular and linear molecules (component II) was determined in an alkaline sucrose density gradient. Usually 0.2 μg of [^{3}H]thymidine-labeled SV40 DNA (10 400 cpm) and the test fraction were incubated at 37 °C for 60 min, and then 50 μl of 0.2 M EDTA was added to stop the reaction. The mixture was layered on top of 5 ml of a linear gradient of 5–20% sucrose in solution containing 0.3 M NaOH, 0.5 M NaCl, and 0.01 M EDTA. The gradients were centrifuged for 3 h at 40 000 rpm in a Hitachi RPS 40 rotor. Fractions were collected from the bottom of the tube and the DNA in each fraction was precipitated with 5% cold Cl_3CCOOH and collected on a Whatman GF-C glass fiber filter to measure radioactivity.

Assay of Ribonuclease H Activity. Ribonuclease H activity was assayed using the standard reaction mixture for RNA polymerase (omitting nucleoside triphosphates, radioactive UTP and DNA) with labeled DNA–RNA hybrid as substrate. The hybrid was synthesized using RNA polymerase II and denatured Ehrlich ascites tumor DNA, as described previously (Natori et al., 1973c). The reaction mixture containing 1200 cpm of DNA–RNA hybrid labeled with [^{3}H]UTP (corresponding to 20 pmol of UMP) was incubated with the test fraction for 10 min at 37 °C. The degree of RNA hydrolysis was determined from the amount of radioactivity released into the acid-soluble fraction.

Analytical Gel Electrophoresis. This was done according to the method of Weber and Osborn (1969). Protein samples were dissociated by heating for 20 min at 75 °C in a solution containing 1% sodium dodecyl sulfate and 2% β-mercaptoethanol before electrophoresis.

Determination of the Molecular Size of RNA Synthesized in Vitro. RNA was synthesized in 0.5 ml of the reaction mixture under the standard conditions. After incubating the mixture for 60 min at 37 °C, 5μg of RNase-free deoxyribonuclease was added and incubation was continued for 15 min more to digest template DNA. Then sodium dodecyl sulfate was added to 0.1% and the mixture was dialyzed against 0.01 M Tris-HCl buffer, pH 7.9, containing 0.01 M $MgCl_2$ for 90 min at room temperature. The dialyzed RNA was layered on top of 12 ml of a gradient of 7 to 25% sucrose containing 0.01 M Tris-HCl, pH 7.4, 0.01 M NaCl, and 0.001 M EDTA and the gradient was centrifuged for 16 h at 34 000 rpm in a Hitachi RPS 40T rotor. Fractions were collected from the bottom of the tube, and the RNA in each fraction was precipitated with 5% cold Cl_3CCOOH and collected on a Whatman GF-C glass fiber filter to measure radioactivity. Each gradient was calibrated internally with an RNA preparation extracted from *Escherichia coli* labeled with [^{14}C]uracil.

Chemicals and Other Procedures. Pancreatic deoxyribonuclease, α-chymotrypsinogen, poly(dAT), and cytochrome *c* were purchased from Sigma Biochemical Co. Calf thymus DNA was purchased from Worthington Biochemical Co. α-Amanitin and SV40 DNA were generously supplied by Drs. T. Wieland and K. Oda, respectively. Protein concentration was determined by the method of Lowry et al. (1951).

Results

Previously we reported two factors which stimulate RNA polymerase II of Ehrlich ascites tumor cells (S-I and S-II). These factors showed distinct template and enzyme specificities (Natori et al., 1973a). To obtain more information about these factors, we tried to purify one of them, S-II. To follow the purification of this factor, we introduced stimulation unit. One stimulation unit was defined as the amount which enhanced the activity of 10 units of RNA polymerase II to 11 units under the standard assay conditions.

Extraction of Stimulatory Factors. All procedures were conducted at between 0 and 4 °C unless otherwise specified. About 30 g of frozen Ehrlich ascites tumor cells, harvested from 20 mice, was thawed in an equal volume of 0.05 M Tris-HCl, pH 7.9, containing 5 mM $MgCl_2$, 0.1 mM EDTA, 0.3 mM dithiothreitol, and 25% glycerol (buffer 1). The cells were disrupted by 60 rapid strokes of a Potter-Elvehjem homogenizer and 1 ml of 4 M ammonium sulfate was added to 15 ml of the resulting homogenate. The solution was sonicated until its viscosity decreased to a point at which it easily formed drops at the tip of a Pasteur pipet. Then 2 volumes of buffer 1 was added, and the mixture was centrifuged for 1 h at 156 500*g* in a Hitachi ultracentrifuge. The precipitate was discarded and 0.42 g of ammonium sulfate per 1 ml was added to the clear supernatant. The mixture was stirred for 20 min and then centrifuged for 1 h at 77 000*g* in a Hitachi ultracentrifuge. The resulting precipitate was dissolved in 30 ml of buffer 1 and dialyzed extensively against the same buffer for 2 h.

