Methods Enzymology 17B, 47.
Mehler, A. H., and Tabor, H. (1953), *J. Biol. Chem. 201,* 775.
Millar, I. T., and Springell, H. D. (1966), Sidgwick's Organic Chemistry of Nitrogen, 3rd ed, Oxford, Clarendon Press, p 505.
Neufeld, E., Harell, A., and Chayen, R. (1971), *Biochim. Biophys. Acta 237,* 465.
Niedrich, H., and Grupe, R. (1965), *J. Prakt. Chem. 27,* 108.
Okamura, H., Nishida, T., and Nakagawa, H. (1974), *J. Biochem. 75,* 139.
Peterkovsky, A. (1962), *J. Biol. Chem. 237,* 787.
Rechler, M. M. (1969), *J. Biol. Chem. 244,* 551.
Rubery, P. H., and Northcote, D. H. (1968), *Nature (London) 219,* 1230.
Schirmer, M. D., and Harper, A. E. (1970), *J. Biol. Chem. 245,* 1204.
Shibatani, T., Kakimoto, T., and Chibata, I. (1975), *Eur. J. Biochem. 55,* 263.
Sletzinger, M., Firestone, R. A., Reinhold, D. F., Rooney, C. S., and Nicholson, W. H. (1968), *J. Med. Chem. 11,* 261.
Smissman, E. E., and Warner, J. D. (1972), *J. Med. Chem. 15,* 681.
Smith, T. A., Cordelle, F. H., and Abeles, R. H. (1967), *Arch. Biochem. Biophys. 120,* 724.
Soutar, A. K., and Hassal, H. (1969), *Biochem. J. 114,* 79P.
Tabor, H., and Mehler, A. H. (1955), *Methods Enzymol. 2,* 228.
Yankeelov, J. A., and Jolley, C. J. (1972), *Biochemistry 11,* 159.
Zannoni, V. G., and LaDu, B. J. (1963), *Biochem. J. 88,* 160.
Zucker, M. (1965), *Plant Physiol. 40,* 779.

Stimulation of Ascites Tumor RNA Polymerase II by Protein Kinase†

Michael E. Dahmus

ABSTRACT: The activity of purified RNA polymerase II from Novikoff ascites tumor cells is stimulated 5–7-fold by a purified protein factor. This protein factor, designated HLF_2, has extensive protein kinase activity and catalyzes the incorporation of γ-^{32}P from ATP into protein under normal RNA polymerase assay conditions. Protein phosphorylation is totally dependent on the presence of HLF_2 and is stimulated 2–3-fold by the presence of highly purified RNA polymerase II. The purification procedure developed for the isolation of the polymerase stimulatory factor resulted in a 4000-fold purification of a protein kinase. Chromatography on carboxymethylcellulose, phosphocellulose, and Sephadex G-100 did not resolve polymerase stimulatory activity from protein kinase activity: Adenylylimidodiphosphate (AMP-PNP), an inhibitor of protein kinases, inhibited the stimulatory activity of purified factor by 80%. The heat denaturation profile of protein kinase was paralleled by the loss of polymerase stimulatory activity. Concentrations of $(NH_4)_2SO_4$ which are known to inhibit polymerase stimulation (Lee and Dahmus, 1973) also inhibit protein kinase activity. The protein kinase activity associated with stimulatory factor catalyzes the phosphorylation of casein or phosvitin but does not catalyze the phosphorylation of basic proteins such as protamine or histone. The protein kinase is not stimulated by cyclic 3′,5′-AMP or -GMP over a concentration range of 10^{-6}–10^{-4} M. Furthermore, protein kinase activity is not inhibited by either the regulatory subunit of rabbit muscle protein kinase or by the heat-stable inhibitor of cyclic 3′,5′-AMP-dependent protein kinases. Protein kinase activity is stimulated by KCl or NH_4Cl and is inhibited by $MnCl_2$. The apparent K_m values, determined in the presence of 4 mM Mg^{2+}, are 0.02 mM for ATP, and 4.1 mM for GTP.

Alterations in gene activity, in a number of instances, have been observed to closely correlate with changes in the phosphorylation of nuclear proteins (Kleinsmith et al., 1966; Langan, 1971; Ahmed, 1971; Jungmann and Schweppe, 1972; Johnson et al., 1974; Karn et al., 1974; Kleinsmith, 1975). Indeed phosphorylated nonhistone chromosomal proteins have been reported to stimulate RNA synthesis in cell-free systems using purified DNA templates (Teng et al., 1971; Shea and Kleinsmith, 1973; Kostraba et al., 1975). This particular class of nonhistone phosphoproteins binds to DNA and stimulates RNA synthesis only in the presence of homologous DNA. The modification of nonhistone chromosomal proteins by phosphorylation is thought to influence their interaction with DNA and thereby lead to alterations in gene activity. It has also been suggested that transcription may in part be regulated by the direct phosphorylation of nuclear RNA polymerase. Jungmann et al. (1974) have reported the stimulation of both calf RNA polymerase I and II, present in partially purified extracts, by the addition of a cAMP-dependent protein kinase from calf ovary cytosol. Martelo and Hirsch (1974) have also reported the stimulation of isolated rat liver polymerase I by a nuclear protein kinase.

Kish and Kleinsmith (1974) have reported the fractionation of nuclear protein kinases from beef liver into 12 distinct fractions. The results of Ahmed and Wilson (1975) also suggest the presence of multiple protein kinases associated with

† From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received October 17, 1975.* This work was supported by the National Institutes of Health (HD 04899).

rat ventral prostate chromatin. Furthermore, both the phosphorylation of prostate nuclear proteins and the level of chromatin associated protein kinases were sensitive to the androgenic status of the animal. Keller et al. (1975) have fractionated chromatin from the oviducts of estrogen-treated chickens and have reported an enrichment of protein kinase in the transcriptionally active fraction.

The existence of multiple DNA-dependent RNA polymerases in eukaryotic cells is firmly established (for review, see Chambon, 1975). One of these, RNA polymerase II, is a nucleoplasmic enzyme that catalyzes the synthesis of heterogeneous nuclear RNA, a portion of which is thought to be the precursor for cytoplasmic messenger RNA. We have previously reported the purification of two protein factors that independently stimulate the activity of purified Novikoff ascites tumor RNA polymerase II 5–7-fold (Lee and Dahmus, 1973). These factors do not stimulate *E. coli* RNA polymerase, nor do they stimulate the activity of homologous polymerases in the presence of denatured DNA. One of these protein factors, designated HLF_2,[1] has been extensively purified. This factor appears to stimulate the initiation of RNA chains and alters the base composition of the RNA transcribed by purified RNA polymerase II (manuscript in preparation). In this paper we present evidence that the stimulation of RNA polymerase II activity by HLF_2 involves protein phosphorylation, possibly of RNA polymerase itself. The protein kinase activity associated with HLF_2 is also partially characterized.

