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Purification and Partial Characterization of a Stimulatory Factor for Lamb Thymus RNA Polymerase II†

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ABSTRACT: A heat-stable protein (HSF) that stimulates the activity of lamb thymus RNA polymerase II has been purified 2500-fold and partially characterized. This factor stimulates the activity of RNA polymerase II up to 13 times and retains complete activity when heated at 90 °C for 5 min. Stimulation is observed only in the presence of RNA polymerase II and requires native DNA as template. The stimulatory factor has a sedimentation coefficient of 2.7 S, a diffusion coefficient of 9.55×10^{-7} cm^2/s, and an isoelectric point of 8.0. Calculated from the sedimentation and diffusion data, the factor has a molecular weight of about 24000. Electrophoresis of the purified factor on polyacrylamide gels in the presence of sodium dodecyl sulfate results in a single band corresponding to a molecular weight of 25000. The number-average length of the RNA synthesized by RNA polymerase II is increased in the presence of the factor. Sedimentation velocity and exclusion chromatography experiments suggest that the stimulatory factor interacts with RNA polymerase II. These results suggest that the factor stimulates RNA synthesis through a direct interaction with RNA polymerase II. The stoichiometry of the HSF–RNA polymerase binding appears to be about 1:1. HSF is located in the nucleus, as determined by cell fractionation studies.

Eucaryotic cells contain three distinct classes of DNA-dependent RNA polymerases, designated I, II, and III, as determined by their order of elution from DEAE-Sephadex and by their sensitivity to α-amanitin [for reviews see Chambon (1974) and Roeder (1976)]. Since RNA polymerase II appears to be involved in the production of hnRNA and mRNA (Roeder, 1976), an understanding of its structure and function is central to an understanding of the mechanism of gene regulation. RNA polymerase II does not appear to transcribe DNA in vitro asymmetrically or with high fidelity (Mandel & Chambon, 1974; Honjo & Reeder, 1974). In attempts to resolve this question, proteins have been described that stimulate the activity of RNA polymerase II in vitro (Stein & Hausen, 1970; Seifart, 1970; Lentfer & Lezius, 1972; Mondal et al., 1972; Lee & Dahmus, 1973; Sugden & Keller, 1973; Chuang & Chuang, 1975; Sekimizu et al., 1976; Benson et al., 1978). Based on ease of denaturation, these stimulatory factors can be separated into those that are stable to heat treatment at 90 °C for 5 min (Stein & Hausen, 1970; Lee & Dahmus, 1973; Stein et al., 1973; Seifart et al., 1973; Chuang & Chuang, 1975) and those that are heat labile (Lentfer & Lezius, 1972; Lee & Dahmus, 1973; Sugden & Keller, 1973; Dahmus, 1976; Sekimizu et al., 1976). This paper reports the purification and partial characterization of a heat-stable factor, designated HSF, from lamb thymus. Stein et al. (1973) and Seifart et al. (1973) have extensively purified factors that have small molecular weights and stimulate the elongation reaction of RNA polymerase II. HSF, a protein that appears to be similar to these proteins, has been characterized with respect

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to physical properties, mode of stimulation, and intracellular location.

Materials and Methods

Materials

Lamb thymus was obtained immediately after slaughter and either used immediately or frozen in liquid nitrogen and stored at −80 °C for periods of up to 2 months. Ribonucleoside triphosphates were purchased from P-L Biochemicals. α-Amanitin was purchased from Boehringer Sohn, Ingelheim, Germany. Ultrapure Tris base, ultra-pure ammonium sulfate, RNase free sucrose, and [^{3}H]UTP were purchased from Schwartz/Mann. Nitrocellulose filters (HAWP, 0.45-μm pore size) were obtained from Millipore. Hydroxylapatite (Bio-Gel HTP) was purchased from Bio-Rad. Sp-Sephadex (C50) was purchased from Pharmacia. [γ-^{32}P]ATP was prepared by a modification (Walsh et al., 1971) of the procedure of Glynn & Chappell (1964).

Methods

Purification of RNA Polymerase II. The solubilization and purification of lamb thymus RNA polymerase II was accomplished by a modification of the procedure desribed by Kedinger et al. (1972) and Kedinger & Chambon (1972). Unless otherwise stated, all work was performed at 4 °C. Lamb thymus (150 g) was homogenized in 750 mL of 50 mM Tris-HCl, pH 7.9, 10 mM thioglycerol, and 0.1 mM dithiothreitol (buffer A) containing 5% glycerol in a Waring Blendor and sonicated as described by Kedinger et al. (1972). The sonicated solution was brought up to 30% saturation of ammonium sulfate to selectively precipitate RNA polymerase II. The solution was centrifuged at 16300*g* for 45 min, and the pellet was dissolved in buffer A containing 30% glycerol in a final volume of 200 mL. Eight milliliters of a 1% solution of protamine sulfate was added, and the resulting precipitate was removed by centrifugation at 250000*g* for 1 h. The supernatant was dialyzed against five volumes of buffer A containing 30% glycerol and chromatographed on DEAE-cellulose (2.5 × 50 cm). RNA polymerase was eluted with two column volumes of a linear gradient of 0.08–0.8 M ammonium sulfate in buffer A containing 30% glycerol. The fractions containing activity were pooled and dialyzed against three volumes of buffer A containing 30% glycerol. RNA polymerase was then loaded onto a phosphocellulse column (1.5 × 20 cm) and eluted with two column volumes of a linear gradient of 0.06–0.8 M ammonium sulfate in buffer A containing 30% glycerol (fraction PC). RNA polymerase was further purified by centrifugation at 131000*g* for 44 h in an SW27 rotor on a linear 5–20% sucrose gradient in buffer A containing 30% glycerol and 1 mM $ZnSO_4$. The specific activity of the gradient-purified enzyme ranged from 20 to 70 units/mg of protein.

