DNA-Dependent RNA Polymerase II Stimulatory Factors from Calf Thymus: Purification and Structural Studies†

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ABSTRACT: Protein factors which stimulate RNA polymerase II but not polymerases I and III on native DNA have been isolated from calf thymus. These factors are basic proteins and exhibit properties which are similar to the stimulatory factor first described by H. Stein & P. Hausen [(1971) *Cold Spring Harbor Symp. Quant. Biol. 35,* 719]. The stimulatory factors are initially purified by the following steps: homogenization, 40–80% ammonium sulfate precipitation, DEAE-Sephadex, CM-cellulose, and Sephadex G-75. Two stimulatory peaks (SF-1 and SF-2) are resolved by Sephadex G-75. SF-1 is resistant while SF-2 is sensitive to heat denaturation at 98 °C. The protein kinase activity present in SF-1 is heat labile, demonstrating that it is not responsible for the stimulation by SF-1. Both factors stimulate transcription on native calf thymus DNA but not denatured calf thymus DNA. SF-2 stimulates while SF-1 does not stimulate transcription on poly[d(A-T)]. Both factors are sensitive to digestion with proteolytic enzymes, indicating that both factors contain a protein which is essential for stimulatory activity. Gel filtration studies show that SF-1 and SF-2 exhibit native molecular weights of 55 000–65 000 and 25 000–35 000, respectively. Upon further purification by phosphocellulose chromatography the SF-1 activity peak was coincident with polypeptides at 27 000 and 36 000–38 000 molecular weights, indicating that SF-1 may be a dimer. SF-2 contains several components; the major polypeptide (90% pure) which is coincident with peak of activity exhibits a molecular weight of 19 000 and is active in unwinding poly[d(A-T)]. Isoelectric focusing indicates that two different stimulatory factors are present in SF-2, the 19 000 molecular weight poly[d(A-T)] unwinding activity and a second peak with polypeptides of 34 000 and 22 000 molecular weight. Using an alternate purification procedure, small quantities of the second stimulatory factor in SF-2 were purified to near homogeneity (95% pure) and it appears to be composed of single polypeptide of 34 000 molecular weight. Interestingly, the minor impurities in SF-1 and SF-2 found in the side fractions of the activity peaks comigrate on sodium dodecyl sulfate–polyacrylamide gels with the four low molecular weight polypeptides of homologous RNA polymerase II.

Different classes of eukaryotic RNA polymerase (Roeder and Rutter, 1969) transcribe the respective classes of cellular RNA. RNA polymerase I is localized in nucleoli (Roeder & Rutter, 1970) and transcribes the rRNA precursor (Blatti et al., 1970; Reeder & Roeder, 1972); RNA polymerase II is responsible for pre-mRNA synthesis (Blatti et al., 1970; Zylber & Penman, 1971); RNA polymerase III transcribes the 4.5S pre-tRNA and 5S rRNA (Weinmann & Roeder, 1974; Weil & Blatti, 1976). Thus the different classes of RNA polymerase may be controlled independently to regulate the amount of transcription of each gene class. Hydrocortisol treatment, administration of estradiol, and partial hepatectomy all result in increased RNA polymerase I activity in the respective target tissues (Blatti et al., 1970) and recently estradiol has been shown to increase RNA polymerase III activity in rat uterus nuclei (Weil et al., 1977). However, it is thought that the primary events after steroid treatment involve the expression of structural genes, presumably by RNA polymerase II. How specific structural genes are selected for expression by RNA polymerase II remains one of the major unsolved problems of eukaryotic biology.

The structural similarity of calf thymus RNA polymerase II with two large subunits and several small subunits (Blatti et al., 1970; Weaver et al., 1971; Kedinger et al., 1974; Hodo & Blatti, 1977) to the prokaryotic polymerase (Burgess, 1969) suggests that similar mechanisms of control may be involved. Thus, dissociable protein factors such as the σ factor (Burgess et al., 1969) and other low molecular weight protein factors (see references in Chamberlain, 1974) which are required for proper initiation and specificity of gene expression in prokaryotes may also be present in eukaryotic transcription systems.

Factors which specifically stimulate transcription by RNA polymerase II on double-stranded DNA templates have been identified in extracts of calf thymus (Stein & Hausen, 1970) and rat liver (Seifart, 1970; Seifart et al., 1972), KB cells (Sugden & Keller, 1973), Novikoff ascites cells (Lee & Dahmus, 1973), and mouse-myeloma cells (Lentfer & Lezius, 1972). These factors stimulate homologous RNA polymerase II four- to tenfold on native DNA but do not stimulate the activity of RNA polymerase I or *E. coli* RNA polymerase. Although some of the stimulatory factors have been highly purified (Sekimizu et al., 1976), most of the factors isolated have not been purified to homogeneity and none have been compared to purified homologous RNA polymerase II. The purpose of this report is to describe the purification and properties of two stimulatory factors SF-1 and SF-2 from calf thymus. We also compare the molecular sizes of the polypeptides present in SF-1 and SF-2 with RNA polymerase II subunits.

Materials and Methods

Materials. All chemicals were reagent grade. 5′-^{3}H-labeled-UTP (>20 Ci/mmol) was purchased from New England

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Nuclear. Calf thymus DNA (grade I) and protease were from Sigma Chemical Co. Crystalline bovine serum albumin was from Pentex-Miles and poly[d(A-T)] from Miles Laboratories. α-Amanitin (A grade), trypsin (A grade), and soybean trypsin inhibitor (B grade) were obtained from Calbiochem. Fresh frozen calf thymus was purchased from Dubuque Pack, Dubuque, Iowa, and stored at −70 °C.

Solutions. All solutions were prepared using reverse-osmosis purified water. Dithiothreitol, when present, was added immediately before use. Buffer A was composed of 1.0 M sucrose, 10 mM Tris-HCl (pH 7.9), 25 mM KCl, and 5 mM $MgCl_2$. Buffer B contained 12% glycerol (v/v), 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol. Buffer C was 33% glycerol (v/v), 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol. Buffer D was the same composition as buffer C except that $MgCl_2$ was omitted. Buffer E contained 15% glycerol (v/v), 10 mM K_2HPO_4 (pH 7.1), 0.1 mM EDTA, and 0.5 mM dithiothreitol. Buffer F was the same composition as buffer E except it contained 10% glycerol (v/v). Buffer G contained 10 mM Tris-HCl (pH 7.4), 6 mM $MgCl_2$, and 2.5 mM dithiothreitol. Buffer H contained 10% glycerol, 5 mM K_2PO_4 (pH 7.8), 0.5 mM $MgCl_2$. Buffer I contained 10 mM Tris (pH 7.9) and 10 mM NaCl. Buffer J contained 50 mM Tris (pH 7.9), 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10 mM KCl. Solutions containing ammonium sulfate were adjusted to the desired concentration by the addition of 4 M ammonium sulfate (pH 7.9), adjusted at 20 °C with NH_4OH.

Determination of $(NH_4)_2SO_4$ and KCl Concentrations. The ammonium sulfate concentration of a solution was determined by measuring the conductivity of a 100-fold dilution using a Radiometer CDM26 conductivity meter. The values obtained were compared with a standard curve constructed using ammonium sulfate solutions of known concentrations. The KCl concentration of a solution was determined in a similar fashion using a 40-fold sample dilution and a standard curve constructed using KCl solutions of known concentration.

