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Phosphorylation of Deoxyribonucleic Acid Dependent RNA Polymerase II by Nuclear Protein Kinase NII: Mechanism of Enhanced Ribonucleic Acid Synthesis†

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ABSTRACT: RNA polymerase II was purified from Morris hepatoma 3924A by a series of ion-exchange and affinity column chromatographic fractionations, followed by sucrose gradient centrifugation in the presence of 0.3 M KCl. Purified RNA polymerase II had a specific activity of greater than 400 nmol of UMP incorporated (30 min)$^{-1}$ (mg of protein)$^{-1}$ by using double-stranded DNA as template. The purified enzyme contained five polypeptides (M_r 214 000, 140 000, 33 000, 25 000, and 21 000) that were present in molar quantities and two additional polypeptides (M_r 19 000 and 18 000) that had a combined molar ratio of 1.0. The cyclic AMP independent nuclear protein kinase NII, also purified from hepatoma 3924A, was able to phosphorylate RNA polymerase II polypeptides of M_r 214 000, 140 000, and 21 000. Phosphorylation of the polymerase was accompanied by enhanced transcription of double-stranded DNA, heat-denatured DNA, and poly[d-(A-T)]. The elevation in RNA polymerase activity was dependent upon the presence of hydrolyzable ATP and resulted from an increased number of RNA molecules synthesized in vitro. The average length of RNA chains was not affected by the kinase. Under similar conditions, protein kinase NII also stimulated homologous RNA polymerase I. In contrast to the phosphorylation of polymerase II, modification of polymerase I resulted in an increase in the average size, but not number, of RNA chains synthesized. The specificity of the NII kinase-catalyzed reaction was demonstrated by the inability of another homologous protein kinase, NI, to phosphorylate or activate RNA polymerase II.

Phosphorylation of chromatin proteins has been correlated with enhanced expression of several genes. Both histone and nonhistone nuclear proteins are phosphorylated in response to biological stimuli [see Krebs & Beavo (1979)]. The modification of nonhistone chromatin proteins is most probably catalyzed by nuclear kinases which are cyclic AMP independent. Two major nuclear protein kinases are found in rat liver and are designated NI and NII according to their elution from DEAE-Sephadex (Desjardins et al., 1972). These kinases have distinct reaction properties and preferentially phosphorylate casein and phosvitin in vitro. Protein kinases NI (Thornburg et al., 1978; Rose & Jacob, 1979) and NII (Thornburg & Lindell, 1977; Rose et al., 1981a) have been extensively purified from rat liver and a rat hepatoma. Although phosphorylation of nuclear proteins by endogenous or exogenous nuclear kinase has been studied in a number of

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laboratories, the function of most of these protein acceptors has not been well-defined.

The modification of the DNA-dependent RNA polymerases (EC 2.7.7.6) has been investigated in an effort to elucidate the role of phosphorylation in gene expression. In vivo, both RNA polymerase I (Bell et al., 1977; L. Pizer and K. Rose, unpublished results) and RNA polymerase II (Bell et al., 1977; Dahmus, 1981b) are phosphorylated. Initial studies by Hirsch & Martelo (1976, 1978) indicated that RNA polymerase I could be phosphorylated in vitro by an associated protein kinase. Recent studies in this laboratory have demonstrated that nuclear protein kinase NII is associated with polymerase I (Rose et al., 1981b,c) and phosphorylates (Rose et al., 1981c; Duceman et al., 1981) as well as activates (Duceman et al., 1981) the polymerase. In contrast to the NII kinase, protein kinase NI does not activate RNA polymerase I (Duceman et al., 1981), although it does phosphorylate (Rose & Jacob, 1979) and activate (Rose & Jacob, 1980) the nuclear enzyme poly(A) polymerase. RNA polymerase II appears to be phosphorylated in vitro by both cyclic AMP dependent (Kranias et al., 1977) and independent (Kranias & Jungmann, 1978; Dahmus, 1981b) protein kinases. To date, there have been no definitive studies in which highly purified RNA polymerase II has been shown to be activated as well as phosphorylated by a well-defined cyclic nucleotide independent protein kinase. To elucidate the role of the nuclear casein kinases on RNA polymerase II catalyzed transcription, we undertook the purification of RNA polymerase II from the rat tumor, Morris hepatoma 3924A. We now report that highly purified RNA polymerase II is phosphorylated and activated by homologous protein kinase NII.

