Perry, R. P., Kelley, D. E., Frederici, K. H., & Rottman, F. (1975) *Cell 4*, 387–394.
Reeder, R. H., & Roeder, R. G. (1972) *J. Mol. Biol. 67*, 433–441.
Rose, K. M., Ruch, P. A., & Jacob, S. T. (1975) *Biochemistry 14*, 3598–3604.
Schibler, U., & Perry, R. P. (1976) *Cell 9*, 121–130.
Schibler, U., & Perry, R. P. (1977) *Nucleic Acids Res. 4*, 4133–4149.
Schibler, U., Kelley, D. E., & Perry, R. P. (1977) *J. Mol. Biol. 115*, 695–714.
Schmincke, C. D., Herrmann, D., & Hausen, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1994–1998.
Sehgal, P. B., Derman, E., Molloy, G. R., Tamm, I., & Darnell, J. E. (1976a) *Science 194*, 431–433.
Sehgal, P. B., Darnell, J. E., Jr., & Tamm, I. (1976b) *Cell 9*, 473–480.
Smith, M. M., & Huang, R. C. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 775–779.
Suzuki, Y., & Giza, P. E. (1976) *J. Mol. Biol. 107*, 183–206.
Tamm, I. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5011–5015.
Tamm, I., Hand, R., & Caliguri, L. A. (1976) *J. Cell Biol. 69*, 229–240.
Verbert, A. P., Sergeant, A., & Krsmanovic, V. (1974) *Biochemie 56*, 1103–1110.
Weinmann, R., & Roeder, R. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1790–1794.
Winicov, I. (1977) *Biochemistry 16*, 4233–4237.
Winicov, I., & Perry, R. (1976) *Biochemistry 15*, 5039–5046.
Wu, G. J., & Zubay, G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1803–1807.
Zylber, E. A., & Penman, S. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 2861–2865.

Purification and Preparation of Antibody to RNA Polymerase II Stimulatory Factors from Ehrlich Ascites Tumor Cells†

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ABSTRACT: An improved method was developed for purification of the protein termed S-II that specifically stimulates RNA polymerase II of Ehrlich ascites tumor cells. The specific activity of the final preparation was 400 000 units/mg of protein, which is about 30-fold higher than that of the previous preparation [Sekimizu, K., et al. (1976) *Biochemistry 15*, 5064]. The final preparation gave a single band on both sodium dodecyl sulfate and nondenaturing gel electrophoresis, and the protein extracted from the band on nondenaturing gel had stimulatory activity. S-II is a basic protein with a molecular weight of 40 500. The fundamental characteristics of S-II determined with the previous preparation were confirmed with completely purified S-II. A specific antibody to S-II was prepared. This antibody inhibited only the stimulatory activity of S-II and did not affect the activity of RNA polymerase II itself. Thus, S-II is probably not a component of the multimeric proteins of RNA polymerase II.

It is likely that many proteins participate in regulation of eukaryotic transcription, but little is known about them, because the transcriptional machinery of eukaryotic cells cells much is much more complicated than that of prokaryotic cells and has not yet been well characterized. In studies on the proteins regulating eukaryotic transcription, attempts have been made to isolate proteins that affect the activity of RNA polymerase II in vitro. Proteins that stimulate homologous RNA polymerase II have been isolated from various organisms (Stein & Hausen, 1970; Seifart, 1970; Natori, 1972; Lentfer & Lezius, 1972; Sugden & Keller, 1973; Lee & Dahmus, 1973), but at present it is difficult to evaluate their significance, because most of them are still impure and it is unknown how they stimulate RNA synthesis. One crucial question is whether these proteins are subunits of RNA polymerase II or not, but to answer this question these proteins must be purified. So far, three groups have reported the purifications from different sources of proteins that stimulate RNA polymerase II (Lee & Dahmus, 1973; Seifart et al., 1973; Benson et al., 1978). However, the criteria of purification used by these groups were different, and it seems difficult to compare the characteristics of these proteins from different sources, because their grades of purity are different.

Previously we reported two proteins from Ehrlich ascites tumor cells, named S-I and S-II, that specifically stimulate the activity of homologous RNA polymerase II in vitro (Natori et al., 1973a), and we described the purification of S-II to near homogeneity (Sekimizu et al., 1976). These protein factors seemed to be candidates for the regulatory protein of transcription, because they showed distinct template specificity, and S-II was found to enhance the formation of the initiation complex with RNA polymerase II and homologous DNA in the presence of nucleoside triphosphates (Sekimizu et al., 1977). However, further study showed that S-II was still not pure, although it gave a single band on sodium dodecyl sulfate gel electrophoresis in Weber and Osborn's system (Weber & Osborn, 1969).

This paper describes an improved method for purification of S-II. We adopted the following criteria for judging the purity of the stimulatory factor. (1) It should give a single band on both sodium dodecyl sulfate and nondenaturing polyacrylamide gel electrophoresis. (2) The protein migrating as a single band on nondenaturing polyacrylamide gel should have activity for stimulating RNA polymerase II. We es-

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tablished a procedure for isolating a preparation of stimulatory factor S-II that fulfilled the above criteria. The specific activity of the final preparation was 400 000 units/mg of protein, which is about 30 times more than that of our previous preparation of S-II. We prepared antibody to S-II and found that it inhibited only the stimulatory activity of S-II without affecting the activity of RNA polymerase II itself. This indicates that S-II is not a component of RNA polymerase II.