Assay of RNA Polymerase Activity. Assays were carried out as previously described (Blatti et al., 1970) but in a final volume of 60 μL. The assays were initiated by the addition of 10 μL of RNA polymerase fraction in either buffer C or D to an assay mixture containing 35 μL of a solution containing: 3 μmol of Tris-HCl (pH 7.9), 100 nmol of $MnCl_2$, 20 μg of DNA, 36 nmol each of GTP, CTP, and ATP, 600 pmol of unlabeled UTP, and 0.5 μCi of ^3H-labeled UTP, and 15 μL of solution C containing the appropriate amount of ammonium sulfate. Unless otherwise indicated polymerase I was assayed at 40 mM ammonium sulfate and polymerases II and III were assayed at 100 mM ammonium sulfate. This reaction mixture contained nonsaturating levels of UTP and was used for routine assays. When units of enzyme activity were determined, saturating levels of 6 nmol of UTP per assay were used. The assay was incubated at 37 °C for 10 min and a 50-μL aliquot was withdrawn and absorbed to a DEAE-cellulose paper disc (DE-81 filter 2.5 cm diameter). The filters were washed six times for 5 min each in 5% Na_2HPO_4 followed by two washes in distilled water, two washes in 95% ethanol, and a final two washes in ether. The filters were dried under an infrared lamp and radioactivity was determined using 10 mL/filter of toluene containing 4 g/L of Omnifluor (New England Nuclear). Tritium counting efficiency using this system was 21%.

One unit of RNA polymerase activity represents 1 nmol of UMP incorporated per 10 min under saturating UTP conditions using native calf thymus DNA as a template. Specific activity was determined under optimal salt conditions for each polymerase. One nanomole of UMP incorporated is equal to 3.7×10^4 cpm.

Assay of Stimulatory Factor Activity. Stimulatory factor activity was routinely assayed in a final volume of 60 μL by the addition of a 10-μL aliquot of the sample to be assayed, usually in buffer F, to 15 μL of RNA polymerase, purified through the second DEAE-Sephadex step, in buffer C containing sufficient ammonium sulfate to result in a final concentration of 20 mM. The reaction was initiated by the addition of 35 μL of the reaction cocktail described above, followed by incubation at 37 °C. After 10 min, 50 μL of the reaction was absorbed to DEAE-cellulose paper discs and the filters were washed and counted as indicated above.

Preparation of Ion-Exchange and Gel Filtration Resins. DEAE-Sephadex A-25 and SP-Sephadex C-25 were obtained from Pharmacia. The resins were suspended in 4 volumes of reverse-osmosis purified water and titrated to pH 7.9 with 2 M Tris base. The resins were washed with 16 volumes of water and 8 volumes of buffer C containing 50 mM ammonium sulfate or with buffer F for A-25 and C-25, respectively. The resins were resuspended in the appropriate buffer. The solutions were made 0.03% toluene as 1:1 (buffer:resin) slurries and stored at 4 °C.

Phosphocellulose (P-11) was obtained from Whatman, Inc. It was washed with 8 volumes of 0.5 N NaOH followed by 6 washes with 8 volumes of reverse-osmosis purified water. The resin was then washed in 8 volumes of 0.5 N HCl followed by 6 washes with 8 volumes of reverse-osmosis purified water. The final solution was titrated to pH 7.9 with KOH and gently stirred overnight. The pH of the solution was readjusted with KOH and the resin washed 4 times in 8 volumes of buffer D. The resin solution was adjusted to 0.03% toluene as a 2:1 (resin:buffer) slurry and stored at 4 °C.

CM-cellulose (CM-52) was obtained from Whatman, Inc. It was suspended in ten times concentrated buffer F and equilibrated overnight. The resin was washed twice in 8 volumes of buffer F, resuspended in buffer F, made 0.03% in toluene as a 1:1 (resin:buffer) slurry and stored at 4 °C.

All resin slurries were warmed to room temperature and vacuum degassed before use. Columns were packed at room temperature, placed at 4 °C, and equilibrated with several volumes of the appropriate buffer.

Sephadex G-75 was suspended in 4 volumes of buffer F containing 400 mM KCl. The slurry was boiled for 3 h and stored at 4 °C for several weeks to allow complete swelling. The slurry was adjusted to a 1:1 (resin:buffer) slurry, warmed to room temperature, and vacuum degassed before packing. The columns were washed at 4 °C with 10 column volumes of buffer F, 400 mM KCl to allow complete packing. Samples in buffer F, 400 mM KCl comprising less than 2% of the total column volume were layered onto the top of the column.

Sodium Dodecyl Sulfate Gel Electrophoresis. Polyacrylamide–sodium dodecyl sulfate gels (10 and 12.5%) were run according to the method of Laemmli & Favre (1973), except that the reservoir buffers contained 0.5 mM dithiothreitol. The samples were either concentrated by precipitation with 5% trichloroacetic acid at 0 °C or dialyzed into 20 mM NH_4HCO_3, 0.05 mM dithiothreitol and lyophilized. The samples were resuspended in 25 mM Tris-HCl (pH 7.9) and 5% (v/v) β-mercaptoethanol. The samples were made 1% in sodium dodecyl sulfate and immediately boiled for 60 s. After adjustment to 15% (v/v) glycerol the samples were layered on the gels. Gels were stained according to the method of Fairbanks et al. (1971).

Protein Determination. Protein was determined by the method of Lowry et al. (1951) after either dialysis against 10

mM Tris-HCl (pH 7.9) or precipitation with cold 10% trichloroacetic acid.

RNA Polymerase II Purification. RNA polymerase II was either purified by the method outlined previously (Weil & Blatti, 1975) through the second DEAE-Sephadex column and then subjected to sucrose density gradient as described by Blatti et al. (1970) or purified by the method of Hodo & Blatti (1977). Both enzymes were stimulated to the same extent by stimulatory factors.

Isolation of RNA Polymerase II Stimulatory Factors. Frozen thymus (1.7 kg) was thawed in 0.15 M KCl and homogenized in a Waring blender for 2 min (40 s each at the low, medium, and high settings) in 3 L of buffer A. All manipulations were performed at 4 °C. The homogenate was adjusted to 0.3 M ammonium sulfate and 0.5 mM β-mercaptoethanol by the addition of the appropriate volume of 4 M ammonium sulfate (pH 7.9) and 100% β-mercaptoethanol. The solution was homogenized for 60 s (20 s each at the low, medium, and high settings), diluted with 6 L of buffer B and homogenized for an additional 60 s at the high setting. The mixture was centrifuged 40 min at 13 000*g*, solid ammonium sulfate (0.234 g/mL of solution) was added to the supernatant fraction, and the mixture was stirred for 90 min. The resulting precipitate was removed by centrifugation for 40 min at 13 000*g*. Ammonium sulfate (0.22 g/mL) was then added to the 40% ammonium sulfate supernatant and the solution was adjusted to pH 8.0 with NH_4OH. The precipitate was collected after 1 h by centrifugation for 60 min at 13 000*g*. The pellet was resuspended in buffer B and dialyzed to 10 mM ammonium sulfate. This solution was clarified by centrifugation at 48 000*g* for 60 min, passed through a layer of glass wool, and added to 100 g (dry weight) of DEAE-Sephadex A-25 equilibrated in buffer B. The resulting slurry was stirred 12 h and poured onto a bed of DEAE-Sephadex making a 5 × 60 cm DEAE-Sephadex column. The effluent was titrated to pH 7.1 with 2 N HCl, diluted with 1 volume of a 10% glycerol solution, and applied to a 5 × 60 cm column of CM-cellulose equilibrated with buffer F containing 10 mM KCl. The column was washed with 2 column volumes of buffer F containing 10 mM KCl and eluted with 2 column volumes of a linear 10 to 400 mM KCl gradient in buffer F. The fractions containing stimulatory activity were pooled and precipitated with solid ammonium sulfate (0.56 g/mL) for 2 h at pH 8.0. The precipitate was collected by centrifugation at 48 000*g* for 30 min, resuspended in buffer F, dialyzed to 400 mM KCl in buffer F, and clarified by centrifugation at 6000*g* for 30 min. The stimulatory activities present were resolved and further purified by gel filtration utilizing a 2.5 × 130 cm G-75 Sephadex column equilibrated in buffer F containing 400 mM KCl. The stimulatory activity eluted in two major peaks termed SF-1 and SF-2 in order of elution.