RNA Polymerase and Stimulatory Assays. The RNA polymerase assay contained, in 0.25 mL, 80 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 4 mM thioglycerol, 2 mM $MnCl_2$, 100 mM ammonium sulfate, 8% glycerol, 0.4 mM each of ATP, GTP, CTP, and [^{3}H]UTP (50 Ci/mol), 30 μg of lamb thymus DNA, and RNA polymerase. Lamb thymus DNA was prepared according to the method of Dahmus & McConnell (1969). Optimal RNA polymerase activity occurred with 80–100 mM ammonium sulfate. Unless otherwise stated, the reaction was incubated at 37 °C for 30 min. To terminate reactions the samples were placed on ice and 0.75 mL of cold water was added, followed by addition of 1 mL of cold 10% trichloroacetic acid. After 15 min the precipitates were collected on nitrocellulose filters and washed three times with 10-mL aliquots of cold 5% trichloroacetic acid. The filters were then dried and counted in a liquid scintillation spectrometer. One unit of RNA polymerase activity is defined as that amount of enzyme that catalyzes the incorporation of 1 nmol of [^{3}H]UMP into RNA in 10 min under the above conditions.

The stimulatory assay was identical with the RNA polymerase assay except that no ammonium sulfate was added and a constant amount of RNA polymerase was added to each tube. Unless otherwise stated, sucrose gradient purified RNA polymerase II was used. One unit of stimulatory activity is defined as that amount of factor that causes a 100% increase in [^{3}H]UMP incorporation into RNA in 30 min by using 0.0125 unit of RNA polymerase II.

Purification of HSF. The supernatant of the 30%-saturated ammonium sulfate solution described in the RNA polymerase II purification above was brought to 50% saturation with solid ammonium sulfate. The solution was stirred for 30 min, and the precipitate was pelleted by centrifugation at 16300*g* for 60 min. The supernatant was then brought to 70% saturation with addition of solid ammonium sulfate, stirred at least 1 h, and then centrifuged at 142800*g* for 1.5 h. The pellet was dissolved with the aid of a motor-driven Teflon-glass homogenizer in buffer A and dialyzed against two changes of 10 volumes of buffer A. A precipitate formed and was removed by centrifugation for 20 min at 17300*g*. The supernatant (fraction P70) was chromatographed on Sp-Sephadex (2.5 × 50 cm column) equilibrated in buffer A. The column was washed with one column volume of buffer A, and HSF was eluted with two column volumes of a linear gradient of 0–0.5 M ammonium sulfate in buffer A. Fractions containing stimulatory activity were pooled and heated to 90 °C for 5 min, and the precipitate was removed by centrifugation at 17300*g* for 20 min. HSF was then placed directly onto a hydroxylapatite column (0.9 × 2 cm) equilibrated in 0.01 M potassium phosphate, pH 7.8, 0.1 mM dithiothreitol, and 10 mM thioglycerol. The column was washed with four column volumes of phosphate buffer and developed with 10 column volumes of a linear gradient of 0.01–0.3 M potassium phosphate, pH 7.8, 0.1 mM dithiothreitol, and 10 mM thioglycerol. The fractions containing stimulatory activity were pooled, dialyzed against 100 volumes of buffer A, and stored at −80 °C.

Protein Determination. Protein was measured by the method of Lowry et al. (1951) following precipitation with trichloroacetic acid. Bovine serum albumin was used as a standard.

Proteolysis of HSF. An aliquot of HSF was incubated in buffer A for 30 min at 37 °C in the presence or absence of 0.5 mg/mL of Enzite protease (Miles-Servac). The solution was stirred every 5 min. Buffer A incubated with Enzite protease served as a control. After proteolysis, the solutions were centrifuged and aliquots of the supernatant assayed for stimulatory activity.

Gel Electrophoresis and Isoelectric Focusing. $NaDodSO_4$–polyacrylamide gels were run as described by Weber & Osborn (1969) or as described by Laemmli & Favre (1973). Isoelectric focusing was carried out in 5.5% polyacrylamide gels containing 1.2% pH 3–10 ampholytes, 0.8% pH 7–9 ampholytes, and 5% glycerol. The gels were polymerized by using 2 μg/mL of riboflavin and a fluorescent light source. HSF, in 1.2% pH 3–10 ampholytes, was loaded from the acid end. The acid electrode buffer was 0.03 M H_2SO_4, and the base electrode buffer was 0.04 N NaOH and 0.02 N $Ca(OH)_2$. Voltage was set at 200 V for 16 h and 300

V for the final 1–2 h. The gels were sliced and incubated overnight in 0.2 mL of buffer A, and the supernatant was assayed for stimulatory activity. A parallel gel was used to determine the pH gradient.

Sepharose 6B Chromatography. A Sepharose 6B column (0.9 × 18 cm) was equilibrated in buffer A containing 25% glycerol. Purified HSF (0.2 mL) was loaded and washed with two 0.2-mL aliquots of buffer A containing 25% glycerol, and then 0.2 mL of fraction PC RNA polymerase II was loaded. When HSF or RNA polymerase was run separately, buffer A containing 25% glycerol was substituted for the sample not included.