DEAE-Cellulose Chromatography. The dialyzed solution was diluted with 4 volumes of buffer 1 and applied to a DEAE-cellulose column (6 × 20 cm) equilibrated with buffer 1. The column was washed with 4 column volumes of buffer 1, and unadsorbed material was collected and stored at −80 °C. This fraction stimulated RNA polymerase II and was used as starting material for purification of stimulatory factors.

Ammonium Sulfate Fractionation. About 1 to 1.5 l. of the unadsorbed fraction from DEAE-cellulose obtained from about 200 mice was used for one experiment. This fraction was mixed with an equal volume of saturated solution of ammonium sulfate, pH 7.9, and stirred for 30 min. The resulting turbid solution was centrifuged for 20 min at 15 000*g* and the precipitate was discarded. Saturated ammonium sulfate solution was added to the clear supernatant to give a final saturation of 85% and stirring was continued for 30 min. The mixture was centrifuged for 20 min at 15 000*g* and the resulting precipitate was dissolved in 50 ml of 0.01 M Tris-HCl, pH 7.9, containing 5 mM β-mercaptoethanol (buffer 2), and dialyzed against the same buffer for 3 h. The precipitate formed during dialysis was removed by centrifugation for 60 min at 77 000*g*.

Phosphocellulose Chromatography. The clear supernatant obtained was mixed with an equal volume of buffer 2 and applied to a column of phosphocellulose (3.8 × 10 cm, Whatman P 11) which had been carefully equilibrated with buffer 2 by washing with more than 100 column volumes of the buffer.

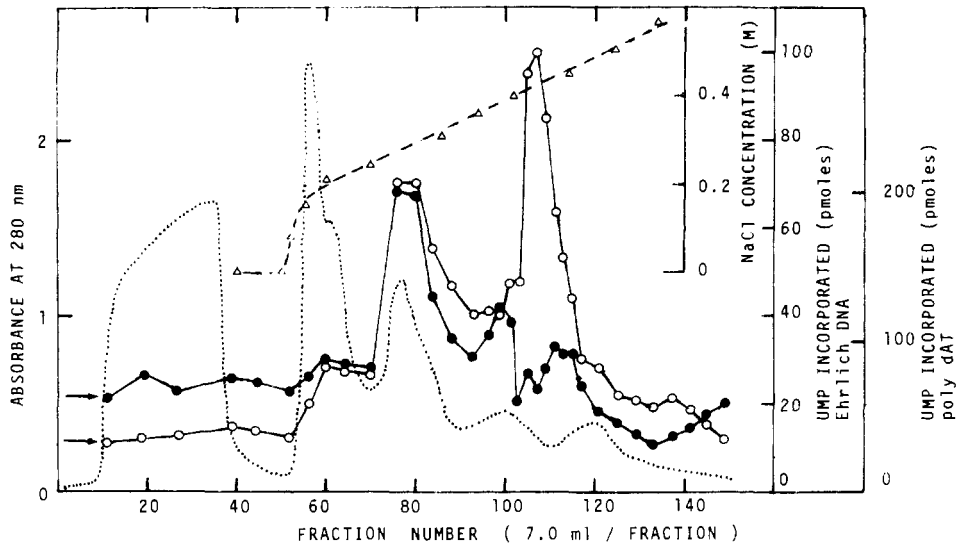

FIGURE 1: Phosphocellulose chromatography of factors stimulating RNA polymerase II. Column size, 3.8 × 10 cm. Flow rate, 40 ml/h. Fraction size, 7 ml. Sample, 50 ml of dialyzed solution containing proteins precipitating between 50 and 85% saturation of ammonium sulfate (see the text). The column was washed with 0.3 M NaCl in buffer 2 (0.01 M Tris-HCl, pH 7.9, 5 mM β-mercaptoethanol) and developed with a linear gradient of 600 ml of 0.3–0.8 M NaCl in buffer 2. Samples of 30 μl of each fraction were assayed for activity to stimulate RNA synthesis under the standard conditions using Ehrlich ascites tumor DNA (○) or poly(dAT) (●) as template. The arrow shows the basal level of 10 units of RNA polymerase II on Ehrlich ascites tumor DNA or poly(dAT). (···) Absorbance at 280 nm; (△- - -△) concentration of NaCl.