SP-Sephadex Chromatography of SF-2. The fractions containing SF-2 activity from the G-75 column were pooled, dialyzed to 10 mM KCl in buffer F, and applied to 2.0 × 23 cm column of SP-Sephadex C-25. The column was washed reverse flow with 6 column volumes of buffer F, 10 mM KCl, followed by 0.6 column volumes of buffer F, 25 mM KCl. The activity was eluted with 2 column volumes of a linear 25 to 400 mM KCl gradient in buffer F.

Alternate Procedure for the Purification of SF-2. The procedure through the CM-cellulose step (Figure 1) was identical with that described above. The peak fractions from CM-cellulose were pooled and dialyzed against 0.01 M KCl, buffer F. A sample containing 80 mg of protein in a volume of 320 mL was adsorbed to a column of SP-Sephadex (bed volume, 150 mL) at 35 mL/h equilibrated in 0.01 M KCl, buffer F. The column was washed with 300 mL of 0.01 M KCl, buffer F, and the stimulatory activity was eluted with buffer F, containing 0.05 M KCl. The factor preparation containing 40 mg of protein in 250 mL was dialyzed to 0.01 M KCl, buffer F and adsorbed to a 200-mL bed volume CM-cellulose column equilibrated in the same buffer. The column washed was 200 mL with buffer F, 10 mM KCl and eluted with a 400 mL of 0.01–0.25 M KCl gradient in buffer F at a flow rate of 80 mL/h. The peak of stimulatory activity which eluted at 55 mM KCl was pooled (85 mL) and concentrated to 6 mL by lyophilization. After dialysis against buffer F, 0.4 M KCl, the sample containing 20 mg of protein in 5 mL was layered onto Sephadex G-75 column and run as described in the other procedure. The void volume was 218 mL and the peak of stimulation eluted at about 400 mL. Only one peak of stimulatory activity was obtained, eluting at a position identical to SF-2. The pooled peak of stimulatory activity contained 3.2 mg of protein in 65 mL. After dialysis against 10 mM KCl, buffer F the sample was loaded onto a 2-mL SP-Sephadex C-25 column equilibrated in the same buffer. The column was washed with 5 column volumes of 0.01 M KCl, buffer F and eluted with a 20-mL 10–300 mM KCl, buffer F gradient at flow rate of 8 mL/h, collecting 0.25-mL fractions. The stimulatory activity found in the 0.01 M KCl wash was lyophilized to 1.2 mL and dialyzed against buffer F containing 5% glycerol, 0.1 M KCl, and 1 mM dithiothreitol. The concentrated stimulatory fraction was layered onto a 5–20% sucrose gradient in the same buffer. After sedimentation in an SW65 rotor for 48 h at 4 °C, the gradients were collected in 0.2-mL fractions.

TABLE I: Purification of Stimulatory Factors SF-1 and SF-2.[a]

Purification step	Total protein (mg)		Total volume (mL)		% recovery[b]	
Tissue homogenate	230 000		14 700			
40% ammonium sulfate supernatant	88 000		16 000			
DEAE-Sephadex flowthrough	2 900		1 800			
CM-cellulose chromatography	235		550		100	
	SF-1	SF-2	SF-1	SF-2	SF-1	SF-2
Sephadex G-75	53	17	50	70	45	55
Heat treatment	11		48		45	
Phosphocellulose chromatography	0.8		17		20	
SP-Sephadex chromatography		8		21		25

[a] SF-1 and SF-2 refer to the two peaks of stimulatory activity obtained when the CM-cellulose activity is chromatographed on G-75. Protein, volume, and % recovery, respectively, are listed for SF-1 and SF-2 material in subsequent purification steps. [b] Recovery was estimated from the amount of factor required to stimulate 0.1 unit of polymerase activity to 0.2 unit of activity.

Results

Purification and Separation of Multiple Forms of Stimulatory Factors. The purification procedure is detailed in Materials and Methods. Disrupted calf thymus tissue is disrupted by homogenization in high salt, diluted to lower salt, and centrifuged to remove chromatin and cellular debris. The stimulatory factor activities are precipitated between 40 and 80% of saturation with ammonium sulfate and the pellet is resuspended and dialyzed. It was found that most of the ribonuclease H contamination could be removed if the preparation

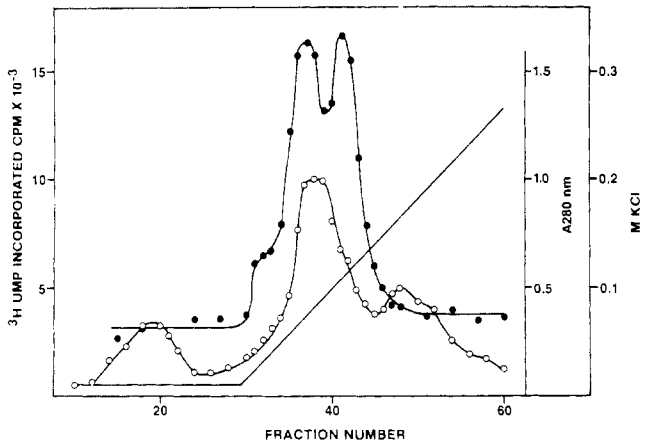

FIGURE 1: Carboxymethylcellulose chromatography of RNA polymerase II stimulatory activity. A 5 × 60 cm CM-cellulose column was loaded with 2.9 g of protein, washed with 2 column volumes of buffer F, 10 mM KCl, and subsequently eluted reverse flow with 2 column volumes of a linear 10 to 400 mM KCl gradient in buffer F at a flow rate of 300 mL per h. Fractions of 24 mL were collected and assayed for stimulatory activity at 20 mM ammonium sulfate using 0.3 unit of polymerase activity. (●) RNA polymerase II activity; (○) absorption at 280 nm; (—) KCl concentration.

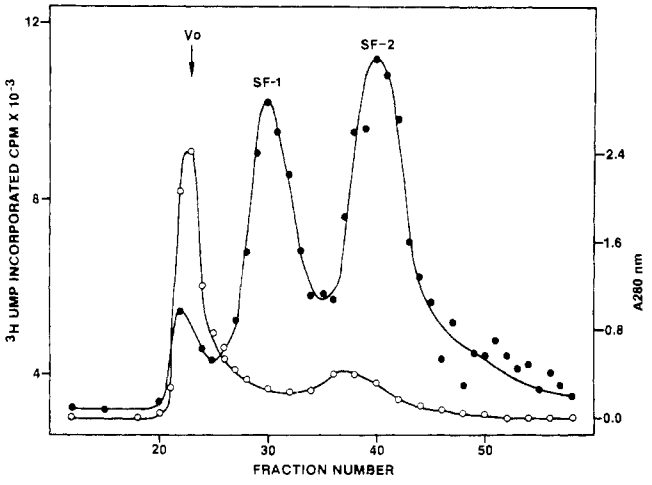

FIGURE 2: Sephadex G-75 filtration of polymerase II stimulatory factors. Peak fractions from CM-cellulose chromatography were concentrated by ammonium sulfate precipitation and a 15-mL sample (15.6 mg/mL) in buffer F; 400 mM KCl was layered onto a 130 × 2.5 cm Sephadex G-75 column previously equilibrated in the same buffer. The flow rate was 40 mL/h. Fractions of 13.7 mL were collected and assayed for stimulatory activity using 0.3 unit of polymerase II per assay at 20 mM ammonium sulfate. (●) Polymerase activity; (○) absorbance at 280 nm.

