degree that the activation free energy for the transition reflects the interaction between rhodopsin and disk phospholipid, one would expect that if a unique protein–phospholipid interaction occurred upon formation of a boundary layer for rhodopsin, either a leveling out or an inflection point would be observed for the dependence of the activation free energy on $\bar{\nu}$. The observed behavior of the activation free energy is, however, linear throughout the range of $\bar{\nu}$ studied; these data encompass the value of $\bar{\nu}$ associated with the formation of a phospholipid boundary layer around rhodopsin. The linear behavior of the activation free energy implies that both a noncooperative and nonspecific mode of interaction exists between rhodopsin and disk membrane phospholipids. Calculation of the activation free energy for rhodopsin with 75 associated phospholipid molecules, the ratio which exists in the disk membrane, yields a value of 13.2 kcal/mol, while the value for the disk membrane, as determined from the data of Applebury et al. (1974), is 14.6 kcal/mol. Hence, while there is no marked increase in the activation free energy associated with the formation of a phospholipid bilayer around rhodopsin, there does appear to be an increase of 1.4 kcal/mol associated with the insertion of rhodopsin into a phospholipid bilayer.

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Stimulation of Messenger Ribonucleic Acid Synthesis in Isolated Nuclei by a Protein That Stimulates RNA Polymerase II†

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ABSTRACT: A factor stimulating RNA polymerase II purified from Ehrlich ascites tumor cells was found to stimulate α-amanitin-sensitive RNA synthesis in nuclei isolated from spleen cells of anemic mice, though less than it stimulated purified RNA polymerase II. The fidelity of the resulting RNA synthesis was monitored by measuring the stimulation of globin mRNA synthesis. Globin mRNA was measured quantitatively by DNA–RNA hybridization by using plasmid DNA containing globin DNA sequences. Results showed that the synthesis of globin mRNA was enhanced in isolated nuclei in the presence of this factor coinciding with an increase of overall α-amanitin-sensitive RNA synthesis. Thus, it was concluded that an externally added factor did not stimulate random transcription but meaningful RNA synthesis in isolated nuclei.

Little information is available about proteins regulating eukaryotic gene expression. However, it is clear that RNA polymerase II or III alone is insufficient for faithful transcription in vitro (Weil et al., 1979a,b; Ng et al., 1979). There are several reports of proteins stimulating the activity of RNA polymerase II in vitro, though the biological functions of these proteins are unknown (Stein & Hausen, 1970; Seifart, 1970; Natori, 1972; Lentfer & Lezius, 1972; Sugden & Keller, 1973; Lee & Dahmus, 1973; Benson et al., 1978; Spindler, 1979; Revie & Dahmus, 1979).

We purified a protein, named S-II, from Ehrlich ascites tumor cells that exclusively stimulates the activity of RNA polymerase II (Sekimizu et al., 1979a). Studies using antibody against S-II showed that S-II is a nuclear protein that is localized in the nucleoplasm and that it is not a component

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of multimeric RNA polymerase II. Since the antibody selectively inhibits RNA synthesis catalyzed by RNA polymerase II in isolated nuclei, it is likely that S-II is an essential protein for eukaryotic transcription catalyzed by RNA polymerase II (Ueno et al., 1979). In addition, proteins like S-II cross-reacting with the antibody against S-II were found to exist in the nucleoplasm of other eukaryotic cells, such as HeLa cells and salivary gland cells of flesh-fly larvae (Sekimizu et al., 1979b). Thus, S-II may be a protein regulating eukaryotic transcription.

This paper describes the effect of S-II on transcription in isolated nuclei. Since spleen cells of anemic mice are known to synthesize hemoglobin actively (Cheng & Kazazian, 1976), nuclei from these cells should synthesize globin mRNA extensively. In this work, we quantitated the amount of globin mRNA in these nuclei as an indicator of meaningful transcription and examined the effect of S-II on RNA synthesis. We found that the synthesis of globin mRNA nearly doubled on addition of S-II to isolated nuclei, with a concomitant increase in overall α-amanitin-sensitive RNA synthesis. Thus, we concluded that exogenously added S-II stimulated meaningful RNA synthesis in isolated nuclei.

This is the first report that an externally added stimulatory factor affects α-amanitin-sensitive RNA synthesis in isolated nuclei.

Materials and Methods

Purification of RNA Polymerase II Stimulatory Factor S-II. S-II was purified from Ehrlich ascites tumor cells harvested from ddY mice by the procedures described before (Sekimizu et al., 1979a). The purified factor gave a single band of protein with a molecular weight of 40 500 on Na-DodSO$_4$[1]-polyacrylamide gel electrophoresis, as reported previously.

