Phosphorylative and Functional Modifications of Nucleoplasmic RNA Polymerase II by Homologous Adenosine 3':5'-Monophosphate-dependent Protein Kinase from Calf Thymus and by Heterologous Phosphatase*

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We have studied the effects of calf thymus nuclear cAMP-dependent protein kinase and of bacterial phosphatase on the phosphorylative and functional modification of calf thymus nucleoplasmic RNA polymerase II. Incubation of highly purified preparations of calf thymus nuclear cAMP-dependent protein kinase and of homologous RNA polymerase II in the presence of [γ-32P]ATP and 1 μM cAMP led to a 3-fold stimulation of RNA polymerase II activity and to an average incorporation of 0.5 mol of [32P]phosphate/mol of RNA polymerase II. Analysis of the 32P-labeled RNA polymerase II by polyacrylamide gel electrophoresis under nondenaturing conditions showed that both forms of RNA polymerase II were phosphorylated. Polyacrylamide gel electrophoresis of the 32P-labeled RNA polymerase II in the presence of 0.1% sodium dodecyl sulfate revealed that the 25,000-dalton polypeptide subunit of RNA polymerase II (subunit B5) served as the principal [32P]phosphate acceptor. In some but not all experiments a minor degree of [32P] incorporation into the 180,000-dalton subunit (subunit B2) was also observed. Ion exchange chromatography of acid-hydrolyzed 32P-labeled RNA polymerase II resulted in the identification of serine and threonine as the [32P]phosphate acceptor amino acids.

Phosphorylation and activation of RNA polymerase II were dependent upon the presence of ATP and of the active catalytic subunit of the nuclear cAMP-dependent protein kinase. Substitution of ATP with adenylyl imidodiphosphate, an inhibitor of protein kinase, prevented protein kinase-mediated activation of RNA polymerase II. Furthermore, selective inhibition of the protein kinase catalytic subunit by a heat-stable protein kinase inhibitor from rabbit muscle led to both an inhibition of RNA polymerase II phosphorylation and to a proportional decrease of the degree of activation of RNA polymerase II. Incubation of 32P-labeled RNA polymerase II with alkaline phosphatase from Escherichia coli resulted in a loss of 32P label with a concomitant decrease of RNA polymerase II activity. The results indicate that phosphorylative and functional modifications of RNA polymerase II by homologous nuclear protein kinases and by heterologous phosphatase may be achieved in vitro.

In recent years, cAMP-dependent and independent protein kinases from nuclei of a number of tissues have been identified and characterized (1-12). The biological role of nuclear protein kinases remains, however, unknown. It is conceivable that in combination with nuclear phosphoprotein phosphatase, nuclear protein kinases of the cAMP-dependent and independent variety act as converter enzymes achieving the phosphorylative and functional modification of proteins that partake in the diversity and complexity of nuclear function.

Alterations in nuclear RNA synthesis correlate closely with changes in the phosphorylation of nuclear nonhistone proteins (13-19), and there is indirect evidence suggesting that control of DNA transcription may be achieved through a phosphorylative and functional modification of RNA polymerases. We have previously reported the activation of RNA polymerases I and II by a cAMP-dependent protein kinase from calf ovary cytosol (20), and we demonstrated a functional modification of calf thymus RNA polymerase II by homologous nuclear cAMP-dependent protein kinase and by phosphatase (21). Additionally, activation of rat liver RNA polymerase I (22) and of ascites tumor RNA polymerase II (23) was reported to occur through the action of homologous protein kinases.

In the light of these reports, it appears a distinct possibility that functional modification of polymerase activity may be achieved through phosphorylation and dephosphorylation of the core RNA polymerase. The following report presents evidence for the subunit phosphorylation and stimulation of the enzymatic activity of RNA polymerase II by a homologous nuclear cAMP-dependent protein kinase from calf thymus in vitro. Additionally, we demonstrate the in vitro dephosphorylation and deactivation of RNA polymerase II through the action of phosphatase.

EXPERIMENTAL PROCEDURES

Materials—All biochemical reagents were purchased from Sigma Chemical Co., St. Louis, Mo. Ultrapure ammonium sulfate and ribonuclease-free sucrose were obtained from Schwarz/Mann, Or-
angeburg, N. Y. Adenosine 5'-yl-[32P]triphosphate, ammonium salt (2 to 10 Ci/mmol), uridine 5'-yl-[32P]triphosphate, ammonium salt (5 to 10 Ci/mmol), uridine 5'-yl-[5,6-3H]triphosphate, tetrasodium salt (35 to 50 Ci/mmol), were purchased from New England Nuclear, Boston, Mass. Whatman phosphocellulose P-11 was prepared according to Gissinger and Chambon (24).