Materials and Methods

Biochemicals. Unlabeled nucleoside triphosphates, CTP, GTP, UTP, ATP, and AMP-PNP, were obtained from P-L Biochemicals. [^{3}H]UTP was obtained from Schwarz-Mann. [γ-^{32}P]ATP was prepared by a modification (Walsh et al., 1971) of the procedure of Glynn and Chappell (1964). [γ-^{32}P]GTP was prepared by an adaptation of the procedure for ATP according to a suggestion by Dr. Earle Davie, University of Washington, Seattle. Phosvitin was obtained from Sigma. Casein, prepared as described by Reimann et al. (1971), was a gift from Dr. D. A. Walsh (Department of Biological Chemistry, University of California, Davis). Bovine serum albumin was purchased from Schwarz-Mann. Protamine was obtained from Calbiochem. Histone 2b, prepared according to method 1 of Johns (1964), was a gift from Drs. W. Palmer and D. A. Walsh. The heat-stable inhibitor protein, purified from rabbit skeletal muscle (Ashby and Walsh, 1974), was a gift from Mr. J. McPherson and Dr. D. A. Walsh. The regulatory and catalytic subunit of cAMP-dependent protein kinase, purified from rabbit skeletal muscle (Beavo et al., 1974), was a gift of Drs. P. J. Bechtel and E. G. Krebs (Department of Biological Chemistry, University of California, Davis). Novikoff ascites tumor DNA was prepared as described by Dahmus and McConnell (1969). Nitrocellulose membrane filters were obtained from either Schleicher and Schuell (type B-6) or Millipore (HAWP 0.45 μ).

Pancreatic RNase, purchased from Sigma, was preincubated at 85 °C for 15 min in 0.01 M Tris-HCl (pH 7.9). Pronase, obtained from Calbiochem, was preincubated for 60 min at 37 °C in 0.01 M Tris-HCl (pH 7.9) at a concentration of 1 mg/ml.

Solutions. Buffer A contained 0.05 M Tris-HCl (pH 7.9), 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol. Buffer B contained 0.05 M Tris-HCl (pH 7.9), 25% glycerol, 0.1 mM EDTA, and 0.5 mM dithiothreitol. Buffer C contained 0.05 M Tris-HCl (pH 7.9), 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 10 mM thioglycerol. Buffer D contained 0.05 M Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol.

Cell Line. The Novikoff ascites tumor line was maintained by serial transplantation in female Sprague-Dawley rats. The cells were harvested and washed as previously described (Lee and Dahmus, 1973).

RNA Polymerase Assay. The standard assay mixture contained in 0.25 ml: 40 mM Tris-HCl, pH 7.9; 4 mM $MgCl_2$; 1 mM $MnCl_2$; 5 mM dithiothreitol; 0.4 mM ATP, GTP, CTP, and [^{3}H]UTP (50–100 Ci/mol); 10 μg of Novikoff ascites tumor DNA; and RNA polymerase. When present, the specific activity of [γ-^{32}P]ATP was 80 Ci/mol. Assay reaction mixtures were incubated at 37 °C for 30 min. The reaction was terminated with the addition of 0.75 ml of cold water and precipitated with 1 ml of cold 10% trichloroacetic acid. After 15 min the precipitates were collected on nitrocellulose filters and washed three times with 10-ml portions of cold 5% trichloroacetic acid. Filters were then dried and counted in a liquid scintillation spectrometer. ^{32}P and ^{3}H counts were separated with the aid of a computer. One unit of RNA polymerase activity is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of UMP into RNA in 10 min under the conditions described above.

Protein Kinase Assay. The assay for protein kinase was carried out as described by Beavo et al. (1974). The standard reaction mixture of 0.08 ml contained: 25 mM Mes buffer, pH 6.9; 4.0 mM magnesium acetate; 0.25 mM EGTA; 0.125 mM [γ-^{32}P]ATP (specific activity, ca. 2×10^8 cpm/μmol); 100 μg of either casein or phosvitin; and enzyme. Unless otherwise indicated reactions were carried out with phosphocellulose purified HLF_2, also referred to as protein kinase. The assays described in Table I were carried out in the presence of 2.5 mM ATP and 0.24 M NH_4Cl. In certain experiments [γ-^{32}P]GTP (specific activity, ca. 5×10^9 cpm/μmol) replaced [γ-^{32}P]ATP as phosphate donor. Reactions were initiated by the addition of enzyme and incubated for 10 min at 37 °C. To stop the reaction, 1 ml of cold water was added followed by the addition of 1 ml of cold 10% trichloroacetic acid. The precipitates were collected on nitrocellulose filters and washed with 5% trichloroacetic acid as described above. One unit of protein kinase activity is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of phosphate from ATP to casein in 1 min under the conditions described above.

Purification of RNA Polymerase II. RNA polymerase II was purified as previously described (Lee and Dahmus, 1973) with the following modifications. Both species of polymerase II were eluted together from DEAE-cellulose with buffer A containing 0.32 M $(NH_4)_2SO_4$. The sample was then dialyzed against buffer A containing 0.05 M $(NH_4)_2SO_4$ and chromatographed on phosphocellulose. Polymerase was eluted with a linear gradient of 0.05–0.6 M $(NH_4)_2SO_4$ in buffer B. The gradient volume was equal to three times the column bed volume. Fractions containing polymerase activity were pooled and precipitated by the addition of solid $(NH_4)_2SO_4$ to a final concentration of 65% saturation (0 °C). After 4 h at 0 °C, the precipitate was removed by centrifugation in the Beckman Ti 60 rotor at 230 000*g* for 1 h. The precipitate was then dissolved

[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N*′-tetraacetate; HLF_1, heat-labile RNA polymerase stimulatory factor from Novikoff ascites tumor cells, fractions 1 from phosphocellulose; HLF_2, heat-labile RNA polymerase stimulatory factor from Novikoff ascites tumor cells; fraction 2 from phosphocellulose; and AMP-PNP, adenylylimidodiphosphate; Mes, 4-morpholineethanesulfonic acid.

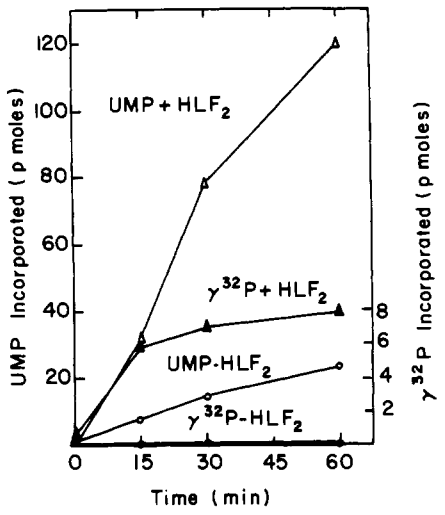

FIGURE 1: Effect of HLF_2 on the incorporation of γ-^{32}P from ATP and [^{3}H]UMP by purified ascites tumor RNA polymerase II. Each reaction contained, in a volume of 0.5 ml: 4 μg of RNA polymerase II, 20 μg of ascites DNA, [^{3}H]UTP (100 Ci/mol), and [γ-^{32}P]ATP in addition to salts and nucleotides as described in Materials and Methods. HLF_2, when present, was at a concentration of 28 μg/0.5 ml. Samples of 0.1 ml were removed, added to 1 ml of water, and precipitated as described in Materials and Methods. Incorporation of [^{3}H]UMP by RNA polymerase II alone (O—O); in presence of HLF_2 (Δ—Δ). Incorporation of γ-^{32}P by RNA polymerase II alone (●—●); in presence of HLF_2 (▲—▲).