Materials and Methods

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

Buffer Solutions. All buffer solutions were prepared using deionized water. Buffer I was composed of 50 mM Tris-HCl, pH 7.9 (25 °C), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.3 mM dithiothreitol, and 25% (v/v) glycerol. Buffer II contained 10 mM Tris-HCl, pH 7.9 (25 °C), 5 mM $MgCl_2$, and 5 mM β-mercaptoethanol. Buffer II′ had the same composition as buffer II, except that 0.1% (v/v) Triton X-100 was added. Buffer III contained 10 mM Tris-HCl, pH 7.9 (25 °C), 10 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol, and 50% glycerol. Buffer IV contained 2 mM Tris-HCl, pH 7.9 (25 °C), 0.02 mM EDTA, 2 mM $MgCl_2$, 2 mM KCl, 1 mM β-mercaptoethanol, 10% glycerol, and 0.1% Triton X-100. Dithiothreitol was added to buffer I immediately before use.

Saturated Ammonium Sulfate Solution. Ammonium sulfate was dissolved in deionized water at 80–100 °C nearly to saturation and then the solution was kept overnight at 4 °C to allow the excess ammonium sulfate to crystallize out. Then the solution was adjusted to pH 7.9 at 20 °C by adding 10% NH_4OH solution and stored at 4 °C.

Preparation and Assay of RNA Polymerase II. RNA polymerase II was partially purified from Ehrlich ascites tumor cells as described before by DEAE-cellulose column chromatography and glycerol-gradient centrifugation (Natori et al., 1973b). One unit of RNA polymerase activity was defined as the amount catalyzing the 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 $MnCl_2$, 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, CTP, and GTP, 0.00625 μmol of UTP, 0.5 μCi of [^{3}H]UTP (20 Ci/mmol), 5 μg of purified Ehrlich ascites tumor DNA, and RNA polymerase II. The mixture was incubated for 60 min at 37 °C, and then 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 each sample was applied to a Whatman GF-C glass fiber filter. The filter was washed with 60 mL of cold 5% Cl_3CCOOH solution containing 0.01 M sodium pyrophosphate and then with 1 mL of 95% ethanol and dried under an infrared lamp. The radioactivity on the filter was counted in a Packard liquid scintillation spectrometer. The specific activity of RNA polymerase II used in this work was usually 1000 to 2000 units/mg of protein.

Assay of the Stimulatory Factor of RNA Polymerase II. Stimulatory activity was routinely assayed under the standard assay conditions for RNA polymerase II in the presence of the test fraction. Incorporation of UMP into the acid-insoluble fraction was compared with that in the reaction mixture without the test fraction. One unit of stimulatory activity was defined as the amount that enhanced the activity of 10 units of RNA polymerase II to 11 units under these conditions.

Sodium Dodecyl Sulfate Gel Electrophoresis. Electrophoresis on polyacrylamide–sodium dodecyl sulfate gel (12.5%) was carried out by the method of Laemmli (1970). Proteins were denatured by heating in 1% sodium dodecyl sulfate solution containing 2% (v/v) β-mercaptoethanol for 20 min at 75 °C. Gels were stained by the method of Fairbanks et al. (1971).

Gel Electrophoresis under Undenatured Conditions. The method of Davis (1964) was used. Two cylindrical 5% polyacrylamide gels (0.5 × 6.5 cm) containing 0.1% Triton X-100 were run simultaneously. A sample of 12 μg of purified stimulatory factor was applied to each gel and electrophoresis was carried out at 5 mA/gel at 4 °C. Then one gel was fixed in a mixture of methanol, acetic acid, and water (5:3:1, v/v) and stained with Coomassie brilliant blue R250 to see protein. The other gel was sliced into 2.7-mm sections and the protein was extracted by incubating each section in 0.3 mL of buffer II′ for 12 h at 4 °C. Stimulatory activity in the extract was assayed under the standard conditions. Before loading on the samples, a current of 2 mA/gel was applied to the gels for 90 min to remove ammonium persulfate.

Protein Determination. Protein was determined by the method of Lowry et al. (1951). For determination of protein in buffers I, III, or IV, which contain glycerol, the protein was first precipitated with cold 5% Cl_3CCOOH solution. For assay of protein in buffer II′, which contains Triton X-100, sodium dodecyl sulfate was added to a concentration of 0.1% just before addition of phenol reagent, because otherwise a precipitate appeared that prevented accurate measurements. As a control, a known concentration of bovine serum albumin was assayed in parallel with the samples under the same conditions.

Preparation of Antibody to Stimulatory Factor S-II. Since stimulatory factor S-II is a basic protein, immunization was done by the method of Stollar & Ward (1970) who made antibody to a histone. First, 0.1 mg of purified S-II was mixed with 0.1 mg of yeast RNA (Sigma Chemical Co.) in buffer III. The solution was then thoroughly mixed with an equal volume of complete Freund's adjuvant and injected into the footpads of male albino rabbits weighing about 3 kg. A booster injection of 0.05 mg of S-II in incomplete Freund's adjuvant was given 14 days later. Animals were bled 7 days after the booster injection and antisera were examined by the immunodiffusion method of Ouchterlony (1958). Immunodiffusion was performed on Ouchterlony plates containing 0.9% NaCl, 0.1% Triton X-100, and 10 mM Tris-HCl (pH 7.9 at 25 °C) in 1% agarose. After overnight incubation at 37 °C, plates were dried and stained with Coomassie brilliant blue R250 by the method of Weeke (1973).

