Difference in Phosphorylation of Two Factors Stimulating RNA Polymerase II of Ehrlich Ascites Tumor Cells†

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ABSTRACT: The structures of two protein factors, S-II and S-II′, that specifically stimulate RNA polymerase II from Ehrlich ascites tumor cells were compared. The two proteins behaved differently on CM-cellulose chromatography and on isoelectric focusing, although they were shown to have common antigenicity. The following findings strongly suggest that S-II and S-II′ have the same primary structure, but that S-II′ is more extensively phosphorylated than S-II: (1) S-II and S-II′ gave identical peptide maps when digested with various proteases. (2) S-II′ that had been treated with alkaline phosphatases had the same mobility on sodium dodecyl sulfate–polyacrylamide gel as S-II, indicating that it could be converted to S-II by hydrolysis of its phosphate residues. (3) S-II′ was phosphorylated more than S-II when Ehrlich ascites tumor cells were labeled in vivo with [^{32}P]orthophosphate.

RNA polymerase II is known to catalyze the synthesis of heterogeneous nuclear ribonucleic acid (RNA) in the nucleoplasm (Roeder, 1976), and so elucidation of the control of its activity should provide useful information on regulation of eukaryotic gene expression. For this reason, there have been many studies on proteins that specifically stimulate (Stein & Hausen, 1970; Natori, 1972; Seifart et al., 1973; Lee & Dahmus, 1973; Sugden & Keller, 1973; Natori et al., 1973; Kostraba et al., 1975; Sekimizu et al., 1976; Nakanishi et al., 1978; Benson et al., 1978; Spindler, 1979; Revie & Dahmus, 1979; Swandogo et al., 1980) or repress (Natori et al., 1974, 1975; Kostraba & Wang, 1975) the activity of RNA polymerase II in vitro. Recently, we purified two protein factors, named S-II and S-II′, from Ehrlich ascites tumor cells which specifically stimulate the activity of RNA polymerase II, and we also raised antibody against S-II (Sekimizu et al., 1979a). S-II and S-II′ seem to have essential roles in messenger RNA synthesis in vivo, for the following reasons: (1) Immunofluorescent studies showed that S-II is localized in the nucleoplasm like RNA polymerase II, not in the nucleoli (Sekimizu et al., 1979b). (2) Proteins having common antigenicity with S-II were found in the nucleoplasm of HeLa cells and in salivary gland cells of flesh-fly larvae, indicating that an S-II-like protein is ubiquitously present in the nucleoplasm of eukaryotic cells (Sekimizu et al., 1979b). (3) Antibody against S-II selectively inhibited α-amanitin-sensitive RNA synthesis in isolated nuclei, but it did not affect the activity of purified RNA polymerase II, suggesting that S-II is a regulatory protein and not a subunit of RNA polymerase II (Ueno et al., 1979).

Antibody against S-II was found to inhibit the activity of both S-II and S-II′, indicating that these two proteins have common antigenicity though they behave differently on CM-cellulose chromatography (Sekimizu et al., 1979a).

This paper describes structural studies on S-II and S-II′, showing that S-II′ is basically the same protein as S-II but differs in its extent of phosphorylation. Thus, the heterogeneity of these stimulatory factors may be partly explained by differences in the extent of modification of a single protein.

Experimental Procedures

Materials. Experimental materials were obtained from the following sources: chymotrypsinogen, chymotrypsin, ficin, myoglobin, conalbumin, phosphoserine, and phosphothreonine from Sigma Chemical Co.; bovine serum albumin (fraction V) from Amersham/Searle; *Staphylococcus aureus* V-8 protease from Miles Laboratories; *Escherichia coli* alkaline phosphatase from Worthington Biochemical Co.; human placental alkaline phosphatase from Dr. K. Matsui (Ichimaru Co., Japan); [^{32}P]orthophosphate (carrier free) from Japan Atomic Energy Research Institute. Before use, [^{32}P]orthophosphate was heated in 0.1 N HCl in boiling water for 30 min and then neutralized with 1 N NaOH.

Purification of S-II and S-II′. Two protein factors, S-II and S-II′, which specifically stimulate RNA polymerase II were purified to homogeneity from Ehrlich ascites tumor cells as described before. One unit of stimulatory activity was defined as the amount that enhanced the activity of 10 units of RNA polymerase II to 11 units under the standard assay conditions. Details of the procedures for purification and assay of these proteins are described previously (Sekimizu et al., 1979a).

Antibody against S-II. Antibody against S-II was prepared as described previously (Sekimizu et al., 1979a). Immunoglobulin G was purified by ammonium sulfate fractionation and DEAE-cellulose chromatography by the method of McCauley & Racker (1973).

Polyacrylamide Gel Electrophoresis in NaDodSO$_4$.[1] Electrophoresis on a NaDodSO$_4$–polyacrylamide slab gel was carried out by the method of Laemmli (1970). Proteins were denatured by heating in 1% NaDodSO$_4$ solution containing 2% β-mercaptoethanol for 20 min at 75 °C. The protein sample (0.2–5 μg) was mixed with a small amount of bromphenol blue as a tracking dye and put into a 4-mm-wide well

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[1] Abbreviations used: NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; PBS, phosphate-buffered saline; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Cl_3CCOOH, trichloroacetic acid.