Materials and Methods

Materials. Crystalline bovine serum albumin (Miles Laboratories) was purified by Affi-Gel Blue chromatography as described in Technical Bulletin 1049 (Bio-Rad Laboratories, 1977). Calf thymus DNA (Worthington Biochemicals Corp.) was extracted with phenol (Gross-Bellard et al., 1973) before use in the RNA polymerase assay. All reagents used in the assay were prepared in sterile glass-distilled water. Glass and plasticware were treated with 0.1% diethyl pyrocarbonate prior to use. Heparin–Sepharose was prepared from cyanogen bromide activated Sepharose 4B by using the procedure of Iverius (1971) modified as described (Rose et al., 1981a). Calf thymus DNA was linked to carboxymethylcellulose by the procedure of Potuzak & Wintersberger (1976). [γ-^{32}P]ATP, 2000 Ci/mmol, and [5,6-^{3}H$_2$]UTP, 40 Ci/mmol were purchased from Amersham (Arlington Heights, IL). Adenylyl 5′-imidodiphosphate was obtained from P-L Biochemicals (Milwaukee, WI).

Enzyme Units. One unit of RNA polymerase activity was that amount of enzyme which catalyzed the incorporation of 1 pmol of UMP into RNA in 30 min at 30 °C. One unit of protein kinase activity was equivalent to 1 pmol of radioactive phosphate transferred from [γ-^{32}P]ATP to casein in 30 min at 30 °C as described by Rose et al. (1981a). One unit of poly(A) polymerase activity was that amount of enzyme catalyzing the incorporation of 1 pmol of AMP into poly(A) in 1 h at 37 °C under conditions described previously (Rose & Jacob, 1976a).

Purification of Enzymes. Nuclear extracts of Morris hepatoma 3924A were prepared as described previously (Rose et al., 1976) and applied to a DEAE-Sephadex column (Rose et al., 1981a). Protein kinase NI and poly(A) polymerase were recovered from the flow-through and 10 mM $(NH_4)_2SO_4$ wash fractions and further purified as described (Rose & Jacob, 1976a, 1979). The specific activities of the purified NI kinase and poly(A) polymerase were 30 nmol (mg of protein)$^{-1}$ (30 min)$^{-1}$ and 2000 nmol (mg of protein)$^{-1}$ (60 min)$^{-1}$, respectively. Protein kinase NII and RNA polymerase I were eluted from DEAE-Sephadex with 0.1 M $(NH_4)_2SO_4$ and purified as described previously (Rose et al., 1981a,c). The specific activities of purified NII kinase and RNA polymerase I were 18 000 and 75 nmol (mg of protein)$^{-1}$ (30 min)$^{-1}$, respectively.

The protocol utilized for the purification of RNA polymerase II was similar to that described for the purification of RNA polymerase I (Rose et al., 1981a,c).

(*A*) *DEAE-Sephadex Chromatography.* After elution of RNA polymerase I from the DEAE-Sephadex column with 0.1 M $(NH_4)_2SO_4$ (Rose et al., 1981a), RNA polymerases II and III were eluted together with 0.4 M $(NH_4)_2SO_4$ in buffer A [50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.9, 25% glycerol, 5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.5 mM dithiothreitol]. The enzyme was then diluted with buffer A to reduce the $(NH_4)_2SO_4$ concentration to 0.18 M and applied to a second DEAE-Sephadex column (0.47 mL of gel/g of tissue) that had been equilibrated with buffer A containing 0.18 M $(NH_4)_2SO_4$. After the column was washed with the equilibration buffer (2 bed volumes), RNA polymerases were eluted with a linear salt gradient [0.18–0.50 M $(NH_4)_2SO_4$ in buffer A] at a flow rate of 7–9 mL/min. Aliquots (30 μL) were then analyzed for polymerase activity in the presence and absence of α-amanitin (1 μg/mL) as described previously (Rose et al., 1976). RNA polymerase II (α-amanitin sensitive) and polymerase III (α-amanitin resistant) activities eluted at 0.25 and 0.30–0.34 M $(NH_4)_2SO_4$, respectively.