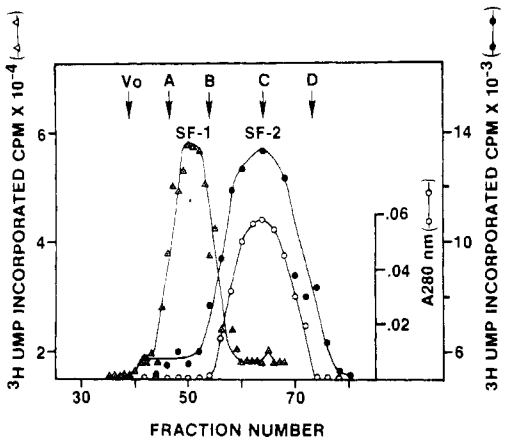

FIGURE 3: Gel filtration of SF-1 and SF-2 stimulatory factors on a calibrated G-75 column. SF-1 (10 mg in 1.5 mL) purified through Sephadex G-75 and SF-2 (5 mg in 1.5 mL) purified through SP-Sephadex were sequentially chromatographed on a Sephadex G-75 column (1.5 × 124 cm) in buffer F, 200 mM KCl. SF-1 and SF-2 were assayed using 1.5 units and 0.3 unit of RNA polymerase II, respectively. The column was subsequently calibrated with protein standards. (V_o) Blue dextran 2000; (△) SF-1; (●) SF-2; (○) absorbance at 280 nm of SF-2. A, B, C, and D represent the positions at which BSA, ovalbumin, chymotrypsinogen, and cytochrome *c* eluted, respectively.

TABLE II: Trypsin Sensitivity of Stimulatory Factors.[a]

Sample	Trypsin	Trypsin inhibitor	cpm
Buffer F	+	+	8 947
Buffer F	−	−	9 031
BSA[a] (250 μg/mL)	+	+	10 363
SF-1	+	+	10 501
SF-2	+	+	10 796
Buffer F	−	+	9 775
SF-1	−	+	32 157
SF-2	−	+	29 756

[a] Each preparation was incubated for 15 min at 37 °C in the presence or absence of trypsin (25 μg/mL). Soybean trypsin inhibitor (250 μg/mL) was then added to all of the samples and the samples were incubated for an additional 5 min at 37 °C. The reactions were then cooled to 0 °C and 10 μL of each was assayed in standard factor assay reaction with 0.5 unit of RNA polymerase per reaction. The bovine serum albumin and stimulatory factor samples were in buffer F, 10 mM KCl. [b] BSA, bovine serum albumin.

was passed through DEAE-Sephadex in buffer containing 20 mM ammonium sulfate and 5 mM $MgCl_2$. As shown in Table I, when the preparation is passed through DEAE-Sephadex most of the nucleic acid and a considerable amount of contaminating protein are removed. Stimulatory activity can be first detected reproducibly in the DEAE flow through fraction, but the presence of nucleases makes the assay unreliable at this stage of purification. After DEAE-Sephadex chromatography the pH of the solution is reduced to 7.1 by titration and the ionic strength is further reduced by dilution. The stimulatory activity is subsequently chromatographed on a column of CM-cellulose. Figure 1 illustrates the activity profile which is obtained from this column. A biphasic peak is routinely obtained at this step; gel analysis, however, does not reveal any significant differences across the peak of activity. Although ribonuclease H assays seldom reveal any activity in these fractions when ribonuclease H activity is present, it is found before the peak of stimulatory activity.

The CM-cellulose purified stimulatory activity is next concentrated by ammonium sulfate precipitation and chromatographed on Sephadex G-75. Two peaks of stimulatory activity are resolved on this column, termed SF-1 and SF-2 by order of elution (Figure 2). During the initial stages of this study it was observed that variable and usually small amounts of SF-1 were obtained at this step. The pH was originally adjusted by dialysis after the DEAE-Sephadex step, but this method proved to be inadequate. The method of titration and dilution to low ionic strength was adopted, resulting in reproducibility of higher yields of SF-1 activity.

To ascertain if the activity in the SF-1 peak might be an association product of the smaller SF-2 species existing as a rapid equilibrium, the activities were each concentrated by ammonium sulfate precipitation and separately rechromatographed on a second Sephadex G-75 column (Figure 3). The results indicate that a rapid interconversion does not exist since SF-1 and SF-2 each rechromatographed as a single peak, eluting at K_{av} values similar to those obtained on the first G-75 column. When the K_{av} values are plotted vs. molecular radius

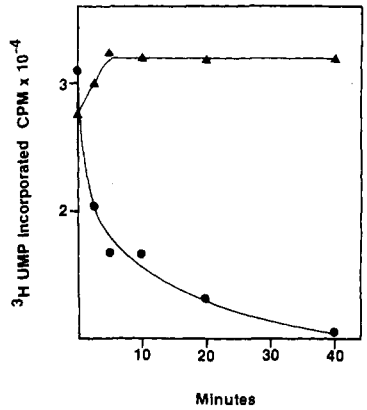

FIGURE 4: Sensitivities of SF-1 and SF-2 to heat inactivation. Samples of SF-1 purified through the Sephadex G-75 step and SF-2 purified through the SP-Sephadex step were separately diluted with buffer F, 10 mM KCl to subsaturating activity levels (each factor stimulated about threefold) and a final protein concentration of 0.9 mg/mL and 0.03 mg/mL for SF-1 and SF-2, respectively, each in 0.5 mL. The samples were incubated at 98 °C and 10-μL samples were removed at the indicated times. The samples were chilled to 0 °C and assayed in a standard assay with 0.4 unit of RNA polymerase II. (▲) SF-1 activity; (●) SF-2 activity.

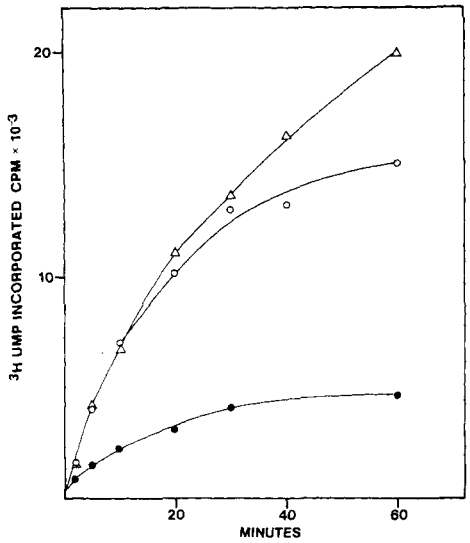

FIGURE 5: Kinetics of the RNA polymerase II reaction in the presence or absence of saturating amounts of SF-1 or SF-2. Standard reaction mixtures contained saturating levels of UTP and 0.4 unit of calf thymus RNA polymerase II with or without saturating levels of SP-Sephadex purified SF-1 or SF-2 in a final volume of 390 μL. The reactions were begun by the addition of enzyme followed by incubation at 37 °C. At the times indicated 40-μL aliquots were removed and processed as described in Materials and Methods. (●) RNA polymerase activity with no additions; (○) enzyme activity in the presence of SF-1; (△) RNA polymerase activity in the presence of SF-2.

according to the relationship of Laurent & Killander (1964), the molecular radius of SF-1 is calculated to be 31 Å and that of SF-2 is 21 Å (data not shown). Assuming these proteins have a globular shape, the data indicate native molecular weights of 55 000–65 000 and 25 000–35 000 for SF-1 and SF-2, respectively.

Multiple peaks of stimulatory activity are also obtained when the factor activity from CM-cellulose is subjected to isoelectric focusing (R. H. Benson and S. P. Blatti, unpublished observations). The peaks of stimulation are isoelectric at pH 9.6, 9.1, 8.6, and 8.0. When SF-1 and SF-2 are each subjected to isoelectric focusing, SF-1 exhibits a major peak at pI = 9.1, while SF-2 exhibits major peaks at 8.8 and 9.4.

