Effect of Protein Kinase on Ribonucleic Acid Polymerase*

Orlando J. Martelo, Savio L. C. Woo, Erwin M. Reimann, and Earl W. Davie

ABSTRACT: Protein kinase from rabbit skeletal muscle and rabbit reticulocytes stimulates the DNA-dependent RNA polymerase of *Escherichia coli* with T4 DNA as template. Additional stimulation of RNA synthesis is observed in the presence of adenosine 3′,5′-monophosphate, but this effect varies from one preparation of protein kinase to another. The increase in RNA synthesis by protein kinase is associated with phosphorylation of the σ component or a σ-like factor present in RNA polymerase. This was indicated by experiments in which the enzyme was labeled with [γ-^{32}P]ATP in the presence of protein kinase and adenosine 3′,5′-monophosphate. Fractionation of the enzyme by chromatography on phosphocellulose columns indicated that phosphorylation occurs in the protein fraction containing the σ component. In further studies with partially purified σ factor and core enzyme, the direct phosphorylation of the fraction containing σ factor by adenosine triphosphate and protein kinase was observed. Under similar conditions, little or/no phosphorylation of the core enzyme occurred. These results suggest that adenosine 3′,5′-monophosphate and protein kinase play an important role in the regulation of RNA synthesis through a phosphorylation mechanism. Furthermore, phosphorylation of the enzyme may account for some of the heterogeneity previously observed with this enzyme.

Deoxyribonucleic acid dependent RNA polymerase purified from *Escherichia coli* by the method of Chamberlin and Berg (1962) as modified by Richardson (1966a) has been employed in many laboratories for enzyme studies. This preparation has a high specific activity, but shows some heterogeneity in chromatographic properties, specific activity, aggregation, and template DNA and tRNA binding (Hayashi *et al.*, 1964; Richardson, 1966b; Bremer *et al.*, 1966; Pettijohn and Kamiya, 1967; Stead and Jones, 1967). With the discovery of σ factor (Burgess *et al.*, 1969), M factor (Davison *et al.*, 1969), ρ factor (Roberts, 1969), and the stimulation of RNA polymerase by DNase (Vogt, 1969), it is clear that many of the differences in the properties of various RNA polymerase preparations were due to variable amounts of these proteins present in the enzyme preparation.

Heterogeneity also may be the result of minor proteolysis of the enzyme (Hara and Mitsui, 1967; Mitsui and Hara, 1967) or the presence of nuclei acid and other proteins such as the ω component (Burgess, 1969a). Structural modification of the polymerase can give rise to heterogeneity as indicated by the change in the polyacrylamide gel patterns and chromatographic profiles of the enzyme before and after T4 phage infection (Walter *et al.*, 1968; Crouch *et al.*, 1969).

Heterogeneity of RNA polymerase also could be due to phosphorylation of the enzyme by an adenosine 3′,5′-monophosphate dependent protein kinase in a manner analogous to the glycogen phosphorylase system (Krebs and Fischer, 1962; Walsh *et al.*, 1968), glycogen synthetase (Friedman and Larner, 1963), and pyruvic dehydrogenase (Wieland and Siess, 1970). Phosphorylation of RNA polymerase could account for recent experiments linking adenosine 3′,5′-monophosphate with the synthesis of a number of enzymes such as β-galactosidase which are sensitive to catabolite repression (Perlman and Pastan, 1968; Ullmann and Monod, 1968; Chambers and Zubay, 1969; Jacquet and Kepes, 1969). In the present communication we wish to report the effects of protein kinase isolated from rabbit tissue on *E. coli* RNA polymerase. A preliminary report of this work has appeared elsewhere (Martelo *et al.*, 1970).

Materials and Methods

Protein kinase from rabbit skeletal muscle was prepared by a modification of the method of Walsh *et al.* (1968). Protein kinase from peak I of the DE-52 column chromatography step was employed in the present studies unless stated otherwise (E. M. Reimann, D. A. Walsh, and E. G. Krebs, manuscript in preparation). Rabbit reticulocyte protein kinase, purified approximately 100-fold, was kindly provided by Dr. M. Tao, University of Illinois College of Medicine, Chicago, Ill. Protein kinase concentrations were determined by optical density measurements at 280 mμ assuming an absorbancy coefficient ($A_{1\,cm}^{1\%}$) at 280 mμ of 10.

RNA polymerase was prepared from *E. coli* B or MRE-600 by the method of Chamberlin and Berg (1962) as modified by Richardson (1966a). The hydroxylapatite enzyme was routinely employed in most experiments reported in this paper. RNA polymerase which was not purified by the hydroxylapatite step bound substantial amounts of ^{32}P from [γ-^{32}P]-ATP in the absence of protein kinase and was not employed in the present studies. When hydroxylapatite enzyme was subjected to electrophoresis in 5% polyacrylamide gels by the general methods of Ornstein (1964) and Davis (1964), a major protein band containing 85–90% of the total protein was observed with the Amido-Schwarz stain. Polyacrylamide gel electrophoresis was also carried out on the protein for enzyme assay, and these gels were sliced into 16 fractions and tested for polymerase activity. RNA polymerase activity was found only in the gel fraction corresponding to the major protein band.

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105, and Department of Biological Chemistry, University of California Medical School, Davis, California 95616. *Received August 7, 1970.* This work was supported by U. S. Public Health Service Grants GM 10793 and TI-AM 05472-5.