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in 1-mm-thick gel. The stacking gel (3% polyacrylamide) was about 1 cm long, and the separating gel (12.5% polyacrylamide) was about 7 cm long. Samples were subjected to electrophoresis at 50 V, and when the tracking dye entered the separating gel the voltage was increased to 100 V. After electrophoresis, the gels were stained by the method of Fairbanks et al. (1971) with Coomassie brilliant blue R250.

Isoelectric Focusing. Isoelectric focusing in 5% polyacrylamide gel was carried out essentially as described by Vesterberg (1972). A 50-mL sample of a solution of 9.7% acrylamide and 0.3% bis(acrylamide) (both from Eastman Kodak Co.) was mixed with 0.5 g of Amberlite MB-1 (Organo Co., Japan) with stirring for 1 h at 20 °C to remove contaminating ionic substances, and then used for making slab gel (6 × 10 × 0.1 cm) containing 2% Ampholine (LKB, pH 3.5–10). Isoelectric focusing was carried out by using an isoelectric focusing apparatus (flatbed apparatus, FBE 3000, Pharmacia Fine Chemicals). Before samples were loaded on, preelectrophoresis was done at 500 V for 30 min. Samples (1–3 μg of protein) in 20 μL of 10 mM Tris-HCl, pH 7.9 (25 °C), 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM KCl, 50% glycerol, 5 mM β-mercaptoethanol, and 0.35% Triton X-100 were applied with a sample applicator, and electrophoresis was carried out successively at 600, 700, 800, and 900 V for 5-min periods. Then the voltage was increased to 990 V, and electrophoresis was continued for 280 min. After electrophoresis, the pH of extracts of gel slices was measured. Gels were stained by Coomassie brilliant blue G250 according to Vesterberg (1972).

Peptide Mapping Analysis by $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The patterns of partial proteolytic digests of S-II and S-II′ were compared in $NaDodSO_4$–polyacrylamide gel by the method of Cleveland et al. (1977). A sample of 2 μg of S-II and S-II′ was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The gel was stained, and regions corresponding to bands of proteins were cut out. Each gel slice was placed in a well of another $NaDodSO_4$–polyacrylamide slab gel consisting of stacking gel (3% polyacrylamide) and separating gel (15% polyacrylamide). Various amounts of peptidases (*Staphylococcus aureus* V-8 protease or chymotrypsin) were added to each well, and electrophoresis was carried out at 20 °C. Proteins were partially digested during electrophoresis, and the resulting peptides were separated on the gel.

Tryptic Peptide Analysis of S-II and S-II′. Peptide analysis was carried out by the method of Elder et al. (1977) by using radioiodinated protein in polyacrylamide gel. Polyacrylamide gel slices (5 × 1 × 1 mm) containing 1 μg of S-II or S-II′ were washed extensively with 25% isopropyl alcohol, and then with 10% methanol to remove $NaDodSO_4$, and dried under a heat lamp. Dried gel slices were put into 20 μL of 0.5 M phosphate buffer (pH 7.5), and proteins were radioiodinated by incubation with 300 μCi of ^{125}I (Amersham/Searle, 15 mCi/μg) and 5 μL of chloramine T (1 mg/mL) for 1 h at 27 °C. Control gel slices containing no protein were treated in the same way to determine the background count. The reaction was stopped by adding 1 mL of sodium bisulfite (1 mg/mL), and the gel slices were washed with 10 mL of 10% methanol, changing the solution 10 times at 2-h intervals. The gel slices were then wrapped in nylon mesh and washed in 1 L of 10% methanol with extensive stirring for 12 h. The washing solution was changed once after 2 h. The gel slices were then dried, homogenized in 0.5 mL of TPCK-treated trypsin (50 μg/mL) in 50 mM triethylamine–bicarbonate buffer (pH 8.0), and incubated for 20 h at 37 °C. The supernatants were removed, lyophilized, and dissolved in 50 μL of a mixture of acetic acid, formic acid, and water (15:5:80). Samples of 1 μL (2×10^5 cpm) were spotted on cellulose-coated TLC plates (20 × 20 cm, Merck) and subjected to electrophoresis at 0 °C in a mixture of acetic acid, formic acid, and water (15:5:80) at 950 V for 50 min. Then the plates were dried, and peptides were chromatographed in a mixture of butanol, pyridine, acetic acid, and water (32.5:25:5:20) for 5 h. The plates were again dried and analyzed by autoradiography with Fuji Rx-s film.

Analysis of Immunoprecipitates Prepared from a Crude Extract of ^{32}P-Labeled Ehrlich Ascites Tumor Cells and Antibody against S-II. [^{32}P]Orthophosphate (5 mCi) was injected into the abdominal cavity of two male ddY mice 8 days after inoculation of 3×10^6 Ehrlich ascites tumor cells. Cells were harvested 4 h later and suspended in 27 mL of cold water to disrupt erythrocytes. Then 3 mL of 10× PBS was added, the mixture was centrifuged for 5 min at 800*g*, and the resulting precipitate of white cells, weighing about 3 g, was washed twice with 30 mL of cold PBS and stored at −80 °C.