Preparation of Nuclei. Nuclei of Ehrlich ascites tumor cells were prepared as described by Marzluff et al. (1973). For isolation of the nuclei from spleen cells of anemic mice, ddY male mice were injected with 0.25 mL of 0.4% β-acetylphenylhydrazine solution 3 times by the method of Cheng et al. (1974), and the spleens were removed 90–100 h after the first injection and washed well with ice-cold phosphate-buffered saline. For isolation of nuclei, four spleens from anemic mice were combined and disrupted, and the cells were dispersed in 40 mL of ice-cold phosphate-buffered saline. The cell suspension was passed through a no. 200 metal mesh filter to remove clumps of tissue. The cells were then washed twice with 40 mL of phosphate-buffered saline by centrifugation at 800g for 5 min and then suspended in hypotonic buffer consisting of 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 6 mM $MgCl_2$, at a final concentration of 1×10^8 cells/mL. The cells were allowed to swell for 5 min on ice and then homogenized in a 70-mL glass homogenizer by four gentle strokes of a Teflon pestle. The crude nuclear fraction was collected by centrifugation at 800g for 5 min. The white nuclear pellet was suspended in 20 mL of solution A [10 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 3 mM $Mg(CH_3COO)_2$, 2 mM $CaCl_2$, 5 mM β-mercaptoethanol, and 0.025% (v/v) Triton X-100] in a glass homogenizer by three gentle strokes of a Teflon pestle, and about 6 mL of the resulting suspension was layered on 15 mL of solution B [10 mM Tris-HCl, pH 7.5, 2 M sucrose, 5 mM $Mg(CH_3COO)_2$, and 5 mM β-mercaptoethanol] and centrifuged for 60 min at 4 °C at 12 000 rpm in a Hitachi RPS-25 rotor.

The supernatant was carefully removed, and the nuclei were suspended in solution C [50 mM Hepes, pH 8.0, 25% (v/v) glycerol, 5 mM $Mg(CH_3COO)_2$, and 12 mM β-mercaptoethanol] to give a concentration of 3×10^8 nuclei/mL. The suspension of nuclei was rapidly frozen in liquid nitrogen and stored at −80 °C. They could be used for at least 2 weeks without any appreciable loss of activity for RNA synthesis.

RNA Synthesis in Isolated Nuclei. The reaction mixture (0.1 mL) contained 25 mM Hepes, pH 8.0, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 2.5 mM $Mg(CH_3COO)_2$, 10% (v/v) glycerol, 150 mM KCl, 12 mM β-mercaptoethanol, 0.25 mM each of ATP, GTP, and CTP, 0.025 mM UTP, 1 μCi[^{3}H]UTP (20 Ci/mmol), and nuclear suspension. The final concentration of Triton X-100 in the reaction mixture was adjusted to 0.018%. For tests on the effect of S-II, nuclei were first preincubated in solution C containing 0.075% Triton X-100 for 5 min on ice in the presence or absence of S-II and then added to the reaction mixture. RNA synthesis was performed at 25 °C, and the reaction was terminated by adding 2 mL of 5% (w/v) Cl_3CCOOH containing 5% sodium pyrophosphate. After 30 min, the mixture was centrifuged for 10 min at 2000g at 4 °C, and the precipitate was washed once with 5% Cl_3CCOOH and dissolved in 0.5 mL of 0.1 M NaOH containing 0.4% sodium deoxycholate. This solution was mixed with 10 mL of scintillation fluid containing 20% (v/v) Triton X-100, and its radioactivity was counted in a liquid scintillation spectrometer.

Globin mRNA and Plasmids Containing Globin Gene Sequences. Globin mRNA was isolated from spleen cells of anemic mice as described by Cheng et al. (1974) and labeled with ^{125}I by the procedure of Gets et al. (1972). Plasmids containing sequences of mouse globin α chain (pCR1αMG) or β chain (pCR1βMG), originally isolated by Rougeon & Mach (1977), were supplied from Dr. Honjo and amplified in *Escherichia coli* χ1776.

Extraction of RNA. The RNA synthesized in isolated nuclei was extracted by the hot phenol method described by Marzluff (1978). It was treated with 200 μg/mL DNase I in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 6 mM $MgCl_2$ for 40 min at 25 °C and extracted again with phenol. This process was repeated twice, and, finally, RNA was passed through a column of Sephadex G-50 (1 × 30 cm) equilibrated with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 0.1% (w/v) NaDodSO$_4$ to remove free [^{3}H]UTP. The excluded fraction was collected, and RNA was precipitated by adding 2.5 volumes of ethanol at −20 °C in the presence of 100 mM NaCl.