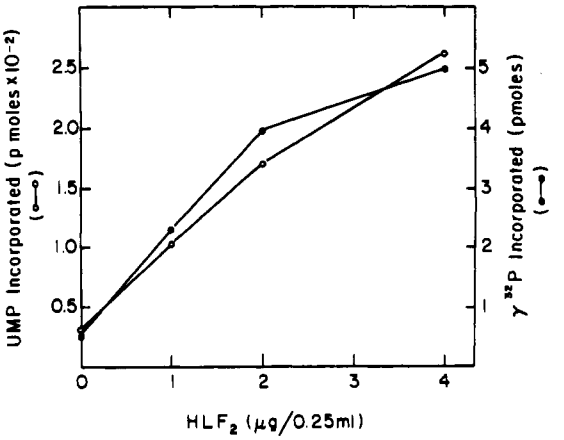

FIGURE 2: Dependence of γ-^{32}P and ^{3}H incorporation on the concentration of HLF_2. Reaction conditions were as specified in Materials and Methods except the reaction was incubated at 20 min at 37 °C. Each reaction contained 1 μg of RNA polymerase II.

in a sufficient volume of buffer C to reduce the final $(NH_4)_2SO_4$ concentration below 0.15 M. The salt concentration was determined with a conductivity meter following a 200-fold dilution of sample with water.

The enzyme was then further purified by glycerol gradient centrifugation as described by Kedinger and Chambon (1972). Sample volumes of up to 2 ml were layered on 35 ml of a 15–30% glycerol gradient prepared in buffer C containing 50 mM $(NH_4)_2SO_4$. Samples were then centrifuged in a Beckman SW 27 rotor at 131 000g for 44 h at 4 °C. One-milliliter fractions were collected through the bottom of the tube and assayed as described above. Fractions containing polymerase activity were pooled and stored at −80 °C.

The specific activity ranged from 20 to 50 units/mg. The molecular weights of the three large subunits are approximately 193 000, 170 000, and 145 000, indicating the presence of both species of type II enzyme (Kedinger and Chambon, 1972).

Purification of Stimulatory Factor HLF_2. The purification of HLF_2 was as previously described (Lee and Dahmus, 1973) with the following modification. Following chromatography on phosphocellulose, fractions containing stimulatory activity were pooled and precipitated by the addition of solid $(NH_4)_2SO_4$ to a final concentration of 70% saturation (4.36 g of $(NH_4)_2SO_4$ per 10 ml of solution). After precipitation at 0 °C for at least 4 h the precipitate was removed by centrifugation at 230 000g for 1 h in the Beckman Ti 60 rotor. The precipitate was dissolved in a small volume (0.25 ml) of buffer D containing 0.05 M NH_4Cl and applied to a 0.9 × 60 cm column of Sephadex G-100 equilibrated in the same buffer. Fractions of 0.25 ml were then collected.

Preparation of Nuclear and Cytoplasmic Fractions. Washed ascites tumor cells were suspended in 5 volumes of 0.01 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.025 M NaCl, and 5 mM dithiothreitol containing 0.5% Triton X-100 and let stand at 0 °C for 45 min. The sample was then centrifuged at 800g for 15 min, and the cell pellet washed with 10 volumes deionized water. The cells were then suspended in 15 volumes of deionized water with the aid of a Teflon homogenizer and allowed to lyse at 0 °C. Nuclei were sedimented by centrifugation at 800g for 20 min. The supernatant was removed (cytoplasmic fraction) and the pellet examined to determine the extent of cell lysis. If unlysed cells accounted for more than 10% of the sample the water wash was repeated.

Protein Determination. Protein concentrations were determined by the method of Lowry et al. (1951) after precipitation of aliquots with 10% trichloroacetic acid.

Results

Association of Protein Kinase Activity with Stimulatory Factor HLF_2. The presence of protein kinase activity associated with RNA polymerase II stimulatory factor, HLF_2, was observed during studies designed to establish its mechanism of action. Indirect evidence derived from inhibition studies with the rifampicin derivative AF/013, a specific inhibitor of chain initiation, as well as kinetic studies suggested that HLF_2 stimulates RNA synthesis by promoting the initiation by RNA chains (manuscript in preparation). In order to study the effect of HLF_2 on the initiation process more directly, its effect on the RNA polymerase II catalyzed incorporation of [γ-^{32}P]ATP was investigated. The polymerase reaction was carried out in the presence of both [γ-^{32}P]ATP and [^{3}H]UTP in order to permit the simultaneous determination of net RNA synthesis. The time course for the incorporation of γ-^{32}P from ATP by ascites tumor RNA polymerase II alone and in the presence of HLF_2 is shown in Figure 1. The presence of HLF_2 increased the rate of γ-^{32}P incorporation dramatically. The time course of [^{3}H]UMP incorporation (Figure 1) demonstrates the effect of HLF_2 on net RNA synthesis. At the end of a 60-min reaction about 5 times the mass of RNA was synthesized when HLF_2 was present. The effect of increasing concentrations of HLF_2 on the incorporation of γ-^{32}P and [^{3}H]UMP is shown in Figure 2. The increase in total RNA synthesis was paralleled by an increase in the incorporation of γ-^{32}P.

The nature of the product was examined to determine the sensitivity of the incorporated ^{32}P to RNase and pronase digestion. RNA was transcribed in the presence of [γ-^{32}P]ATP and [^{3}H]UMP, as described in the legend to Figure 1, and the reaction terminated by heating at 85 °C for 2 min and divided into three aliquots. These samples were then incubated for an additional 30 min at 37 °C in the presence of either buffer

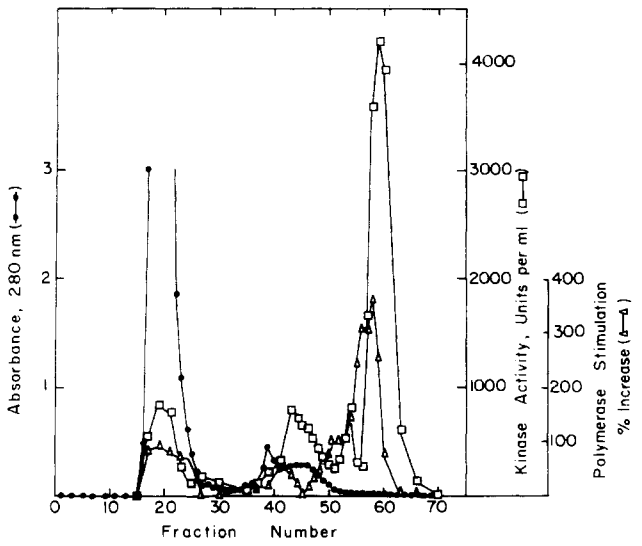

FIGURE 3: CM-cellulose chromatography of stimulatory factor HLF_2. HLF_2 (ca. 100 mg of protein from 50 g of ascites tumor cells) was applied to a 2.5 × 40 cm column of CM-cellulose (Whatman CM-52) equilibrated with buffer D. Protein was eluted with a 500-ml linear gradient of 0–0.8 M NH_4Cl in buffer D and 7-ml fractions were collected. Aliquots of 0.02 ml were assayed to determine polymerase stimulation or protein kinase activity. Polymerase stimulation was assayed in the presence of 0.010 unit of DEAE-cellulose purified RNA polymerase II. Units of protein kinase activity are as defined in Materials and Methods except that they are based on incorporation of phosphate into phosvitin.