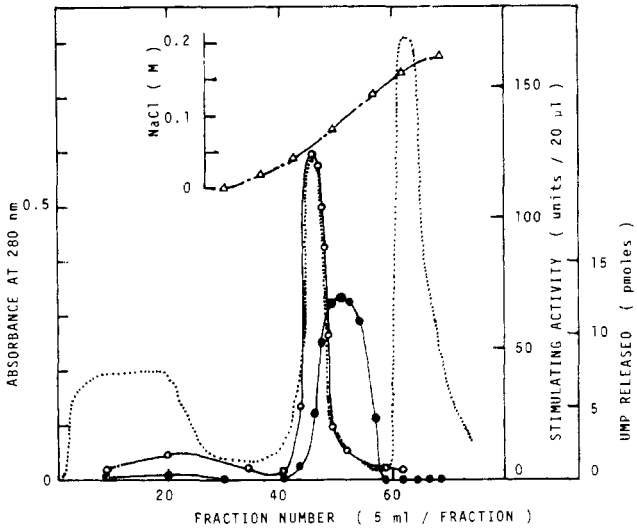

FIGURE 2: CM-Cellulose chromatography of S-II. Column size, 2 × 4 cm. Flow rate, 30 ml/h. Fraction size, 5 ml. Sample, about 100 ml of dialyzed samples. The column was developed with 200 ml of a linear gradient of 0 to 0.2 M NaCl in buffer 2. Samples of 20 μl of each fraction were assayed for activity to stimulate RNA synthesis or for ribonuclease H activity under the standard conditions described in the Materials and Methods. (○—○) Stimulation of RNA synthesis; (●—●) ribonuclease H activity; (···) absorbance at 280 nm; (△- - -△) concentration of NaCl.

After application of the sample, the column was washed with the buffer until the absorbance at 280 nm was less than 0.1. Then the adsorbed material was eluted with 0.3 M NaCl in buffer 2. When the absorbance of the eluate at 280 nm had decreased to below 0.3, the column was developed with a linear gradient of 600 ml from 0.3 to 0.8 M NaCl in buffer 2. Stimulation of RNA synthesis by each fraction was measured under the standard conditions. As shown in Figure 1, two peaks of material stimulating RNA synthesis on Ehrlich ascites tumor DNA were detected. The first fraction (S-I) stimulated RNA synthesis on poly(dAT), whereas the second fraction (S-II) did not. The second fraction was dialyzed against buffer 2 for 3 h and the precipitate formed during dialysis was removed by centrifugation for 60 min at 77 000*g*.

CM-Cellulose Chromatography. The clear supernatant obtained (about 100 ml) was applied to a column of CM-cellulose (2 × 4 cm, Whatman CM 52) equilibrated with buffer 2. At this step it was important to use 1 ml of CM-cellulose per 4 mg of protein to obtain good recovery. The column was washed extensively, and then adsorbed material was eluted with 200 ml of a linear gradient of 0 to 0.2 M NaCl in buffer 2. The elution profile is shown in Figure 2. The activity stimulating RNA synthesis was eluted with 0.04 M NaCl, coinciding with a protein peak. Ribonuclease H activity was found to be separated from the activity for stimulation of RNA synthesis under these chromatographic conditions. This is a significant finding because it was previously not possible to obtain stimulatory factors free from ribonuclease H activity (Natori et al., 1973c) and it was suggested that ribonuclease H might participate in stimulation of RNA synthesis in vitro (Sekeris et al., 1972). However, the present result shows that ribonuclease H activity is not related to the stimulation of RNA synthesis.

Concentration. The activity eluted from CM-cellulose was concentrated by one-step elution from phosphocellulose followed by dialysis against buffer 2 containing 0.3 M NaCl and 50% glycerol. The fractions eluted from CM-cellulose were combined (200 ml), dialyzed against buffer 2 for 3 h, and then applied to a column of phosphocellulose (1.5 × 2 cm, Whatman P 11) equilibrated with the same buffer. The column was washed with 3 to 4 column volumes of buffer 2, and then adsorbed activity was eluted with 3 ml of 0.8 M NaCl in buffer 2. This solution was dialyzed for 4 h against buffer 2 containing 0.3 M NaCl and 50% glycerol. The recovery of the activity by this process was usually 30 to 50%.

Bio-Gel P-60. About 1 ml of the concentrated solution was applied to Bio-Gel P-60 column (1 × 60 cm) equilibrated with 0.3 M NaCl in buffer 2. Fractions with activity recovered from the column in a single peak were combined and concentrated by dialysis against 0.01 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$,

TABLE I: Purification of the Stimulatory Factor S-II.