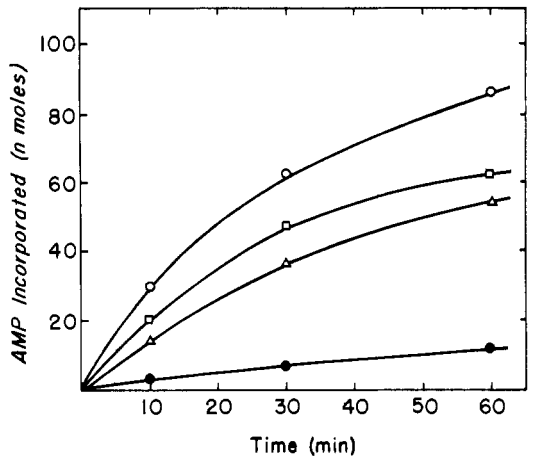

FIGURE 1: The effect of increasing concentrations of rabbit skeletal muscle protein kinase on RNA synthesis by RNA polymerase. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.8); 1.25 $\times$ 10^{-2} M $MgCl_2$; 1 $\times$ 10^{-3} M GTP, UTP, and CTP; 1 $\times$ 10^{-3} M [^3H]ATP (containing 10 μCi/μmole); 5 $\times$ 10^{-4} M glycerol; 0.2 M KCl; T4 DNA (50 μg/ml); RNA polymerase (25 μg/ml); and increasing amounts of muscle protein kinase. (●——●) control, (△——△) 12.5 μg/ml of protein kinase, (□——□) 25 μg/ml of protein kinase, and (○——○) 50 μg/ml of protein kinase. Samples were incubated at 37° for various time intervals and a 0.1-ml aliquot removed for assay as described in Materials and Methods.

For some experiments, the hydroxylapatite enzyme was further fractionated into core enzyme and σ factor by column chromatography on phosphocellulose by a modification of the method of Burgess (1969b). Polyacrylamide gel electrophoresis patterns of the native σ factor on 5% polyacrylamide gels showed four protein bands. In the presence of 0.1% sodium dodecyl sulfate, two protein bands were obtained.

The various RNA polymerase preparations were stored in 0.2 M KCl, 0.05 M Tris-HCl buffer (pH 7.8), 5 $\times$ 10^{-3} M dithiothreitol, 1 $\times$ 10^{-4} M ethylenediaminetetraacetate, and 50% glycerol at $-80°$. Polymerase concentration was determined by optical density measurements at 280 mμ assuming an absorbancy coefficient, $A^{1\%}_{1\,cm}$, at 280 mμ of 16.9 (Richardson, 1966b).

[^3H]ATP and [^{32}P]P_i were purchased from New England Nuclear Corp., Boston, Mass., and unlabeled nucleoside triphosphates from Pabst Laboratories, Milwaukee, Wis. Adenosine 3′,5′-monophosphate was purchased from Schwarz BioResearch, Orangeburg, N. Y. γ-^{32}P-labeled ATP was prepared by a modification of the method of Glynn and Chappell (1964). T4 DNA and *E. coli* DNA were isolated by a modification of the procedure of Marmur (1961). Calf thymus DNA was purchased from Calbiochem, Los Angeles, Calif. *E. coli* B, grown to half-log phase on enriched media, was purchased from Grain Processing Corp., Muscatine, Iowa.

Enzyme Assay. The typical reaction mixture for an RNA polymerase assay contained 0.1 M Tris-HCl buffer (pH 7.8); 1.25 $\times$ 10^{-2} M magnesium chloride; 1 $\times$ 10^{-3} M GTP, UTP, and CTP; 1 $\times$ 10^{-3} M [^3H]ATP (nonuniform label, specific activity 10 μCi/μmole); 1 $\times$ 10^{-2} M β-mercaptoethanol; 5 $\times$ 10^{-4} M glycerol; 0.2 M KCl; T4 DNA (50 μg/ml); and RNA polymerase (1–5 μg/ml) in a final volume of 0.2 ml. Reaction mixtures were incubated at 37° for 10 min. An aliquot (0.1 ml) of the reaction mixture was spotted on Whatman No. 3MM filter paper and washed four times with cold 5% trichloroacetic acid and once with cold 95% ethanol. The filter was dried and counted in a TriCarb liquid scintillation counter using Omni-Fluor toluene scintillant (So *et al.*, 1967).

σ factor was measured in the regular polymerase assay in the presence of core enzyme and T4 DNA. This assay usually contained about 5 μg/ml of σ factor and 10 μg/ml of core enzyme. Maximal stimulation by saturating levels of σ factor with T4 DNA as template was 50–60-fold.