Sucrose Gradient Centrifugation. Hydroxylapatite-purified HSF (0.2 mL) was layered on the top of a 5–20% linear sucrose gradient in buffer A and centrifuged at 256000*g* for 52 h in the SW40 rotor. Fractions of 0.3 mL were collected from the bottom and assayed for stimulatory activity. Standards were run either in the same tube or in parallel tubes.

When RNA polymerase and HSF were centrifuged together, hydroxylapatite-purified HSF (0.3 mL), in 25% glycerol and 3% sucrose in buffer A, was layered on top and overlayed with 0.3 mL of fraction PC RNA polymerase II in buffer A. The tubes were centrifuged at 256000*g* for 44 h. When HSF or RNA polymerase was run alone, 0.3 mL of the appropriate buffer was layered in place of the fraction omitted.

Incorporation of [γ-^{32}P]ATP and [^{3}H]UMP into RNA. An RNA polymerase assay 2.5 times the normal size was run by using 0.2 mM [γ-^{32}P]ATP (1340 cpm/pmol). At the end of the normal polymerase reaction, samples were incubated for an additional 30 min with either 20 μg/mL of proteinase K (EM Laboratories), 20 μg/mL of RNase A (Sigma Chemical Co.), or S1 nuclease (obtained by the method of Harris et al., 1975). The reactions were stopped by heating to 80° C for 5 min, the volume was brought up to 0.975 mL with cold water, and 0.025 mL of 20% $NaDodSO_4$ was added. The solution was then extracted with phenol and chloroform by the procedure of Penman (1969). After extraction, 0.9 mL of the aqueous layer was removed, precipitated with 1 mL of cold 10% trichloroacetic acid, filtered, and counted.

Cell Fractionation. Lamb thymus cells were separated into nuclei and cytoplasm by a modification of the method of Kellas et al. (1977). Fresh lamb thymus (150 g) was ground up in a food mill (Foley Manufacturing Co., Minneapolis, MN), and the volume was brought up to 750 mL with 2.4 M sucrose, 25 mM KCl, 3 mM $MgCl_2$, and 0.1 mM dithiothreitol. The solution was passed three times through a motor-driven Teflon-glass homogenizer. The resulting solution was centrifuged at 73000*g* for 1 h to pellet the nuclear fraction. The nuclear fraction was homogenized in the same buffer as above except it contained 1 mM $MgCl_2$. The volume was brought up to 280 mL and centrifuged as before. The supernatants of the two centrifugations were combined and constituted the cytoplasmic fraction. The pellet of the second centrifugation constituted the nuclear fraction. The nuclear fraction was suspended in buffer A containing 5% glycerol, and RNA polymerase and HSF were purified as described above. HSF was purified through Sp-Sephadex while RNA polymerases I and II were separated by salt precipitation. The cytoplasmic fraction was dialyzed against 10 volumes of buffer A containing 5% glycerol, and RNA polymerase and HSF were purified as described above.

Assays other than for RNA polymerase and HSF were performed on nuclear and cytoplasmic fractions without further purification. DNA concentrations were measured by the diphenylamine method of Burton (1968) or by the methyl green DNA binding assay of Peters & Dahmus (1979). Protein was measured by the amido-Schwartz method of Schaffner & Weissman (19733) or by the Coomassie blue G250 method of Bradford (1976). Glyceraldehyde-3-phosphate dehydrogenase was measured by the method of Bruening et al. (1970), alkaline phosphatase was measured as described by Weiser (1973), and cytochrome *c* reductase was measured as described by Fleischer & Fleischer (1967).

Table I: Table of Purification[a]

stage of purification	volume (mL)	protein (mg)[b]	units of stimulatory act.[c]	sp act. (units/mg)	% yield
homogenate	1000	8100	18 100[d]	2.2	100
50–70% ammonium sulfate precipitate	280	1240	9 520[d]	7.7	53
Sp-Sephadex	90	27.5	11 500	417	64
heated Sp-Sephadex peak	90	12	11 400	950	63
hydroxylapatite	10	1.6	8 700	5440	48

[a] The table is based on 150 g of lamb thymus as starting material. [b] Determined as described under Methods. [c] All fractions were dialyzed to low salt to determine activity. The unit of stimulatory activity is defined under Methods. [d] The fraction was heated and then clarified by centrifugation prior to assay.

Determination of Salt Concentration. Salt concentration was determined on a Yellow Spring Instruments Model 31 conductivity bridge following a 200-fold dilution of sample with water.

Determination of RNase H Activity. DNA–RNA hybrids were formed by using RNA polymerase II to transcribe denatured DNA under the standard assay conditions in the presence of labeled [^{3}H]UTP. Single-stranded RNA was digested with RNase A. The [^{3}H]UMP-labeled RNA–DNA hybrid was incubated with HSF for 30 min at 37 °C under standard RNA polymerase assay conditions. Trichloroacetic acid precipitable counts were determined at the end of the reaction as described above.

Results

Purification of HSF. Thymus glands were homogenized and sonicated to solubilize RNA polymerase as well as other proteins that tightly bind DNA. The proteins were then separated into three fractions by precipitation with ammonium sulfate. RNA polymerase II precipitated between 0 and 30% saturation, whereas RNA polymerase I precipitated between 30 and 50%. The fraction that precipitated between 50 and 70% ammonium sulfate contained RNA polymerase II stimulatory activity (HSF). Greater than a 50-fold purification of HSF was obtained by chromatography in Sp-Sephadex. HSF was then pooled, heated at 90 °C for 5 min, and centrifuged to remove precipitated protein. Over 90% of the stimulatory activity remained after heating for 5 min at 90 °C. This step removes most high molecular weight polypeptides. HSF was then bound directly onto a hydroxylapatite column and eluted with a gradient of phosphate buffer. The heat treatment can be repeated at this stage, if necessary, to remove contaminating high molecular weight polypeptides. This procedure results in a 2500-fold purification of HSF with an overall yield of about 50%. The specific activity and recovery of HSF at the various stages of purification are shown in Table I.