Purification Step	Vol (ml)	Protein (mg)	Act.[a] (units)	Spec. Act. (units/mg)	Yield (%)
DEAE-Cellulose unadsorbed fraction	1330	4260	2 020 000	474	100
Ammonium sulfate ppt	172	1460	1 300 000	890	64
Phosphocellulose[b]	120	48.6	178 000	3 660	8.8
CM-Cellulose	18	6.9	72 500	10 400	3.6
Bio-Gel P-60	1.1	1.0	14 700	14 700	0.7

[a] Activity was calculated from calibration curves obtained with amounts of each fraction which stimulated RNA synthesis linearly under the standard conditions. [b] Two factors, S-I and S-II, were separated at this step.

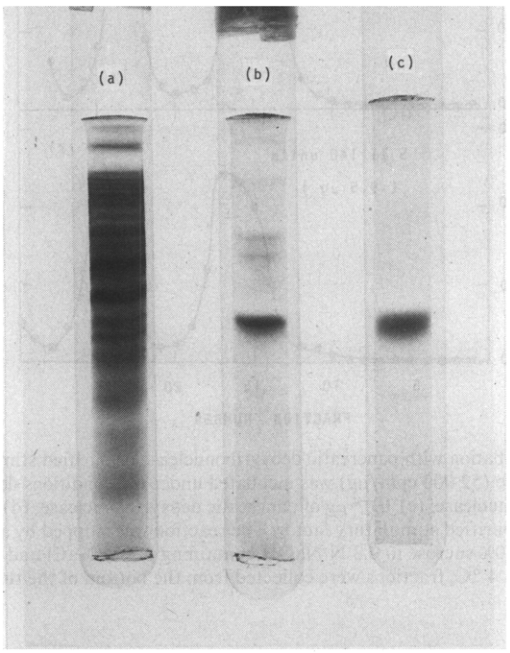

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of various fractions obtained during purification of S-II. (a) DEAE-Cellulose flow-through fraction; (b) CM-cellulose fraction; (c) Bio-Gel P-60 fraction. Each fraction contained 50 units of stimulatory activity. Electrophoresis was performed as described in the Materials and Methods.

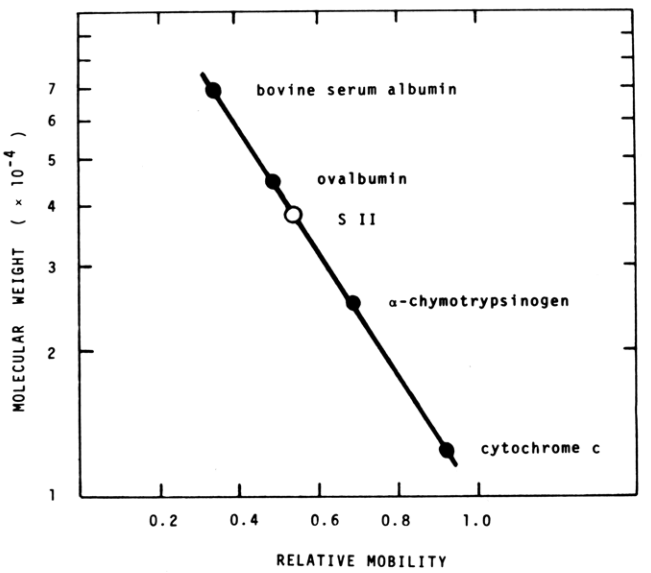

FIGURE 4: Determination of the molecular weight of S-II by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Marker proteins were run separately under the same conditions.

0.01 M KCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol, and 50% glycerol for 5 h. The activity of the final preparation was stable for at least 1 month on storage at −20 °C.

Summary of Purification and Molecular Characterization of S-II. A typical purification is shown in Table I. Stimulation of RNA synthesis was assayed using 10 units of RNA polymerase II and concentrations of the fractions in the ranges which stimulated RNA synthesis linearly under the standard conditions. The activity was calculated from the calibration curves obtained for each fraction. Usually about 30-fold purification over the unadsorbed fraction from DEAE-cellulose was achieved with a recovery of less than 1% of the initial activity. The final preparation eluted from Bio-Gel P-60 gave almost a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as shown in Figure 3. A similar electrophoretic pattern was obtained with the active fraction from CM-cellulose. The molecular weight of this factor was determined to be 38 000 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, as shown in Figure 4. A similar value (34 000) was obtained by gel filtration through Bio-Gel P-60.