TABLE III: Effect of SF-1 and SF-2 on the Activity of RNA Polymerases I, II, and III.[a]

Enzyme	cpm
Polymerase I	30 000
Polymerase I + SF-1	31 000
Polymerase I + SF-2	29 000
Polymerase II	22 000
Polymerase II + SF-1	70 000
Polymerase II + SF-2	90 000
Polymerase III	2 500
Polymerase III + SF-1	2 300
Polymerase III + SF-2	2 600

[a] Standard reactions were carried out with subsaturating UTP at 20 mM ammonia sulfate. SF-1 (0.9 mg/mL) was purified through the Sephadex G-75 step and SF-2 (0.13 mg/mL) was purified through the SP-Sephadex step. RNA polymerase I was chromatographed on DEAE-Sephadex (Weaver et al. 1971). RNA polymerase III was purified as described by Weil & Blatti (1975) except that the phosphocellulose step was omitted; polymerase III was assayed in the presence of 4.8 mg/mL α-amanitin.

TABLE IV: Sensitivity of Protein Kinase Activity in SF-1 to Heat Denaturation.[a]

	Protein kinase act. [γ-^{32}P]ATP incorp/10 s	Stimulation ratio
SF-1 (G-75)	95 400	1.9
SF-1 (20 min at 95 °C)	100	2.0
SF-2 (G-75)	3 500	2.2
SF-2 (20 min at 95 °C)	100	1.1

[a] Protein kinase activity was assayed as previously described (Bell et al., 1977). The specific activity of [γ-^{32}P]ATP was 2000 dpm/pmol.

Sensitivity of SF-1 and SF-2 to Heat and Proteolytic Enzymes. Table II presents the results obtained when SF-1 and SF-2 are incubated with trypsin for 10 min at 37 °C. RNA polymerase II activity was measured after inactivation of trypsin by soybean trypsin inhibitor. As can be seen, trypsin treatment completely abolishes the stimulatory activity of both SF-1 and SF-2. The activities of SF-1 and SF-2 are also sensitive to treatment with protease K or autodigested protease. The data indicate that the stimulatory activities observed have an essential protein moiety as part of their structure.

The stability of SF-1 and SF-2 against heat denaturation at 98 °C was tested. As can be seen in Figure 4, SF-1 activity is completely stable at 98 °C for 40 min. In contrast, SP-Sephadex purified SF-2 activity is completely inactivated by incubation at 98 °C for 40 min.

Effect of SF-1 and SF-2 on RNA Polymerase II Activity. The effect of SF-1 and SF-2 on the kinetics of RNA incorporation by RNA polymerase II using calf thymus DNA as template was investigated. Only about a twofold increase in RNA polymerase activity is observed during the first few minutes of RNA synthesis (Figure 5). However, the fold stimulation increases at later times due to the rapid decline in the rate of RNA synthesis which occurs in the absence of the factors. Although the ammonium sulfate optima for maximum RNA synthesis remains at 100 mM in the presence of SF-2, the maximum level of stimulation is obtained at 10 to 20 mM ammonium sulfate. Therefore, the assays of stimulatory activity were routinely performed at 20 mM ammonium sulfate.

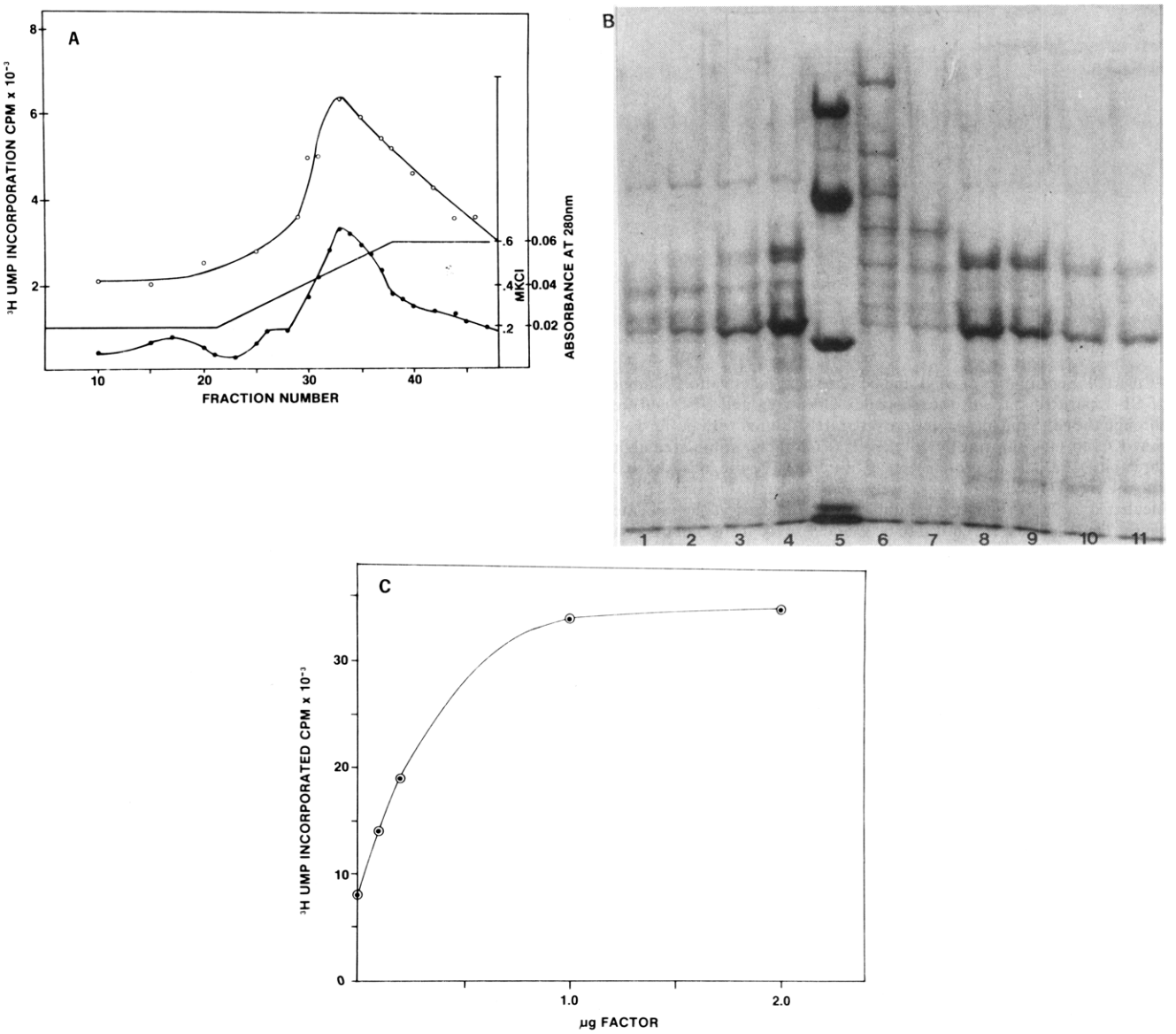

FIGURE 6: (A) Phosphocellulose chromatography of SF-1. The fractions which contained SF-1 activity were pooled, dialyzed to 0.2 M KCl in buffer F, and heated at 80 °C for 2 min. After centrifugation at 27 000*g* for 30 min, the supernatant containing 11 mg of protein in 50 mL was adsorbed to a 0.6 × 20 cm phosphocellulose column (6 mL bed volume) equilibrated in 0.2 M KCl, buffer F. The column was washed with 2 column volumes of 0.2 M KCl, buffer F and the factor activity was eluted with 42 mL of 0.2-0.8 M KCl, buffer F gradient at a flow rate of 15 mL/h. Fractions of 2.5 mL were collected and assayed for stimulatory activity in the presence of 0.2 unit of RNA polymerase II. (○) RNA polymerase activity; (●) absorbance at 280 nm; and (—) M KCl concentration. (B) Sodium dodecyl sulfate–polyacrylamide (10%) slab gel of fractions across the peak of stimulatory activity from phosphocellulose. Lanes 1–4 and 8–11 were respectively from 0.125 mL each of the even numbered fractions 24–40 from the phosphocellulose column. Lane 5 contains standards bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* with cytochrome *c* moving at the dye front. Lanes 6 and 7 contain 20 µg of SF-1 (G-75) before the heat step and 10 µg of SF-1 (G-75) after the heat step, respectively. (C) Dependence of protein concentration on RNA polymerase II stimulation by SF-1. A sample from a phosphocellulose column was concentrated by lyophilization and dialyzed against buffer F. Increasing amounts of protein were assayed in the presence of 0.8 unit of RNA polymerase II.