To establish whether or not the stimulatory activity of HSF is dependent on protein, HSF was incubated with Enzite protease as described under Methods and reassayed for stimulatory activity. After proteolysis, stimulation of RNA

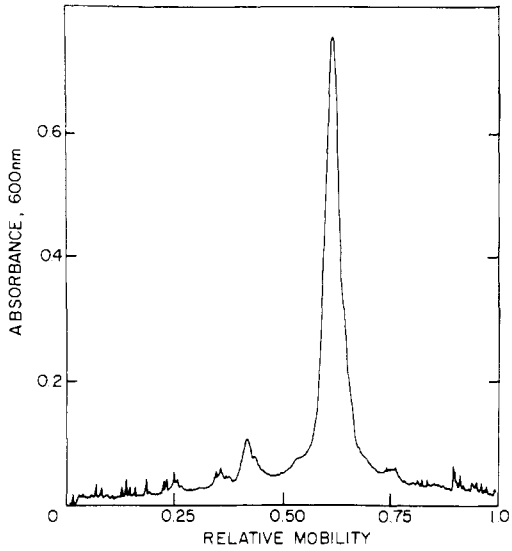

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of HSF. HSF (15 μg) was electrophoresed on an 8% $NaDodSO_4$–polyacrylamide gel according to the procedure of Weber & Osborn (1969). The gel was scanned at 600 nm on a Gilford recording spectrophotometer.

polymerase II decreased from 700% to a value just above background. The activity of HSF therefore appears to reside in protein.

In order to relate HSF to previously described stimulatory proteins (Stein & Hausen, 1970; Lentfer & Lezius, 1972; Lee & Dahmus, 1973; Sekimizu et al., 1976), it was chromatographed at pH 7.9 on both DEAE-cellulose and CM-cellulose. The activity appeared in the flow-through peak of each column. The fact that HSF does precipitate in high salt, does not bind to DEAE-cellulose or CM-cellulose at pH 7.9, and is heat stable suggests it is similar to the stimulatory factor first described by Stein & Hausen (1970).

Physical Characterization of HSF. HSF was run on a sucrose gradient as described under Methods, and its sedimentation constant relative to standard proteins was determined. The activity sedimented as a single peak corresponding to an *s* value of 2.7. The diffusion coefficient (*D*) was estimated by gel filtration on Sephadex G-100 (Andrews, 1964). A plot of log *D* against the elution position of stimulatory activity relative to the elution position of standard proteins indicates HSF has a diffusion coefficient of 9.55×10^{-7} cm^2/s. A Stokes radius (*S*) of 22.3 Å was determined for HSF by using the relationship determined by Holmes & Johnstone (1973). By use of the *S* and *D* values determined above and a partial specific volume of 0.725 cm^3/g (Siegel & Monty, 1966), the molecular weight of HSF was calculated to be about 24 000.

Polyacrylamide gel electrophoresis of purified HSF in the presence of sodium dodecyl sulfate results in a single major band (Figure 1). The molecular weight, determined by mobility relative to standard proteins, is 25 000. The molecular weight determined by polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ is therefore in good agreement with that determined by velocity sedimentation and exclusion chromatography. Furthermore, the stimulatory activity is coincident with the presence of this protein following chromatography in Bio-Gel P60.

Purified HSF was electrofocused on polyacrylamide gels, and the activity across the gel was determined as described under Methods. A single peak of activity was observed corresponding to an isoelectric point of 8.0 and coincident with the major peak of protein. Electrophoresis of this protein in a second dimension in the presence of $NaDodSO_4$ results in a single band corresponding to a molecular weight of about 25 000. It therefore appears that the stimulatory activity resides in a single polypeptide of about 25 000 daltons. A single band was also observed when purified HSF was run on nondenaturing gels as described by Reisfeld et al. (1962) or Hoffman & Chalkely (1976). No stimulatory activity could be recovered from such gels, however.

Table II: Effect of HSF on Activity of RNA Polymerases I and II and *E. coli* RNA Polymerase

	[^{3}H]UMP incorporated (pmol)[a]		% increase
	−HSF	+HSF[b]	
RNA polymerase I	9.9	11.4	15
RNA polymerase II	11.4	63.4	460
E. coli RNA polymerase	11.4	12.3	8

[a] Assayed as described under Methods. The specific activity of RNA polymerase I was 0.05, of RNA polymerase II was 27, and of *E. coli* RNA polymerase was 300. [b] Five micrograms of hydroxyapatite purified HSF was added per assay.