Tissue-Thymus glands from 4- to 5-month-old calves were obtained at the time of slaughter. The tissues were immediately frozen in dry ice, stored at -80°C, and used for experimentation within 4 weeks after their collection from the slaughter house.

Preparation and Purification of Calf Thymus Nuclear CAMP-dependent Protein Kinase and of RNA Polymerase II—Nuclear non-histone proteins were solubilized and extracted from purified calf thymus nuclei by a modification of the method of Roeder and Rutter (25) which was previously described by us (20). The nuclear nonhistone protein fraction was applied onto a column of Whatman DE52 DEAE-cellulose equilibrated in 20 mM Tris, 5 mM MgCl2, 0.1 mM EDTA, 0.5 mM dithiothreitol, 25% (v/v) glycerol, pH 7.4 (Buffer A) containing 50 mM ammonium sulfate (Buffer B). The column was washed with 5 bed volumes of Buffer B, Elution was carried out with a linear gradient between solutions of 0.05 and 0.5 M ammonium sulfate in Buffer A. Aliquots of the fractions eluted from the column were assayed for protein content, RNA polymerase, and protein kinase activities (for elution profile see Ref. 20 (25)).

The fractions containing RNA polymerase II activity were pooled, dialyzed against 20 mM Tris, 0.1 mM EDTA, 0.5 mM dithiothreitol, 95% (v/v) glycerol, pH 7.4 (Buffer C) containing 50 mM ammonium sulfate (Buffer D). The dialyzed sample was applied onto a column of Whatman phosphocellulose P-11 equilibrated in Buffer D. The column was subsequently washed with 5 bed volumes of Buffer D and eluted with a linear gradient between solutions of 0.05 and 0.5 M ammonium sulfate in Buffer C. The fractions containing RNA polymerase II activity eluted with 0.27 M ammonium sulfate. They were pooled, dialyzed against Buffer B, and concentrated with the Minisart, a single hollow fiber microconcentrator (MMA Sciatic, Inc., Park Ridge, Ill.). The RNA polymerase II preparation was either used immediately for the phosphorylation studies or it was stored at -80°C after adjustment of the glycerol concentration in Buffer B to 60% (v/v). Under these conditions no loss of enzyme activity was detected for a storage period of up to 4 weeks. Purity and homogeneity of the RNA polymerase II preparations were ascertained by polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions revealed a characteristic banding pattern of RNA polymerase II subunits as described by Kedinger and Chambon (26). No other contaminating proteins were detected. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the characteristic banding pattern of RNA polymerase II subunits as described by Kedinger and Chambon (26). Under these nondenaturing conditions the contaminating protein bands and protein kinase activity were eluted from the DEAE-cellulose column (for DEAE-cellulose elution profile see Ref. 21) as described in the previous findings (26). No other contaminating proteins were detected. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the characteristic banding pattern of RNA polymerase II subunits as described by Kedinger and Chambon (26). Under these nondenaturing conditions the contaminating protein bands and protein kinase activity were eluted from the DEAE-cellulose column (for DEAE-cellulose elution profile see Ref. 21) as described in the previous findings (26).

Preparation of RNA Polymerase II Activity—RNA polymerase assays were performed as previously described by us (90) but with 6 nmol of radioactive UTP as substrate in a total reaction volume of 0.15 ml. Reactions were initiated by the addition of RNA polymerase II to the assay mixtures, and incubation was carried out at 37°C for 15 min as described in the text. The reaction was terminated by the addition of 1 ml of 10 mM Tris-HCl, pH 8.0, 200 μg of RNA, 200 μg of bovine serum albumin, 1 mM UTP and of 0.5 M sodium dodecyl sulfate. The reaction mixtures were thoroughly mixed on a Vortex mixer. After addition of 2 ml of 10% trichloroacetic acid in ice for 30 min and then filtered using Millipore filters (0.3 μ). The filters were washed five times with 8 ml of ice-cold 5% trichloroacetic acid in 0.01 M sodium pyrophosphate, dried, dissolved in 10 ml of phase-combining system (Amersham/Searle), and analyzed for radioactivity. Under the experimental conditions incorporation of radioactive UTP into acid-insoluble material proceeded linearly up to 60-min incubation time. Heat-denatured calf thymus DNA was prepared from native DNA by heat treatment at 100°C for 5 min and subsequent cooling in ice. Denatured DNA was prepared freshly before each experiment.

Assay of Protein Kinase Activity—Protein kinase activity was assayed with protamine as substrate as previously described by us (31). When RNA polymerase II was the substrate, phosphorylation reactions were carried out in Buffer A/40 mM ammonium sulfate buffer with 1 mM cAMP. Reaction volume, quantities of RNA polymerase II, protein kinase, and [γ-32P]ATP varied for the various experiments and are given in the text. Incubations were carried out for 30 min at 30°C. The reaction was terminated by the addition of 2 ml of 20% trichloroacetic acid containing 1% sodium dodecyl sulfate. The samples were filtered using Millipore filters (0.3 μ). The filters were washed with several eight times with 4 ml of 20% trichloroacetic acid/1% sodium dodecyl sulfate, dried, dissolved in 10 ml of phase-combining system, and analyzed for radioactivity. Under the experimental conditions incorporation of [32P] into protein substrate proceeded linearly up to 30-min incubation time in the absence and presence of cAMP.