Specificity of Stimulatory Factor SF-1 and SF-2. Stimulatory factors SF-1 and SF-2 were tested for their ability to stimulate the activity of the various classes of calf thymus RNA polymerase (Table III). The stimulatory factors have no effect on the activity of RNA polymerase I or III, but specifically stimulate the activity of RNA polymerase II. This stimulation plateaus at about 4.5-fold increase in activity. This plateau indicates that the mode of stimulation by SF-2 is distinct from that of the polyamines, which inhibit RNA synthesis at higher concentrations.

The specificity of SF-1 and SF-2 activity for stimulation on various DNA templates was also tested. SF-2 stimulates enzyme II activity on poly[d(A-T)] 22-fold and on native calf thymus DNA 2.7-fold. It does not stimulate activity on single-stranded DNA templates. The data suggest that, while SF-2 stimulation is specific for double-stranded templates, it is not species DNA specific as are the acidic chromosomal protein stimulatory factors. SF-1 is similar to SF-2 since it stimulates synthesis by RNA polymerase II 3.2-fold on native calf thymus DNA but not denatured DNA. However, SF-1 does not stimulate transcription of poly[d(A-T)].

Stimulatory Factors SF-1 and SF-2 Are Not Protein Kinases. Dahmus (1976) has recently shown that an RNA polymerase II stimulatory factor, HLF_2, from Novikoff heptatoma contains a protein kinase. He suggests that the factor stimulates RNA polymerase II by phosphorylation. We therefore tested SF-1 and SF-2 for protein kinase activity. SF-1 was found to contain a very active protein kinase activity, whereas very low levels of protein kinase activity were found in SF-2 (Table IV). Since SF-1 is stable to heat denaturation,

TABLE V: Comparison of the Molecular Weights and Isoelectric Points of Yeast and Calf Thymus RNA Polymerase II Subunits and Polypeptides Present in SF-1 and SF-2.[a]

Mol wt × 10^{-3} (p*I*)				
Yeast RNA polymerase II	Calf-thymus RNA polymerase II	Calf-thymus SF-1	Calf-thymus SF-2	Calf-thymus SF-2 (alternate procedure)
	(a) 210			
185	(b) 180 (7.0–8.8)			
145	(c) 145 (7.0–8.8)			
45 (4.45)	(d) 45 (5.0)			
32 (4.95)	(e) 36–38 (5.0	36–38*	34	34
27 (9.1)		27	27	27
23 (4.5)	(f) 25 (6.15–6.4)			
	(g, h) 20, 18.5 (5.9, 4.4)		19 (7.6)	
16.5 (7.6)	(i) 16 (5.0)	16*		
14.5 (4.6)	(j) 15		15*	15*
12.6 (5.3)	(k) 12–14 (5.3)		13*	
	(l) 11.5		11.5*	11.5*

[a] Yeast RNA polymerase II subunit molecular weights and p*I* values were obtained from Buhler et al. (1976). The 27 000, p*I* 9.1 and 23 000, p*I* 4.5 subunits (Buhler et al., 1976) and possibly the 14 500, p*I* 4.6 subunit (Bell et al., 1977) of yeast polymerase II are common to yeast RNA polymerase I, II, and III. All p*I* values for calf-thymus RNA polymerase II subunits were determined in the presence of 9 M urea as described by Hodo & Blatti (unpublished observations). Those components with asterisks comigrate with RNA polymerase II subunits.

we also tested this fraction for protein kinase after heating. As shown in Table IV, after the heat treatment (20 min at 95 °C) stimulatory activity is not affected, whereas the protein kinase activity is completely inactivated. These data are in agreement with data by Kuroiwa et al. (1977) which demonstrate that protein kinase does not copurify with Ehrlich ascites tumor cell RNA polymerase II stimulatory factor S-II.

Studies on SF-1 Stimulatory Factor. Since SF-1 from Sephadex G-75 was resistant to heat denaturation (Figure 4), a heat step was used before further purification by phosphocellulose chromatography. Figure 6A shows the phosphocellulose activity profile and the sodium dodecyl sulfate–polyacrylamide gel pattern of fractions across the peak of activity (Figure 6B). Polypeptides of 27 000, 36 000, and 38 000 were coincident with the peak of stimulatory activity. These polypeptides were quantitated using densitometer scans with bovine serum albumin as a standard. The molar ratios were determined to be about 27 000 (2), 36 000 (1), and 38 000 (1). These data together with the native molecular weight suggest that SF-1 is a dimer of the 27 000 and/or 36 000–38 000 molecular weight polypeptides. Further attempts to purify SF-1 activity without complete loss of activity were unsuccessful. When SF-1 was electrophoresed on 12.5% sodium dodecyl sulfate slab gel together and separately with calf thymus RNA polymerase II (Figure 7), the 36 000 and/or 38 000 molecular weight subunits migrate at about the same position as subunit e_1e_2 (36 000–38 000) doublet of RNA polymerase II,[1] while the 27 000 molecular weight species clearly migrates in a different position from any subunits of RNA polymerase II. In addition, a minor component with a molecular weight of 16 000 is present which comigrates on sodium dodecyl sulfate gels with subunit (i) of RNA polymerase II (Table V). However, this component is not coincident with the peak of stimulatory activity. Whether the components (36 000, 38 000, and 16 000) are identical to the subunits (e, i) of RNA polymerase II or result from secondary modifications must await further biochemical and charge density analysis.

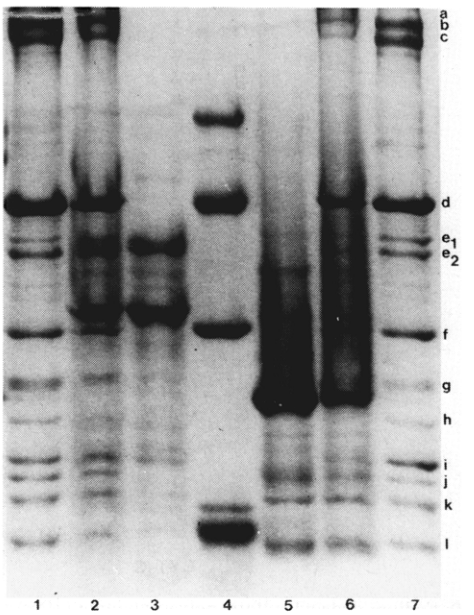

FIGURE 7: $NaDodSO_4$–polyacrylamide (12.5%) gel electrophoresis of SF-1, SF-2, and RNA polymerase II. (Lane 1) Thirty micrograms of RNA polymerase; (lane 2) 10 μg of SF-1, fraction 34 from P-cellulose together with 30 μg of RNA polymerase II; (lane 3) 10 μg of SF-1, fraction 34 from P-cellulose; (lane 4) protein markers bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c*; (lane 5) 40 μg of SF-2, peak tube from SP-Sephadex; (lane 6) 20 μg of SF-2, peak tube from SP-Sephadex together with 30 μg of RNA polymerase II; and (lane 7) 30 μg of RNA polymerase II. RNA polymerase II subunits are labeled a–l; the corresponding molecular weights are given in Table V.