Protein kinase activity with RNA polymerase as substrate was measured by the incorporation of ^{32}P from [γ-^{32}P]ATP into trichloroacetic acid precipitable material. The incubation mixture contained 0.025 M Tris-HCl buffer (pH 7.1), 2.5 $\times$ 10^{-4} M [γ-^{32}P]ATP (specific activity 0.1–0.7 mCi/μmole), 5 $\times$ 10^{-3} M magnesium chloride, 5 $\times$ 10^{-4} M dithiothreitol, 5 $\times$ 10^{-4} M glycerol, 300 μg/ml of RNA polymerase, and protein kinase (10–50 μg/ml) in a final volume of 0.2 ml. The reaction mixtures were incubated for various times at 37° and an aliquot (0.05 ml) was spotted on Whatman No. 3MM filter paper. The filters were then washed for 45 min with gentle swirling in a small volume of 10% trichloroacetic acid at 0° in the presence of 5 $\times$ 10^{-2} M ATP, and three additional times at 0° for 30 min each in 5% trichloroacetic acid in the presence of 0.01 M K_3PO_4 and 0.01 M $K_4P_2O_7$. Additional washings were made with an ethanol–acetone solution (equal volumes of each) and with acetone alone. The filters were dried and counted in a TriCarb liquid scintillation counter as above. Blanks generally ranged from 200 to 300 cpm per sample.

Protein kinase activity with σ factor as a substrate was measured in a manner similar to that with RNA polymerase as substrate. In this case, the reaction mixture generally contained 0.1 M Tris-HCl buffer (pH 7.8); 0.2 M KCl; 1 $\times$ 10^{-3} M GTP, CTP, and UTP; 1 $\times$ 10^{-3} M [γ-^{32}P]ATP (specific activity 0.1–0.7 mCi/μmole); 1.25 $\times$ 10^{-2} M magnesium chloride; 5 $\times$ 10^{-4} M dithiothreitol; 5 $\times$ 10^{-4} M glycerol; 20 μg/ml of σ factor; and skeletal muscle protein kinase (20 μg/ml) in a final volume of 0.2 ml. In some experiments, the phosphorylation of σ factor was carried out in the absence of KCl, UTP, CTP, and GTP. Reaction mixtures were incubated and assayed as described above for the complete enzyme.

Results

Stimulation of RNA Synthesis. The stimulation of RNA polymerase by increasing amounts of skeletal muscle protein kinase is shown in Figure 1. In these experiments, T4 DNA was employed as a template. The protein kinase, which contained no polymerase activity itself, stimulated the incorporation of radioactive AMP into RNA about 8-fold at the highest concentration employed. The same stimulation was observed when the reaction was followed by labeling each of the four nucleoside triphosphates. Stimulation by the skeletal muscle protein kinase varied from one preparation of RNA polymerase to another and ranged from 2- to 13-fold. The stimulation also varied with the stage of purification of the RNA polymerase. For instance, in one preparation made by the method of Chamberlin and Berg (1962), the stimulation was 0-, 5.8-, 11-, and 13-fold for the crude supernatant, the 30–

TABLE I: Effect of Protein Kinase on RNA Synthesis by RNA Polymerase.[a]

Expt	Additions	AMP Incorp (nmoles/10 min) T4 DNA	Calf Thymus DNA	*E. coli* DNA
1	Polymerase	8.2	2.3	2.6
2	Polymerase + muscle protein kinase	16	2.7	2.6
3	Polymerase + muscle protein kinase + adenosine 3′,5′-monophosphate	20	2.3	2.6
4	Polymerase	4.5		
5	Polymerase + red cell protein kinase	13		
6	Polymerase + red cell protein kinase + adenosine 3′,5′-monophosphate	27		

[a] The reaction mixture is essentially the same as that described under Figure 1 except as follows: T4 DNA (50 μg/ml); calf thymus DNA (400 μg/ml) or *E. coli* DNA (400 μg/ml) as shown; muscle protein kinase (50 μg/ml) in expt 2 and 3, and red cell protein kinase (45 μg/ml) in expt 5 and 6; adenosine 3′,5′-monophosphate at a concentration of 1×10^{-6} M in expt 3, and 1×10^{-5} M in expt 6. Samples were incubated for 10 min at 37°, and 0.1-ml aliquots were removed for assay as described in Materials and Methods.

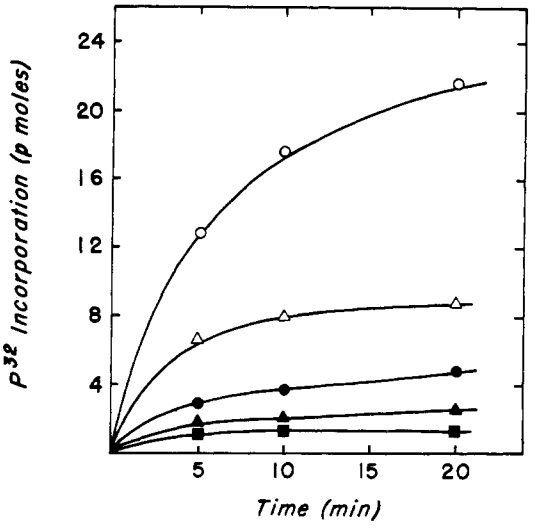

FIGURE 2: Effect of rabbit skeletal muscle protein kinase on ^{32}P incorporation into RNA polymerase. The reaction mixture contained 0.05 M Tris-HCl buffer (pH 7.8), 2.5×10^{-4} M [γ-^{32}P]ATP (specific activity 400 μCi/μmole), 5×10^{-3} M $MgCl_2$, 5×10^{-4} M dithiothreitol, 5×10^{-4} M glycerol, 300 μg/ml of RNA polymerase, and 30 μg/ml of muscle protein kinase. (■——■) RNA polymerase and adenosine 3′,5′-monophosphate; (▲——▲) protein kinase; (●——●) protein kinase and adenosine 3′,5′-monophosphate; (△——△) RNA polymerase and protein kinase; (○——○) RNA polymerase, protein kinase, and adenosine 3′,5′-monophosphate (1×10^{-6} M). Reaction mixtures were incubated at 37° and aliquots (0.05 ml) were removed and assayed for acid-precipitable ^{32}P as described in Materials and Methods. Samples with RNA polymerase alone were identical with those of RNA polymerase in the presence of adenosine 3′,5′-monophosphate.