Preparation of Heat-stable Protein Kinase Inhibitor Protein—Heat-stable inhibitor protein was prepared from rabbit skeletal muscle by the method of Walsh et al. (27). Partial purification of the inhibitor protein was carried out by heat treatment of the muscle tissue at 90°C for 3 min and subsequent cooling in ice. The preparation was carried out using DEAE-cellulose chromatography as described by Walsh et al.

Phosphorylative Modifications of RNA Polymerase II

Acid Hydrolysis of [32P]labeled RNA Polymerase II and Determination of [32P]Phosphoserine and [32P]Phosphothreonine—After labeling of the RNA polymerase II with [32P]Phosphate in the presence of [32P]cAMP-dependent protein kinase, the kinase reaction mixture was applied onto DEAE-cellulose under the conditions of Fig. 2. The eluted [32P]-labeled RNA polymerase II was precipitated by the addition of 10% trichloroacetic acid, 0.5% sodium
dodecyl sulfate and recovered by centrifugation. The precipitate was resuspended and washed twice in 10% trichloroacetic acid, 0.5% sodium dodecyl sulfate and recovered by centrifugation. The resulting pellet was extracted twice with diethyl ether and dried in vacuo. After suspension of the precipitate in 6 N HCl, hydrolysis was carried out for 4 h at 110°C. The hydrolysate was lyophilized and redisolved in 0.2 ml of 0.05 M HCl containing 25 mM phosphoserine and 25 mM phosphothreonine. This solution was applied onto a column of Dowex AG50W-5X (200 to 400 mesh; Bio-Rad Laboratories) equilibrated in 0.05 M HCl. Elution was carried out with 0.05 M HCl (32). Fractions (0.5 ml) were collected at a flow rate of 1.5 ml/h. Aliquots (5-μl) were spotted on Whatman No. 1 paper, and the paper was treated with 1% ninhydrin in acetone containing 0.76 M acetic acid and 3.7 mM cadmium acetate to detect phosphoserine and phosphothreonine eluted from the column (33). Aliquots (200-μl) of each fraction were also dissolved in 10 ml of phase-combining system and subsequently analyzed for 32P radioactivity.

**Phosphoprotein Phosphatase Assay—** Phosphoprotein phosphatase activity was measured with alkaline phosphatase (Escherichia coli, electrophoretically purified, Worthington Biochemical Co.) using either [32P]protamine sulfate (400 cpm of 32P/μg) or 32P-labeled RNA polymerase II as substrate in a solution of Buffer A (34, 35). Incubation was carried out at 35°C for 30 min. The reaction was stopped by the addition of 2 ml of 30% trichloroacetic acid and 1% sodium dodecyl sulfate, and the samples were filtered on Millipore filters. The loss of 32P label from [32P]protamine sulfate or 32P-labeled RNA polymerase II after incubation was used as index of phosphatase activity. Under the experimental conditions phosphatase activity with [32P]protamine as substrate was linear up to 35-min incubation time.

**Polyacrylamide Gel Electrophoresis—** Polyacrylamide gel electrophoresis under nondenaturing conditions was carried out in a discontinuous system according to a modification of the procedure of Davis (36) and Beckman and Frenkel (37). The stacking gel (dimensions 6.5 × 1 cm) was photopolymerized from a solution containing 2.5% acrylamide in Tris buffer, pH 6.9. The separating gel (dimensions 6.5 × 8 cm) was polymerized from a solution containing 7% acrylamide, 25% glycerol (v/v) or 5% acrylamide, 25% glycerol (v/v). Determination of RNA polymerase activity in gel slices was carried out as described previously (37).

Polyacrylamide gel electrophoresis under denaturing conditions was carried out at pH 7.1, in the presence of 0.1% sodium dodecyl sulfate according to the methods of Maizel (38) and Weber and Osborn (39). The gels (dimensions 6.5 × 10 cm) contained 5% acrylamide in the upper half and 10% acrylamide in the lower half of the gel (mixed gels). Protein samples were prepared for electrophoresis by incubation at 90°C for 3 min in 10 mM sodium phosphate buffer, pH 7.1, containing 3% mercaptoethanol and 1% sodium dodecyl sulfate.