Figure 6C demonstrates that SF-1 stimulatory activity increases linearly with protein concentration at low protein concentrations and that saturation is reached at about 1 μg of SF-1. Using a specific activity of 200 units/mg for RNA polymerase II, we calculate that about 2–4 mol of SF-1 (molecular weight 60 000) stimulate 1 mol of RNA polymerase II.

Studies on SF-2 Stimulatory Activity. Two methods were

[1] When the e subunit of RNA polymerase splits into two subunits labeled e_1 and e_2, the SF-1 components in this molecular weight range also split into a doublet. However, when splitting is not observed in the gel, the SF-1 component migrates at 38 000 molecular weight and RNA polymerase II subunit e migrates as a 36 000 molecular weight species, indicating that, although these components may be structurally related, they are not identical.

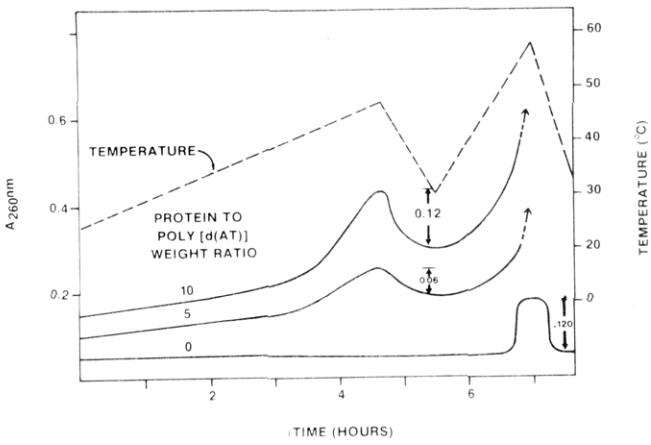

FIGURE 8: Effect of SF-2 on the thermal melting of poly[d(A-T)]. Each sealed cuvette contained 435 μL of buffer H, 5 μL of poly[d(A-T)] (1 mg/mL) in buffer I, and 60 μL of stimulatory factor SF-2. The sample was obtained from the peak of activity of SP-Sephadex which was further purified over Sephadex G-50 column. The buffers were vacuum degassed before mixing. Samples contained as indicated poly[d(A-T)] only, plus 25 μg factor or plus 50 μg factor to give respective protein/poly[d(A-T)] ratios of 0, 5, or 10. A companion cuvette standard containing buffers only was monitored and any OD change was subtracted from the experimental values.

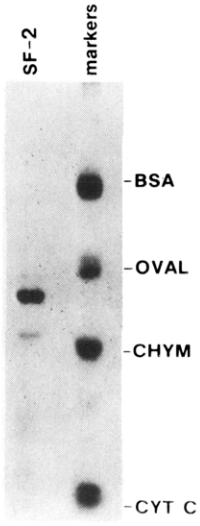

FIGURE 9: Sodium dodecyl sulfate-polyacrylamide (10%) gel of SF-2, purified by the alternate procedure. SF, 20 μg of protein from the peak tube of a sucrose gradient. Protein markers are bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c*.

utilized to isolate SF-2 activity. In the first method, the pooled SF-2 fractions from G-75 (Figure 2) were further chromatographed on SP-Sephadex as described in Materials and Methods. Figure 7 (gel 5) shows the polypeptides present in the peak fractions from SP-Sephadex. The minor proteins present migrate with molecular weights of 34 000, 27 000, 15 000, 14 000, and 11 500. The 34 000 and 27 000 molecular weight components are eluted in early fractions before the peak of stimulation while the low molecular weight species 15 000, 14 000, and 11 500 come off the column after the peak of stimulation. As shown in Figure 7 (gel 6), the three smallest polypeptides comigrate with subunits j, k, and l of RNA polymerase II. The major protein (90% pure) which was coincident with peak of stimulatory activity exhibits a molecular weight of 19 000. This protein is similar, if not identical, to UP-1 protein described by Herrick & Alberts (1976), since its amino acid composition is very similar to UP-1 and it exhibits a pI of 7.6–7.8 upon isoelectric focusing in 8 M urea. As shown in Figure 8, SF-2 lowers the T_m of poly[d(A-T)]. This effect is heat labile and sensitive to oxidation.

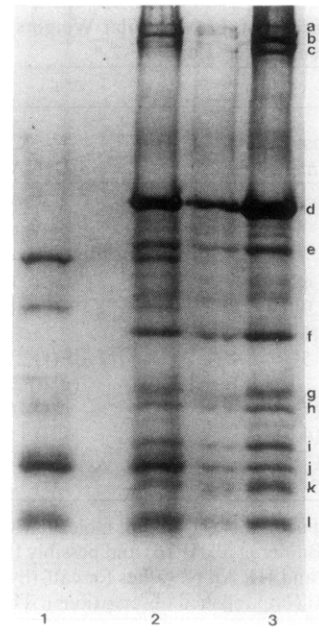

FIGURE 10: Sodium dodecyl sulfate–polyacrylamide (12.5%) gels of SF-2 and RNA polymerase II. The sample of SF-2 used for these gels (1 and 2) was obtained by pooling the entire peak of stimulatory activity from a sucrose gradient as described for the alternative procedure. The low molecular weight polypeptides which comigrate with RNA polymerase II subunits j and l were present on the low molecular weight side of the activity peak. (Lane 1) twenty micrograms of SF-2, pooled peak; (lane 2) 20 μg of SF-2, pooled peak, together with 30 μg of RNA polymerase II; and (lane 3) 30 μg of RNA polymerase II. The molecular weights of the RNA polymerase II subunits a–l are given in Table V.

In the alternate procedure for SF-2 purification (detailed in Materials and Methods), the final step in the procedure is sucrose density gradient sedimentation. The peak of stimulatory activity after electrophoresis in sodium dodecyl sulfate–polyacrylamide gels (10%) is shown in Figure 9. The major protein observed is a 34 000 polypeptide and a minor protein component at 27 000 molecular weight.[2] When the entire peak is pooled and compared with RNA polymerase II subunits on 12.5% sodium dodecyl sulfate gels (Figure 10), two additional proteins are observed. The molecular weights of these polypeptides are 15 000 and 11 500 and they comigrate with RNA polymerase II subunits j and 1. Since only small quantities of SF-2 factor were obtained by this procedure, we were unable to test for poly[d(A-T)] unwinding activity.

Discussion

Resolution of Multiple Stimulatory Factors. Two distinct RNA polymerase II stimulatory proteins were extensively purified from calf thymus tissue. This procedure utilized ammonium sulfate precipitation, DEAE-Sephadex, CM-cellulose, and finally Sephadex G-75 before two peaks of stimulatory activity (SF-1 and SF-2) are resolved. Rechromatography of each peak on Sephadex G-75 demonstrated that the two species do not exist in a rapid equilibrium under these conditions.

The elution volume of SF-1 and SF-2 activity on Sephadex G-75 indicates, respectively, native molecular weights of

[2] The 19 000 component described above was not present in the material applied to the sucrose density gradient. This protein was retained during the 10 mM KCl wash of the SP-Sephadex column and, upon elution, gave less than twofold stimulation.

55 000–65 000 and 25 000–30 000. Sodium dodecyl sulfate–polyacrylamide gels of SF-1 after the heat step and phosphocellulose chromatography reveal three proteins with molecular weights of 38 000–36 000 and 27 000 which were coincident with peak of activity. Thus a structure of SF-1 with native molecular weight of 55 000–65 000 may be a dimer composed of the 27 000 and/or 36 000–38 000 components.

Isoelectric focusing of RNA polymerase II subunits in 9 M urea demonstrates the conspicuous absence of any basic polypeptides. As Table V shows, yeast but not calf thymus RNA polymerase II contains a pI = 9.1, 27 000 polypeptide. Thus, it is possible that a 27 000 molecular weight component is lost from the calf thymus enzyme during purification. On the other hand, the 36 000 molecular weight subunit (e) of RNA polymerase II is known to be an acidic protein with a pI = 5.0 (see Table V). Therefore, if the 27 000 and 36 000–38 000 components exist as a dimer, and both are lost from the enzyme, the interaction must be between a very basic protein and an acidic protein.