50% ammonium sulfate fraction, the DEAE-cellulose fraction, and the hydroxylapatite fraction, respectively. Preincubation of T4 DNA with rabbit muscle protein kinase did not result in greater stimulation of RNA synthesis. Thus, the possibility of a template modification as a mechanism for RNA synthesis stimulation by protein kinase seems unlikely.

Stimulation of RNA polymerase was observed by protein kinase throughout its purification from skeletal muscle. Furthermore, a general correlation was observed between RNA polymerase stimulation and phosphorylation of casein by the crude muscle extract, the pH 5.5 supernatant, the 78,000*g* supernatant, and the purified fractions from DE-52 column chromatography (E. M. Reimann *et al.*, manuscript in preparation).

The effects of adenosine 3′,5′-monophosphate on RNA polymerase in the presence of protein kinase are shown in Table I. At low levels of skeletal muscle protein kinase, the synthesis of RNA was increased about 2-fold with T4 DNA as template. This was further increased in the presence of 10^{-6} M adenosine 3′,5′-monophosphate. The effect of adenosine 3′,5′-monophosphate, however, was variable, and with some muscle protein kinase preparations, no additional stimulation was observed with adenosine 3′,5′-monophosphate. With other muscle protein kinase preparations, however, complete dependency on adenosine 3′,5′-monophosphate was observed. Adenosine 3′,5′-monophosphate at a concentration of 1×10^{-6} M has no effect on the polymerase by itself. With calf thymus or *E. coli* DNA as template, little stimulation of RNA synthesis was observed at this concentration of protein kinase.

A similar stimulation of RNA synthesis by *E. coli* RNA polymerase was observed with a protein kinase isolated from rabbit reticulocytes (Table I). With this protein kinase preparation, the rate of RNA synthesis was increased about 2-fold in the presence of adenosine 3′,5′-monophosphate.

^{32}P Incorporation into RNA Polymerase. The stimulation of RNA synthesis by protein kinase suggests that phosphorylation of the RNA polymerase may be occurring. This possibility was tested employing [γ-^{32}P]ATP as a substrate. The formation of acid-precipitable ^{32}P with time following the incubation of RNA polymerase with skeletal muscle or red cell protein kinase is shown in Figures 2 and 3. Substantial amounts of radioactive phosphorous became protein bound in the presence of protein kinase and RNA polymerase, and this was greatly increased in the presence of adenosine 3′,5′-monophosphate. Phosphorylation of protein was indicated by the fact that digestion of the trichloroacetic acid precipitate with trypsin and chymotrypsin resulted in solubilization of ^{32}P. Some incorporation of radioactive phosphate into an acid-insoluble form occurred in the controls, which contained protein kinase and adenosine 3′,5′-monophosphate, or polymerase and adenosine 3′,5′-monophosphate. These levels, however, were always far below that found in the complete reaction mixtures.

Further identification of the phosphorylation site was made

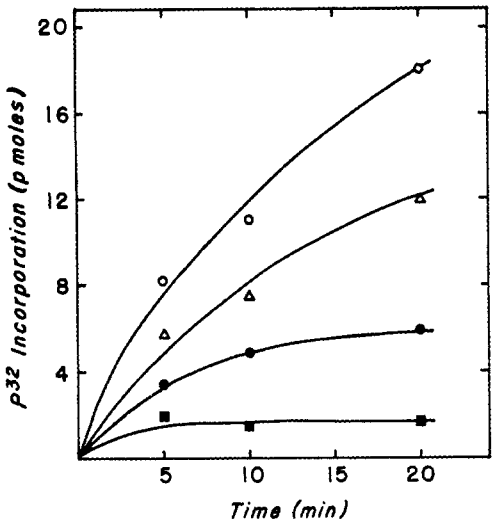

FIGURE 3: Effect of red cell protein kinase on ^{32}P incorporation into RNA polymerase. The reaction mixture contained 0.125 M Tris-HCl buffer (pH 7.5), 20 mM $MgCl_2$, 1×10^{-5} M adenosine 3',5'-monophosphate, 2.5×10^{-4} M [γ-^{32}P]ATP (specific activity 400 μCi/μmole), 60 μg of RNA polymerase, and 20 μg of red cell kinase in a final volume of 0.2 ml. (■——■) RNA polymerase and adenosine 3',5'-monophosphate; (●——●) red cell kinase and adenosine 3',5'-monophosphate; (△——△) RNA polymerase and red cell kinase; (○——○) RNA polymerase, red cell kinase, and adenosine 3',5'-monophosphate. Reaction mixtures were incubated at 37° and aliquots (0.05 ml) were removed and assayed for acid-precipitable ^{32}P as described in Materials and Methods.