Sucrose Density-Gradient Centrifugation. The RNA sample was heated at 80 °C for 5 min, then layered on top of a 5-mL linear gradient of 5% to 20% sucrose in a solution of 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, and 0.1% NaDodSO$_4$, and centrifuged for 1.5 h at 64 000 rpm in a Hitachi RPS-65 rotor at 25 °C. Fractions were collected from the bottom of the gradient, and the RNA in each fraction was precipitated with cold 5% Cl_3CCOOH and collected on a Whatman GF-C glass-fiber filter for counting its radioactivity.

Hybridization of [^{3}H]RNA to DNA Filters. Plasmid DNA was immobilized on a nitrocellulose filter by the method of McKnight & Palmiter (1979). DNA was dissolved in 2 M NaCl containing 200 mM NH_4OH and heated at 100 °C for 1 min, and then 4-μL samples (containing 4 μg of DNA) were spotted on Schleicher & Schüll filter disks (BA 85) and baked.

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); NaDodSO$_4$, sodium dodecyl sulfate; SSC, standard saline citrate; Tris, tris(hydroxymethyl)aminomethane.

Under these conditions, more than 90% of the plasmid DNA bound to the filters and remained attached during subsequent hybridization and washing.

[^{3}H]RNA [(1.5–2.5) × 10^5 cpm] and/or [^{125}I]globin mRNA (2000 cpm) was first heated in a glass vial at 80 °C in 20 μL of 50% (v/v) formamide, 40 mM Pipes, pH 6.5, 500 mM NaCl, and 1 mM EDTA for 5 min to denature double-stranded sequences, and then it was transferred to a small vinyl bag containing one DNA filter. The vinyl bags were sealed, kept at 4 °C for 2 h, and then incubated at 41 °C for 15 h. Preincubation at 4 °C increased the efficiency of hybridization. After incubation, the filters were washed twice at 41 °C with 10 mL of 1.4 × SSC for 1 h and then twice with 10 mL of 0.1 × SSC for 20 min. Finally, they were treated with RNase A (20 μg/mL) in 1 mL of 2 × SSC for 1 h at 25 °C and then washed with 2 × SSC for 20 min. For measurement of the radioactivity of [^{125}I]RNA, the filters were each put into a glass vial and counted in an Aloka auto-well γ counter. For measurement of the radioactivity of [^{3}H]RNA, the filters were solubilized with 1 mL of Cellosolve, mixed with 10 mL of Aquasol solution, and counted in a liquid scintillation spectrometer.

Heat Stability of DNA–RNA Hybrid. DNA–RNA hybridization was performed on DNA filters as described above. After incubation for 15 h at 41 °C, the filters were rinsed with 0.1 × SSC containing 0.1% (v/v) diethyl pyrocarbonate and then washed repeatedly with chloroform and air-dried. They were then each put into a small vial containing 0.5 mL of 0.1 × SSC and heated for 5 min at 45 °C. Then each filter was transferred to another vial kept at 50 °C and incubated for 5 min. This process was repeated with 5 °C increase in incubation temperature each time, from 45 to 80 °C. The labeled RNA released into the incubation medium was precipitated by adding 25 μL of 100% Cl_3CCOOH with 100 μg of yeast RNA as a carrier, and the radioactivity in the precipitate was counted. Melting curves of DNA–RNA hybrids were constructed from results on the radioactivity released from the DNA filters at various temperatures. This procedure was essentially the same as that of Kunkel & Weinberg (1978).

Results

Effect of S-II on RNA Synthesis in Isolated Nuclei. It is known that when S-II is added to a transcriptional system in vitro consisting of purified RNA polymerase II and deproteinized DNA, RNA synthesis is enhanced severalfold. The enhancement depends on the amount of S-II added, and, with an increase in S-II, the synthesis increases to more than 10 times the level of the control without S-II (Sekimizu et al., 1976). Since antibody against S-II inhibits α-amanitin-sensitive nuclear RNA synthesis, it is highly likely that S-II participates in the transcriptional process in the nucleoplasm (Sekimizu et al., 1979b; Ueno et al., 1979). However, to prove that S-II is essential for nucleoplasmic transcription, it is necessary to show directly that exogenously added S-II enhances mRNA synthesis in isolated nuclei. Thus, we examined the effect of S-II on RNA synthesis in isolated nuclei.

We expected that externally added S-II would not affect nuclear RNA synthesis significantly since isolated nuclei should contain endogenous S-II. However, as shown in Figure 1a, S-II significantly enhanced α-amanitin-sensitive RNA synthesis in isolated nuclei of spleen cells from anemic mice. RNA synthesis proceeded linearly for at least 10 min under our experimental conditions, and, with S-II, it was almost twice the control level. This stimulation of RNA synthesis was probably not due to stimulation of RNA polymerase II released from the nuclei, because microscopic examination showed that almost all the nuclei remained intact during incubation. RNA synthesis in the supernatant after centrifuging the nuclei was very low, and no globin mRNA synthesis was detected. Thus, it seems that S-II is effective not only for in vitro transcription but also for RNA synthesis in isolated nuclei, though it causes less stimulation of isolated nuclei. Probably externally added S-II penetrates into the nuclei and affects the transcriptional machinery functioning on chromatin.