(*B*) *DNA–Cellulose Chromatography.* Fractions from the second DEAE-Sephadex column containing RNA polymerase II were pooled and dialyzed overnight against buffer B (same as buffer A except that the glycerol concentration was 50%). The enzyme was diluted with buffer A (without glycerol) to reduce the glycerol concentration to 15% and applied to a DNA–cellulose column (0.13 mL of packed cellulose/g of tissue) previously equilibrated with buffer C (same as buffer A except the glycerol concentration was 15%) containing 20 mM NaCl. The column was washed with 2 bed volumes of buffer C containing 20 mM NaCl followed by elution of the enzyme with a linear salt gradient (20 mM–1.0 M NaCl in buffer C) at a flow rate of 4–5 mL/min. Samples (30 μL) of each fraction were then analyzed for RNA polymerase activity. The peak of enzyme activity eluted from the column at 0.28 M NaCl.

(*C*) *Heparin–Sepharose Chromatography.* RNA polymerase II containing fractions from the DNA–cellulose column were pooled and dialyzed overnight against buffer A. Following dialysis, the enzyme was applied to a heparin–Sepharose column (0.04 mL of gel/g of tissue) equilibrated with buffer A containing 0.25 M NH_4Cl. The column was then washed with the equilibration buffer (2 bed volumes) and the enzyme eluted with 2 bed volumes of buffer A containing 0.8 M NH_4Cl at a flow rate of 1.5 mL/min. Fractions (20-μL aliquots) were analyzed for polymerase II activity, and those containing the enzyme were pooled and dialyzed overnight under vacuum in buffer A (no EDTA) containing 0.3 M KCl.

(*D*) *Sucrose Gradient Centrifugation.* Prior to gradient centrifugation, the enzyme from the heparin–Sepharose column was dialyzed for an additional 3 h in buffer D (50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 0.3 M KCl, 0.5 mM dithiothreitol, and 0.5 mM β-mercaptoethanol) conaining 5% (w/v) sucrose. After dialysis, the enzyme (1 mL) was layered

onto a 10–30% (w/v) sucrose gradient (32 mL) prepared in buffer D and then centrifuged as described by Rose et al. (1981a). Aliquots (20 μL) of fractions (0.8 mL) were assayed for RNA polymerase activity. Enzyme recovered from the sucrose gradients was dialyzed overnight against 50 mM Tris-HCl (pH 7.9), 50% glycerol, and 0.5 mM dithiothreitol and stored at −70 °C.

RNA Polymerase Assay. RNA polymerases were incubated for 15 min at 30 °C in the presence or absence of protein kinase in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 30 μM ATP, 12.5 mM $(NH_4)_2SO_4$, and 250 μg/mL bovine serum albumin in a total volume of 100 μL. Cofactors necessary for RNA synthesis were then added so that the reaction contained 125 μg/mL calf thymus DNA, 600 μM each of GTP, CTP, and ATP, 30 μM [^{3}H]UTP (45 cpm/pmol), 6 mM KCl, 2.5 mM NaF, 0.6 mM dithiothreitol, and 50 mM $(NH_4)_2SO_4$ in a final volume of 120 μL. After an additional 30 min at 30 °C, reactions were terminated, and the incorporation of radioactive label was determined as described previously (Rose et al., 1976). Reactions utilizing [^{3}H]GTP instead of [^{3}H]UTP contained 30 μM [^{3}H]GTP and 600 μM UTP. In assays containing RNA polymerase I, the $(NH_4)_2SO_4$ concentration was 12.5 mM throughout the assay.

For RNA synthesis in the absence of reinitiation, a high salt concentration was used (Hyman & Davidson, 1970; Cedar, 1975; Leonard & Jacob, 1977). Preliminary experiments indicated that 275 mM $(NH_4)_2SO_4$ completely inhibited initiation by hepatoma RNA polymerase II. Reaction mixtures (100 μL) containing enzyme, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 12.5 mM $(NH_4)_2SO_4$, 250 μg/mL bovine serum albumin, 150 μg/mL calf thymus DNA, 625 μM each of ATP and GTP, 7 mM KCl, 3 mM NaF, and 0.5 mM dithiothreitol were incubated for 15 min at 30 °C. After formation of initiation complexes, $(NH_4)_2SO_4$, CTP, and [^{3}H]UTP were added to final concentrations of 275 mM, 600 μM, and 30 μM, respectively, bringing the final volume to 120 μL. Incubation at 30 °C was then continued for an additional 30 min. Reactions were processed as described above. For reactions utilizing [^{3}H]GTP instead of [^{3}H]UTP, the [^{3}H]GTP and UTP concentrations were 30 and 600 μM, respectively.