The immunoglobulin G fraction was obtained from sera by ammonium sulfate precipitation and DEAE-cellulose chromatography according to the method of McCauley & Racker (1973).

Results

Previously we reported the presence of two proteins that stimulate RNA polymerase II in Ehrlich ascites tumor cells

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

and described a procedure for purifying one of these proteins named S-II. The final preparation was shown to have a specific activity of 15000 units/mg of protein and to give a single band on polyacrylamide–sodium dodecyl sulfate gel electrophoresis by Weber and Osborn's procedure. However, in further studies on purified S-II, we found that purification was still incomplete and that the final preparation was a mixture of stimulatory factor S-II and a contaminating protein of the same molecular weight. Therefore, we reexamined each step of the purification and established a new procedure for obtaining pure stimulatory factor S-II in higher yield.

Solubilization of Stimulatory Factors. All subsequent procedures were performed between 0 and 4 °C unless otherwise specified. About 30 g of frozen Ehrlich ascites tumor cells was thawed in 30 mL of buffer I and then 5% (w/v) sodium deoxycholate solution was added to give a final concentration of 0.1%. This concentration was found to be optimal for solubilizing stimulatory proteins, and only half the stimulatory activity could be extracted in the absence of deoxycholate. Cells were disrupted by 30 rapid strokes in a Potter-Elvehjem homogenizer with a Teflon pestle and then 4 mL of saturated ammonium sulfate solution was added to give a final concentration of 0.25 M, which was found to be optimal. The solution was sonicated in a Branson B-12 sonifier using a microtip at a power setting of 5 (90 W), until the viscosity decreased to a point at which the solution easily formed drops at the tip of a Pasteur pipet; usually the mixture was sonicated six times for 2-min periods with intervals of 1–2 min. Then 60 mL of buffer I was added, and the mixture was homogenized well and centrifuged for 1 h at 170000*g*. The precipitate was discarded and the clear supernatant was stored at −80 °C (sonic extract).

Ammonium Sulfate Fractionation. To 600 mL of sonic extract, prepared from about 90 g of packed cells, was added an equal volume of saturated ammonium sulfate solution and the mixture was stirred for 30 min. The resulting precipitate was removed by centrifugation for 30 min at 14000*g* and 252 g of solid ammonium sulfate was added to the clear 50% ammonium sulfate supernatant. The mixture was stirred for 60 min and the precipitate was collected by centrifugation for 30 min at 14000*g*. The pellet was dissolved in 58 mL of buffer I and dialyzed against 2.5 L of buffer I for 15–18 h. Usually a precipitate appeared in the dialysis tubing during this process. After dialysis the preparation was promptly frozen and stored at −80 °C (1st ammonium sulfate fraction).

First DEAE-cellulose Column Chromatography. About 150 mL of the first ammonium sulfate fraction, prepared from 180 g of packed cells, was centrifuged for 30 min at 15000*g* and the precipitate was discarded. The resulting clear supernatant was applied to a column of DEAE-cellulose (3.5 × 20 cm) that had been equilibrated with buffer I. The column was washed with 4 column volumes of buffer I and the absorbance of the eluate at 280 nm was monitored. The fractions containing unadsorbed material were combined (380 mL) and stored at −80 °C (DEAE-cellulose unadsorbed fraction).

Ammonium Sulfate Precipitation. To 380 mL of DEAE-cellulose unadsorbed fraction were added 2 volumes of saturated ammonium sulfate solution and then 160 g of solid ammonium sulfate and the mixture was stirred for 30 min. This mixture contained nearly the saturating amount of ammonium sulfate. It was found that, when a saturating amount of solid ammonium sulfate was added all at one time, the recovery of stimulatory factors was significantly less. The resulting precipitate was collected by centrifugation for 60 min at 14000*g*, dissolved in 15 mL of buffer II, and dialyzed against

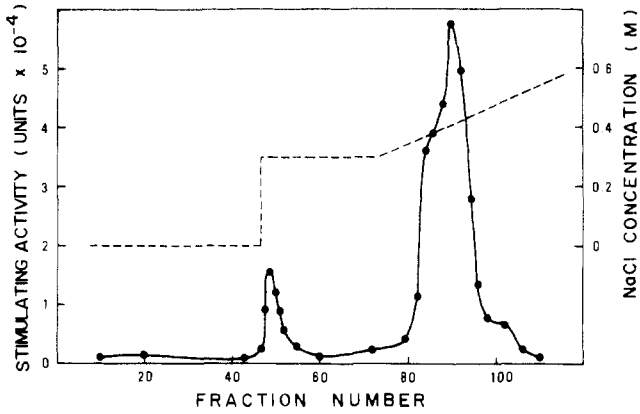

FIGURE 1: Phosphocellulose chromatography of RNA polymerase II stimulatory factors. A 2 × 4 cm phosphocellulose column (Whatman P-11) was loaded with 75 mL (165 mg of protein) of the ethanol fraction (see the text) and washed with 5 column volumes of buffer II′ and then with 4 column volumes of 0.3 M NaCl in buffer II′. Stimulatory factor S-I was eluted at this step. The column was then developed with a linear gradient of 200 mL of 0.3–0.8 M NaCl in buffer II′ at a flow rate of 30 mL/h. Fractions of 3 mL were collected and samples of 10 μL of each fraction were assayed for activity to stimulate RNA synthesis under the standard conditions with 10 units of RNA polymerase II. (●—●) Stimulating activity per fraction; (---) concentration of NaCl.