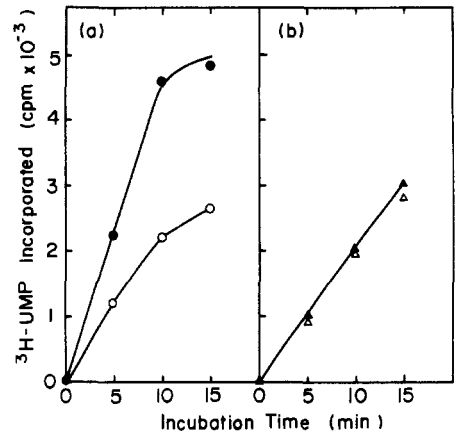

FIGURE 1: Time course of RNA synthesis in isolated nuclei prepared from spleen cells of anemic mice. RNA synthesis was performed with 5 × 10^7 nuclei at 25 °C in the presence or absence of 0.5 μg/mL α-amanitin, and α-amanitin-sensitive (a) and -resistant (b) RNA synthesis are plotted separately. α-Amanitin-sensitive RNA synthesis was determined by subtracting α-amanitin-resistant RNA synthesis from RNA synthesis in the absence of α-amanitin. (●, ▲) 200 units (0.5 μg) of purified S-II was added to 0.1 mL of reaction mixture at time 0; (○, △) RNA synthesis without S-II. One unit of S-II is the amount that increases 10 units of RNA polymerase II to 11 units under the standard assay conditions [see Sekimizu et al. (1979a)].

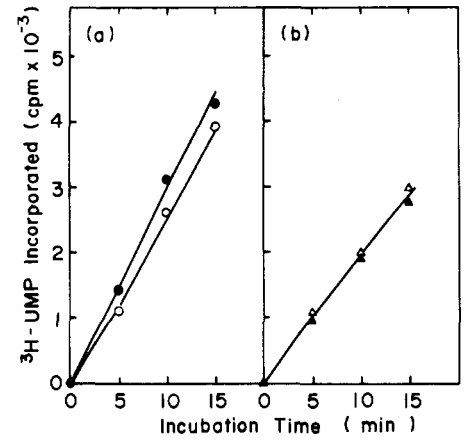

FIGURE 2: Time course of RNA synthesis in isolated nuclei prepared from Ehrlich ascites tumor cells. RNA synthesis was performed with 1 × 10^7 nuclei at 25 °C in the presence or absence of 0.5 μg/mL α-amanitin. (a) α-Amanitin-sensitive RNA synthesis; (b) α-amanitin-resistant RNA synthesis. (●, ▲) 200 units of purified S-II was added to the reaction mixture at time 0; (○, △) RNA synthesis without S-II.

Since S-II was originally isolated from Ehrlich ascites tumor cells, the effect of S-II on the nuclei of Ehrlich ascites tumor cells was examined. As shown in Figure 2a, the stimulation of RNA synthesis by S-II was much less than that of spleen cells from anemic mice. The difference between the responses to S-II of the two types of nuclei may be due to the amount of S-II originally present in the nuclei: the nuclei of Ehrlich ascites tumor cells may contain more S-II than the spleen cells.

As shown in Figures 1b and 2b, externally added S-II had little effect on α-amanitin-resistant RNA synthesis, which is

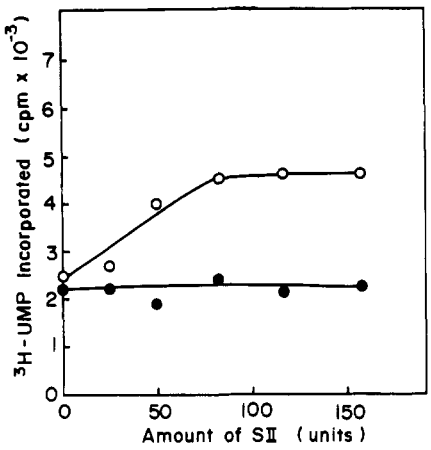

FIGURE 3: Dose–response curve of S-II on RNA synthesis in isolated nuclei prepared from spleen cells of anemic mice. Increasing amounts of S-II were added to the reaction mixture, and RNA synthesis was performed at 25 °C for 10 min in the presence or absence of 0.5 μg/mL α-amanitin. (●) α-Amanitin-resistant RNA synthesis; (○) α-amanitin-sensitive RNA synthesis.

thought to be due to RNA polymerases I and III. This specificity of the effect of S-II was the same as that observed with purified RNA polymerase II in vitro.