Absence of Endonuclease Activity in the S-II Preparation. In studies on factors stimulating RNA synthesis in vitro, it is important to determine that the stimulation is not due to contaminating endonuclease, because it is known that endonuclease apparently stimulates RNA synthesis in vitro by making nonspecific nicks in template DNA (Vogt, 1969; Chambon et al., 1970). To test for endonuclease, the purified preparation of S-II was incubated with 0.2 μg of [^{3}H]thymidine-labeled SV40 DNA to see if it converted circular coiled DNA (component I) to single-stranded, circular or linear molecule (component II) by making nicks under the conditions described in the Materials and Methods. As shown in Figure 5b and c, 10^{-5} to 10^{-4} μg of authentic pancreatic deoxyribonuclease (RNase-free, code DPFF) partially or completely converted component I to component II under these conditions. However, the sedimentation profiles of SV40 DNA treated with 0.95 μg (14 units) or 9.5 μg (140 units) of purified S-II were identical with that of control DNA, as shown in Figure 5d, e, and f. Maximum stimulation of RNA synthesis by deoxyribonuclease was observed when 10^{-2} μg of the enzyme was added to the reaction mixture for RNA synthesis, as shown in Figure 6, and 10^{-5} μg of the enzyme did not affect the transcription. Thus since the amount of deoxyribonuclease contaminating 9.5 μg of purified S-II, if any, was estimated as well below 10^{-5} μg, it was concluded that the stimulation of RNA synthesis by S-II was not due to contaminating endonuclease.