1 L of buffer II for 15–18 h, changing the outer solution after 1 h. The precipitate that appeared during dialysis was removed by centrifugation for 60 min at 77000*g*, and the clear supernatant was recovered (2nd ammonium sulfate fraction).

Ethanol Fractionation. The stimulatory factors in the second ammonium sulfate fraction were precipitated by adding chilled ethanol. For this, about 26 mL of 99.5% (v/v) ethanol that had been kept at −20 °C was added to 53 mL of the 2nd ammonium sulfate fraction to give a final concentration of 33% ethanol. The mixture was kept for 60 min at −20 °C to complete the precipitation and then centrifuged for 30 min at 14000*g*. The resulting white pellet was suspended in 76 mL of buffer II′, which contains 0.1% of Triton X-100. The suspension was homogenized by 10 slow strokes of the Teflon pestle to solubilize stimulatory factors and then centrifuged for 60 min at 77000*g*. The resulting clear supernatant contained stimulatory factors (ethanol fraction).

Phosphocellulose Chromatography. About 75 mL of the ethanol fraction was applied to a column of phosphocellulose (2 × 4 cm, Whatman P-11) that had been equilibrated with buffer II′. After application of the sample, the column was washed successively with 5 column volumes of buffer II′ and 4 column volumes of 0.3 M NaCl in buffer II′. The stimulatory factor named S-I was eluted in the latter washing fluid. Then stimulatory factor S-II was eluted with a linear gradient of 200 mL of 0.3–0.8 M NaCl in buffer II′, as shown in Figure 1. The fractions containing S-II were combined and dialyzed extensively for 9 h against 2.5 L of buffer II′ to remove NaCl. The chromatographic profile was essentially the same as that reported before (Sekimizu et al., 1976).

CM-cellulose Chromatography. The dialyzed fraction (about 35 mL) was applied to a column of CM-cellulose that had been equilibrated with buffer II′. The column was washed with 5 column volumes of buffer II′ and then S-II was eluted with 100 mL of a linear gradient of 0–0.1 M NaCl in buffer II′. A typical elution profile is shown in Figure 2. Two distinct peaks of activity were eluted, the second peak usually being larger than the first one. With the previous purification procedure a single peak with stimulatory activity was eluted at this step; the improvement in the step of solubilization of stimulatory factors in the present method probably results in

Table I: Summary of Purifications of S-II and S-II′

purification step	vol (mL)	protein (mg)	act.[a] (units)	sp act. (units/mg)	yield (%)
DEAE-cellulose unadsorbed fraction	380	950	1 860 000	2 000	100
second $(NH_4)_2SO_4$ fraction	53	1200	1 990 000	1 700	107
ethanol fraction	75	165	1 080 000	6 500	58
phosphocellulose S-II	35	7.7	440 000	57 000	24
CM-cellulose S-II′	3.5	0.42	58 000	140 000	3.1
CM-cellulose S-II	3.0	1.1	200 000	180 000	11
final preparation S-II′	11	0.07	44 000	600 000	2.4
final preparation S-II	6.4	0.3	110 000	400 000	5.9

[a] Activity was calculated from calibration curves obtained with amounts of each fraction that stimulated RNA synthesis linearly under the standard conditions.

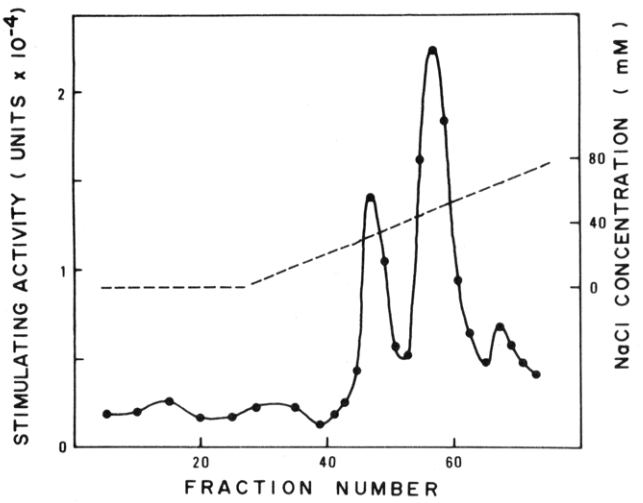

FIGURE 2: CM-cellulose chromatography of S-II. Peak fractions from the phosphocellulose column were combined, dialyzed well, and applied to a column of CM-cellulose (1 × 4 cm). Sample: 35 mL (7.7 mg of protein). Flow rate: 30 mL/h. Fraction size: 2 mL. The column was developed with 100 mL of a linear gradient of 0–0.1 M NaCl in buffer II′. Samples of 5 μL of each fraction were assayed for activity to stimulate RNA synthesis under the standard assay conditions with 10 units of RNA polymerase II. (●—●) Stimulating activity per fraction; (---) concentration of NaCl.