The dose–response curve of the stimulatory effect of S-II on RNA synthesis in isolated nuclei prepared from spleen cells of anemic mice is shown in Figure 3. With an increase in the amount of S-II added, α-amanitin-sensitive RNA synthesis increased gradually to a plateau at nearly 2-fold the control level, but α-amanitin-resistant RNA synthesis did not change appreciably. These externally added S-II proteins enhanced α-amanitin-sensitive nuclear RNA synthesis. For evaluation of the stimulatory effect of S-II, it is important to know whether S-II stimulates meaningful transcription in isolated nuclei. Therefore, we next analyzed the RNA synthesized in the presence of a saturating amount of S-II.

Analysis of RNA Synthesized in Isolated Nuclei. Since the spleen cells of anemic mice synthesize considerable hemoglobin, the isolated nuclei prepared from these cells should have the ability to synthesize globin mRNA. Therefore we measured the amount of globin mRNA synthesized in isolated nuclei as an indicator of meaningful RNA synthesis and tested whether S-II stimulated its production. First, we analyzed the size of RNA synthesized in isolated nuclei by sucrose density gradient centrifugation. This is important, because the size of RNA affects the efficiency of subsequent DNA–RNA hybridization experiments. RNA was extracted from nuclei labeled in vitro with [^{3}H]UTP in the presence and absence of a saturating amount of S-II, and the sedimentation profiles of the two preparations in a 5%–20% sucrose gradient were compared. As shown in Figure 4a,b, addition of S-II did not affect the size of the RNA but nearly doubled the overall incorporation of radioactivity. Most of the RNA sedimented between 4 S and 23 S, indicating that the size of the RNA was sufficient to use for subsequent DNA–RNA hybridization experiments.

As is evident from Figure 4c, the RNA synthesized in the presence of 0.5 μg/mL of α-amanitin, which is supposed to be synthesized by RNA polymerases I and/or III, also sedimented between 4 S and 23 S. Since the size of the RNA synthesized in isolated nuclei was almost the same in the presence and absence of α-amanitin, it was impossible to separate the products of RNA polymerase II from those of RNA polymerases I and/or III. Therefore, in subsequent experiments, RNA synthesis was carried out in the absence of α-amanitin.

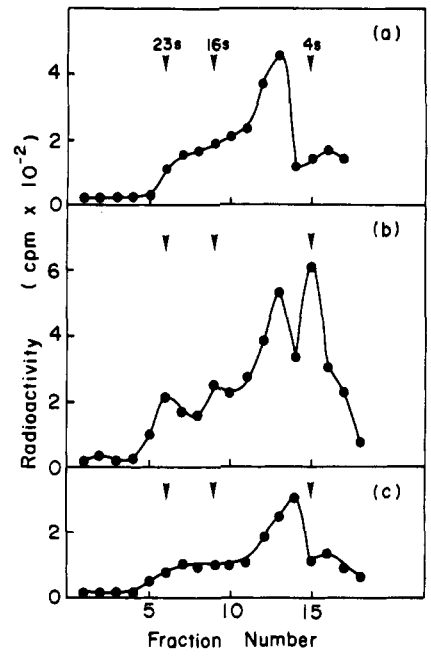

FIGURE 4: Sedimentation profiles in a 5%–20% sucrose density gradient of RNA synthesized in isolated nuclei of spleen cells from anemic mice. *E. coli* RNA labeled with [^{14}C]uridine, as a positional marker, was cosedimented with the sample RNA. (a) RNA synthesized in the absence of S-II; (b) RNA synthesized in the presence of a saturating amount of S-II; (c) RNA synthesized in the presence of 0.5 μg/mL of α-amanitin. Sedimentation was from right to left. Arrows indicate the positions of 23S, 16S, and 4S RNA.

Effect of S-II on the Synthesis of Globin mRNA. The amount of globin mRNA in RNA synthesized in the presence of S-II in isolated nuclei was measured to see if S-II stimulates meaningful RNA synthesis. The globin mRNA was measured by hybridization to plasmid DNA integrating globin DNA sequences. Plasmids integrating mouse globin DNA corresponding to the α chain and β chain, respectively, were amplified in *E. coli* χ1776 and purified extensively. These plasmids, pCR1αMG and pCR1βMG, were fixed on nitrocellulose membrane filters, and the radioactivity of RNA remaining on the filter after hybridization and washing was measured.

Suitable conditions for hybridization were determined by using ^{125}I-labeled globin mRNA. As shown in Figure 5a, saturation of hybridization was attained by incubating the DNA filters for 15 h with globin mRNA at 41 °C. The hybridization profiles were similar with the two plasmids, except that the saturation level was lower with pCR1αMG. Under these conditions, the hybridization increased linearly with the amount of input globin mRNA, as shown in Figure 5b, indicating that DNA was in excess. Thus, the subsequent hybridization experiments were performed under the condition of DNA excess where 37% and 22% of the input globin mRNA were hybridized to pCRβMG and αpCRMG filters, respectively.