Specificity of the Purified S-II. In the assay of this factor,

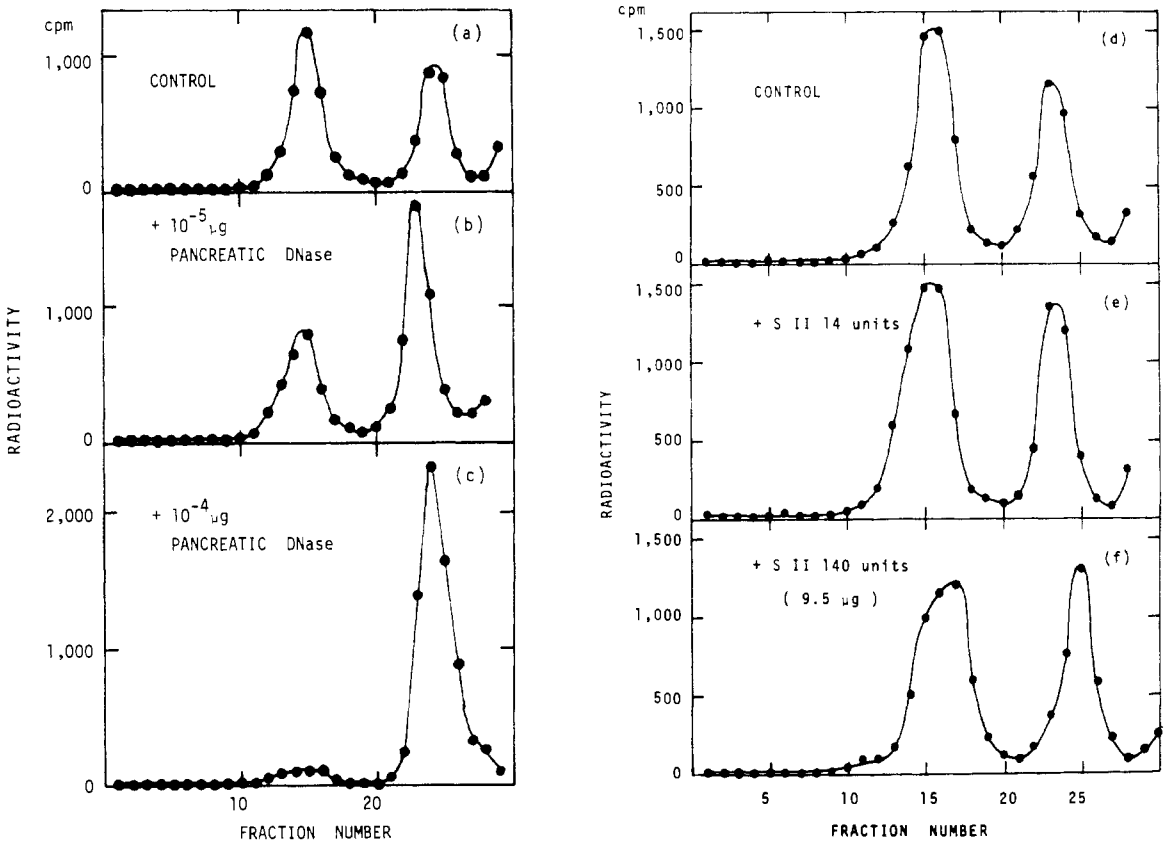

FIGURE 5: Alkaline sucrose density gradient centrifugation of SV40 DNA after incubation with pancreatic deoxyribonuclease or purified stimulatory factor. Supercoiled, double-stranded, SV40 DNA (0.2 μg) labeled with [^{3}H]thymidine (52 000 cpm/μg) was incubated under the conditions described in the Materials and Methods. (a) Control DNA; (b) 10^{-5} μg of pancreatic deoxyribonuclease; (c) 10^{-4} μg of pancreatic deoxyribonuclease; (d) control DNA; (e) 14 units (0.95 μg) of purified stimulatory factor; (f) 140 units (9.5 μg) of purified stimulatory factor. The reaction was stopped by addition of 0.05 ml of 0.2 M EDTA. The samples were then layered on 5-ml gradients of 5–20% sucrose in 0.3 N NaOH containing 0.5 M NaCl and 10 mM EDTA. After centrifugation for 180 min at 40 000 rpm in a Hitachi RPS-40 rotor at 4 °C, fractions were collected from the bottom of the tubes and acid-insoluble radioactivity was counted.

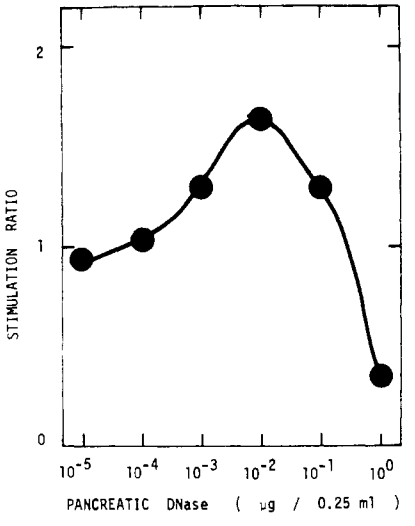

FIGURE 6: Effect of pancreatic deoxyribonuclease on RNA polymerase II activity. RNA synthesis was assayed under the standard conditions using 10 units of RNA polymerase II and various amounts of pancreatic deoxyribonuclease.

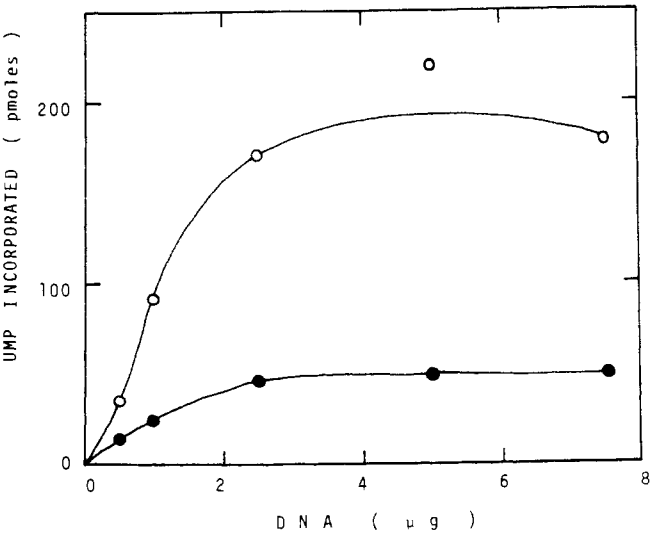

FIGURE 7: Effect of DNA on the stimulation of RNA synthesis by S-II. The reaction mixture contained 43 units of RNA polymerase II and 42 units of purified S-II with various amounts of DNA. Incubation conditions were the same as described in the Materials and Methods. (●—●) RNA polymerase II alone; (○—○) RNA polymerase II with S-II.

it was shown that the stimulation of RNA synthesis by the factor depended on the amount of DNA present in the assay system, as shown in Figure 7. As shown in Figure 8, this factor specifically stimulated RNA polymerase II in the presence of double-stranded DNA as template. It did not affect the activity of RNA polymerase I. Moreover, it did not affect *E. coli* RNA polymerase, as reported before (Natori et al., 1973a). Thus this factor seems to be strictly specific for homologous RNA polymerase II. The purified factor did not stimulate RNA