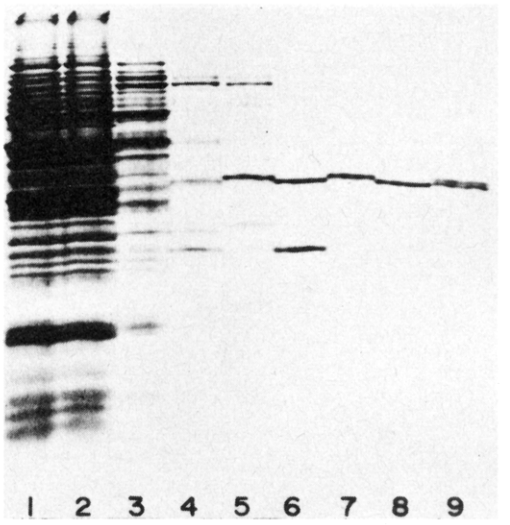

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide (12.5%) slab gel electrophoresis of fractions obtained at each step of purification. (Lane 1) DEAE-cellulose unadsorbed fraction, 35 units; (lane 2) 2nd ammonium sulfate fraction, 38 units; (lane 3) ethanol fraction, 43 units; (lane 4) phosphocellulose S-II fraction, 68 units; (lane 5) CM-cellulose S-II′, 150 units; (lane 6) CM-cellulose S-II, 120 units; (lane 7) final preparation of S-II′, 140 units; (lane 8) final preparation of S-II, 93 units; (lane 9) mixture of S-II′ and S-II, 120 units. The amount of protein varied from 0.3 to 20 μg.

a biphasic peak at this step. The first and second peaks differed slightly in mobility on sodium dodecyl sulfate gel, as shown in Figure 3. On the basis of the NaCl concentration required for elution, the second peak was identified as S-II. The first peak was tentatively termed S-II′. The fractions in each peak were combined separately, concentrated by dialysis against buffer III, which contains 50% glycerol, for 14 h, and stored at −20 °C.

Second DEAE-cellulose Chromatography. The final step of purification was to pass the active fraction from CM-cellulose through DEAE-cellulose. S-II and S-II′ were each diluted with 4 volumes of distilled water and applied to a column of DEAE-cellulose (1 × 1.5 cm) that had been equilibrated with buffer IV. The column was washed with 4 column volumes of buffer IV. The stimulatory activity was recovered in the flow-through fraction. The active fractions were combined, dialyzed extensively against buffer III, and stored at −20 °C.

Purity of Stimulatory Factors. The results of a typical purification are shown in Table I. The specific activity of purified S-II was 400 000 units/mg of protein, which is about 30-fold higher than that of our previous preparation. The electrophoretic patterns of the preparations at each purification step are shown in Figure 3 with the stimulatory activity in each sample used for electrophoresis given in the legend. Both S-II and S-II′ from the 2nd DEAE-cellulose column gave a single band on 12.5% sodium dodecyl sulfate slab gel with a molecular weight of about 41 000. S-II migrated a little faster than S-II′ and a mixture of S-II and S-II′ gave two close, but distinct bands. The molecular weights of S-II′ and S-II were determined as 41 000 and 40 500, respectively, by coelectrophoresis with bovine serum albumin, ovalbumin, α-chymotrypsinogen, and five subunits of *Escherichia coli* ATPase (Futai et al., 1974).

It was important to show that the purified protein contains stimulatory activity. For this purpose, samples of 3700 units (12 μg) of purified S-II were subjected to electrophoresis in two cylindrical gels, under conditions causing no denaturation, as described under Materials and Methods. After electrophoresis, one of the gels was sliced and the protein was extracted from each section and assayed for stimulatory activity. The other gel was stained to locate the protein. As shown in Figure 4, a single band of protein was detected on the gel and the peak of stimulatory activity coincided well with this band. Since this protein gave a single band on sodium dodecyl sulfate gel, it may be safely concluded that the purified S-II is a single protein. The amount of S-II′ available was too small to permit analysis of S-II′ under similar conditions. However, judging from the specific activity of S-II′, it should be the same grade of purity as S-II.

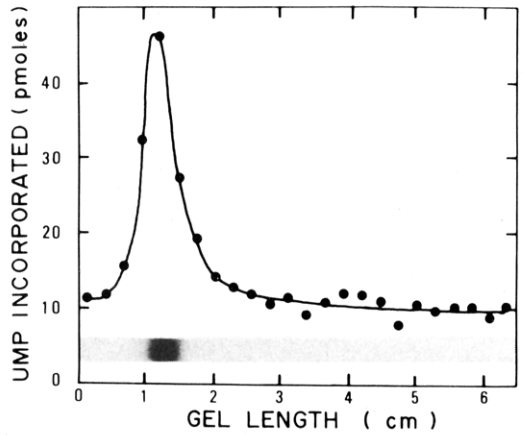

FIGURE 4: Polyacrylamide gel electrophoresis of S-II. The final preparation of S-II was subjected to electrophoresis under conditions not causing denaturation. Electrophoresis was performed using two cylindrical gels, and 12 μg (3700 units) of purified S-II was applied to each gel. After electrophoresis, one gel was sliced into 24 sections and protein was extracted from each section in 0.3 mL of buffer II′ and assayed for stimulatory activity using 10 units of RNA polymerase II and 90 μL of each fraction. The other gel was stained to locate protein (see Materials and Methods).