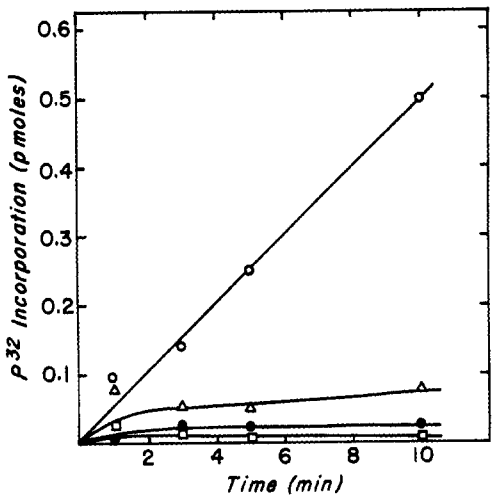

FIGURE 4: Effect of rabbit skeletal muscle protein kinase on ^{32}P incorporation into partially purified σ factor. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.8); 0.2 M KCl, 1×10^{-3} M [γ-^{32}P]ATP (specific activity 500 μCi/μmole); 1×10^{-3} M GTP, CTP, and UTP; 1.25×10^{-2} M $MgCl_2$; 5×10^{-4} M dithiothreitol; 5×10^{-4} M glycerol; 20 μg/ml of partially purified σ factor; and 20 μg/ml of skeletal muscle protein kinase in a final volume of 0.2 ml. (□——□) Protein kinase and adenosine 3',5'-monophosphate; (●——●) partially purified σ factor and adenosine 3',5'-monophosphate; (△——△) partially purified σ factor and protein kinase; (○——○) σ factor, protein kinase, and adenosine 3',5'-monophosphate (1×10^{-6} M). Reaction mixtures were incubated at 37° and aliquots (0.02 ml) were removed and assayed for acid-precipitable ^{32}P as described in Materials and Methods.

by chromatography of the phosphorylated polymerase on phosphocellulose. This procedure separates σ factor from the core enzyme (Burgess *et al.*, 1969). In these experiments, RNA polymerase was incubated with [γ-^{32}P]ATP, adenosine 3',5'-monophosphate, and skeletal muscle protein kinase. After a 45-min incubation at 30°, the sample was subjected to gel filtration on a Sephadex G-75 column (1.0 × 60 cm) to remove the unreacted [γ-^{32}P]ATP. The polymerase which now contained radioactive phosphate was subjected to column chromatography on phosphocellulose. After chromatography, the major portion of the protein-bound ^{32}P was present in the fraction containing σ factor, and little radioactivity was in the core enzyme fraction. Therefore, the direct phosphorylation of σ factor by protein kinase was attempted. In the experiments shown in Figure 4, the reaction conditions were similar to those in the RNA polymerase assay, while those in Figure 5 represent conditions more optimal for the protein kinase reaction. It can be seen that protein kinase from skeletal muscle stimulates the incorporation of ^{32}P from [γ-^{32}P]ATP into a partially purified RNA polymerase σ factor in the presence of adenosine 3',5'-monophosphate. At the lower protein kinase concentrations employed in these experiments, little radioactive phosphate became protein bound in the controls.

In other experiments, protein kinase and [γ-^{32}P]ATP were incubated with core polymerase in the presence of adenosine 3',5'-monophosphate. In these studies, little or no ^{32}P becomes protein bound. Thus, RNA polymerase is phosphorylated by ATP in the presence of protein kinase and adenosine 3',5'-monophosphate, and this phosphorylation occurs with the protein fraction containing the σ component.

Activation of Partially Purified σ Factor by Muscle Protein Kinase. The phosphorylation of the partially purified σ factor preparation suggests that preincubation of this fraction with ATP and protein kinase may lead to activation when it is assayed in a second incubation reaction mixture with core enzyme and T4 DNA as template. The results of these experiments are shown in Table II.

In tube 1, the buffer control containing nucleoside triphosphates and adenosine 3',5'-monophosphate was preincubated for 5 min. An aliquot (0.02 ml) was then added to an RNA assay mixture containing core enzyme and T4 DNA. This diluted the original sample 10-fold in the assay reaction mixture. The second tube containing σ factor was also preincubated for 5 min, and an aliquot added to a second polymerase assay. In this case, nearly a 6-fold stimulation of RNA synthesis occurred when an aliquot of the preincubated σ factor preparation was added to the final RNA assay containing core polymerase (0.73 *vs.* 4.1). In tube 3, the preincubation mixture contained protein kinase in addition to σ factor, and, in this case, a 10-fold stimulation of RNA synthesis was found. This activity is nearly 2-fold greater than the σ factor preparation preincubated in the absence of protein kinase (4.1 *vs.* 7.5). In these experiments, protein kinase was diluted 10-fold in the polymerase assay and at this concentration had no effect on the synthesis of RNA in the final assay. A similar effect is shown in tubes 4, 5, and 6 where protein kinase also stimulated the σ factor activity following preincubation with protein kinase.

Discussion

The results reported in this communication show that RNA polymerase of *E. coli* is stimulated by protein kinase from

either rabbit skeletal muscle or rabbit reticulocytes. The stimulation of RNA synthesis is associated with a phosphorylation of a σ or σ-like component of RNA polymerase by ATP and protein kinase.