A crude cell extract was prepared from these cells as described previously (Sekimizu et al., 1979a). A sample of 2 mg of protein, at the step of the second ammonium sulfate precipitation, was mixed with 2.5 mg of antibody against S-II or immunoglobulin G from nonimmunized rabbit sera in 200 μL of 10 mM Tris-HCl, pH 7.9 (25 °C), 5 mM NaCl, 2.5 mM β-mercaptoethanol, and 5% glycerol and incubated for 2 h at 25 °C. The resulting precipitate was collected by centrifugation for 5 min at 1500*g*, washed 3 times with 2 mM Tris-HCl, pH 6.8 (25 °C), 0.2 M NaCl, and 1% Triton X-100, and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. Autoradiography was done by using Fuji Rx-s film with an intensifying screen at −80 °C.

Treatment of S-II and S-II′ with Alkaline Phosphatase. A sample of 0.2 μg of S-II or S-II′ was incubated with alkaline phosphatase from *E. coli* (0.25 unit) or from human placenta (0.14 unit) in 40 μL of 10 mM Tris-HCl, pH 7.9 (25 °C), 0.1 mM EDTA, 10 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 50% glycerol, and 0.3% Triton X-100 at 37 °C for 2 h. One unit of activity was defined as the amount which hydrolyzed 1 μmol of *p*-nitrophenyl phosphate under the conditions described by Garen & Levinthal (1960). After incubation, the reaction mixture was chilled on ice, mixed with 0.5 mL of 5% Cl_3CCOOH, and centrifuged at 8800*g* for 15 min. The resulting precipitate was dissolved in 50 μL of 1% $NaDodSO_4$, 2% β-mercaptoethanol, 200 mM Tris-HCl, pH 9 (37 °C), 0.05 mM EDTA, and 12% glycerol, heated for 2 min at 100 °C, and then subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis.

Determination of Phosphorylated Amino Acids. [^{32}P]-Orthophosphate (5 mCi) was injected into the abdominal cavity of four male ddY mice 8 days after inoculation of 3×10^6 Ehrlich ascites tumor cells. After 4 h, the cells were harvested, and S-II and S-II′ were isolated from the cells as described previously (Sekimizu et al., 1979a). Partially purified S-II′ was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, and the band corresponding to S-II′ was cut out, washed with 10% methanol, and dried. The gel slice was then pulverized in 50 mM triethylamine–bicarbonate buffer (pH 8.0) and incubated first for 2 h at 41 °C and then for 12 h at 37 °C to extract phosphorylated S-II′. Then the mixture was centrifuged at 15000*g* for 3 min, and S-II′ was precipitated from the resulting clear supernatant by adding Cl_3CCOOH at a final concentration of 20%. The precipitate was washed 3 times with a mixture of ethanol and ether (1:1) to remove Cl_3CCOOH and then hydrolyzed in 4 N HCl at

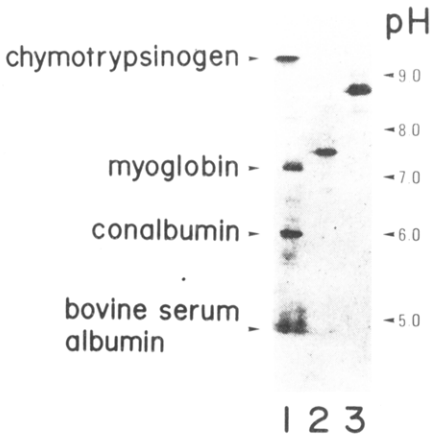

FIGURE 1: Isoelectric focusing of S-II and S-II′. Samples of 1 μg of purified S-II′ (lane 2) and S-II (lane 3) were subjected to electrophoresis in pH gradients with various marker proteins (lane 1). After electrophoresis, the gel was stained with Coomassie brilliant blue G250. The pH of the extracts of gel slices was also measured.

110 °C for 4 h in a sealed glass vial. The hydrolysate was diluted with water, lyophilized, dissolved in 10 μL of electrophoresis buffer consisting of formic acid, acetic acid, and water (25:87:887), and spotted on cellulose-coated TLC plates with standard phosphorylated amino acids. Electrophoresis was carried out in a cold-plate apparatus at 950 V for 1.5 h. ^{32}P-labeled compounds were identified by autoradiography by using Fuji Rx-s film with an intensifying screen at −80 °C as reported before (Segawa et al., 1977).

Protein Determination. Protein was determined by the method of Lowry et al. (1951). For assay of protein in the presence of Triton X-100, $NaDodSO_4$ was added at a concentration of 0.1% just before addition of phenol reagent, because otherwise a precipitate appeared that prevented accurate measurements. As a control, a known concentration of bovine serum albumin was assayed in parallel with the samples under the same conditions.

Results

Differences in the Isoelectric Points of the Two Stimulatory Proteins. Previously, we purified two proteins specifically stimulating RNA polymerase II from Ehrlich ascites tumor cells. Since the antibody raised against one of them, S-II, cross-reacted with the other protein, S-II′, these two proteins have common antigenicity. However, they behaved differently on CM-cellulose chromatography, being eluted as two distinct peaks when the column was developed with a linear gradient of NaCl. On $NaDodSO_4$–polyacrylamide gel electrophoresis, S-II′ migrated a little slower than S-II, and the molecular weights of the proteins were estimated to be 41 000 and 40 500, respectively (Sekimizu et al., 1979a). From these findings, we concluded that these two proteins probably have different ionic properties although they have similar molecular weights. If this conclusion is correct, the two proteins should behave differently in isoelectric focusing.