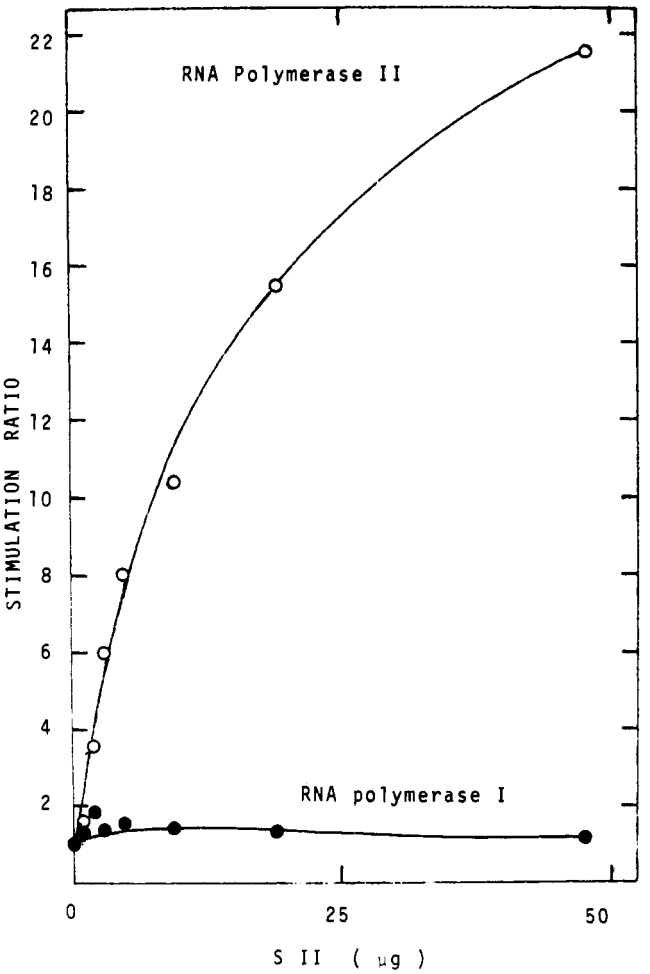

FIGURE 8: Enzyme specificity of the purified S-II. RNA synthesis was examined using 10 units of RNA polymerase I (●) or II (○) under the standard conditions with increasing amounts of purified S-II. For the assay of RNA polymerase I, 0.5 μg of α-amanitin was added per reaction mixture. Stimulation was calculated on the basis of RNA synthesis without the factor.

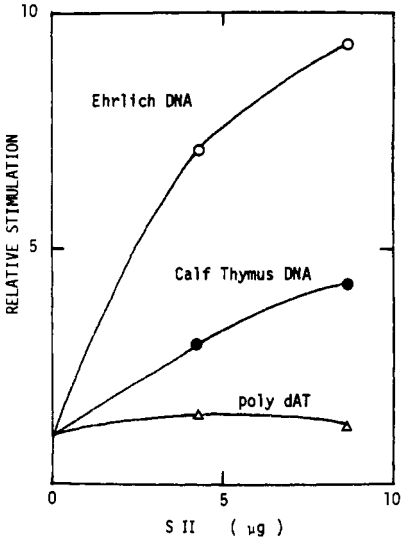

FIGURE 9: Template specificity of the purified S-II. RNA synthesis was examined using 10 units of RNA polymerase II on various templates with purified S-II under the standard conditions. The stimulation was calculated on the basis of RNA synthesis without the factor. (○—○) Ehrlich ascites tumor DNA; (●—●) calf thymus DNA; (△—△) poly(dAT).

TABLE II: Effect of the Factor on Incorporation of [γ-^{32}P]GTP and [^{3}H]UTP.[a]

Factor	[^{3}H]UTP Incorp (pmol)	[γ-^{32}P]GTP Incorp (pmol)
−	49.3	0.05
+	205.0	0.11

[a] The reaction mixture was as described in the Materials and Methods, except that 0.1-fold decrease of the amount of cold GTP was adopted. After incubation for 60 min at 37 °C, the samples were heated at 85 °C for 3 min to terminate the reaction. These samples were then incubated for an additional 40 min at 37 °C in the presence of 80 μg/ml of proteinase K (Boehringer Mannheim GmbH) and then the radioactivity incorporated into acid-insoluble fraction was counted. Each reaction contained 43 units of RNA polymerase II and, when present, 32 units of purified S-II.

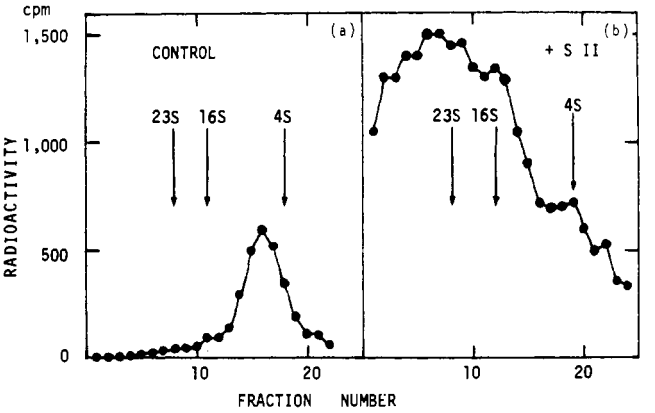

FIGURE 10: Sucrose gradient analysis of RNA synthesized in vitro. RNA samples were layered on the top of 12 ml of a gradient of 7 to 25% sucrose containing 0.01 M Tris-HCl buffer, pH 7.4, 0.01 M NaCl, and 1 mM EDTA. Gradients were centrifuged for 16 h at 34 000 rpm in a Hitachi RPS-40T rotor, fractions were collected from the bottom of the tubes, and acid-insoluble radioactivity was counted. (a) RNA synthesized by RNA polymerase II alone on Ehrlich ascites tumor DNA; (b) RNA synthesized in the presence of purified S-II on Ehrlich ascites tumor DNA. Each gradient was calibrated internally with *E. coli* RNA.