Phosphorylation of a σ or σ-like factor by a bacterial protein kinase similar to that reported by Kuo and Greengard (1969) provides a simple mechanism for the control of the amount and type of RNA synthesized in bacteria. In *E. coli*, σ factor participates in RNA chain initiation (Travers and Burgess, 1969; Dunn and Bautz, 1969) and is released from the polymerase–DNA complex once RNA initiation occurs. Thus, a phosphorylation–dephosphorylation series of reactions could shift chain initiation from one site to another on the DNA template and thus influence the type and amount of various RNAs which are synthesized. It is also possible that more than one σ factor exists in RNA polymerase, only one of which is phosphorylated. This may also affect the synthesis of numerous classes of RNAs by changing the initiation site for the RNA polymerase.

Phosphorylation of a σ or σ-like factor could provide a simple explanation for the synthesis of a number of enzymes which are sensitive to catabolite repression. This type of cellular regulation occurring in microorganisms was originally called the glucose effect (Epps and Gale, 1942) since carbon sources such as glucose repress the synthesis of various inducible enzymes such as β-galactosidase. When the supply of glucose is exhausted in these cells, there is a rapid appearance of adenosine 3′,5′-monophosphate (Makman and Sutherland, 1965). From the studies of Perlman and Pastan (1968), Ullman and Monod (1968), and Chambers and Zubay (1969), it is clear that the synthesis of mRNA for β-galactosidase is associated with the appearance of adenosine 3′,5′-monophosphate. These studies are summarized in an excellent review recently published by Pastan and Perlman (1970). The experiments of Jacquet and Kepes (1969) with rifampicin suggest that the adenosine 3′,5′-monophosphate control is at the level of transcription. Thus, the presence of a protein kinase in *E. coli* which is activated by adenosine 3′,5′-monophosphate (Kuo and Greengard, 1969) could lead to the phosphorylation of RNA polymerase and the transcription of the lac operon. Recently, Zubay *et al.* (1970) and Emmer *et al.* (1970) have partially purified an adenosine 3′,5′-monophosphate binding protein from *E. coli* which stimulates β-galactosidase synthesis. Whether this adenosine 3′,5′-monophosphate binding protein is related to a protein kinase such as that described by Kuo and Greengard (1969) has not been established.

In the present studies, little or no stimulation of RNA synthesis by protein kinase was observed with either *E. coli* or calf thymus DNA as template. This is not surprising since protein kinase does not exert its effect directly on the core enzyme. Since σ factor has little effect on net RNA synthesis with core enzyme and calf thymus DNA as template (Burgess *et al.*, 1969) or with *E. coli* DNA as template (S. L. C. Woo, unpublished results), its modification by protein kinase would not influence the total amount of RNA synthesis with these DNAs employed as template. It is possible, however, that a different RNA product was synthesized in the presence of protein kinase with these DNAs, and this difference was not detected by changes in the total synthesis of RNA. Other procedures such as hybridization techniques will be required to show whether any difference exists in the products synthesized with these DNA templates. Studies of this type are necessary in order to show whether any relationship exists between protein kinase and catabolite repression.

TABLE II: Activation of Partially Purified σ Factor by Muscle Protein Kinase.[a]

Tube	Additions	AMP Incorp in the Presence of Core Polymerase (nmoles/10 min)
1	Control	0.73
2	σ factor	4.1
3	σ factor + protein kinase	7.5
4	Control	0.7
5	σ factor	1.2
6	σ factor + protein kinase	2.6

[a] The preincubation reaction mixture contained: 0.1 M Tris-HCl buffer (pH 7.8); 0.2 M KCl; 1×10^{-3} M ATP, GTP, UTP, and CTP; 1.25×10^{-2} M magnesium chloride; 1×10^{-6} M adenosine 3′,5′-monophosphate; 10 μg/ml of rabbit skeletal muscle protein kinase; and partially purified σ factor in a volume of 0.3 ml. Tubes 2 and 3 contained 20 μg σ factor/ml, and tubes 5 and 6 contained 10 μg of σ factor/ml. After preincubation at 37° for 5 min, a 0.02-ml sample was removed and added to an RNA polymerase assay reaction mixture containing T4 DNA and core polymerase (see Materials and Methods). The control preincubation reaction mixtures contained buffer alone or σ factor in the absence of protein kinase.

Phosphorylation of RNA polymerase provides another explanation for the heterogeneity of this enzyme influencing both its biological activity and its physical–chemical properties. Thus, some of the changes in the specific activity and chromatographic behavior of this enzyme may be due to variations in the growth conditions of the cells employed for isolating the enzyme. For instance, *E. coli* cells, which have utilized all of their glucose and are rapidly synthesizing adenosine 3′,5′-monophosphate (Makman and Sutherland, 1965), may contain polymerase in a highly phosphorylated stage. This enzyme could differ substantially from polymerase isolated from cells grown on enriched media.

In 1969, Davison and coworkers described an RNA polymerase stimulatory factor, designated M factor, from an *E. coli* subcellular fraction rich in ribosomes. This factor stimulates 30–50-fold the *in vitro* transcription of DNA exerting the largest effect with T4 DNA as template. Whether the M factor is a bacterial protein kinase and exerts its influence in a manner similar to the rabbit skeletal muscle protein kinase is not known. It appears to be different from σ factor since the stimulatory effect of M factor is not restricted to the use of core enzyme prepared by phosphocellulose chromatography.