**Polyacrylamide Gel Analysis—** After electrophoresis the gels were stained and destained according to Weber and Osborn (39) and scanned at 550 nm in a Gilford model 2000 spectrophotometer with a gel scanning device. After scanning the gels were sliced into 1- or 2-mm sections. The sections were placed into counting vials and extracted for 24 h at 20°C in 0.5 ml of Protosol (New England Nuclear Corp.). After addition of 10 ml of phase-combining system, the samples were counted for the determination of radioactivity.

**Determination of Protein—** Protein concentration was determined by the method of Lowry et al. (40) with bovine serum albumin as reference standard.

**Determination of Radioactivity—** Samples collected on Millipore filters were dissolved in 10 ml of phase-combining system (Amersham/Beckman) which was used as the scintillation fluid. All samples were counted in a model 3375 Packard Tri-Carb liquid scintillation spectrometer as previously reported (20).

**RESULTS**

**Phosphorylation of Calf Thymus Nucleoplasmic RNA Polymerase II by Homologous CAMP-dependent Protein Kinase—** Upon incubation of nucleoplasmic RNA polymerase II with increasing concentrations of CAMP-dependent protein kinase increasing amounts of [32P]phosphate became incorporated into RNA polymerase II (Fig. 1). Incorporation of [32P]phosphate into RNA polymerase II appeared to be linear with protein kinase concentrations up to 20 μg but leveled off at higher concentrations of protein kinase (between 20 and 60 μg).

Incorporation of [32P]phosphate into RNA polymerase II depended upon the presence of CAMP-dependent protein kinase. When RNA polymerase II was incubated with 32P-ATP in the absence of protein kinase, essentially no 32P radioactivity was recovered in the trichloroacetic acid-precipitated RNA polymerase II. However, some autophosphorylation of the CAMP-dependent protein kinase occurred during its incubation with [γ-32P]ATP. Therefore, the degree of autophosphorylation of the CAMP-dependent protein kinase was determined under the experimental conditions of Fig. 1. The cpm 32P representing the autophosphorylation of the protein kinase were subtracted from the total cpm 32P incorporated into RNA polymerase II in the presence of protein kinase. The data of Fig. 1, therefore, reflect the degree of [32P]phosphate incorporation into the RNA polymerase II only.

The total amount of [32P]phosphate that was incorporated per μg of RNA polymerase II to achieve complete saturation of all phosphate acceptor sites varied with the different polymerase preparations. The reason for this variation is unknown but may be related to the fact that RNA polymerase II may be partially phosphorylated in vivo or may have undergone phosphorylative modification by nuclear protein kinases and phosphatases during its isolation from the nuclear extracts. Assuming a molecular weight of 700,000 for calf thymus RNA polymerase II (20), an average value of 0.5 mol of [32P]phosphate incorporated/mol of RNA polymerase II at saturation levels was determined.

Phosphorylation of RNA polymerase II under the conditions of Fig. 1 was neither stimulated by the addition of an ATP regenerating system, nor by the addition of native DNA (up to 200 μg/assay). The phosphorylation reaction was, however, progressively inhibited in reaction media of increasing ionic strength. All phosphorylation reactions were routinely carried out in the presence of 40 mM ammonium sulfate at which concentration the CAMP-dependent protein kinase exhibited 75% of its optimal catalytic activity observed in the absence of ammonium sulfate.

To determine that phosphorylation of RNA polymerase II was mediated by the catalytic protein kinase subunit, the
Effect of a heat-stable protein kinase inhibitor protein on the phosphorylation of RNA polymerase II was investigated. It has been shown that the heat stable inhibitor protein inhibits cAMP-dependent protein kinase activity by directly interacting with the free catalytic subunit of cAMP-dependent protein kinase (27-30). The data of Table I show that in the presence of cAMP-dependent protein kinase, cAMP, ATP, and increasing amounts of inhibitor protein incorporation of [32P]phosphate into RNA polymerase II was progressively inhibited.