synthesis on poly(dAT) and it had less effect on RNA synthesis on calf thymus DNA than that on Ehrlich ascites tumor DNA, as shown in Figure 9.

In order to determine whether the action of this factor on RNA synthesis is on chain initiation or on chain elongation, RNA chain initiation was assayed by measuring the incorporation of γ[^{32}P]GTP into RNA. As shown in Table II, in the presence of this factor, RNA chain initiation was increased at least twofold, whereas chain elongation determined by the incorporation of [^{3}H]UTP into RNA was found to be increased more than fourfold under the same conditions. These results indicate that this factor stimulates both initiation and elongation of RNA chains. The RNA synthesized in the presence of purified S-II was examined. As shown in Figure 10, the RNA synthesized by RNA polymerase II on native Ehrlich ascites tumor DNA in vitro without S-II was homogeneous in size, sedimenting in the region of about 6 S, whereas that synthesized in the presence of purified S-II was much larger and was heterogeneous. These results are similar to those obtained previously with the crude factor (Natori et al., 1973a, c).

Discussion

This paper describes a method for purifying a factor, named S-II, from Ehrlich ascites tumor cells which stimulates the activity of RNA polymerase II on native DNA in vitro. There are several reports on protein factors stimulating eukaryotic RNA polymerase in vitro (Biswas et al., 1975), but none on quantitative measurements on their purification. This is the first report of quantitative measurements of purification of one of these factors.

It was found that this factor is a basic protein. Its molecular weight was estimated as 38 000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and as 34 000 by measuring activity eluted from Bio-Gel P-60, indicating that the protein has no subunit structure.

It is extremely important to show that the apparent stimulation of RNA polymerase by such factors is not due to contaminating endonuclease because nicks or gaps incroduced into template DNA by endonuclease may enhance the apparent activity of RNA polymerase, as pointed out by Dezélée et al. (1974). Therefore, we carefully tested for endonuclease activity in the purified preparation. We concluded that the stimulation of RNA polymerase was not due to contaminating endonuclease, because the endonuclease activity of 9.5 μg of purified S-II, if any, was well below the 10^{-5} μg equivalent of pancreatic deoxyribonnuclease, and 10^{-5} μg of deoxyribonuclease when added to the reaction mixture was shown to have no effect on RNA synthesis. Moreover, deoxyribonuclease caused at most 2-fold stimulation of RNA synthesis, whereas purified S-II caused more than 20-fold stimulation under the same conditions. Furthermore, Dezélée et al. (1974) showed that endonuclease stimulates both RNA polymerase I and II, whereas this factor stimulated RNA polymerase II only. These observations also show that the stimulation of RNA synthesis by this factor is quite specific.

The characteristic effect of this factor is to cause marked increase in size of product RNA when added to the reaction mixture. In this it differs from the factor SF-B found in KB cells by Sugden and Keller (1973).

Previously we reported that the stimulatory fraction contained ribonuclease H activity (Natori et al., 1973a). However, as shown in this paper, ribonuclease H activity was separated from the peak of stimulatory factor by CM-cellulose chromatography, showing that ribonuclease H is not related to stimulation of RNA synthesis in vitro. It should be pointed out that purified factor shows template specificity. Ehrlich ascites tumor DNA is a much better template for RNA polymerase II than calf thymus DNA when assayed in the presence of this factor although, in its absence, the two DNA's have about the same template activities for RNA polymerase II. The mechanism by which this factor stimulates RNA synthesis is unknown. However, it is shown that this factor stimulates both initiation and elongation of RNA chains, though the effect on chain elongation is much more than that on chain initiation. It is possible that the factor recognizes specific sequences on template DNA and makes it possible for RNA polymerase II to initiate stable RNA synthesis, resulting in production of high-molecular-weight RNA. Calf thymus DNA may contain fewer of these specific sequences than Ehrlich ascites tumor DNA and poly(dAT) may contain none.

This paper reports the purification of a factor stimulating RNA polymerase II and results suggesting that its stimulatory activity is quite specific. Elucidation of the mechanism of stimulation of RNA synthesis by this factor may provide more information about the regulation of transcription in eukaryotic cells.

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