Figure 1 shows the isoelectric focusing patterns of S-II and S-II′ with various marker proteins. S-II and S-II′ each gave a single band on the gel, and their p*I* values were determined to be 8.7 and 7.5, respectively, by measuring the pH of extracts of gel slices.

There are two possible explanations of why S-II′ is more negatively charged than S-II. One is that S-II and S-II′ may be completely different proteins although they have common antigenicity and that S-II′ may contain more acidic amino acids than S-II. The other possibility is that S-II and S-II′ may be essentially the same protein, but S-II′ may be modified to be more acidic.

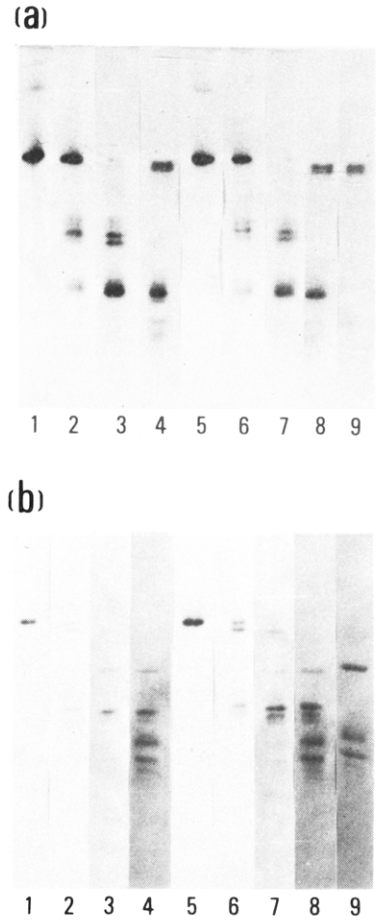

FIGURE 2: Partial digestion of S-II and S-II′ with two proteases. Samples of 2 μg of S-II and S-II′ were partially digested with various concentrations of *Staphylococcus aureus* V-8 protease (a) or chymotrypsin (b) in $NaDodSO_4$–polyacrylamide gel by the method of Cleveland et al. (1977). The gel was stained after electrophoresis. (a) Lanes 2–4, peptides released from S-II; lanes 6–8, peptides released from S-II′. Lanes 1 and 5, S-II and S-II′ without enzyme; lanes 2–4 and 6–8, peptides released from S-II and S-II′, respectively, by digestion with 10 ng, 100 ng, and 1 μg of V-8 protease. Lane 9, electrophoretic pattern of 1 μg of V-8 protease. (b) Lanes 2–4, peptides released from S-II; lanes 6–8, peptides released from S-II′. Lanes 1 and 5, S-II and S-II′ without enzyme; lanes 2–4 and 6–8, peptides released from S-II and S-II′, respectively, by digestion with 0.2, 1, and 5 μg of chymotrypsin. Lane 9 shows the electrophoretic pattern of 5 μg of chymotrypsin.

Partial Digestion of S-II and S-II′ by Various Proteases. To test the structural similarity of S-II and S-II′, we compared the patterns of their hydrolysis products on $NaDodSO_4$–polyacrylamide gels by the method of Clevelend et al. (1977). As shown in Figure 2, *Staphylococcus aureus* V-8 protease and chymotrypsin both hydrolyzed S-II and S-II′, liberating specific proteolytic fragments. The electrophoretic patterns of these peptides on $NaDodSO_4$–polyacrylamide gels changed with the concentrations of proteases used, but with fixed concentrations of proteases, the patterns of the products of S-II and S-II′ were almost indistinguishable. For instance, comparison of tracks 3 and 7 in Figure 2a for the products of S-II and S-II′, respectively, shows that on treatment with 100 ng of V-8 protease the bands of S-II and S-II′ disappeared with formation of three distinct bands of proteolytic fragments. These peptides were not those of V-8 protease itself, because 1 μg of the enzyme gave only one band, as shown in track 9. Since the distributions and densities of these bands of the products of S-II and S-II′ were almost identical, these two

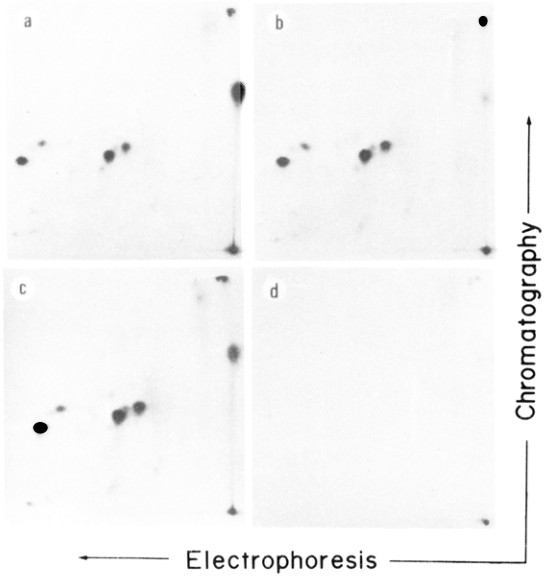

FIGURE 3: Comparison of tyrosine-containing tryptic peptides of S-II and S-II′. A 1-μg sample of S-II or S-II′ was subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis. The band of protein was cut out from the gel. Protein was radioiodinated in the gel slice, digested with trypsin, and analyzed by electrophoresis followed by thin-layer chromatography. Autoradiograms are shown: (a) S-II; (b) S-II′; (c) mixture of S-II and S-II′; (d) background, i.e., a gel slice containing no protein treated in the same way.