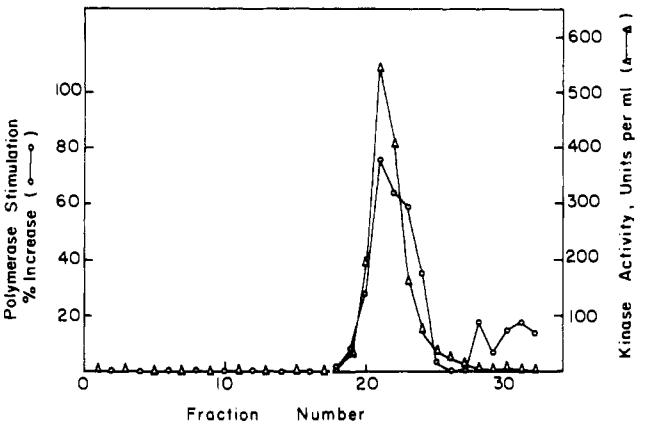

FIGURE 4: Phosphocellulose chromatography of stimulatory factor HLF_2. HLF_2 was applied to a phosphocellulose column (Whatman P-11), of 2-ml bed volume, equilibrated in buffer B. Protein was eluted with an 8-ml linear gradient of 0–0.6 M $(NH_4)_2SO_4$ in the presence of buffer B. Fractions of 0.3 ml were collected. Aliquots of 0.02 ml were assayed to determine polymerase stimulation and protein kinase activity. Polymerase stimulation was assayed in the presence of 0.011 unit of DEAE-cellulose purified RNA polymerase II. Protein kinase activity was determined as specified in Materials and Methods with casein as phosphate acceptor.

(control), RNase, or pronase. Greater than 99% of the [^{3}H]UMP incorporated by RNA polymerase II in the presence of HLF_2 was solubilized by treatment with RNase. None of the ^{3}H incorporated was solubilized by treatment with pronase. In addition to providing evidence that the [^{3}H]UMP was indeed incorporated into RNA, this experiment demonstrates that the pronase was free of interfering nuclease activity. The ^{32}P incorporated in the presence of HLF_2 was resistant to RNase treatment but was susceptible to pronase digestion. This suggests that the stimulation of γ-^{32}P incorporation by HLF_2 was due to protein phosphorylation rather than to an increased frequency of RNA chain initiation.

The observation that the incorporation of γ-^{32}P from ATP was not dependent upon the presence of the other ribonucleoside triphosphates, GTP, CTP, and UTP, or on the presence of DNA provided additional evidence that the γ-^{32}P was not incorporated into RNA. Incorporation of γ-^{32}P showed a nearly complete dependence on the presence of HLF_2. The incorporation of γ-^{32}P decreased more than 95% when HLF_2 was omitted from the reaction mixture. The omission of RNA polymerase II resulted in a 60% reduction in the incorporation of γ-^{32}P.

Copurification of Protein Kinase and Polymerase Stimulatory Activity. The purification procedure for HLF_2 was developed using the stimulation of RNA polymerase II as an assay. It was, therefore, of interest to determine the effect of this purification procedure on the fractionation or purification of protein kinase.

Protein kinase activity was determined, as described in Materials and Methods, at each step in the purification. Activity was determined in the absence of cAMP with casein or phosvitin as phosphate acceptor. Since the procedure for the purification of HLF_2 has been described in detail elsewhere (Lee and Dahmus, 1973), it will be discussed in outline form only. The first step in the procedure involves suspension of the ascites tumor cells in a high sucrose buffer and sonication in the presence of 0.3 M $(NH_4)_2SO_4$. The lysed cells were then diluted with buffer and centrifuged at 230 000g for 2 h. The supernatant was precipitated with 30% saturated $(NH_4)_2SO_4$ and the precipitate discarded. The $(NH_4)_2SO_4$ concentration was then increased to 50% saturation and the precipitate dialyzed and chromatographed on DEAE-cellulose. About 60% of the protein kinase activity did not bind to DEAE-cellulose and was recovered in the flow through peak. This fraction contains HLF_2 in addition to a number of other proteins capable of stimulating the activity of RNA polymerase II (Lee and Dahmus, 1973).

The DEAE-cellulose flowthrough was then fractionated by $(NH_4)_2SO_4$ precipitation (45–70% saturation), dialyzed, and chromatographed on carboxymethylcellulose (CM-cellulose). Protein was eluted with a linear gradient of NH_4Cl in the presence of buffer D and fractions were assayed for both RNA polymerase stimulatory activity and protein kinase activity. Stimulatory activity was determined by assaying an aliquot of each fraction in the presence of a fixed amount of RNA polymerase II. Protein kinase activity was measured in the presence of [γ-^{32}P]ATP with phosvitin as a phosphate acceptor as described in Materials and Methods. The elution profile from CM-cellulose is shown in Figure 3. Three peaks of protein kinase activity were observed. The major peak of protein kinase activity, however, eluted in the same region as the polymerase stimulatory activity. Both activities eluted in a region that contained only a small fraction of the total protein applied.

The major peak of protein kinase activity was pooled, dialyzed, and adsorbed onto phosphocellulose. The column was developed with a linear gradient of $(NH_4)_2SO_4$ in buffer B. Fractions were assayed for both polymerase stimulatory activity and protein kinase activity as described above. The elution profile from phosphocellulose is presented in Figure 4. Nearly coincident peaks of polymerase stimulatory activity and protein kinase activity were observed. Similar results were obtained by chromatography on HLF_2 on DNA cellulose.

Table I summarizes the results obtained for a typical preparation. The procedure developed for the purification of polymerase stimulatory factor HLF_2 results in a 4000-fold purification of protein kinase with an overall yield of 18%. Since the fraction of protein kinase which bound to DEAE-

TABLE I: Summary of Purification of Novikoff Ascites Tumor Protein Kinase.[a]

Step	Volume (ml)	Protein (mg)	Units	Specific Act.	Yield (%)
(1) Cell lysate	128	1750	441 000	251	100
(2) 230 000*g* supernatant	113	1100	459 000	417	104
(3) 30–50% saturated ammonium sulfate	20.8	364	278 000	765	63
(4) Concentrated DEAE-cellulose flowthrough	6.0	54	122 000	2 260	28[b]
(5) CM-cellulose	17.0	0.375	95 600	256 000	22
(6) Phosphocellulose	6.6	0.086	78 200	910 000	18

[a] This preparation utilized 25-ml packed cells. Protein kinase activity was determined as described in Materials and Methods except for the presence of 2.5 mM ATP and 0.24 M NH_4Cl. [b] The apparent poor yield at this step in the purification is due to the fractionation of protein kinase and the subsequent purification of only that fraction which did not bind to DEAE-cellulose.