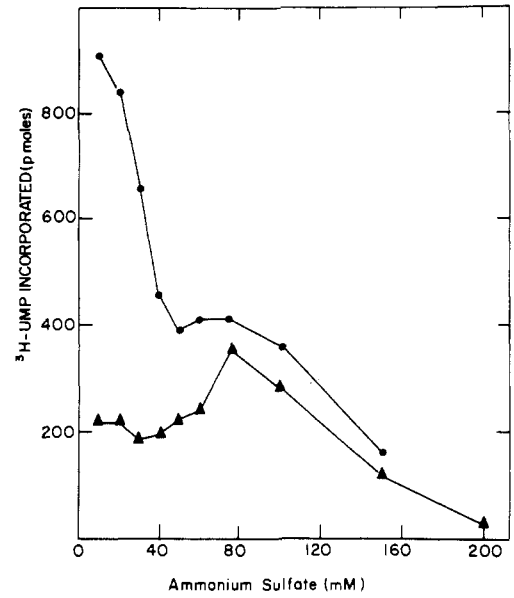

FIGURE 2: Effect of ionic strength on stimulatory activity of HSF. RNA polymerase II was assayed as described under Methods at various concentrations of ammonium sulfate in the presence (●) or absence (▲) of 10 μg of hydroxylapatite-purified HSF.

General Properties of Stimulatory Reaction. The ability of HSF to stimulate the activity of lamb thymus RNA polymerases I and *Escherichia coli* RNA polymerase was determined. The results presented in Table II show that neither *E. coli* RNA polymerase nor RNA polymerase I was significantly stimulated by HSF under conditions that result in a nearly fivefold increase in RNA polymerase II activity. HSF was also tested for stimulatory activity in the presence of RNA polymerase II, utilizing either native or heat-denatured DNA. Little or no stimulation was observed when denatured DNA served as a template.

The effect of ionic strength on the ability of HSF to stimulate RNA polymerase II is shown in Figure 2. Maximal stimulation is observed at low ionic strength with little or no stimulation above 60 mM ammonium sulfate. Ribonuclease H has been shown to stimulate RNA polymerase under certain conditions (Sekelis et al., 1972; Dezelee et al., 1977). Despite the copurification of some stimulatory factors and ribonuclease

Table III: Incorporation of [^{3}H]UMP and [γ-^{32}P]ATP into RNA[a]

time RNA polymerase added[b]	time HSF added	total incubation time	treatment[c]	pmol of [γ-^{32}P]ATP incorporated	pmol of [^{3}H]UMP incorporated	number-average length of RNA in nucleotides[d]
	0	60		0	1.69	
0		30		5.34	345	129
0		60		5.95	347	123
0	0	30		8.20	1425	348
0	0	60		11.59	1567	270
0	30	60		7.40	1440	389
0	0	60	proteinase K	12.20	1321	217
0	0	60	RNAse A	3.53	9.36	
0	0	60	RNAse A + S1 Nuclease	0.47	2.76	

[a] Assay conditions as described under Methods. [b] Each reaction contained 4.2 μg of RNA polymerase II (sp act. of 27 units/mg). Assuming 80% purity and a molecular weight of 550000, we added about 7 pmol of RNA polymerase II. [c] Proteinase K, RNase A, and S1 nuclease were added as described under Methods after the 60 min incubation. [d] Calculated by assuming the RNA has 25% U and equivalent A and G at the 5′ end.

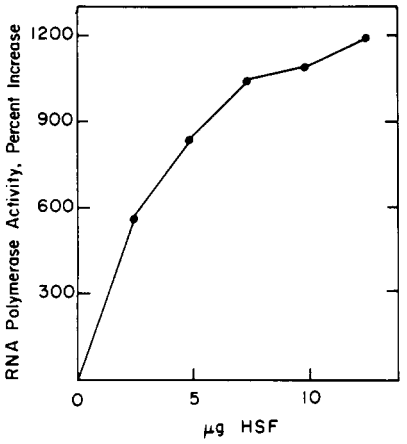

FIGURE 3: Effect of increasing concentrations of HSF on RNA polymerase II activity. Increasing amounts of dialyzed HSF were added to reactions containing 0.015 units of RNA polymerase. Activity was measured as described under Methods. Each assay contained 10 mM ammonium sulfate.

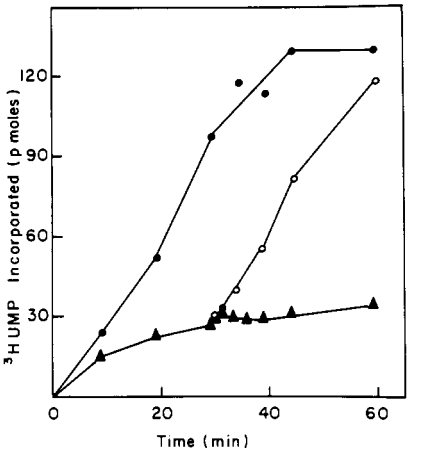

FIGURE 4: Time course of HSF stimulation of RNA polymerase II. RNA polymerase was assayed as described under Methods. Aliquots of 0.25 mL were removed at the indicated times, and the Cl_3AcOH precipitable counts were determined. RNA polymerase alone (▲); 10 μg of HSF added at 0 min (●) or at 30 min (○).

H at early stages, when these factors have been more extensively purified, a separation of stimulatory activity from ribonuclease H activity has been observed (Sekimizu et al., 1976). When HSF was incubated with [^{3}H]UMP-labeled RNA–DNA hybrids prepared as described under Methods, no significant degradation of the RNA moiety of these hybrids was observed. It is therefore unlikely that the stimulatory activity of HSF is due to RNase H activity.

(*1*) *Saturation of RNA Polymerase II by HSF.* The activity of RNA polymerase II in the presence of increasing concentrations of HSF is shown in Figure 3. Although saturation has not been obtained, stimulation of 1200%, corresponding to 13 times the original activity, was observed. This concentration of HSF represents a molar ratio of HSF to RNA polymerase II of about 500:1. Increasing the amount of DNA in the reaction does not affect the HSF saturation curve. If a constant amount of HSF is added to increasing amounts of RNA polymerase II, the percent of stimulation decreases, but the total increase in RNA synthesis remains nearly constant.