Determination of Average RNA Chain Length. Assays were carried out under standard conditions or in the absence of reinitiation by using either [^{3}H]UTP or [^{3}H]GTP. Reactions were pooled, precipitated with trichloroacetic acid, and hydrolyzed with 0.33 M KOH as described by Duceman et al. (1981). Radioactively labeled UMP and uridine or GMP and guanosine residues were then separated by two-dimensional thin-layer chromatography on poly(ethylenimine)–cellulose (Randerath & Randerath, 1967) as reported by Rose & Jacob (1976b). The solvent systems used for the first and second dimensions were glass-distilled water and 1.6 M LiCl, respectively.

Results

Purification of RNA Polymerase II. RNA polymerases were solubilized from isolated nuclei of Morris hepatoma 3924A as described previously (Rose et al., 1976). Due to the large amounts of starting material, purification of the enzymes was modified from that reported earlier (Rose et al., 1976). RNA polymerases II and III were separated from polymerase I by step elution on DEAE-Sephadex. RNA polymerases II and III were resolved by rechromatography on DEAE-Sephadex, using a shallow salt gradient for elution. RNA polymerase II was then further purified as shown in Table I. The specific activity of polymerase II from the sucrose gradient ranged from 400 to 800 nmol of UMP (30 min)$^{-1}$ (mg of protein)$^{-1}$ and was comparable to that of homogeneous enzyme from other mamalian sources [see Chambon (1975) and Jacob & Rose (1978)]. Sucrose gradient purified polymerase II was completely sensitive to α-amanitin (1 μg/mL) and had similar salt, spermine, and divalent ion optima as described for the partially purified enzyme (Rose et al., 1976). The polypeptide composition of purified polymerase II was determined by polyacrylamide gel electrophoresis under denaturing conditions (Figure 1). Five polypeptides (M_r 214 000, 140 000, 33 000, 25 000, and 21 000) were present with molar ratios (determined by densitometry) approximately equal to 1.0. The two smallest polypeptides (M_r 19 000 and 18 000) had a combined molar ratio of 1.0. This composition compared favorably to that obtained in other laboratories (Weaver et al., 1971; Ingles, 1973; Kedinger et al., 1974).

Phosphorylation of RNA Polymerase II. Hepatoma nuclear protein kinase NII is capable of phosphorylating homologous RNA polymerase I in vitro (Duceman et al., 1981; Rose et al., 1981b,c). To ascertain whether protein kinase NII could also phosphorylate RNA polymerase II, we incubated the enzymes in reaction mixtures containing [γ-^{32}P]ATP and

Table I: Purification of RNA Polymerase II from Morris Hepatoma 3924A[a]

fraction	protein (mg)	enzyme (units)	sp act. (units/mg)
first DEAE-Sephadex	163.63	2.94×10^6	18.0×10^3
second DEAE-Sephadex	25.20	2.18×10^6	86.3×10^3
DNA–cellulose	3.36	0.84×10^6	249.0×10^3
heparin–Sepharose	1.30	0.37×10^6	281.0×10^3
sucrose gradient	0.35	0.17×10^6	477.0×10^3

[a] RNA polymerase II was purified from 350 g of Morris hepatoma 3924A as described under Materials and Methods. Because the enzyme from the first DEAE-Sephadex column contains both RNA polymerases II and III, the enzyme units given for this fraction represent α-amanitin (1 μg/mL) sensitive polymerase activity.