Nuclei were incubated in the presence and absence of a saturating amount of S-II, and RNA was labeled with [^{3}H]UTP. As a control, RNA was synthesized in the presence of 0.5 μg/mL of α-amanitin. These RNAs were mixed with ^{125}I-labeled globin mRNA and incubated with DNA filters. Under these conditions, 20%–37% of the ^{125}I-labeled globin mRNA hybridized to DNA filters, indicating that hybridization occurred in the condition of DNA excess. For determination of the background count, the same amount of RNA was hybridized to filters containing pCR1 plasmid DNA only. The results of experiments on hybridization of RNA

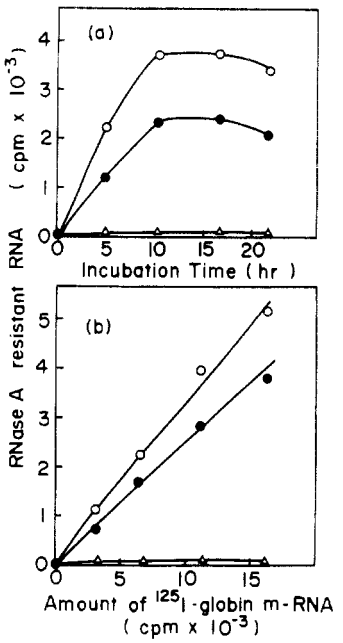

FIGURE 5: Time-course and dose–response curves of hybridization of globin mRNA to DNA immobilized on nitrocellulose filters. (a) ^{125}I-Labeled globin mRNA (10^4 cpm) (1.5×10^7 cpm/μg) was incubated at 41 °C with DNA filters containing pCR1αMG (●), pCR1βMG (○), and pCR1(△) DNA. At the times indicated, filters were removed, washed well, and treated with RNase A. The radioactivity of RNA resistant to RNase is plotted against the incubation time of hybridization. (b) Dose–response curve of hybridization. Increasing amounts of ^{125}I-labeled globin mRNA were incubated with DNA filters containing pCR1αMG (●), pCR1βMG (○), and pCR1 (△) DNA for 15 h at 41 °C. Filters were removed, washed well, and treated with RNase A. The radioactivity resistant to RNase digestion is plotted against the input of globin mRNA.

Table I: Hybridization to cDNA of Globin mRNA of RNA Synthesized in Isolated Nuclei of Spleen Cells from Anemic Mice

condition of RNA synthesis	input RNA (cpm)	hybridized RNA (cpm)[a] pCR1-αMG[b]	pCR1-βMG[b]	content of globin mRNA (%) α-chain mRNA	β-chain mRNA
+S-II	2.25×10^5	401	625	0.180	0.290
−S-II	1.46×10^5	195	298	0.133	0.204
+α-amanitin	1.04×10^5	8	14	0.008	0.014

[a] These values were corrected by subtracting the background radioactivity that bound to filters containing pCR1 DNA from the observed values. The background count was usually 40–70 cpm. [b] The efficiency of hybridization was simultaneously checked by mixing 2000 cpm of [^{125}I]globin mRNA with sample RNA; 23% and 37% of the input radioactivity were hybridized to pCR1αMG DNA filters, respectively.

synthesized in siolated nuclei to globin DNA are summarized in Table I. When $1.5–2.5 \times 10^5$ cpm of radioactive RNA was employed, significant radioactivity was trapped on DNA filters containing pCR1αMG or pCR1βMG. Under the same conditions, the background levels bound to filters containing plasmid DNA were 40–70 cpm. These results indicate that RNA synthesized in isolated nuclei contained a significant amount of RNA that could hybridize with globin DNA. This RNA must be synthesized by RNA polymerase II, because little hybridization was detected by using RNA synthesized in the presence of α-amanitin.

The content of globin mRNA in the total input RNA was calculated from the radioactivity hybridized to globin DNA sequences. As is evident from Table I, the contents of α-chain and β-chain mRNA in the synthesized RNA were 0.133% and 0.204%, respectively, in the absence of S-II and 0.180% and 0.290%, respectively, in the presence of S-II. This increase is probably not due to selective stimulation of globin mRNA but to stimulation of overall RNA synthesis by RNA polymerase II. Using these values, we determined the stimulation of globin mRNA synthesis by S-II. RNA synthesis by nuclei of spleen cells of anemic mice was performed in the presence and absence of S-II, and then the RNA was extracted. Simultaneous monitoring of α-amanitin-sensitive RNA synthesis showed that S-II stimulated RNA synthesis 1.78-fold.