TABLE II: Effect of Adenylylimidodiphosphate on the Stimulation of Novikoff Ascites Tumor RNA Polymerase II by HLF_2.[a]

Nucleotide	Polymerase Activity pmol of UMP Incorporated −HLF_2	Polymerase Activity pmol of UMP Incorporated +HLF_2	Polymerase Stimulation (% increase)
ATP	14.5	94.3	550
AMP-PNP	4.8	9.6	100

[a] The assay was as described in Materials and Methods. Each reaction contained 0.01 unit of RNA polymerase II and, when present, 6.4 μg of HLF_2.

cellulose (ca. 40%) was not purified further, the recovery of protein kinase is actually greater than 18%. CM-cellulose chromatography is an especially efficient step since it alone results in a 100-fold purification with a yield of about 80%.

The construction of a similar purification table for the stimulatory factor HLF_2 was not possible. The presence of nucleases, proteases, and numerous proteins that can interact with the template DNA in a variety of ways makes it impossible to assay polymerase stimulation in crude extracts. The difficulty in determining the specific activity and recovery of HLF_2 is further complicated by the fact that the DEAE-cellulose flowthrough contains at least two other polymerase stimulatory factors. We have, however, estimated that chromatography on CM-cellulose and phosphocellulose results in approximately a 50-fold and 4-fold purification, respectively.

Electrophoresis of phosphocellulose purified enzyme on polyacrylamide gels in the presence of sodium dodecyl sulfate, according to the method of Weber and Osborn (1969), results in the resolution of two bands. The molecular weight of the major band, based on mobility relative to proteins of known molecular weight, is 85 000 daltons. This is in agreement with the previously reported molecular weight for purified HLF_2 (Lee and Dahmus, 1973). Most of the remaining protein (ca. 10%) appears in a faster moving band with a molecular weight of about 37 000 daltons.

In a further effort to resolve the protein kinase and polymerase stimulatory activity, phosphocellulose purified HLF_2 was concentrated by precipitation with $(NH_4)_2SO_4$ and chromatographed on Sephadex G-100. Protein kinase and polymerase stimulatory activity were assayed as described above. The elution profile from Sephadex G-100 is presented in Figure 5. Both the protein kinase activity and stimulatory

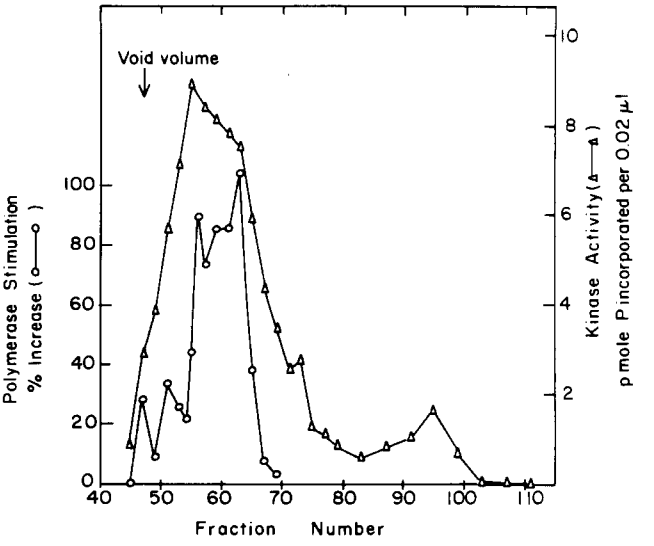

FIGURE 5: G-100 Sephadex gel filtration of stimulatory factor HLF_2. A 0.25-ml sample was applied to a 0.9 × 60 cm column of Sephadex G-100 equilibrated with buffer D containing 0.05 M NH_4Cl. Polymerase stimulation and protein kinase activity were determined as described in the legend to Figure 4 and in Materials and Methods. Polymerase stimulation was assayed in the presence of 0.010 unit of DEAE-cellulose purified RNA polymerase II.

activity eluted together at a position slightly behind the void volume of the column.

Effect of Adenylylimidodiphosphate on Polymerase Stimulation. The ATP analogue, adenylylimidodiphosphate (AMP-PNP), in which the β-γ bridge oxygen is replaced with an NH group, is an effective inhibitor of enzymes which cleave the β-γ linkage (Yount et al., 1971). This analogue should have less of an effect on the RNA polymerase reaction, however, since this reaction results in hydrolysis of the bond between the α and β phosphates. The effect of AMP-PNP on the activity of RNA polymerase and the activity of HLF_2 was therefore determined. The data presented in Table II show that there is a significant inhibition of RNA polymerase activity by the ATP analogue. The ability of HLF_2 to stimulate polymerase activity was, however, reduced by 80%.

Heat Denaturation of Protein Kinase and Polymerase Stimulatory Activity. To further test the hypothesis that a functional relationship exists between the activity of protein kinase and the stimulation of RNA polymerase II, aliquots of HLF_2 were heated at different temperatures for 10 min and the protein kinase and polymerase stimulatory activity was determined. In this experiment protein kinase activity was

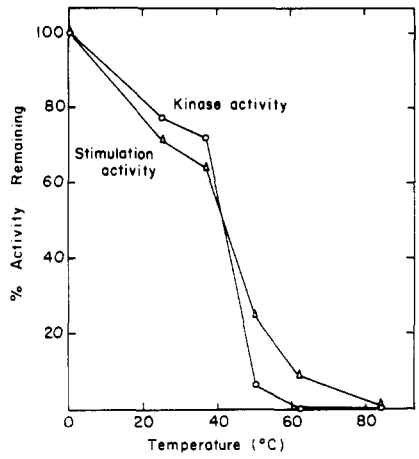

FIGURE 6: Heat inactivation of protein kinase and polymerase stimulatory activity. Each incubation (0.05 ml) contained 2.6 μg of HLF_2 in buffer D. Following a 10-min incubation at the indicated temperature, samples were cooled to 0 °C and centrifuged at 9400*g* for 15 min. No precipitate was visible. Aliquots of 0.02 ml were assayed for protein kinase and polymerase stimulatory activity as described in Materials and Methods. DEAE-cellulose purified RNA polymerase II was used to measure polymerase stimulation. RNA polymerase alone incorporated 27 pmol of UMP. In the presence of untreated HLF_2, polymerase activity increased to 60 pmol of UMP incorporated. Protein kinase activity was determined with casein as phosphate acceptor. Untreated samples contained 82 units of protein kinase. Protein kinase activity (O—O); polymerase-stimulation activity (Δ—Δ).

determined with casein as a phosphate acceptor. The heat denaturation profile of both protein kinase and polymerase stimulatory activity was nearly identical (Figure 6).

Subcellular Localization of Protein Kinase. Ascites tumor cells were fractionated into a cytoplasmic and nuclear fraction, as described in Materials and Methods, and both fractions subjected to the standard purification procedure for HLF_2. Each fraction was purified through the CM-cellulose chromatography step. Greater than 90% of the protein kinase and polymerase stimulatory activity were associated with the nuclear fraction. Furthermore, the yield (units of protein kinase per milliliter of packed cells) was nearly equivalent to that obtained when protein kinase was isolated from whole cells.