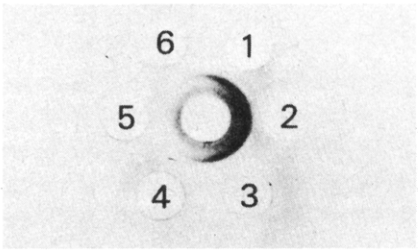

FIGURE 5: Immunodiffusion pattern of antibody to S-II. Immunodiffusion was carried out in 1% agarose gel containing 10 mM Tris-HCl, pH 7.9, 0.9% NaCl, and 0.1% Triton X-100. Purified S-II (1 μg) was placed in the center well. Wells 1–3 contained 100 μg of immunoglobulin G prepared from immunized serum; wells 4–6 contained 100 μg of immunoglobulin G prepared from nonimmunized rabbit serum.

Previously we described several characteristics of S-II: it specifically stimulates RNA polymerase II but does not affect RNA polymerase I; it increases the molecular size of RNA synthesized by RNA polymerase II; and it shows some template specificity and does not stimulate RNA synthesis on poly[d(AT)] (see Natori et al., 1973a,c; Sekimizu et al., 1976). All these characteristics were confirmed with S-II purified by the new procedure.

Antibody to S-II. Antibody was prepared against purified S-II using Freund's adjuvant, as described in Materials and Methods. After the booster injection, appreciable antibody activity was detected in the serum of both rabbits used. IgG was prepared from immunized serum according to the method of McCauley & Racker (1973). As shown in Figure 5, IgG prepared from immunized serum gave only one precipitin line with purified S-II. Its effect on the stimulatory activity of S-II was examined. As is evident from Figure 6, the stimulatory activity of purified S-II was almost completely inhibited by the antibody, whereas normal IgG prepared from nonimmunized rabbit serum did not affect the stimulatory activity of S-II. This result indicates that the antibody is specific for S-II.

It is important to point out that neither the antibody against S-II nor normal IgG affected the activity of RNA polymerase II itself. There has been some discussion about whether the stimulatory factor is an intrinsic component of RNA polymerase II that is detached from the enzyme during purification or a separate entity (Roeder, 1976). The present results clearly demonstrate that our RNA polymerase II did not contain S-II. Our preparation of RNA polymerase II was a partially purified preparation that should retain a more intact structure than completely purified enzyme. The fact that the antibody to S-II did not inhibit the activity of RNA polymerase II itself may indicate that S-II is not a component of RNA polymerase II.

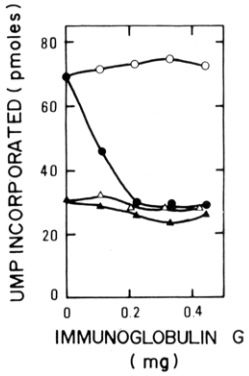

FIGURE 6: Effect of immunoglobulin G on the stimulatory activity of S-II. Increasing amounts of immunoglobulin G prepared from immunized and nonimmunized rabbit sera were mixed with 15 units of S-II and incubated for 1 h at 25 °C. The reaction mixture contained the following components: 8.3 mM Tris-HCl, pH 7.9, 0.017 mM EDTA, 1.7 mM $MgCl_2$, 1.7 mM KCl, 170 mM NaCl, 0.83 mM β-mercaptoethanol, and 15% glycerol. Then the mixture was added to the standard assay mixture for RNA polymerase II and assayed for stimulatory activity. (●—●) Immunized immunoglobulin G; (○—○) nonimmunized immunoglobulin G. The effect of immunoglobulin G on RNA polymerase II was examined by adding increasing amounts of immunoglobulin G directly to reaction mixture containing 30 units of RNA polymerase II. (▲—▲) Immunized immunoglobulin G; (△—△) nonimmunized immunoglobulin G.

This antibody was also found to inhibit the stimulatory activity of S-II′ (data not shown). Thus, S-II and S-II′ seem to have a common antigenic site, although they differ in mobility on sodium dodecyl sulfate gel.

Discussion

This paper describes an improved method for purification of stimulatory factor S-II from Ehrlich ascites tumor cell. By this method the factor can be obtained in a homogeneous state. In the previous paper (Sekimizu et al., 1976), we reported the purification of S-II to a state giving a single band on sodium dodecyl sulfate gel electrophoresis by the method of Weber & Osborn (1969). However, judging from the specific activity of the present preparation of S-II, the purity of the preparation described previously must have been less than 5%.

In this work three main improvements were made in the purification method. First, 0.1% deoxycholate was used to solubilize the stimulatory factors. Deoxycholate seems to be very effective for solubilizing these factors, because using deoxycholate the total activity of the fraction not adsorbed on DEAE-cellulose was twofold more than that of the fraction prepared without the detergent from the same number of cells. Second, ethanol fractionation was introduced. This step removed a protein with a molecular weight of 38 000 that contaminated the previous S-II preparation. Third, 0.1% Triton X-100 was added to all the chromatographic buffers. This greatly improved the recovery of stimulatory factors from the columns.

We chose the following two criteria for assessing purification. (1) The final preparation should give a single band on both sodium dodecyl sulfate and nondenaturing polyacrylamide gel electrophoresis. (2) The protein that migrated

as a single band on nondenaturing polyacrylamide gel should have activity to stimulate RNA polymerase II. The fact that the factor gives a single band on sodium dodecyl sulfate gel is not alone an adequate criterion for concluding that the preparation is pure, because it may contain several proteins with the same molecular weight. Thus, it is necessary to show that contaminating protein is not detectable on nondenaturing gel. Moreover, in order to conclude that the activity is due to the main protein, it is important to show that the protein migrating as a single band on nondenaturing gel has stimulatory activity. The final preparation of S-II conformed to both criteria and, thus, we concluded that it is pure. The grade of purity was to the extent that the preparation was free of major contaminants of greater than about 5%, judging from the electrophoretic patterns shown in Figure 3.