(*2*) *Time Course of Stimulation.* The kinetics of RNA synthesis supported by RNA polymerase II in the presence or absence of HSF is shown in Figure 4. The rate of UMP incorporation by RNA polymerase alone reaches a plateau after about 20 min of incubation. When HSF is added at zero time, the reaction is nearly linear for about 40 min. If HSF is added at 30 min, the rate of UMP incorporation increases to a rate nearly equal to that observed when HSF is added at zero time. Addition of 4 μg/mL of α-amanitin or 20 μg/mL of rifampicin AF/013 at 20 min to either RNA polymerase or RNA polymerase in the presence of HSF results in the cessation of RNA synthesis (data not shown).

(*3*) *Incorporation of* [γ-^{32}P]*ATP and* [^{3}H]*UMP into RNA.* To determine if HSF has a specific effect on the initiation or elongation step of the polymerase reaction, RNA polymerase II was incubated in the presence of [γ-^{32}P]ATP and [^{3}H]UTP in the presence or absence of HSF. The results presented in Table III show that, under conditions that resulted in a fourfold increase in [^{3}H]UMP incorporation, less than a twofold increase in the incorporation of γ-^{32}P was observed. To establish that the ^{32}P and ^{3}H were incorporated into RNA, the sensitivity of the product to various enzymes was determined. RNase A solubilized greater than 99% of the ^{3}H counts but did not completely solubilize the ^{32}P counts. This result could be explained by a high purine content at the 5′ end of the chains initiated in vitro. S1 nuclease does eliminate these resistant ^{32}P counts. Proteinase K solubilized neither ^{32}P nor ^{3}H counts, indicating that the incorporation of either was not onto protein.

Under the conditions of the experiment, HSF increases the number-average length of RNA about three times with only a slight increase in the frequency of initiation. Since HSF increases the average size of the RNA and increases the length of time the RNA polymerase assay is linear, it appears to

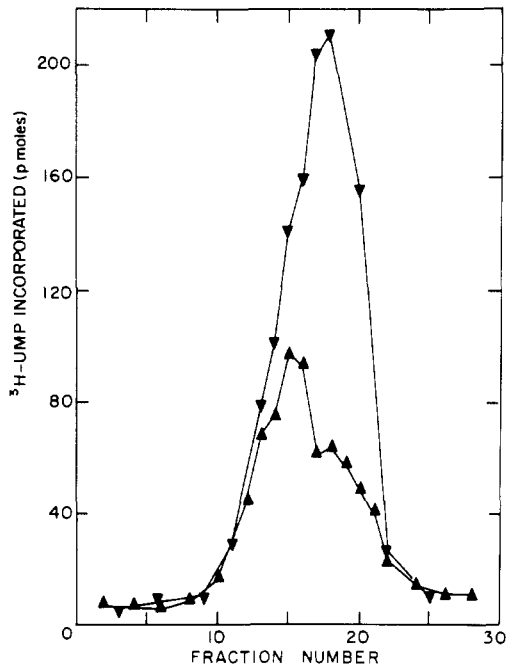

FIGURE 5: Sedimentation of RNA polymerase II in sucrose gradients in the presence and absence of HSF. Five to twenty percent sucrose gradients were run as described under Methods in a SW40 rotor. Activity of PC-purified RNA polymerase (250 μg) centrifuged in the absence (▲) or presence (▼) of 12 μg of HSF. HSF run alone does not sediment past the first several fractions. Fractions of 0.4 mL were collected.

stimulate primarily the elongation reaction. The small increase in initiation observed is probably not a primary effect of HSF but may represent the reinitiation of RNA chains.

Evidence for a Direct Interaction between HSF and RNA Polymerase. (1) Sucrose Gradient Centrifugation. RNA polymerase II was run on 5–20% sucrose gradients in the presence or absence of HSF. HSF containing 3% sucrose and 25% glycerol was layered on top of the gradient and then overlayered with RNA polymerase containing 25% glycerol. Since HSF has a sedimentation constant of 2.7, whereas RNA polymerase II has an *s* value of about 15, the RNA polymerase must pass through the HSF layer during sedimentation. Figure 5 shows that the activity of RNA polymerase is increased several-fold in the gradient containing HSF relative to the gradient containing polymerase alone. Under these conditions and in the absence of RNA polymerase, HSF does not sediment past the first two fractions of the gradient. Total recovery of RNA polymerase activity was 60% in the absence of HSF and 120% in the presence of HSF.

(2) Gel Filtration. The interaction of HSF and RNA polymerase II was also investigated by chromatography on Sepharose 6B. RNA polymerase elutes in fractions 12–16 (Figure 6), whereas HSF elutes in fractions 28–32. When HSF and RNA polymerase were run together, they were loaded in a manner similar to that used for sucrose gradient centrifugation. HSF was loaded and washed with two small aliquots of buffer A, before the application of RNA polymerase. As shown in Figure 6, when RNA polymerase and HSF were run together, the activity of the RNA polymerase peak increased dramatically. When run alone, the yield of RNA polymerase activity was 64%; when run with HSF the yield of polymerase activity was 169%. To determine whether or not the RNA polymerase chromatographed in the presence of HSF could be further stimulated, the column fractions were also assayed in the presence of a constant amount of HSF. Figure 6 shows that addition of HSF stimulated the RNA