Substrate Specificity of Protein Kinase. The ability of protein kinase to phosphorylate a variety of proteins was determined (Table III). Of the proteins tested, casein was the preferred substrate. Phosvitin was also an acceptable substrate, although the extent of phosphorylation was less than 50% of that observed with casein. Histone 2b, protamine, bovine serum albumin, and egg white lysozyme were not phosphorylated to an appreciable extent.

Experiments were also carried out to compare the efficiency of [γ-^{32}P]ATP and [γ-^{32}P]GTP as phosphate donors. At equimolar concentrations of either ATP or GTP, GTP was less than 0.5% as effective a phosphate donor as was ATP. The preference for ATP was not affected by the nature of the phosphate acceptor.

The kinetic parameters of protein kinase were determined using casein and either [γ-^{32}P]ATP or [γ-^{32}P]GTP as substrates (Table IV). Initial rates were determined for each substrate concentration and the data analyzed according to Lineweaver and Burk (1934). In the presence of 4 mM Mg^{2+}, the apparent K_m for ATP was 0.02 mM, about 200 times lower than the apparent K_m for GTP. The V_{max} determined in the presence of ATP was about 25 times that determined in the presence of GTP.

Effect of Cyclic 3′,5′-AMP and -GMP and Regulatory Proteins on Protein Kinase. Protein kinase was assayed alone or in the presence of increasing concentrations of cAMP or cGMP. No change in activity was observed over a concentration range of 10^{-6}–10^{-4} M by either cAMP or cGMP.

To investigate the possibility that protein kinase constituted the free catalytic subunit of a cAMP-dependent protein kinase, we examined its sensitivity to the heat-stable inhibitor of cAMP-dependent protein kinases (Ashby and Walsh, 1974) and the regulatory subunit of rabbit muscle protein kinase (Beavo et al., 1974). No inhibition of ascites tumor protein kinase was observed at heat-stable inhibitor concentrations which resulted in greater than 95% inhibition of the free catalytic subunit of rabbit muscle protein kinase. Furthermore, no inhibition of ascites tumor protein kinase was observed at concentrations of regulatory subunit sufficient to inhibit the homologous catalytic subunit by greater than 95%.

Effect of Increasing Salt Concentration on Protein Kinase. The salt stimulations of casein and phosvitin kinases have been reported in a number of cases (Desjardins et al., 1972; Traugh and Traut, 1974; Rodnight and Lavin, 1964), but the effect is apparently not common to all protein kinases of this type (Goldstein and Hasty, 1973; Lerch et al., 1975). The effect of increasing concentrations of NH_4Cl or $(NH_4)_2SO_4$ on the activity of protein kinase was determined in the presence of 4 mM Mg^{++} (Figure 7). An increase in NH_4Cl concentration from 0 to 0.24 M resulted in a 3-fold increase in protein kinase activity. Similar results were obtained in the presence of increasing concentrations of KCl. Maximum stimulation by $(NH_4)_2SO_4$ was obtained at about 0.04 M; higher concentrations resulted in inhibition of activity.

Effect of RNA Polymerase Assay System on Protein Kinase. The assay of protein kinase under conditions of RNA

TABLE III: Phosphorylation of Various Protein Substrates by Protein Kinase[a]

Protein Substrate	Protein Kinase Activity (pmol of ^{32}P incorporated per 10 min)	Relative Activity
Casein	123	100
Phosvitin	61.3	49.8
Histone 2b	0	0
Protamine	0.86	0.70
Bovine serum albumin	0.64	0.05
Egg white lysozyme	0.79	0.06

[a] The assays were performed under standard protein kinase assay conditions in the presence of 100 μg of each protein substrate. Background values of less than 2 pmol were subtracted for each substrate except histone 2b (4.4 pmol) and protamine (3.6 pmol).

TABLE IV: Kinetic Parameters of Protein Kinase.[a]

Nucleotide	K_m (nucleotide)	K_m (casein)	V_{max}
ATP	0.02 mM	0.106 mM	123 pmol/min
GTP	4.1 mM	—	5.0 pmol/min

[a] The apparent K_m's for ATP and GTP were determined in the presence of 0.1 μg of protein kinase, 2.5 mg of casein/ml, and 4 mM Mg^{2+}. The apparent K_m's for casein were determined in the presence of 2.5 mM ATP. All reactions contained 0.24 M NH_4Cl.

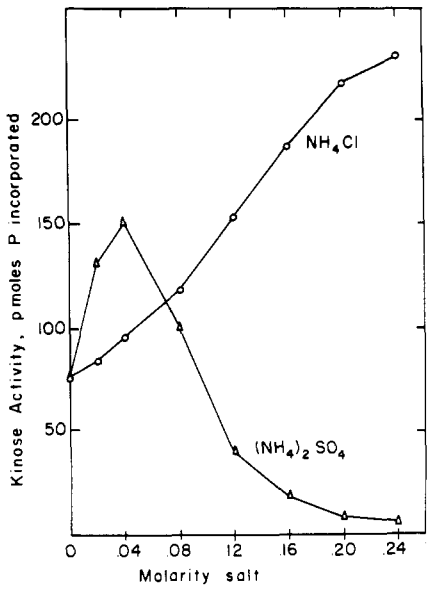

FIGURE 7: Effect of NH_4Cl and $(NH_4)_2SO_4$ on protein kinase. Increasing concentrations of either NH_4Cl or $(NH_4)_2SO_4$ were added to the standard assay mixtures before initiation of the reaction with protein kinase. Casein was used as protein substrate.

synthesis resulted in greater than 90% inhibition of protein kinase activity. One of the major differences in the two assay systems was the presence of 1 mM $MnCl_2$ in the RNA polymerase assay. This consideration, in addition to the fact that phosvitin kinase from rooster liver is inhibited by Mn^{2+} (Goldstein and Hasty, 1973), suggested that Mn^{2+} may account for the reduced activity. The effect of removing Mn^{2+} from standard RNA polymerase assay systems, as well as the inclusion of Mn^{2+} in the standard protein kinase assay system, was determined and is shown in Table V. The addition of Mn^{2+} to the protein kinase assay system resulted in greater than 90% inhibition of protein kinase activity. Furthermore, the removal of Mn^{2+} from the standard polymerase assay system increased protein kinase activity more than 10-fold.

TABLE V: Effect of $MnCl_2$ on Protein Kinase Activity.[a]

Reaction Conditions	Protein Kinase Activity (pmol of ^{32}P incorporated per 10 min)
RNA polymerase assay system	
Complete	35.4
$-Mn^{2+}$	422
Protein kinase assay system	
Complete	387
$+Mn^{2+}$	29.2

[a] The standard RNA polymerase assay system was modified in that RNA polymerase, DNA, UTP, GTP, and CTP were omitted from the reaction mixture. Each reaction contained 0.10 μg of HLF_2.