Recently, Stein and Hausen (1970) isolated a stimulatory factor from calf thymus which increased the activity of calf thymus RNA polymerase about 10-fold. This stimulatory factor did not, however, influence the rate of RNA synthesis

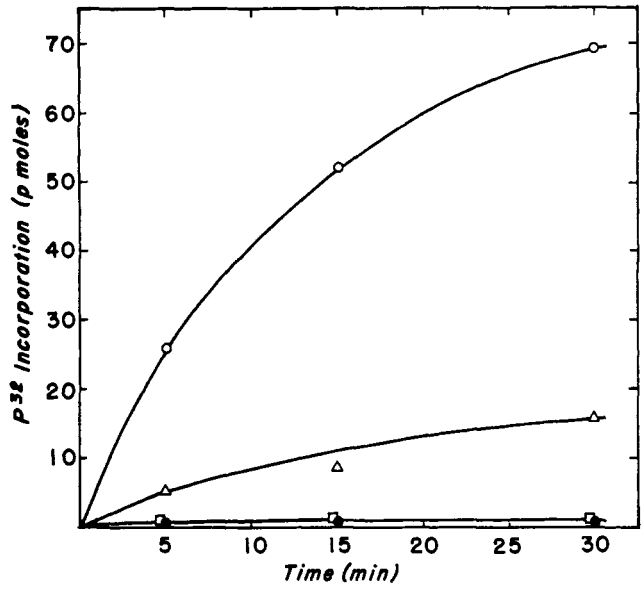

FIGURE 5: Effect of rabbit skeletal muscle protein kinase on ^{32}P incorporation into partially purified σ factor. The reaction mixture contained 0.05 M Tris-HCl buffer (pH 7.8); 2.5×10^{-4} M [γ-^{32}P]ATP (specific activity 400 μCi/μmole); 5×10^{-3} M $MgCl_2$; 5×10^{-4} M dithiothreitol; 5×10^{-4} M glycerol; 100 μg/ml of partially purified σ factor; and 40 μg/ml of muscle protein kinase. (□——□) σ factor and adenosine 3′,5′-monophosphate; (●——●) protein kinase and adenosine 3′,5′-monophosphate; (△——△) σ factor and muscle protein kinase; (○——○) σ factor, muscle protein kinase, and adenosine 3′,5′-monophosphate (1×10^{-6} M). Reaction mixtures were incubated at 37° and aliquots (0.05 ml) were removed and assayed for acid-precipitable ^{32}P as described in Materials and Methods.

catalyzed by the *E. coli* RNA polymerase. Therefore, it appears likely that either this stimulatory factor is not a protein kinase or its substrate specificity is not as broad as the protein kinase from skeletal muscle.

The present studies add another substrate to the list of proteins which can be phosphorylated by skeletal muscle protein kinase. In previous studies, phosphorylation of phosphorylase *b* kinase, casein, and protamine (Walsh *et al.*, 1968), and histone and glycogen synthetase (Soderling *et al.*, 1970) has been demonstrated. Thus, it is clear that this mammalian enzyme has a broad substrate specificity. In the case of polymerase, however, higher concentrations of protein kinase are required for maximal activity as compared to substrates such as phosphorylase *b* kinase.

The extent of the phosphorylation of σ factor by protein kinase has not been established since a careful analysis of phosphate binding will require pure σ factor in sizable amounts. Phosphorylation of σ factor under conditions used for RNA synthesis but not optimal for protein kinase phosphorylation is shown in Figure 4. Under these conditions and using only 20 μg/ml of σ factor as substrate, little but significant phosphorylation occurred. In experiments with incubation conditions optimal for protein kinase phosphorylation, σ factor was phosphorylated to the same extent as RNA polymerase (Figure 5). Also, the degree of phosphorylation of the σ factor when isolated from *E. coli* must be established. Whether the phosphorylation reaction directly parallels the activation of σ factor as measured by net RNA synthesis is also of interest. In the case of phosphorylase *b* kinase from rabbit skeletal muscle, phosphorylation continues after maximal activation of the enzyme by protein kinase, ATP, and adenosine 3′,5′-monophosphate (Riley *et al.*, 1968).

Covalent modification of the sigma component of RNA polymerase *via* phosphorylation differs from that occurring in the core enzyme following T4 phage infection. In this case, there is a structural modification of the α subunit (Walter *et al.*, 1968) and the host σ factor disappears (Seifert, 1969). However, a new bacteriophage σ factor can be isolated from infected cells, and this component is responsible for the synthesis of early messenger (Travers, 1969, 1970). Whether the phage T4 σ factor is related to host σ factor has not been established. It is possible that following phage infection phosphorylation of the host σ factor gives rise to the phage T4 σ factor.

The possibility that RNA polymerase in mammalian cells may be under the control of adenosine 3′,5′-monophosphate and protein kinase is interesting since many hormones, such as estrogens and ACTH, elevate the adenosine 3′,5′-monophosphate levels in various organs (Handler *et al.*, 1965; Savard *et al.*, 1965; Bitensky and Burstein, 1965; Garren *et al.*, 1966; Taunton *et al.*, 1967; Hechter *et al.*, 1967; Chase and Aurbach, 1970). Subsequently, there is the appearance of new protein synthesized *de novo* presumably involving the synthesis of new mRNA. In these cells, such a mechanism may involve a direct influence of adenosine 3′,5′-monophosphate and protein kinase on a nuclear RNA polymerase.