DEAE-cellulose Chromatography and Polyacrylamide Gel Electrophoresis of 32P-labeled RNA Polymerase II—To obtain convincing evidence that [32P]phosphate was specifically associated with RNA polymerase II and to demonstrate that the binding of [32P]phosphate to polymerase protein was covalent in nature, 32P-labeled RNA polymerase II was subjected to chromatographic and electrophoretic analysis, and the presence of [32P]phosphoserine and [32P]phosphothreonine was determined. To achieve this RNA polymerase II was incubated with cAMP-dependent protein kinase, cAMP, and [γ-32P]ATP under conditions which achieved maximum phosphorylation of the polymerase (for conditions see Fig. 2). To determine the amount of protein kinase autophosphorylation, cAMP-dependent protein kinase was incubated under identical conditions in the absence of RNA polymerase II. Both preparations were subsequently subjected to chromatography on DEAE-cellulose to separate protein kinase and RNA polymerase II. Fig. 2, A and B, shows the 32P elution profiles of the 32P-labeled mixture consisting of protein kinase and RNA polymerase II (Fig. 2A) and of the 32P-labeled protein kinase without RNA polymerase II (Fig. 2B). The elution profile of Fig. 2A reveals the elution of 32P radioactivity in Fractions 56 to 66 at an ionic strength of 0.18 M ammonium sulfate, and in Fractions 72 to 87 at an ionic strength of 0.31 M ammonium sulfate. The latter peak of 32P radioactivity coincided with the RNA polymerase II activity which eluted, as expected, at 0.31 M ammonium sulfate. Fig. 2B revealed the elution of 32P radioactivity in Fractions 66 to 68 at an ionic strength of 0.18 M ammonium sulfate but not at the higher concentrations of ammonium sulfate indicating that the radioactivity associated with RNA polymerase II (in Fig. 2A) did not arise from the 32P-labeled protein kinase or phosphorylated protein kinase breakdown products. The chemical nature of the 32P radioactivity eluted in Fractions 56 to 66 at 0.18 M ammonium sulfate is unknown. Some protein kinase activity was associated with that 32P radioactivity but the majority of protein kinase activity applied onto the column eluted at low ionic strength in fractions which did not adsorb onto DEAE-cellulose in accordance with our previous findings (21).

Analysis of the 32P-labeled RNA polymerase II eluted from DEAE-cellulose (see Fig. 2A) by electrophoresis on polyacrylamide gels under nondenaturing conditions revealed the presence of two forms of RNA polymerase II in agreement with previous data (26) (Fig. 3). Both protein bands exhibited RNA polymerase activity (data not shown) and were associated with significant amounts of 32P radioactivity. To determine the pattern of RNA polymerase II subunit phosphorylation, 32P-labeled RNA polymerase II eluted from DEAE-cellulose

TABLE I

Effect of heat-stable protein kinase inhibitor protein on phosphorylation of calf thymus RNA polymerase II by calf thymus nuclear cAMP-dependent protein kinase

Ten micrograms of RNA polymerase II were incubated for 30 min at 30° with 22 µg of cAMP-dependent protein kinase in the presence of 4.4 nmoi (1 µCi) of [γ-32P]ATP, 1 µM cAMP, and the indicated amounts of protein kinase inhibitor in a total volume of 0.22 ml of protein kinase assay buffer. The incorporation of [32P]phosphate into acid-insoluble material was determined. Composition of the assay buffer and further experimental details are given under “Experimental Procedures.” The values shown are the arithmetic means of three determinations.

| Inhibitor | [32P]Phosphate incorporated | Inhibition |
|-----------|-----------------------------|-----------|
| (µg) | pmol/µg RNA polymerase II | % |
| 0.16 | 251.1 | 30 |
| 0.40 | 137.4 | 62 |
| 0.80 | 50.4 | 86 |

Fig. 2. DEAE-cellulose chromatographic elution profiles of 32P radioactivity and RNA polymerase II activity. A, 100 µg of RNA polymerase II were incubated with 513 µg of cAMP-dependent protein kinase, 1 µM CAMP, and 96 nmoi (960 µCi) of [γ-32P]ATP in a total reaction volume of 4.8 ml of protein kinase assay buffer. Composition of the assay buffer is given under “Experimental Procedures.” Incubation was carried out at 30° for 30 min, after which 8 ml of ice-cold Buffer A were added. The reaction mixture was dialyzed against 1000 ml of Buffer A which was changed three times. The 32P-labeled protein fraction was subjected to chromatography on DEAE-cellulose (column dimensions, 0.8 X 15 cm). Elution of Fractions 1 to 40 was carried out with 20 ml of Buffer A; all subsequent fractions were eluted with 30 ml of a linear gradient between a solution of Buffer A and 0.5 M ammonium sulfate in Buffer A. Fractions (0.5 ml) were collected at a flow rate of 4 ml/h. Aliquots (0.1-ml) of all fractions were assayed for RNA polymerase activity and for 32P radioactivity. B, 513 µg of cAMP-dependent protein kinase were incubated with 1 µM CAMP and 96 nmoi (960 µCi) of [γ-32P]ATP in a total reaction volume of 4.8 ml of protein kinase assay buffer. Incubation and all subsequent steps were carried out as described above. ---, 32P radioactivity; O—O, RNA polymerase II activity; ——, ammonium sulfate (M).
Phosphorylative Modifications of RNA Polymerase II

**Fig. 3 (left)**. Polyacrylamide gel electrophoresis of phosphorylated RNA polymerase II under nondenaturing conditions. Fractions 72 to 87 eluted from the DEAE-cellulose column (see Fig. 2) were pooled, dialyzed against Buffer B, and concentrated with the MD-X single hollow fiber microconcentrator. The concentrated protein fraction was used as the source of $^{32}P$-labeled RNA polymerase II. $^{32}P$-labeled RNA polymerase II (20 μg) was applied onto a 5% polyacrylamide gel and subjected to electrophoresis as described under "Experimental Procedures." Electrophoresis was carried out for 4 h at 3 mA/gel at 4°C. The gel was stained, destained, scanned at 550 nm, and subsequently sliced into 1-mm sections. The $^{32}P$ radioactivity content of the slices was determined by liquid scintillation counting. ---, gel scan at 550 nm; O--O, $^{32}P$ radioactivity.