proteins are probably structurally very similar. Similar results were obtained with another protease, ficin (data not shown). However, since the number of detectable proteolytic peptides is small, it is difficult to conclude from this type of experiment that S-II and S-II′ are structurally identical.

Peptide Map of Radioiodinated S-II and S-II′. To examine the structural similarity of S-II and S-II′ more precisely, we digested the radioiodinated proteins with trypsin and compared the resulting peptide maps. For this purpose, S-II and S-II′ were radioiodinated in polyacrylamide gel, extracted from gel slices, and digested with trypsin. The resulting tryptic peptides were then analyzed by electrophoresis followed by thin-layer chromatography. As shown in Figure 3a,b, more than ten spots are detectable. However, the distributions of the spots obtained from S-II and S-II′ were almost identical, indicating that these two proteins are very similar. Moreover, a mixture of the tryptic peptides of S-II and S-II′ gave a pattern of spots that was fundamentally indistinguishable from that of S-II or S-II′, as shown in Figure 3c. It was important to determine the background level in these experiments to confirm the origin of the tryptic peptides. As shown in Figure 3d, no significant spots corresponding to those in a, b, and c could be detected when a gel slice containing no protein was treated in the same way.

Thus, we conclude that S-II and S-II′ are fundamentally the same protein, and that when radioiodinated and digested with trypsin the two proteins gave qualitatively identical tryptic peptides.

Treatment of S-II and S-II′ with Alkaline Phosphatases. Since the above findings showed that S-II and S-II′ are essentially the same protein, the question arose of why S-II′ is more acidic. The most probable explanation seemed to be that S-II′ is a phosphorylated form of S-II, because if some of the serine or threonine residues are phosphorylated the protein would be more acidic. If this is so, S-II′ should be converted to S-II on treatment with alkaline phosphatase. We examined this possibility and found that S-II′ was in fact converted to a protein with the same mobility as S-II on NaDodSO$_4$–polyacrylamide gel electrophoresis when it was incubated with alkaline phosphatase. This conversion of S-II′ to S-II occured on treatment of S-II′ with *E. coli* or human placental alkaline phosphatase, and it was completely inhibited by the presence of 7 mM sodium phosphate, which is a potent inhibitor of these enzymes.

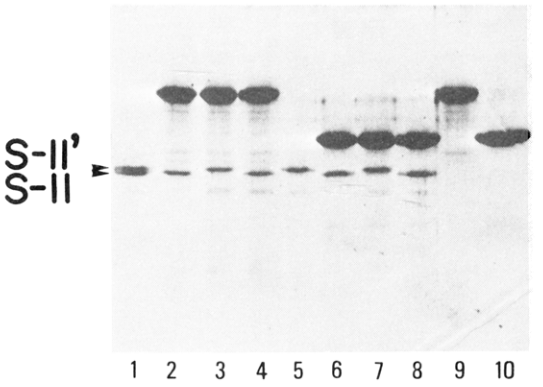

FIGURE 4: Treatment of S-II and S-II′ with alkaline phosphatases. A 0.2-μg sample of S-II or S-II′ was incubated with alkaline phosphatase from *E. coli* (0.25 unit) or from human placenta (0.14 unit) for 2 h at 37 °C and then subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis. Lane 1, mixture of S-II and S-II′; lane 2, S-II treated with placental enzyme; lane 3, S-II′ treated with placental enzyme in the presence of 7 mM sodium phosphate; lane 4, S-II′ treated with placental enzyme; lane 5, S-II′ alone; lane 6, S-II treated with *E. coli* enzyme; lane 7, S-II′ treated with *E. coli* enzyme in the presence of 7 mM sodium phosphate; lane 8, S-II′ treated with *E. coli* enzyme; lane 9, placental alkaline phosphatase alone; lane 10, *E. coli* alkaline phosphatase alone.

The results in Figure 4 show that S-II′, which migrates a little slower than S-II, changed to a form migrating in the same position as S-II on treatment with alkaline phosphatases. The bands of S-II′ and the product of S-II′ were clearly distinguishable from those of the alkaline phosphatases. When S-II was treated with alkaline phosphatases, its mobility on NaDodSO$_4$–polyacrylamide gel did not change significantly. Thus, there seem to be few, if any, phosphorylated amino acids in S-II.

Since the primary structures of S-II and S-II′ are quite similar, it seemed likely from the results described above that S-II′ is a phosphorylated form of S-II. If this is true, it would explain why S-II′ was eluted from CM-cellulose at a lower salt concentration than S-II and why the isoelectric point of S-II′ is lower than that of S-II. As reported before, both S-II and S-II′ have activity to stimulate RNA polymerase II (Sekimizu et al., 1979a). We also tested the stimulatory activity of S-II′ after treatment with alkaline phosphatase and found that it was not lost, indicating that the phosphate residues in S-II′ are not directly related to the activity of this protein.