Three groups have reported the purifications from various sources of stimulatory factors of RNA polymerase II (Lee & Dahmus, 1973; Seifart et al., 1973; Benson et al., 1978). However, the grades of purity of their preparations were different and so it is difficult to compare the characteristics of the factors at the same level. The specific activity of the final preparation of S-II was 400 000 units/mg of protein. Recently, Benson et al. (1978) reported the purification from calf thymus of two factors stimulating RNA polymerase II. The specific activity of their factors, calculated on the basis of our definition of a stimulatory unit, was reported to be 400 000 units/mg of protein, which is the same value as that of S-II.

Two peaks of activity were eluted from CM-cellulose, and these were named S-II′ and S-II in order of their elution. These proteins were not separated on a column of phosphocellulose, from which the stimulatory activity was eluted as a single peak with 0.4 M NaCl, but they were separated into two peaks on subsequent CM-cellulose chromatography. At this step in our previous procedure, we obtained only one peak of activity, which corresponded to the second peak, judging from the NaCl concentration for its elution. Thus S-II′ seems to be solubilized only in the presence of 0.1% deoxycholate. As pointed out in this paper, both S-II and S-II′ were inhibited by the antibody to S-II. Therefore, S-II and S-II′ probably contain the same antigenic site or sites, although their mobilities on sodium dodecyl sulfate gel are different. Several groups have reported the heterogeneity of stimulatory factors; the reason for this heterogeneity is not known at molecular level (Dahmus, 1976; Lukacs & Stein, 1976). It may be possible that these factors function independently at different steps of transcription (Nakanishi et al., 1978).

Specific antibody to S-II was shown to inhibit only the stimulatory activity of S-II and not to affect the activity of RNA polymerase II itself. This is an important finding, because it indicates that RNA polymerase II does not contain S-II. We used partially purified RNA polymerase II, which should be more intact than completely purified RNA polymerase II, in terms of subunit structure. The results obtained here strongly suggest that S-II and possibly S-II′ are independent of RNA polymerase II. Antibody to S-II may be useful for studying the structure and function of these stimulatory factors.

A critical question is whether these stimulatory factors really participate in the regulation of transcription in vivo. We showed that S-II enhanced the formation of the initiation complex with RNA polymerase II and DNA in the presence of nucleoside triphosphates (Sekimizu et al., 1977). We also showed that RNA polymerase II contains a protein that interacts with S-II (Kuroiwa et al., 1977). Moreover, we found that various mouse tissues contain different amounts of S-II (Natori et al., 1974) and a significant amount of S-II was shown to be induced in the spleen of mice on injection of bacterial lipopolysaccharide, a mitogen of bone-marrow-derived lymphocytes (Tsuda et al., 1977). These findings are strong grounds for suggesting that S-II participates in the transcriptional process in some way and regulates gene expression. However, to demonstrate that these factors really participate in regulation of transcription in vivo it will be necessary to show that they are directly involved in the transcriptional process. Experiments with the antibody to S-II may be useful for this purpose and should provide more information about the function of these stimulatory factors.

Added in Proof

After submitting this paper we found that this antibody specifically inhibited α-amanitin-sensitive RNA synthesis in isolated nuclei of Ehrlich ascites tumor cells (Ueno et al., 1979).

References

Benson, R. H., Spindler, S. R., Hodo, H. G., & Blatti, S. P. (1978) *Biochemistry 17*, 1387.

Dahmus, M. E. (1976) *FEBS Lett. 61*, 227.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.

Futai, M., Sternweis, P. C., & Heppel, L. A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2725.

Kuroiwa, A., Mizuno, D., & Natori, S. (1977) *Biochemistry 16*, 5687.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Lee, S. C., & Dahmus, M. E. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1383.

Lentfer, D., & Lezius, A. G. (1972) *Eur. J. Biochem. 30*, 278.

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

Lukacs, N., & Stein, H. (1976) *FEBS Lett. 69*, 295.

McCauley, R., & Racker, E. (1973) *Mol. Cell. Biochem. 1*, 73.

Nakanishi, Y., Sekimizu, K., Mizuno, D., & Natori, S. (1978) *FEBS Lett. 93*, 357.

Natori, S. (1972) *J. Biochem. (Tokyo) 72*, 1291.

Natori, S., Takeuchi, K., Takahashi, K., & Mizuno, D. (1973a) *J. Biochem. (Tokyo) 73*, 879.

Natori, S., Takeuchi, K., & Mizuno, D. (1973b) *J. Biochem. (Tokyo) 73*, 345.

Natori, S., Takeuchi, K., & Mizuno, D. (1973c) *J. Biochem. (Tokyo) 74*, 1177.

Natori, S., Sekimizu, K., & Mizuno, D. (1974) *J. Biochem. (Tokyo) 76*, 695.

Ouchterlony, O. (1958) *Prog. Allergy 5*, 1.

Roeder, R. G. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) p 285, Cold Spring Harbor Laboratory, New York.

Seifart, K. H. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 719.

Seifart, K. H., Juhasz, P. P., & Benecke, B. J. (1973) *Eur. J. Biochem. 33*, 181.