(Fig. 2A) was subjected to electrophoresis on polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate. The characteristic arrangement of six RNA polymerase II subunits was identified (Fig. 4). The subunits were designated B1 through B6 in the order of decreasing molecular weight and increasing mobility in the gels as defined by Kedinger and Chambon (26) (Fig. 4). Using both 5% acrylamide gels (data not shown) and mixed gels (5% acrylamide in the upper half and 10% acrylamide in the lower half of the gel), the 25,000 molecular weight subunit (B5) of RNA polymerase II was identified as the principal $^{32}P$-phosphate acceptor protein. In about 30% of the experiments low levels of $^{32}P$ radioactivity (about 20% of the $^{32}P$ radioactivity seen in the B5 subunit) were also found associated with the 180,000 molecular weight subunit B2.

To identify the $^{32}P$ phosphate acceptor amino acid, $^{32}P$-labeled RNA polymerase II eluted from DEAE-cellulose (see Fig. 2A) was hydrolyzed and the hydrolysate was subjected to ion exchange chromatography on Dowex AG50W-5X. The amino acid elution profile shown in Fig. 5 reveals the presence of $^{32}P$ phosphorylserine and of $^{32}P$ phosphorylthreonine with free inorganic phosphate eluting in the early fractions. It was consistently observed that serine was the major $^{32}P$ phosphate acceptor amino acid. Additional confirmation that the phosphoryl groups attached to RNA polymerase II corresponded to those of phosphorylserine and phosphorylthreonine was obtained when the acid and base stability of the phosphate-RNA polymerase II linkage was tested. Low stability was observed after treatment of the $^{32}P$-labeled RNA polymerase for 1 h at 37°C in 1 N NaOH. No hydrolysis of the phosphate-RNA polymerase linkage was observed after treatment of the $^{32}P$-labeled RNA polymerase for 1 h at 100°C in 0.1 N HCl (data not shown).

**Effects of cyclic AMP-dependent Protein Kinase on RNA Polymerase II Activity**—To test the effect of phosphorylation on polymerase activity, RNA polymerase II was preincubated with cyclic AMP-dependent protein kinase, CAMP, and saturating concentrations of radioactive or nonradioactive ATP (100 μM) under conditions achieving complete phosphorylation of RNA polymerase II. At the end of the preincubation period the samples were assayed for RNA polymerase II activity and $^{32}P$-phosphate incorporation. Fig. 6 correlates the degree of phosphorylation and activation of RNA polymerase II with the increasing concentrations of cyclic AMP-dependent protein kinase present in the assays. A good correlation between protein kinase-mediated RNA polymerase II phosphorylation and activation is demonstrated in Fig. 6, and both phosphorylation and activation of RNA polymerase II were essentially proportional to the concentration of cyclic AMP-dependent protein kinase in the assays. At the highest concentration of protein kinase (18.75 g) 0.25 pmol of $^{32}P$ phosphate became incorporated into 1.2 μg of RNA polymerase II which resulted in a concomitant 3-fold stimulation of RNA polymerase II activity.

**Effects of Adenylyl Imidodiphosphate and Protein Kinase**
Inhibitor on RNA Polymerase II Activation — To demonstrate that phosphorylation of RNA polymerase II occurs concomitantly with or causes RNA polymerase II activation, we attempted to prevent RNA polymerase II activation under conditions where incorporation of phosphate into the RNA polymerase is inhibited. When the phosphate donor ATP is replaced with the ATP analogue adenylylimidodiphosphate, transfer of the y-phosphate group to the RNA polymerase II acceptor amino acids is prevented, because CAMP-dependent protein kinase is unable to cleave the terminal phosphate linkage in AMP-P(NH)P (41). The results of these experiments are shown in Table II. The results indicate that in the presence of increasing concentrations of CAMP-dependent protein kinase and ATP, RNA polymerase II activity increased as a function of protein kinase concentration. No comparable increase of RNA polymerase II activity was observed when ATP was omitted and replaced with AMP-P(NH)P. Addition of AMP-P(NH)P to the reaction mixtures led to a 44% decrease of the unstimulated RNA polymerase II activity presumably due to the fact that AMP-P(NH)P was not as efficiently used as substrate by the polymerase as ATP. The experiment nevertheless indicates that hydrolysis of the y-phosphate linkage is required for successful activation of RNA polymerase II and implicates phosphorylation of RNA polymerase as the mechanistic step for RNA polymerase activation.