Analysis of Immunoprecipitates. Is S-II′ really a phosphorylated protein? To answer this question, we labeled Ehrlich ascites tumor cells with [^{32}P]orthophosphate to see if the radioactivity is incorporated into S-II′. For this, [^{32}P]orthophosphate was injected into the abdominal cavity of tumor-bearing mice, and 4 h later the ascites tumor cells were harvested and fractionated according to our previous procedures. The antibody against S-II was added to the S-II fraction, which should also contain S-II′, at the step of the second ammonium sulfate precipitation. The resulting immunoprecipitates were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis and autoradiography. As shown in Figure 5a, the immunoprecipitates contained both S-II and S-II′. In addition to these two proteins, several distinct bands

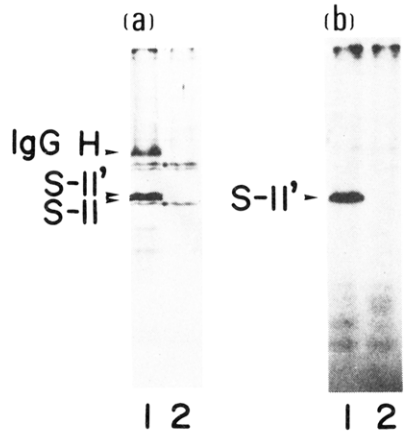

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of the immunocomplex (a) and corresponding autoradiogram (b). Crude S-II fraction (second ammonium sulfate precipitate fraction; Sekimizu et al., 1979a) from ^{32}P-labeled Ehrlich ascites tumor cells was mixed with immunoglobulin G. The resulting precipitate was washed well and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. The gel was stained and then autoradiographed. Lane 1, complex with the antibody; lane 2, complex with nonimmunized rabbit immunoglobulin G. Under these electrophoretic conditions, the light chain of immunoglobulin G diffused into the gel.

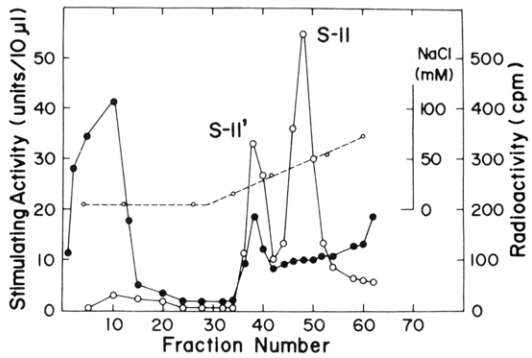

FIGURE 6: CM-cellulose chromatography of S-II and S-II′. S-II and S-II′ were partially purified from 200 g of ^{32}P-labeled Ehrlich ascites tumor cells and separated on a column of CM-cellulose (1 × 4 cm). Fractions of 1.8 mL were collected in the region of linear-gradient elution, and samples of 10 µL were assayed for activity to stimulate RNA polymerase II (O—O). Radioactivity in 1 mL of each fraction was measured in a liquid scintillation counter (●—●). (O---O) NaCl concentration.

of proteins that precipitated nonspecifically (probably insoluble proteins) were also present.

The autoradiogram corresponding to Figure 5a was shown in Figure 5b. As expected, only one band corresponding to S-II′ was found to be phosphorylated. It is premature to conclude from this experiment that S-II is not phosphorylated, but the results show clearly that when Ehrlich ascites tumor cells were labeled with [^{32}P]orthophosphate under fixed conditions S-II′ was phosphorylated and that its extent of phosphorylation was much greater than that of S-II.

Identification of Phosphorylated Amino Acids in S-II′. The amount of S-II′ recovered from immunoprecipitates was too small to determine the chemical nature of the labeled compounds. Therefore, we partially purified S-II and S-II′ from ^{32}P-labeled Ehrlich ascites tumor cells. The elution profiles of these two stimulatory factors from CM-cellulose are shown in Figure 6. Two distince peaks with activity to stimulate RNA polymerase II were eluted with an NaCl gradient as reported before, and the radioactivity mainly coincided with the peak of S-II′ which was eluted at lower salt concentration. Significant radioactivity was also detected in the region of S-II,

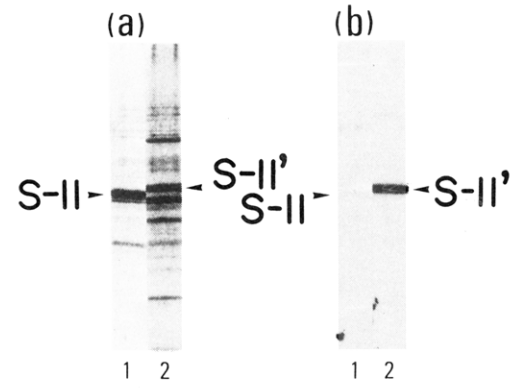

FIGURE 7: Evidence that S-II and S-II′ are phosphorylated proteins. Fractions 36–40 (S-II′) and 46–50 (S-II) in Figure 6 were separately combined and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis followed by autoradiography. (a) Staining pattern of S-II and S-II′ fractions; (b) corresponding autoradiogram. Lane 1, S-II fraction; lane 2, S-II′ fraction. Arrows indicate the positions of S-II and S-II′.