Discussion

The activity of purified Novikoff ascites tumor RNA polymerase II is stimulated 5–7-fold by a protein factor, HLF_2. This factor also stimulates the incorporation of γ-^{32}P from ATP into protein in the standard RNA polymerase assay system. This result suggests the interesting possibility that the mechanism by which HLF_2 stimulates RNA synthesis involves protein phosphorylation. In an effort to establish whether or not a functional relationship exists between protein phosphorylation and the stimulation of RNA polymerase II activity, we have: (1) attempted to physically resolve the protein kinase activity from the stimulatory activity by a variety of separation techniques; (2) tried to determine the effect of specific inhibition of protein kinase activity on the ability of HLF_2 to stimulate polymerase activity; and (3) determined the effects of agents that are known to inhibit polymerase stimulation on the activity of protein kinase.

The procedure developed for the purification of HLF_2 resulted in a 4000-fold purification of a protein kinase. The specific activity of the protein kinase obtained was about 1 μmol of phosphate transferred per min per mg of protein. This specific activity is comparable to a number of purified protein kinases (Goldstein and Hasty, 1973; Beavo et al., 1974; Rubin et al., 1974) but considerably below the specific activity reported by Lerch et al. (1975) for purified casein kinase from baker's yeast (ca. 10 μmol phosphate transferred per min per mg of protein). The purification procedure developed from HLF_2 appears to be a good procedure for the purification of the major nuclear protein kinase of this type.

If protein phosphorylation were required for the stimulation of polymerase II activity, stimulation would be observed only under conditions which allow both protein phosphorylation and RNA transcription to proceed. These conditions are likely to be more stringent than those required for protein phosphorylation alone. Of a variety of separation techniques employed, CM-cellulose chromatography alone resulted in an apparent partial resolution of protein kinase and stimulatory activity. This could be the result of a differential effect of salt in the two reactions or to the partial resolution of HLF_1 and HLF_2. HLF_1 elutes slightly ahead of HLF_2 and can be completely resolved by chromatography on phosphocellulose (Lee and Dahmus, 1973). The fact that protein kinase activity and polymerase stimulatory activity could not be resolved by chromatography on phosphocellulose, DNA cellulose, and Sephadex G-100 suggests that protein phosphorylation may play a key role in polymerase stimulation.

A more critical test of the hypothesis that protein phosphorylation is required for the stimulation of polymerase activity would be a demonstration that the specific inhibition of protein kinase activity resulted in an inhibition of polymerase stimulation. It is difficult to obtain such conditions, however, since most means of inhibiting protein kinase activity also result in an inhibition of RNA polymerase activity. The ATP analogue, AMP-PNP, was, however, partially successful. Although it caused a significant inhibition of polymerase activity it reduced the stimulatory activity of HLF_2 by 80%. If the effect of AMP-PNP on RNA polymerase was simply to either decrease the rate of nucleotide polymerization or inhibit the initiation of chains initiated with ATP, one might expect HLF_2 to cause the same relative increase in polymerase activity. The analogue, on the other hand, had a greater effect on the stimulatory activity of HLF_2 than on the activity of RNA polymerase alone. The small amount of stimulation that was observed could be due to the utilization of one of the other ribonucleoside triphosphates as phosphate donor.

The fact that the heat-denaturation profile of protein kinase was paralleled by the loss of polymerase stimulation activity is also consistent with the idea that protein phosphorylation is required. We cannot, however, eliminate the possibility that the two activities are unrelated but have similar heat inactivation profiles. The fact that both protein kinase and polymerase stimulatory activity were recovered, with high yield, from isolated nuclei is also consistent with the idea that protein

kinase may be involved in the regulation of RNA polymerase II activity.

We have previously reported that the maximum stimulatory activity of HLF_2 was obtained at $(NH_4)_2SO_4$ concentrations of about 10–20 mM (Lee and Dahmus, 1973). The activity rapidly declined as the $(NH_4)_2SO_4$ concentration increased. The effect of $(NH_4)_2SO_4$ on the ability of HLF_2 to stimulate RNA polymerase II, therefore, closely parallels the effect of $(NH_4)_2SO_4$ on the activity of protein kinase.

Incubation of HLF_2 in the absence of RNA polymerase resulted in the incorporation of γ-^{32}P from ATP. This incorporation could arise from either a direct phosphorylation of the protein kinase or the phosphorylation of another protein in this fraction. A highly purified phosvitin kinase from calf brain has been reported to contain a tightly bound protein which was phosphorylated upon incubation with [γ-^{32}P]ATP (Wålinder, 1973). The stimulation of γ-^{32}P incorporation, by the addition of purified RNA polymerase II, suggests that a direct phosphorylation of the enzyme may be involved in the stimulation of activity. Although the polymerase is extensively purified, we cannot presently eliminate the possibility that a contaminating protein is serving as phosphate acceptor.

Rutter and associates (1973) have reported the phosphorylation of purified rat liver RNA polymerase II with a cyclic AMP-dependent protein kinase from both rat liver or rabbit muscle. The major fraction of phosphate incorporated was into a protein of 25 000 molecular weight, presumably a subunit of RNA polymerase. No transcriptional changes were noted between the phosphorylated and nonphosphorylated enzyme. Jungmann et al. (1974) have reported the stimulation of ovarian RNA polymerase II by a partially purified cyclic AMP-dependent protein kinase from calf ovary cytosol. The activity of polymerase II was increased 9-fold whereas the activity of polymerase Ia and Ib was stimulated about 3-fold. An increase in the phosphorylation of the crude enzyme fraction accompanied the increase in polymerase activity. The phosphorylation of the unpurified enzyme fractions is difficult to interpret because of the excess of nonpolymerase proteins which may serve as phosphate acceptors. The differential sensitivity of polymerase I and II to stimulation by the partially purified kinase is strikingly similar to the differential stimulation of ascites tumor polymerases I and II by HLF_2 (manuscript in preparation). The protein kinase activity associated with HLF_2 does not, however, resemble the cyclic AMP-dependent protein kinase activity reported by Jungmann et al. (1974). The kinases differ most dramatically in their dependency on cAMP, molecular weight, and substrate specificity.

The phosphorylation of purified *E. coli* RNA polymerase with beef skeletal protein kinase has also been reported (Martelo et al., 1974). Recently the in vivo phosphorylation of *E. coli* RNA polymerase has been demonstrated in bacteriophage T_7 infected cells (Zillig et al., 1975). After infection with T_7 the β' subunit and to a lesser extent the β subunit of *E. coli* RNA polymerase are phosphorylated by a phage coded protein kinase. The fact that mutants that lack early transcriptional control were also deficient in protein kinase suggests the phosphorylation of RNA polymerase may be the mechanism by which early transcription is regulated.

Ascites tumor protein kinase exhibits a clear preference for casein over a variety of proteins examined. Phosvitin is also an acceptable substrate, but basic proteins such as histones and protamine are not phosphorylated to an appreciable extent. ATP is the preferred phosphate donor although GTP can also be utilized by the enzyme. The apparent K_m for ATP is about 200 times greater than that for GTP. It is not inhibited either by the regulatory subunit of rabbit muscle protein kinase (Beavo et al., 1974) or by a heat-stable inhibitor of a variety of cAMP-dependent protein kinases (Ashby and Walsh, 1974). These results suggest that ascites tumor protein kinase is not the free catalytic subunit of a cAMP-regulated protein kinase.