Table III shows the effect of the heat-stable protein kinase inhibitor protein on the protein kinase-mediated activation of RNA polymerase II. In the presence of increasing amounts of CAMP-dependent protein kinase on the incorporation of 32P-labeled ATP into RNA polymerase II and on the activation of RNA polymerase II. RNA polymerase II (1.2 μg) was incubated with the indicated amounts of CAMP-dependent protein kinase in the presence of 1 μM cAMP and either 11 nmol of nonradioactive ATP or 11 nmol (3.1 μCi) of [γ-32P]ATP in a total reaction volume of 0.11 ml of protein kinase assay buffer (for composition of protein kinase assay buffer see "Experimental Procedures"). The samples containing radioactive and nonradioactive ATP were both incubated for 30 min at 30°. The 32P-labeled RNA polymerase II was subsequently precipitated by the addition of trichloroacetic acid, and 32P radioactivity was determined. The picomoles of [32P]phosphate/sample plotted are corrected data and were obtained after subtraction of the cpm of 32P/sample contributed by the autophosphorylation of the CAMP-dependent protein kinase from the total cpm of 32P/sample obtained after incubation of RNA polymerase II with the various amounts of CAMP-dependent protein kinase. The samples with nonradioactive ATP were cooled in ice. To measure the RNA polymerase activity in the samples, 6 nmol (500 μCi) of [α-32P]UTP and all other reactants for the RNA polymerase assay were added in 40 μl. Incubations were carried out for 60 min at 37°. At the end of the incubation period trichloroacetic acid-insoluble 32P-labeled product was determined as described under "Experimental Procedures." The values shown are the arithmetic means of three determinations. O---O, 32P radioactivity; ▪-▪, RNA polymerase II activity.

**Table II**

| CAMP-dependent protein kinase | RNA polymerase II activity | Stimulation |
|-------------------------------|---------------------------|-------------|
| ATP                           | ATP | AMP-P(NH)P | ATP | AMP-P(NH)P |
| (μg)                          | (pmol) | [α-32P]UTP incorporated/sample | % |
| 4.3                           | 57.5 | 32.4 | 82 | 8 |
| 8.2                           | 108.0 | 37.1 | 88 | 14 |
| 16.5                          | 109.2 | 37.3 | 89 | 15 |
| 26.4                          | 110.7 | 37.8 | 93 | 17 |

The abbreviation used is: AMP-P(NH)P, adenylylimidodiphosphate.
**Phosphorylative Modifications of RNA Polymerase II**

**Tables III and IV**

**Table III**

| Inhibitor | RNA polymerase II activity | RNA polymerase II + protein kinase Inhibition |
|-----------|---------------------------|--------------------------------------------|
| (µg)      | (pmol [3H]UMP incorporated/sample) |               |
| 0.15      | 743.2 | 268.6 | 5.3 |
| 0.5       | 743.4 | 2205.6 | 18 |
| 0.6       | 745.9 | 1466.6 | 46 |
| 1.0       | 745.8 | 1035.6 | 62 |

*The percentage of inhibition of RNA polymerase II activity is calculated from the activity exhibited by RNA polymerase II incubated with protein kinase.*

**Table IV**

| Alkaline phosphatase | RNA polymerase II activity | Deactivation | 32P-labeled RNA polymerase remaining | Dephosphorylation |
|----------------------|---------------------------|-------------|-----------------------------------|------------------|
| (µg)                 | (pmol [3H]UMP incorporated/sample) | %          | pmol | %   |
| 0.05                 | 21.7 | 0.065 |
| 0.10                 | 17.5 | 0.053 | 18.5 |
| 0.15                 | 12.9 | 0.044 | 32.3 |
| 0.20                 | 10.4 | 0.058 | 41.5 |

**Discussion**

Eukaryotic DNA transcription can be regulated either by controlling the availability of the DNA template through various nuclear repressor and activator proteins, by changing the total or relative levels of the various RNA polymerases, or by modulation of the activity of RNA polymerases. Modulation of RNA polymerase activity has been observed in both eukaryotic and prokaryotic cells which experience developmental and physiological changes (20, 41–50). These activity changes occur rapidly in the absence of protein synthesis and presumably involve a precisely controlled modulation of the activity of existing RNA polymerase molecules rather than modification of the synthetic and catabolic rates of RNA polymerase. There is good evidence that regulatory systems exist which provide control of enzymatic activity through covalent modification of key enzymes. Many enzymes for instance occur in a dephospho-phospho form and are interconverted from the inactive to the active state by the coordinated action of specific protein kinases and phosphoprotein phosphatases (21, 51).