Table II: Effect of S-II on Globin mRNA Synthesis in Isolated Nuclei

condition of RNA synthesis	total RNA synthesized[a] (cpm)	α-amanitin-sensitive RNA synthesis[b] (cpm)	amount of globin mRNA[c] α-chain mRNA	β-chain mRNA
−S-II	8.5×10^5	3.7×10^5 (1.00)	1130 (1.00)	1730 (1.00)
+S-II	10.7×10^5	6.6×10^5 (1.78)	1930 (1.71)	3120 (1.80)

[a] Radioactivity of overall RNA synthesized. [b] α-Amanitin-sensitive RNA synthesis in *a*. [c] The amount of globin mRNA was calculated on the basis of the content of globin mRNA shown in Table I.

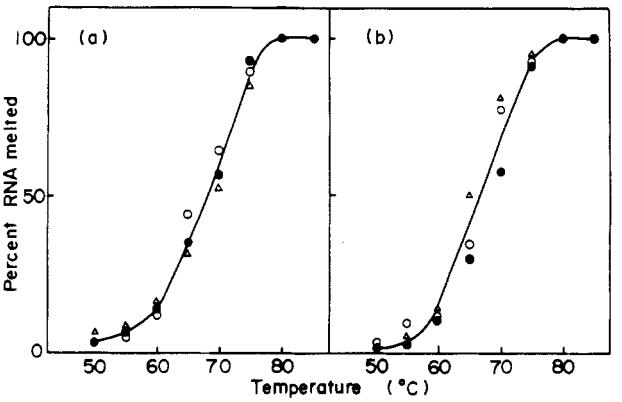

FIGURE 6: Heat stability of DNA–RNA hybrids. [^{3}H]RNA synthesized in isolated nuclei or [^{125}I]globin mRNA hybridized to pCR1αMG (a) and pCR1βMG (b) DNA were eluted from the filters at increasing temperatures as described under Materials and Methods, and thermal-elution curves were constructed by measuring the radioactivity of RNA eluted at various temperatures. (○) RNA synthesized in the presence of S-II; (●) RNA synthesized in the absence of S-II; (△) globin mRNA.

The radioactivities corresponding to α-chain and β-chain mRNA were calculated from the globin mRNA content in Table I, and the results were summarized in Table II. Clearly, the amounts of α-chain and β-chain mRNA synthesized in isolated nuclei in the presence of S-II increased: S-II stimulated synthesis of α-chain mRNA 1.71-fold and that of β-chain mRNA 1.80-fold. These values coincided well with that for its stimulation of overall α-amanitin-sensitive RNA synthesis. The results indicate that when S-II was added externally to isolated nuclei meaningful transcription, meaured by monitoring globin mRNA, increased with increase in overall RNA synthesis by RNA polymerase II. Here we measured only globin mRNA as a model of meaningful transcription. However, it is possible that S-II stimulates the synthesis of many other mRNAs as well as globin mRNA. Thus, it is likely that in isolated nuclei meaningful transcription occurs and externally added S-II enhances it.

Stability of DNA–RNA Hybrids. Before evaluating these results, it was necessary to determine whether the DNA-RNA hybridization detected was specific for the globin gene. As

an indicator of correct matching, we measured the heat stabilities of the hybrids and compared them with that of globin DNA–globin mRNA hybrid. Experiments performed by using pCR1αMG and pCR1βMG plasmids gave the same results. As is evident from Figure 6, the melting profiles of the globin DNA–RNA hybrids coincided well with that of the globin DNA–globin mRNA hybrid, indicating that matching of RNA and globin DNA is as specific as that of globin mRNA and globin DNA. Similar results were obtained with RNA preparations synthesized in the presence and absence of S-II. Thus, it is certain that globin mRNA was actually synthesized in isolated nuclei and that this synthesis was stimulated by externally added S-II, a factor specifically stimulating RNA polymerase II in vitro. However, it is not certain whether the synthesis of globin mRNA in isolated nuclei represents de novo synthesis or simply elongation of RNA chains that had been initiated in vivo.

Discussion

There are several reports of proteins that stimulate RNA polymerase II in various eukaryotic ells (Wang & Kostraba, 1978). However, the biological significance of these proteins is totally obscure. We purified one of these proteins, named S-II, from Ehrlich ascites tumor cells. This protein was found to be localized in the nucleoplasm, not the nucleoli. Antibody against S-II specifically inhibited α-amanitin-sensitive RNA synthesis in isolated nuclei, but it did not affect the activity of purified RNA polymerase II. Thus, we have suggested that for faithful transcription in vitro S-II should be included in a transcription system composed of purified components (Ueno et al., 1979).