The stimulation of protein kinase activity by low salt is characteristic of a number of protein kinases that phosphorylate acidic proteins (Desjardins et al., 1972; Traugh and Traut, 1974; Rodnight and Lavin, 1964). The activity of protein kinase is stimulated severalfold by relatively low concentrations of NH_4Cl and KCl. $(NH_4)_2SO_4$ had a greater stimulatory effect at concentrations below 0.04 M but resulted in inhibition of enzyme activity at higher concentrations. Sulfate would, therefore, appear to be inhibitory to protein kinase activity. Protein kinase resembles phosvitin kinase, purified from rooster liver, in that it is inhibited by low concentrations of $MnCl_2$ (Goldstein and Hasty, 1973).

Ascites tumor protein kinase has properties similar to rat liver nuclear casein kinase NI reported by Desjardins et al. (1972). Both enzymes are insensitive to cAMP, are stimulated by low salt, and have similar chromatographic properties on phosphocellulose and DEAE substituted resins. Ascites tumor protein kinase also resembles the rat liver nuclear protein kinase IV described by Ruddon and Anderson (1972), as well as the activity associated with rat liver chromatin described by Takeda et al. (1971).

We have presented evidence that the activity of eukaryotic RNA polymerase II may in part be regulated by a protein kinase. Although phosphorylation of the enzyme has not been conclusively demonstrated, the fact that highly purified RNA polymerase II stimulates γ-^{32}P incorporation suggests the enzyme itself may be serving as phosphate acceptor. Resolution of the phosphorylated enzyme by gel electrophoresis and an identification of the phosphorylated protein as a subunit of RNA polymerase are required to establish this point. Such experiments are in progress. A demonstration of enzyme phosphorylation, however, is not sufficient to establish a causal relationship between protein phosphorylation and the stimulation of polymerase activity. The establishment of such a relationship would require a demonstration that the selective inhibition of protein kinase inhibits polymerase stimulation and that the extent of stimulation is dependent on the extent of enzyme phosphorylation. A careful analysis of the extent of the in vivo phosphorylation of RNA polymerase II is also required.

Acknowledgments

I wish to thank Drs. E. G. Krebs, D. A. Walsh, and B. E. Kemp for many helpful discussions during the course of this investigation and for their critical review of this manuscript. I also gratefully acknowledge the technical assistance of Ms. Grace Dahmus and Ms. Kathleen Esh. I thank Ms. Jeanette Natzle for assistance in the cell fractionation studies.

References

Ahmed, K. (1971), *Biochim. Biophys. Acta 243,* 38–48.

Ahmed, K., and Wilson, J. (1975), *J. Biol. Chem. 250,* 2370–2375.

Ashby, C. D., and Walsh, D. A. (1974), *Meth. Enzymol. 38,* 350–358.

Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1974), *Meth. Enzymol. 38,* 299–308.

Chambon, P. (1975), *Annu. Rev. Biochem. 44,* 613–638.

Dahmus, M. E., and McConnell, D. J. (1969), *Biochemistry*

8, 1524–1534.
Desjardins, P. R., Lue, P. F., Liew, C. C., and Gornall, A. G. (1972), *Can. J. Biochem. 50,* 1249–1259.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90,* 147–149.
Goldstein, J. L., and Hasty, M. A. (1973), *J. Biol. Chem. 248,* 6300–6307.
Johns, E. W. (1964), *Biochem. J. 92,* 55–59.
Johnson, E. M., Karn, J., and Allfrey, V. G. (1974), *J. Biol. Chem. 249,* 4990–4999.
Jungmann, R. A., Hiestand, P. C., and Schweppe, J. S. (1974), *J. Biol. Chem. 249,* 5444–5451.
Jungmann, R. A., and Schweppe, J. S. (1972), *J. Biol. Chem. 247,* 5535–5542.
Karn, J., Johnson, E. M., Vidali, G., and Allfrey, V. G. (1974), *J. Biol. Chem. 249,* 667–677.
Kedinger, C., and Chambon, P. (1972), *Eur. J. Biochem. 28,* 282–290.
Keller, R. K., Socher, S. H., Krall, J. F., Chandra, T., and O'Malley, B. W. (1975), *Biochem. Biophys. Res. Commun. 66,* 453–459.
Kish, V. M., and Kleinsmith, L. J. (1974), *J. Biol. Chem. 249,* 750–760.
Kleinsmith, L. J. (1975), *J. Cell. Physiol. 85,* 459–475.
Kleinsmith, L. J., Allfrey, V. G., and Mirsky, A. E. (1966), *Science 154,* 780–781.
Kostraba, N. C., Montagna, R. A., and Wang, T. Y. (1975), *J. Biol. Chem. 250,* 1548–1555.
Langan, T. A. (1971), *Ann. N.Y. Acad. Sci. 185,* 166–180.
Lee, S. C., and Dahmus, M. E. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1383–1387.
Lerch, K., Muir, L. W., and Fischer, E. H. (1975), *Biochemistry 14,* 2015–2023.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56,* 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.
Martelo, O. J., and Hirsch, J. (1974), *Biochem. Biophys. Res. Commun. 58,* 1008–1015.
Martelo, O. J., Woo, S. L. C., and Davie, E. W. (1974), *J. Mol. Biol. 87,* 685–696.
Reimann, E. M., Walsh, D. A., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1986–1995.
Rodnight, R., and Lavin, B. E. (1964), *Biochem. J. 93,* 84–91.
Ruddon, R. W., and Anderson, S. L. (1972), *Biochem. Biophys. Res. Commun. 46,* 1499–1508.
Rutter, W. J., Morris, P. W., Goldberg, M., Paule, M., and Morris, R. W. (1973), in The Biochemistry of Gene Expression in Higher Organisms, Pollack, J. K., and Lee, J. W., Ed., Boston, Reidel Publishing Co., pp 89–104.
Rubin, C. S., Erlichman, J., and Rosen, O. M. (1974), *Meth. Enzymol. 38,* 308–315.
Shea, M., and Kleinsmith, L. J. (1973), *Biochem. Biophys. Res. Commun 50,* 473–477.
Takeda, M., Yamamura, H., and Ohga, Y. (1971), *Biochem. Biophys. Res. Commun. 42,* 103–110.
Teng, C. S., Teng, C. T., and Allfrey, V. G. (1971), *J. Biol. Chem. 246,* 3597–3609.
Traugh, J. A., and Traut, R. R. (1974), *J. Biol. Chem. 249,* 1207–1212.
Wålinder, O. (1973), *Biochim. Biophys. Acta 293,* 140–149.
Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S., and Krebs, E. G. (1971), *J. Biol. Chem. 246,* 1968–1976.
Weber, K., and Osborn, M. (1969) *J. Biol. Chem. 244,* 4406–4412.
Yount, R. G., Babcock, D., Ballantyne, W., and Ojala, D. (1971), *Biochemistry 10,* 2484–2489.
Zillig, W., Fujiki, H., Blum, W., Janekovic, D., Schweiger, M., Rahmsdorf, H. J., Ponta, H., and Hirsch-Kauffmann, M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2506–2510.