Acknowledgments

The authors express their sincere thanks to Dr. Edwin G. Krebs for his help and suggestions which have made this project possible. Thanks are also due to Drs. Shirley Su Gillam, Edmond H. Fischer, and Richard Haschke, and Mrs. Carol King for their assistance in these studies.

References

Bitensky, M. W., and Burstein, S. R. (1965), *Nature (London) 208*, 1282.

Bremer, H., Yegian, C., and Konrad, M. (1966), *J. Mol. Biol. 16*, 94.

Burgess, R. R. (1969a), *J. Biol. Chem. 244*, 6168.

Burgess, R. R. (1969b), *J. Biol. Chem. 244*, 6160.

Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969), *Nature (London), 221*, 43.

Chamberlin, M., and Berg, P. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 81.

Chambers, D. A., and Zubay, G. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 118.

Chase, L. R., and Aurbach, G. D. (1970), *J. Biol. Chem. 245*, 1520.

Crouch, R. J., Hall, B. D., and Hager, G. (1969), *Nature (London) 223*, 476.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Davison, J., Pilarski, L. M., and Echols, H. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 168.

Dunn, J. J., and Bautz, E. K. F. (1969), *Biochem. Biophys. Res. Commun. 36*, 925.

Emmer, M., deCrombrugghe, B., Pastan, I., and Perlman, R. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 480.

Epps, H. M. R., and Gale, E. F. (1942), *Biochem. J. 36*, 619.
Friedman, D. L., and Larner, J. (1963), *Biochemistry 2*, 669.
Garren, L. D., Davis, W. W., Crocco, R. M., and Ney, R. L. (1966), *Science 152*, 1386.
Glynn, I. M., and Chappell, J. B. (1964), *Biochem. J. 90*, 147.
Handler, J. S., Butcher, R. W., Sutherland, E. W., and Orloff, J. (1965), *J. Biol. Chem. 240*, 4524.
Hara, K., and Mitsui, H. (1967), *J. Biochem. (Tokyo) 61*, 359.
Hayashi, M., Hayashi, M. N., and Spiegelman, S. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 351.
Hechter, O., Yoshinaga, K., Halkerston, I. D. K., and Birchall, K. (1967), *Arch. Biochem. Biophys. 122*, 449.
Jacquet, M., and Kepes, A. (1969), *Biochem. Biophys. Res. Commun. 36*, 84.
Krebs, E. G., and Fischer, E. H. (1962), *Advan. Enzymol. 24*, 263.
Kuo, J. F., and Greengard, P. (1969), *J. Biol. Chem. 244*, 3417.
Makman, R. S., and Sutherland, E. W. (1965), *J. Biol. Chem. 240*, 1309.
Marmur, J. (1961), *J. Mol. Biol. 3*, 208.
Martelo, O. J., Woo, S. L. C., Reimann, E. M., and Davie, E. W. (1970), Pacific Slope Biochemical Conference, La Jolla, Calif., June, p 22.
Mitsui, H., and Hara, K. (1967), *J. Biochem. (Tokyo), 61*, 647.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Pastan, I., and Perlman, R. L. (1970), *Science 169*, 339.
Perlman, R. L., and Pastan, I. (1968), *J. Biol. Chem. 243*, 5420.
Pettijohn, D., and Kamiya, T. (1967), *J. Mol. Biol. 29*, 275.
Richardson, J. P. (1966a), *Proc. Nat. Acad. Sci. U. S. 55*, 1616.
Richardson, J. P. (1966b), *J. Mol. Biol. 21*, 115.
Riley, W. D., DeLange, R. J., Bratvold, G. E., and Krebs, E. G. (1968), *J. Biol. Chem. 243*, 2209.
Roberts, J. W. (1969), *Nature (London) 224*, 1168.
Savard, K., Marsh, J. M., and Rice, B. F. (1965), *Recent Progr. Hormone Res. 21*, 285.
Seifert, W. (1969), *in* RNA-Polymerase and Transcription, Lepetit Colloquia on Biology and Medicine, Silvestri, L., Ed., Amsterdam, North-Holland Publishing Co., p 158.
So, A. G., Davie, E. W., Epstein, R., and Tissieres, A. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1739.
Soderling, T. R., Hickenbottom, J. B., Reimann, E. M., Hunkeler, F. L., Walsh, D. A., and Krebs, E. G. (1970), *J. Biol. Chem.* (in press).
Stead, N. W., and Jones, O. W. (1967), *J. Mol. Biol. 26*, 131.
Stein, H., and Hausen, P. (1970), *Eur. J. Biochem. 14*, 270.
Taunton, O. D., Roth, J., and Pastan, I. (1967), *Biochem. Biophys. Res. Commun. 29*, 1.
Travers, A. A. (1969), *Nature (London) 223*, 1107.
Travers, A. A. (1970), *Nature (London) 225*, 1009.
Travers, A. A., and Burgess, R. R. (1969), *Nature (London) 222*, 537.
Ullmann, A., and Monod, J. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2*, 57.
Vogt, V. (1969), *Nature (London) 223*, 854.
Walsh, D. A., Perkins, J. P., and Krebs, E. G. (1968), *J. Biol. Chem. 243*, 3763.
Walter, G., Seifert, W., and Zillig, W. (1968), *Biochem. Biophys. Res. Commun. 30*, 240.
Wieland, O., and Siess, E. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 947.
Zubay, G., Schwartz, D., and Beckwith, J. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 104.