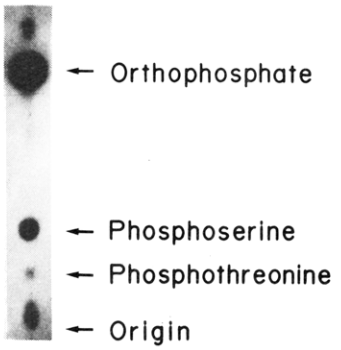

FIGURE 8: Identification of phosphorylated amino acids in S-II′. The band of protein corresponding to S-II′ in Figure 7 was cut out from the gel and extracted. The S-II′ obtained was hydrolyzed in 4 N HCl. Phosphorylated amino acids were analyzed by thin-layer chromatography with phosphoserine and phosphothreonine as markers. The spots of ^{32}P-labeled compounds were detected by autoradiography and those of authentic phosphorylated amino acids with ninhydrin stain.

but it did not show a clear peak, suggesting that it was due to contaminating phosphorylated proteins.

The fractions with stimulatory activity were combined separately and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and then autoradiography. The results are shown in Figure 7. When the gel was stained, bands of S-II and S-II′ were clearly detected, but the radioactivity in the corresponding autoradiogram mainly coincided with S-II′.

Slight radioactivity was also detected in the band of S-II. This is not due to contaminating S-II′, because S-II′ has a different mobility. It is very likely that S-II itself also contains some phosphorylated amino acids, although their content is much less compared to S-II′.

The band of S-II′ was cut out from the polyacrylamide gel, and S-II′ was extracted with triethylamine–bicarbonate buffer and hydrolyzed in 4 N HCl at 110 °C. The resulting amino acids were analyzed by thin-layer chromatography followed by autoradiography. As standards, phosphoserine and phosphothreonine were applied to the thin-layer plates. As shown in Figure 8, four distinct spots were detected on the autoradiogram, and three of these were identified as inorganic phosphate, phosphoserine, and phosphothreonine. A spot migrating faster than inorganic phosphate may be pyrophosphate, but it was not identified. There was much more phosphoserine than phosphothreonine, indicating that most of

the phosphorylated amino acid residues in S-II′ are serine residues. Significant radioactivity, possibly due to unhydrolyzed protein, was also detected at the origin. A similar experiment on S-II was not carried out because the radioactivity in S-II was too low.

Discussion

In this work, we examined the structures of two proteins, from Ehrlich ascites tumor cells, S-II and S-II′, which specifically stimulate the activity of RNA polymerase II. Since the yields of these proteins are low (about 1 mg of S-II and 0.3 mg of S-II′ from 1 kg of Ehrlich ascites tumor cells), we mainly used the radiolabeling technique and $NaDodSO_4$–polyacrylamide gel electrophoresis for analysis.

We carried out this work because we had previously found that the two proteins showed different behaviors on CM-cellulose chromatography and on $NaDodSO_4$–polyacrylamide gel electrophoresis, but had common antigenicity. From the results of this work, we concluded that S-II and S-II′ have basically the same primary structure, but S-II′ is more extensively phosphorylated. In connection with this conclusion, several points require discussion.

Experiments on partial proteolysis and peptide analysis of S-II and S-II′ clearly showed that S-II and S-II′ have basically the same structure but did not show whether the primary structures of S-II and S-II′ are completely the same: a few amino acids of S-II and S-II′ may differ, and these differences may not be detectable by the analytical procedures employed. This point should be confirmed by an analysis of the N-terminal amino acids of these proteins, but it was impossible because only very small amounts of proteins were available.

We found that S-II′ was phosphorylated more than S-II, but we could not detect any appreciable difference between the peptide maps of S-II and S-II′. In our experiment, only peptides which contained radioiodinated amino acids were detectable. Thus, if phosphorylated amino acids were not present in these peptides, they would not be detectable, and, thus, there would be no apparent difference in the peptide maps. It is also possible that phosphorylated peptides were too weakly labeled to be detectable in this experiment.

When S-II′ was treated with alkaline phosphatases, the mobility of its product coincided with that of S-II on a $NaDodSO_4$–polyacrylamide gel. This change was probably not due to proteolysis by proteases contaminating the alkaline phosphatases because *E. coli* and human placental alkaline phosphatases both gave the same result. Moreover, this reaction was completely inhibited by inorganic phosphate, which specifically inhibits alkaline phosphatases. Thus, we believe that the change in mobility of S-II′ on polyacrylamide gel after treatment was due to dephosphorylation. However, we could not directly detect the release of inorganic phosphate from S-II′ on treatment with alkaline phosphatases.