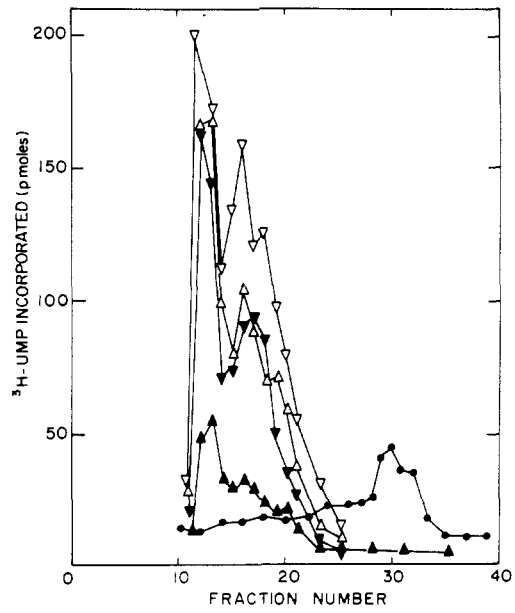

FIGURE 6: Chromatography of RNA polymerase II on Sepharose 6B in the presence and absence of HSF. A Sepharose 6B column (0.9 × 18 cm) was run as described under Methods. Fractions of 0.3 mL were collected and assayed as described under Methods. HSF alone (30 μg) (●); PC-purified RNA polymerase II (250 μg) in the absence (▲) or presence (▼) of 8 μg of HSF. The activity of RNA polymerase II chromatographed in the absence (△) or presence (▽) of HSF was then assayed in the presence of 5 μg of exogenous HSF.

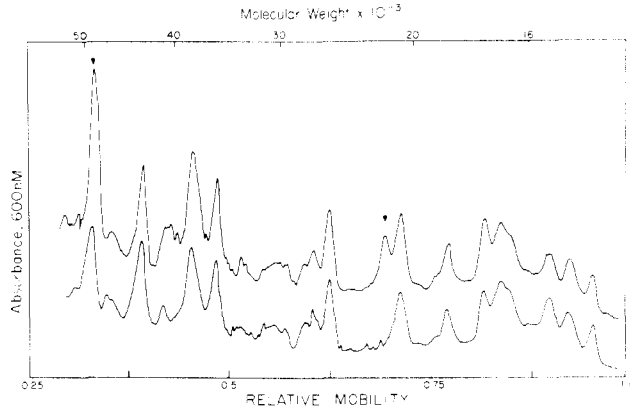

FIGURE 7: $NaDodSO_4$ gel electrophoresis of RNA polymerase chromatographed on Sepharose 6B in the presence and absence of HSF. Fractions 12–14 from the Sepharose column presented in Figure 6 were pooled, concentrated, and electrophoresed in the presence of $NaDodSO_4$ as described in the text. The gels were scanned in a Gilford recording spectrophotometer. Only the scans of the bottom portion of the gels are shown. The remaining portion of the gels were identical. Lower scan: RNA polymerase run alone. Upper scan: RNA polymerase chromatographed in the presence of HSF. Arrows indicate the positions of the 49 000-dalton peptide and HSF.

polymerase chromatographed alone several times, whereas only a small increase was observed in the activity of RNA polymerase chromatographed with HSF. As a control, HSF was chromatographed in the presence of phosphorylase *b* which elutes at fraction 15. The elution of HSF was not influenced by the presence of phosphorylase *b*. On both Sepharose 6B and sucrose gradients, RNA polymerase separates into two peaks. We do not know the basis of the separation, but it does not appear to be a resolution into forms IIa and IIb (data not shown).

These results could be explained by either an association of HSF with RNA polymerase or an irreversible modification of the enzyme so that its activity remains elevated even in the absence of HSF. In an effort to distinguish between these two

Table IV: Subcellular Distribution of HSF[a]

	percentage in cytoplasmic fraction	percentage in nuclear fraction
HSF	35	65
protein	69	31
DNA	6	94
RNA polymerase I	59	41
RNA polymerase II	25	75
glyceraldehyde-3-phosphate dehydrogenase	89	11
alkaline phosphatase	87	13
cytochrome *c* reductase	94	6

[a] The nuclear and cytoplasmic fractions were purified as described under Methods.

possibilities, we have examined the RNA polymerase chromatographed with and without HSF by $NaDodSO_4$–polyacrylamide gel electrophoresis. Fractions 12–14 from the Sepharose 6B column were pooled, dialyzed against 10 volumes of 10 mM Tris-HCl, pH 7.9, lyophilized, and run on 12% $NaDodSO_4$–polyacrylamide gels according to the method of Laemmli & Favre (1973). The results presented in Figure 7 show that the polymerase run in the presence of HSF contains a new polypeptide at a position identical with that of purified HSF in a parallel gel. The stoichiometry of this protein, presumbaly HSF, relative to the 140000-dalton subunit of RNA polymerase is 1.2:1. In addition, the enzyme run in the presence of HSF also contains more of a 49000-dalton protein than does the control. This protein was not present in the HSF sample.

(*3*) *Cell Fractionation.* Lamb thymus cells were fractionated into a nuclear fraction and a cytoplasmic fraction as described under Methods in order to determine the intracellular location of HSF. The results of this fractionation are presented in Table IV. The cell fractionation procedure used hypertonic sucrose as an initial homogenization buffer to reduce RNA polymerase leakage from nuclei (Kellas et al., 1977). RNA polymerase II was found in the nuclear fraction in significantly greater amounts than was RNA polymerase I. As is evident from Table IV, HSF was predominantly localized in the nuclear fraction, and only DNA and RNA polymerase II were found to a greater extent in this fraction.