SF-2 Activity Peak from G-75 Contains Two Stimulatory Activities. The SF-2 stimulatory peak when further purified by SP-Sephadex exhibited a 19 000 molecular weight species which was coincident with the activity peak. After further purification by Sephadex G-50, the 19 000 polypeptide was about 90% pure. This species is active in lowering the T_m for poly[d(A-T)]. Amino acid analysis of the 19 000 molecular weight species indicates a striking similarity to the UP-1 DNA unwinding protein described by Herrick & Alberts (1976). Both contain 1–1.5 cysteines per 20 000 molecular weight. The isoelectric point of the 19 000 molecular weight species in 8 M urea is 7.6–7.8 which is the pI of UP-1 (Herricks & Alberts, 1976). Both the poly[d(A-T)] unwinding activity and the stimulatory activity were reversibly inactivated upon dialysis in the absence of reducing agents and both were reactivated by dithiothreitol. In addition, the unwinding and stimulation activities were heat labile and both were stabilized, at least in part, against heat denaturation in the presence of poly[d(A-T)].

Isoelectric focusing of the SF-2 peak from G-75 under nondenaturing conditions gave two peaks of stimulatory activity, one broad peak, at pI = 8.8 containing the 19 000 molecular weight species and a more sharply defined peak at pI = 9.4 containing a 34 000 and a 22 000 molecular weight species. None of these polypeptides comigrated with any RNA polymerase II subunits.

Using the alternate procedure for purification of stimulatory factors, we obtain a peak of activity which elutes at the position of SF-2 on Sephadex G-75. Upon further purification by SP-Sephadex and sucrose gradient sedimentation, the stimulatory activity is nearly coincident with a 34 000 molecular polypeptide (95% pure). Thus, one of the stimulatory factors in SF-2 is most likely a single subunit protein of 34 000 molecular weight. We estimate that between 0.5–1.0 μg of SF-2 (34 000 mol wt) maximally stimulate RNA polymerase II. Bovine serum albumin at this or higher concentrations does not stimulate the enzyme. The minor components present in side fractions of SF-1 and SF-2 comigrate with the low molecular weight polypeptides (i–l) of RNA polymerase II.

Comparison of Calf-Thymus SF-1 with S-II from Ehrlich Ascites Tumor Cells. Sekimizu et al. (1976) have purified an RNA polymerase II stimulatory factor, S-II, from Ehrlich ascites tumor cells. The factor appears to be a single subunit of 38 000 molecular weight. Since the RNA polymerase II used in those studies exhibits a specific activity which is about 1000-fold lower than the calf thymus enzyme II used in our studies, the large stimulation which they observe (20-fold) may be the result of a reactivation of a "crippled" enzyme which has lost 95% of its 36 000 molecular weight subunit. Alternately, the difference in maximum stimulation, 20-fold vs. 4-fold, may simply arise from the respective incubation times used to assay S-II (60 min) and SF-1 (10 min). At the lower ionic strength used to assay the enzyme in the stimulation assay, the enzyme when assayed alone begins to terminate transcription after the first few minutes, whereas in the presence of saturating stimulatory factor, transcription is linear (Figure 5). Thus longer incubation times would result in the increase in apparent stimulatory activity.

Specific Activity and Purity of Stimulatory Factors. Sekimizu et al. (1976) define a unit of stimulatory activity as the increase of 1 pmole of activity of RNA polymerase II. Their final specific activity is 14.7 pmol/μg of S-II. In contrast, using the same definition for a unit of stimulatory activity, our specific activity is calculated to be about 400 pmol/μg for SF-1 or SF-2. For a 60-min assay our specific activity is increased to over 1500 pmol/μg. Thus if the SF-1 and S-II factors are similar, the definition of this unit may not be valid for factor quantitation. Alternatively, the S-II factor may be only about 1–5% pure.

Specificity of Stimulatory Factors SF-1 and SF-2 for RNA Polymerase II and Native DNA. Both factors stimulate homologous or heterologous RNA polymerase II but not homologous or heterologous RNA polymerase I and III. Although this suggests a unique function of these factors for enzyme II, the result does not preclude a similarity in function or the presence of these factors with enzymes I and III. The latter enzymes may simply retain these components while enzyme II loses them during the purification. Alternatively, different polypeptides in enzymes I and III may be responsible for this function. Detailed biochemical and charge density characterization of subunits of all three classes of calf thymus RNA polymerase and stimulatory factor should answer this question.

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Partial Purification and Characterization of Two Hen Oviduct Protein Synthesis Initiation Factors Capable of Initiation Complex Formation†

J. Fielding Hejtmancik and John P. Comstock*,‡

ABSTRACT: Two initiation factors aIF-2A and aIF-2 capable of binding Met-tRNA$_f$ to form an initiation complex in vitro have been isolated from the hen oviduct. Both factors were obtained from a 0.5 M KCl wash of a nuclear-microsomal fraction of the oviduct homogenate. Four purification steps were carried out according to the protocol for the purification of rabbit reticulocyte eIF-2A before a high molecular weight ribonuclease was removed by molecular sieve chromatography and aIF-2A activity was detected. This oviduct factor has been characterized with respect to its activity in stimulating poly(U)-directed polyphenylalanine synthesis, lowering the Mg^{2+} concentration optimum required for polyphenylalanine synthesis, and stimulating phenylalanyl-puromycin synthesis. In these assays aIF-2A was able to substitute for the corresponding rabbit reticulocyte factor. Factor aIF-2A was capable of binding fMet-tRNA$_f$ and Met-tRNA$_f$ to 40S ribosomal subunits programmed with the AUG codon. The molecular weight and Stokes radius of aIF-2A were estimated by molecular sieve chromatography to be 110 000 and 42.5 Å, respectively. The sedimentation coefficient was 3.5 S as measured by sedimentation analysis on sucrose density gradients. Combining these data, the calculated molecular weight of aIF-2A was 62 500. These values are similar to those reported for the highly asymmetric reticulocyte factor. The activity of the second oviduct factor aIF-2 was detected during gradient elution chromatography of the nuclear-microsomal salt wash on DEAE-cellulose. This factor bound Met-tRNA$_f$ in a binary complex since GTP was not required and did not further increase the binding. This may be caused by the aging of the factor and/or its partial degradation. Similar characteristics have been reported for the corresponding reticulocyte factor, eIF-2. Nevertheless, aIF-2 was capable of forming an 80S initiation complex as determined by the stimulation of methionyl-puromycin synthesis upon the addition of ribosomes and ribosomal subunit joining factors, eIF-5 and eIF-4C and D. The molecular weight of aIF-2 was estimated by molecular sieve chromatography to be 102 000 corresponding to the value reported for the monomer form of the reticulocyte factor.

There is evidence that sex steroid hormones increase the rate of protein synthesis (Palmiter et al., 1970, 1971; Palmiter, 1972a,b; Comstock et al., 1972) as well as increasing the cellular concentration of translatable mRNA in the oviduct (Rosen & O'Malley, 1975; Schimke et al., 1975; Harris et al., 1975). We are interested in learning the mechanisms by which protein synthesis is regulated at the translation level in this tissue and whether hormones act directly or indirectly in this control. We have chosen to use for these investigations the estrogen-stimulated chicken oviduct, a widely used model system for studying the actions of sex steroid hormones. Although both initiation and elongation factors have been well studied in several mammalian and eukaryotic systems, it is necessary systematically to isolate and characterize these factors in the oviduct in order to study the molecular events by which hormones affect protein synthesis.

Recently our laboratory reported on the properties of two protein synthesis initiation factors designated A2A and A2B

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