A protein kinase-mediated activation of eukaryotic RNA polymerases in vitro has been reported by several laboratories. In addition to our own studies demonstrating the activation of calf ovary and calf thymus RNA polymerases I and II by partially purified homologous cAMP-dependent protein kinase (20, 21), Martelo and Hirsch observed activation of rat liver RNA polymerase I by a homologous cAMP-dependent nuclear protein kinase (22). Recently, Dauhaus (23) reported the activation of ascites tumor RNA polymerase II by a homologous cAMP-independent protein kinase. Some preliminary evidence was also presented suggesting that phosphorylation of RNA polymerase subunits may have occurred as a result of protein kinase action (20–22, 52). However, a clear interpretation of the observed phosphorylation of partially purified RNA polymerase is difficult, because phosphorylation of contaminating nuclear nonhistone protein may have occurred which is known to lead to a stimulation of RNA polymerase activity (19, 53–55). Several reports have appeared convincingly demonstrating phosphorylation of RNA polymerase sub-
units. Zillig et al. (56) demonstrated phosphorylation of the β and β′ subunits of E. coli RNA polymerase from bacteriophage T7 infected E. coli cells. Bell et al. (57, 58) and Buhler et al. (59) identified phosphorylation of several subunits of yeast RNA polymerases. Hirsch and Martelo (60) reported the incorporation of [32P]phosphate into serine and threonine residues of several subunits of the nucleolar RNA polymerase I from rat liver when phosphorylation was carried out with intact nuclear preparations. Under their experimental conditions no phosphorylation of RNA polymerase II was observed, but Rutter et al. (52) have previously reported the in vitro phosphorylation of purified rat liver RNA polymerase II with a cAMP-dependent protein kinase from both rat liver and rabbit muscle.

The results of our experiments demonstrate that a highly purified nuclear cAMP-dependent protein kinase carries out the in vitro phosphorylation of the 25,000-dalton polypeptide subunit of the homologous nucleoplasmic RNA polymerase II. In several experiments a minor degree of [32P]incorporation into the 180,000-dalton subunit was observed. The inconsistency of the 180,000-dalton subunit phosphorylation may indicate that the subunit underwent some phosphorylative modification in vivo or during its isolation. The identification of only two RNA polymerase subunits as phosphate acceptors does not exclude the possibility that other RNA polymerase subunits may become phosphorylated as well. The subunits may exist in an extensively phosphorylated state which makes the identification of additional [32P]phospho incorporation impossible. Similarly, the observed average incorporation of only 0.5 mol of [32P]phosphate/mol of RNA polymerase may reflect a partially phosphorylated state of the purified RNA polymerase.

A causal relationship between RNA polymerase II phosphorylation and activation was at least partially confirmed by four experiments. Firstly, as shown in Fig. 6, there is a good correlation between the extent of phosphorylation and activation of RNA polymerase II. Secondly, selective inhibition of the protein kinase catalytic subunit by the heat-stable protein kinase inhibitor led to both an inhibition of RNA polymerase II phosphorylation and to a proportional decrease of the degree of activation of RNA polymerase II (Tables I and III). Thirdly, substitution of ATP and the ATP-analogue adenylyl imidophosphate, whose terminal phosphate group cannot be utilized and transferred to a protein substrate, prevented cAMP-dependent protein kinase-mediated activation of RNA polymerase II (see Table II). Finally, treatment of [32P]labeled RNA polymerase II with bacterial phosphatase led to both a dephosphorylation and concomitant deactivation of RNA polymerase II (Table IV).

The 25,000-dalton RNA polymerase II subunit (B5 subunit) was identified as the major phosphate acceptor polypeptide. Since phosphorylation of the B5 subunit was accompanied by a stimulation of RNA polymerase II activity, it may be conceivable that the B5 subunit plays a crucial role in determining the enzymatic activity of RNA polymerase II. Recently, Valenzuela et al. (61) showed that the molar ratio of the yeast RNA polymerase I 24,000-dalton subunit, a subunit which can become phosphorylated by yeast protein kinase (57, 59), is requisite for yeast RNA polymerase I activity. Although we have not demonstrated a relationship between the molar ratio of the B5 subunit and RNA polymerase II activity, we tentatively conclude from our data that phosphorylation of the B5 subunit polypeptide may conceivably participate in the regulation of polymerase activity.

The major question of whether phosphorylation of RNA polymerase II subunits by nuclear protein kinases modulates RNA polymerase activity in vivo remains unsettled. The precise role of cAMP in modulating RNA polymerase activity in vivo can only be subject to speculation. Although it appears clear from the present study that the polymerase activity is modulated in vitro by cAMP through its action on cAMP-dependent protein kinases, cAMP-independent protein kinases are similarly implicated in the control of RNA polymerase activity (21, 23). Conceivably, both types of protein kinase participate in the regulation of RNA polymerase activity in vivo but in response to different physiological stimuli.

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