Discussion

The procedure developed for the purification of a heat-stable RNA polymerase II stimulatory factor results in a 2500-fold purification with an overall yield of about 50%. HSF has a molecular weight of 24000 as determined by velocity sedimentation and gel fiiltration. Following isoelectric focusing or electrophoresis on $NaDodSO_4$–polyacrylamide gels, greater than 90% of the protein is located in a single band and corresponds to a molecular weight of 25000. The close agreement between the molecular weights determined under native and denaturing conditions, in addition to the fact that the minor polypeptides observed in the $NaDodSO_4$ gel have molecular weights greater than 25000, suggests that the stimulatory factor is composed of a single polypeptide of 25000 daltons.

The extent of stimulation of RNA polymerase II generally varies between 10–15-fold. HSF stimulates RNA polymerase II only in the presence of native DNA and does not increase the activity of RNA polymerase I or *E. coli* RNA polymerase, irrespective of the nature of the DNA template. The fact that HSF stimulates the incorporation of [^{3}H]UMP several-fold with only a slight increase in the incorporation of [γ-^{32}P]ATP suggests that its primary effect is on elongation. Stimulatory factors with properties similar to HSF have been isolated from calf thymus (Stein et al., 1973) and from rat liver (Seifart et al., 1973). These proteins also have molecular weights in the range of 20000–30000 and stimulate the elongation of RNA chains catalyzed by RNA polymerase II.

RNA polymerase II, exposed to HSF and subsequently separated from free HSF by sedimentation or gel filtration, has an elevated activity. These data suggest that either HSF has bound to the enzyme, and can therefore stimulate activity in the subsequent reaction, or HSF has caused a stable modification of the enzyme that results in its elevated activity. The fact that electrophoresis of the enzyme following exposure to HSF shows the presence of a new protein with an electrophoretic mobility indistinguishable from HSF strongly suggests a direct interaction between the two. The elution of HSF with RNA polymerase on Sepharose 6B does not necessarily imply a functional interaction. We cannot eliminate the possibility that the copurification results from nonspecific associations. However, the calculated stoichiometry for HSF to RNA polymerase II of 1.2:1 suggests a specific interaction, as unusual molar ratios would be the expected result of nonspecific interactions. The presence of HSF also causes an increase in the amount of a 49000-dalton protein. Since this protein is not present in HSF, its increase must be due to a stabilization of its interaction with RNA polymerase by HSF. If this subunit was required for catalytic activity, the stimulatory activity of HSF may result from its ability to stabilize the interaction of this subunit with RNA polymerase.

That HSF is localized in the nucleus lends support to the possibility that it may in part regulate RNA polymerase activity in vivo. The real significance of HSF in the regulation of transcription can, however, only be ascertained by utilizing defined cell-free systems in which the specific initiation and termination of known genes can be monitored.

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Isolation of *Euglena gracilis* Chloroplast 5S Ribosomal RNA and Mapping the 5S rRNA Gene on Chloroplast DNA†

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ABSTRACT: Ribosomal RNA (5S) from *Euglena gracilis* chloroplasts was isolated by preparative electrophoresis, labeled in vitro with ^{125}I, and hybridized to restriction nuclease fragments from chloroplast DNA or cloned chloroplast DNA segments. *Euglena* chloroplast 5S rRNA is encoded in the chloroplast genome. The coding region of 5S rRNA has been positioned within the 5.6 kilobase pair (kbp) repeat which also codes for 16S and 23S rRNA. There are three 5S rRNA genes on the 130-kbp genome. The order of RNAs within a single repeat is 16S–23S–5S. The organization and size of the *Euglena* chloroplast ribosomal repeat is very similar to the ribosomal RNA operons of *Escherichia coli*.

The 5S ribosomal RNAs from many organisms have been well characterized. At least 36 5S rRNAs[1] have been completely sequenced (Erdmann, 1978). This RNA is an integral component of both procaryotic and eucaryotic ribosomes. Its exact role in protein synthesis has not been determined, but it may serve to recognize and position transfer RNAs (Monier, 1974). The sequence of 5S rRNA has been well conserved throughout evolution. Sequence data have been used to estimate the evolutionary divergence of various species (Schwarz & Dayhoff, 1978). Prior to this report, the only chloroplast 5S rRNAs which have been mapped with respect to chloroplast DNA restriction nuclease site maps were in *Zea mays* (Bedbrook et al., 1977) and spinach (Whitfield et al., 1978).

Euglena is a versatile unicellular eucaryote which can grow on either phototrophic or organotrophic media. Chloroplasts from this organism contain DNA, which has been extensively studied. Depending on the growth conditions, 500–2000 copies of the 130-kbp genome are present in each cell (Rawson & Boerma, 1976; Chelm et al., 1977). This DNA codes for 16S and 23S rRNA (Scott & Smillie, 1967; Stutz & Rawson, 1970) and approximately 25 tRNAs (McCrea & Hershberger, 1976; Schwartzbach et al., 1976). In addition, 50 kb of RNA of yet unknown function is also transcribed from this genome in vivo (Chelm et al., 1978). The coding region for 16S and 23S rRNAs has been located on a 5.6-kbp DNA segment which is repeated three times in tandem (Gray & Hallick,

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[1] Abbreviations used: rRNA, ribosomal RNA; kbp, kilobase pair; tRNA, transfer RNA; kb, kilobase; G+C, guanine and cytosine; bp, base pair; nt, nucleotides.

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