In this paper, we showed that S-II enhances globin mRNA synthesis in isolated nuclei of spleen cells from anemic mice. This finding is important for several reasons. One reason is that the protein factor stimulating RNA synthesis in vitro was also shown to stimulate RNA synthesis in isolated nuclei, which is a fundamentally different system from the transcriptional system reconstituted in vitro from deproteinized DNA and purified RNA polymerase II. This is the first report that addition of a stimulatory factor externally actually affected transcription by RNA polymerase II in isolated nuclei.

Another reason is that the enhanced RNA synthesis was not random, but reflected RNA synthesis in the original nuclei, demonstrated as an increase in globin mRNA synthesis. Thus, externally added S-II stimulated meaningful RNA synthesis in isolated nuclei.

Since S-II is a nucleoplasmic protein (Sekimizu et al., 1979b), isolated nuclei should contain endogenous S-II. However, externally added S-II increased α-amanitin-sensitive RNA synthesis in the nuclei, although the increased level was at most only twice the original level. This stimulation by externally added S-II may have been because some S-II leaked out of the nuclei during their preparation or because they did not initially contain a saturating amount of S-II.

Our finding that increase in globin mRNA synthesis by S-II coincided with stimulation of overall α-amanitin-sensitive RNA synthesis in isolated nuclei is important, because it indicates that the stimulatory effect of S-II was not a mere artifact but of biological significance.

The molecular mechanism of stimulation of RNA synthesis by S-II is unknown. However, it is found that the RNA produced was larger when S-II was present in the reaction mixture in vitro (Natori et al., 1973). It is also known from in vitro experiments that the amount of the initiation complex increases in the presence of S-II (Sekimizu et al., 1977). But, it seems unlikely that conditions in isolated nuclei are the same as in the transcriptional system in vitro, because nuclei in situ contain endogenous S-II. As shown in Figure 4, S-II did not change the size of the RNA. Moreover, it is known that new initiation of RNA synthesis does not take place in isolated nuclei (Bitter & Roeder, 1978). Thus, at the moment, we cannot deduce anything about the mechanism of the stimulation of RNA synthesis by S-II in isolated nuclei, though it is clear that S-II enhances α-amanitin-sensitive, meaningful RNA synthesis significantly. It is possible that S-II has multiple functions: it may be necessary for initiation of RNA synthesis in the nuclei, and it may also be necessary for RNA polymerase II to continue RNA synthesis without falling from the template.

The present results suggested that S-II is involved in transcription of heterogeneous nuclear RNA in the nucleoplasm. One of the short-term goals of studies on eukaryotic transcription is to reconstruct a faithful transcriptional system in vitro from various components necessary for RNA synthesis. Such a system would contribute greatly to our understanding of the regulation of eukaryotic gene espression. Weil et al. (1979a) showed that, in addition to RNA polymerase II, some protein fractions are necessary for correct transcription of adenovirus late mRNA in vitro. Kuehn et al. (1979) demonstrated that a phosphorylated nonhistone protein stimulated rRNA synthesis in vitro. It was also demonstrated that some protein fraction was essential for transcription of 5S RNA gene (Ng et. al., 1979; Weil et al., 1979b). These experiments strongly suggest that purified RNA polymerase alone is not sufficient for faithful transcription in vitro, although RNA synthesis proceeds with RNA polymerase alone. For reconstruction of a faithful transcriptional system, much attention must be paid to proteins that affect transcription in vitro, and S-II is the first protein found to participate in transcription in isolated nuclei.

Before drawing this conclusion, experimental procedures should be carefully evaluated. Here, we assayed the amount of globin mRNA as an indicator of meaningful transcription. It is clear that the RNA synthesized in isolated nuclei contained globin mRNA sequences, because the heat-denaturation profiles of DNA–RNA hybrids were exactly the same as that of the DNA–globin mRNA hybrid. We carried out DNA–RNA hybridization under conditions of DNA excess where hybridization was proportional to the input of RNA. In order to ensure that hybridization was measured correctly, we always added a small amount of ^{125}I-labeled globin mRNA to the ^{3}H-labeled RNA synthesized in isolated nuclei and monitored hybridization of [^{125}I]RNA to the DNA filter. Since this hybridization was consistently between 22% and 37% and did not decrease in the presence of [^{3}H]RNA, it can be safely concluded that hybridization was measured under the condition of DNA excess.

As is evident from Figure 5, the efficiencies of hybridization with plasmids containing α-chain and β-chain sequences, respectively, were different. This may be partly due to the difference in the lengths of globin gene sequences integrated into the plasmids. Similar results should be obtained when the contents of α-chain and β-chain mRNA are different in our globin mRNA preparation. However, this difference in the efficiency of hybridization should not disturb our conclusion, because we compared hybridization of two RNA samples which were synthesized in the presence and absence of S-II under fixed conditions.

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