Sekimizu, K., Kobayashi, N., Mizuno, D., & Natori, S. (1976) *Biochemistry 15*, 5064.

Sekimizu, K., Mizuno, D., & Natori, S. (1977) *Biochim. Biophys. Acta 479*, 180.

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

Stollar, B. D., & Ward, M. (1970) *J. Biol. Chem. 245*, 1261.
Sugden, B., & Keller, W. (1973) *J. Biol. Chem. 248*, 3777.
Tsuda, M., Mizuno, D., & Natori, S. (1977) *Infect. Immun. 16*, 537.
Ueno, K., Sekimizu, K., Mizuno, D., & Natori, S. (1979) *Nature (London) 277*, 145.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Weeke, B. (1973) *Scan. J. Immunol. 2, Suppl. 1*, 15.

Cell-Free Protein Synthesis in Lysates of *Drosophila melanogaster* Cells†

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ABSTRACT: A procedure is described for preparing cell-free protein synthesizing lysates from *Drosophila melanogaster* tissue culture cells and embryos. Preparation of translationally active lysates from tissue culture cells is dependent on the presence of rat liver supernatant during cell lysis to inhibit ribonuclease activity. After micrococcal nuclease treatment of the lysate, protein synthesis is dependent on the addition of exogenous messenger RNA. The fidelity of translation is very high. The conditions for optimal translation have been determined. In addition, the effects on translation of a variety of supplements, including rat liver supernatant, have been analyzed. The products of translation by the *Drosophila* lysate have been compared with those of wheat germ extracts and of micrococcal nuclease treated rabbit reticulocyte lysates. Translation in vitro of bovine parathyroid hormone messenger RNA yielded two products tentatively identified as preproparathyroid hormone and proparathyroid hormone, as well as an unidentified third product. This result suggests that insect enzymes can accurately process mammalian precursor proteins.

The use of cell-free protein synthesizing systems has facilitated two types of analysis. First, the protein synthesizing system can be dissected and reconstituted, using defined messenger RNAs and well-characterized products, in order to understand how ribosomes and messenger RNA interact with the various controlling signals and factors to synthesize proteins. Second, in vitro translation systems are important tools for determining the coding capacity of purified messenger RNAs. Primary translation products as well as processing and posttranslational modifications can be analyzed.

In general, the more closely the in vitro products made by a translation system resemble the in vivo protein(s) made from the same messenger RNA, the better the in vitro system is judged to be. Two cell-free protein synthesizing systems that have been particularly useful in this regard are translation extracts made from wheat germ embryo (Roberts & Paterson, 1973) and micrococcal nuclease treated rabbit reticulocyte lysates (Pelham & Jackson, 1976; Villa-Komaroff et al., 1974). It is often the differences, however, between in vivo and in vitro protein products that are most interesting, particularly differences that may be due to the ability of the translation system components to recognize signals encoded in the sequence or modifications of the messenger RNA. The capability to interpret (or misinterpret) such signals could have species or even tissue specificity.

Drosophila melanogaster is especially attractive for studies of gene control because of the extensive genetic studies conducted over the past half century, the small size of the genome, and the growing body of knowledge about the developmental biology of this species. In studying certain aspects of gene expression, we realized the potential usefulness of an in vitro translation system prepared from *Drosophila*. We report here the development of such a system from *Drosophila* tissue culture cells and embryos. In both cases the method is simple and the in vitro products closely resemble proteins synthesized in vivo. In addition, the system is responsive to exogenous mRNA[1] and becomes dependent upon such RNA after treatment with micrococcal nuclease.

Materials and Methods

RNA and Protein Extraction. Vesicular stomatitis virus (VSV) messenger RNA was prepared by phenol–chloroform extraction of infected Chinese hamster ovary (CHO) cells (Lodish & Froshauer, 1977). [^{35}S]Methionine-labeled VSV protein from infected CHO cells was a gift from Susan Froshauer. *Drosophila* RNA was extracted and purified from Schneider L-2 suspension culture cells by guanidine hydrochloride extraction (Strohman et al., 1977) as modified by Bruce Paterson (personal communication).

Five hundred milliliters of cultured cells was pelleted, resuspended in 5 mL of 8 M Gdn·HCl, 0.025 M NaAc, and sheared in a Dounce homogenizer. RNA was precipitated at −20 °C by the addition of 0.5 volume of ethanol. The precipitate was collected by a 10-min, 3000*g* centrifugation. The pellet was dissolved in 5 mL of 8 M Gdn·HCl, then brought to 25 mM EDTA, pH 7, and to 50 mM NaAc, pH 5. The ethanol precipitation and resuspension procedure were repeated. The resulting pellet was resuspended in 0.025 M EDTA, pH 7, and extracted with chloroform/butanol (4:1) twice. The RNA was then precipitated with 2 volumes of 4.5 M NaAc, pH 6, at 4 °C overnight.

Yeast transfer RNA was purchased from Boehringer-Mannheim. Yeast and wheat germ transfer RNA synthetases purified by DEAE-cellulose chromatography were gifts from Arnaud Ducruix. Purified wheat germ transfer RNA was

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[1] Abbreviations used: mRNA, messenger RNA; RLS, rat liver supernatant; $NaDodSO_4$, sodium dodecyl sulfate; VSV, vesicular stomatitis virus; RNase, ribonuclease; EDTA, ethylenediaminetetraacetate; Gdn·HCl, guanidine hydrochloride.

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