We showed that both S-II and S-II′ are phosphorylated proteins and that the extent of phosphorylation of S-II′ was much greater than that of S-II. This difference in phosphorylation can explain why the two proteins behave differently on isoelectric focusing and on CM-cellulose chromatography: it is reasonable that S-II′ has a more acidic isoelectric point and that it is eluted from CM-cellulose at a lower salt concentration than S-II. Labeling experiments showed that S-II was phosphorylated, but its extent of phosphorylation was too small to be detectable by analysis of immunoprecipitates. Moreover, after treatment with alkaline phosphatase, S-II did not show any detectable change in mobility on a $NaDodSO_4$–polyacrylamide gel. Thus, the phosphate residues in S-II may be protected in some way from attack by alkaline phosphatases. It is also possible that the difference in the molecular weights of S-II before and after hydrolysis of phosphate residues is too small to be detectable by $NaDodSO_4$–polyacrylamide gel electrophoresis.

We identified the phosphorylated amino acids in S-II′ mainly as serine with a small amount of phosphothreonine. Possibly phosphothreonine is common to S-II and S-II′, and in addition S-II′ contains phosphorylated serine residues. But since a significant amount of inorganic phosphate was released during hydrolysis of ^{32}P-labeled S-II′, as shown in Figure 8, the observed ratio of phosphoserine to phosphothreonine may not represent the true ratio of these amino acids in S-II′.

Since we found that the main structural difference between S-II and S-II′ is in their extent of phosphorylation, the question arises of the biological significance of phosphorylation of these factors. Recently, Kuehn et al. (1979) reported a change in the activity of a factor stimulating ribosomal RNA synthesis of the slime mold *Physarum polycephalum* by phosphorylation and dephosphorylation. We found that S-II and S-II′ both stimulated RNA polymerase II in vitro, but in an assay of RNA synthesis we used the total DNA extracted from Ehrlich ascites tumor cells as a template, and this probably contained very many initiation points of transcription. Recently, participation of specific proteins was suggested to be necessary for accurate transcription by RNA polymerase II or III, in vitro (Weil et al., 1979; Engelke et al., 1980). Jolly & Bogorad (1980) showed preferential transcription of cloned maize DNA sequences by homologous RNA polymerase in the presence of a specific protein. Elucidation of the physiological role of phosphorylation and dephosphorylation of these factors stimulating RNA polymerase II should provide useful information for understanding the regulation of eukaryotic gene expression.

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Sequential Translocation of Two Phenobarbital-Induced Polysomal Messenger Ribonucleic Acids from the Nuclear Envelope to the Endoplasmic Reticulum†

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ABSTRACT: Quantitation of rat liver messenger ribonucleic acids (mRNAs) coding for epoxide hydratase and NADPH–cytochrome *c* oxidoreductase was accomplished with a rabbit reticulocyte protein synthesizing system in conjunction with immunoprecipitation with monospecific antibodies. Translatable levels of both mRNAs were found associated with nuclear envelope bound, rough endoplasmic reticulum bound, and rapidly sedimenting endoplasmic reticulum bound polysomes. Soon after a single dose of phenobarbital, translatable levels of these mRNAs rose in all three membrane fractions; however, the rates of increase were markedly different. Elevated levels of these mRNAs first appeared in the nuclear envelope bound polysomes and then in the polysomes bound to the rough endoplasmic reticulum. Maximal levels that were approximately 3- and 5-fold above the control level for oxidoreductase and epoxide hydratase mRNAs, respectively, were reached for both membrane systems 4–6 h after administration of the drug. These levels then declined concomitantly with an increase in the translatable levels of these mRNAs associated with rapidly sedimenting endoplasmic reticulum, which peaked and stabilized at a level approximately 5-fold above the control value 14–20 h after administration of phenobarbital. Increased in vivo synthesis of nuclear envelope epoxide hydratase and oxidoreductase paralleled closely the levels of their mRNAs associated with the rough endoplasmic reticulum but did not correlate with the rapid increase in their nuclear envelope mRNA levels. The increased synthesis in vivo of these enzymes in the microsomal membrane, however, paralleled closely the increased levels of their respective polysomal mRNAs associated with rapidly sedimenting endoplasmic reticulum. These data strongly indicate that newly synthesized phenobarbital-induced mRNAs first become associated with the nuclear envelope and the adjacent rough endoplasmic reticulum. Polysomes containing these mRNAs are then translocated via the rough endoplasmic reticulum to a region of the endoplasmic reticulum morphologically characterized by closely positioned parallel arrays of rough-surfaced membrane, where the messages are stabilized.

The nuclear envelope is composed of an inner and outer membrane, joined via the pore complex structure. A unique structural element of the envelope is the pore complex lamina, a lipid-free subfraction composed primarily of three polypeptides (Dwyer & Blobel, 1976). Topological interrelationships exist between two of these polypeptides, and two show extensive sequence homology (Lam & Kasper, 1979). The physicochemical and biochemical features of the nuclear envelope have been reviewed (Kay & Johnston, 1973; Franke & Scheer, 1974; Kasper, 1974b; Wunderlich et al., 1976; Maul, 1977; Harris, 1978; Zbarski, 1979); however, much remains to be learned about its biogenesis and its role in nucleocytoplasmic transport. In these regards, the phosphorylation of a pore complex lamina associated protein by an endogenous nuclear envelope protein kinase has been described (Lam & Kasper, 1978) which may play a role in RNA transport (Agutter et al., 1979) as well as in the polymerization and depolymerization of the nuclear envelope during mitosis (Gerace & Blobel, 1980).

Biosynthesis and regulation of enzymes associated with the nuclear envelope are poorly understood. It is interesting to

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