FIGURE 1: Polyacrylamide gel electrophoresis of RNA polymerase II under denaturing conditions. Purified RNA polymerase II (35 μg) was precipitated with trichloroacetic acid and subjected to electrophoresis on a linear polyacrylamide gradient (2–16%) gel under denaturing conditions (Laemmli, 1970) as described by Rose et al. (1981a). Following electrophoresis, the gel was stained with Coomassie Blue R-250. Proteins of known molecular weight were run on parallel gel tracks and used to estimate the molecular weights. The molecular weight markers included myosin (M_r 200 000, R_f 0.26), β-galactosidase (M_r 130 000, R_f 0.32), phosphorylase B (M_r 94 000, R_f 0.45), bovine serum albumin (M_r 67 000, R_f 0.53), ovalbumin (M_r 43 000, R_f 0.65), carbonic anhydrase (M_r 30 000, R_f 0.76), trypsin inhibitor (M_r 20 000, R_f 0.85), and β-lactalbumin (M_r 14 400, R_f 0.94).

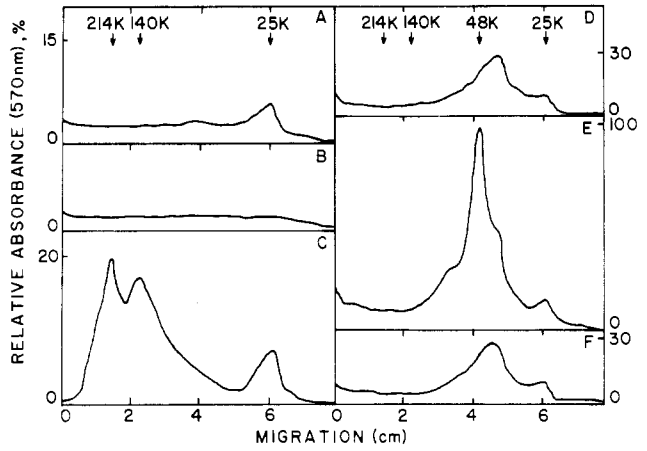

FIGURE 2: Phosphorylation of RNA polymerase II by protein kinase NII. Enzymes were incubated for 5 min at 30 °C in 300 μL of reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 50 μCi of [γ-^{32}P]ATP. Reactions were terminated by the addition of trichloroacetic acid to a final concentration of 10%. Bovine serum albumin (15 μg) was added as carrier. After 16 h at 4 °C, the precipitated proteins were collected by centrifugation, washed with 5% trichloroacetic acid, and subjected to electrophoresis under denaturing conditions on linear polyacrylamide gradient (2–16%) slab gels as described by Rose et al. (1981a,c). Autoradiograms of the gels (Kodak XAR film; Du Pont Cronex Lightning Plus XL screen) were analyzed with a Transidyne 2955 scanning densitometer (570 nm). (A) Protein kinase NII (50 units); (B) RNA polymerase II (30 units); (C) RNA polymerase II plus protein kinase NII (30 and 50 units, respectively); (D) protein kinase NI (50 units); (E) poly(A) polymerase (10 units) plus protein kinase NI (50 units); (F) RNA polymerase II (30 units) plus protein kinase NI (50 units). Following autoradiography, the gels were stained with Coomassie Blue R-250, and the molecular weights (as indicated) were estimated from proteins of known molecular weight run in parallel gel tracks as described in the legend to Figure 1.

$MgCl_2$. After acid precipitation, the phosphopeptides were subjected to polyacrylamide gel electrophoresis under denaturing conditions. As shown in Figure 2 (panel C), the largest polypeptides (M_r 214000 and 140000) of RNA polymerase II were phosphorylated by the NII kinase. Unlike RNA polymerase I, with its endogenous kinase activity (Rose et al., 1981b,c), polymerase II was not capable of autophosphorylation (panel B). Because of the autophosphorylation of the smaller NII kinase polypeptide [M_r 25000; Figure 2, panel A; see also Rose et al. (1981a)], it was not readily apparent whether the polymerase II polypeptides of similar molecular weight served as phosphate acceptors. However, the larger area under the densitometry peak in this region when the polymerase was present (panel C) indicated that one or more of the smaller polymerase II polypeptides were phosphorylated by the NII kinase. For determination of which of these RNA polymerase II polypeptides were modified by protein kinase NII, sucrose density gradient centrifugation was utilized to separate the phosphorylated polymerase (which sediments at 15 S) from the kinase [which sediments at 7 S (Rose et al., 1981a)]. Gradient fractions containing RNA polymerase II activity were pooled, and the enzyme was precipitated with trichloroacetic acid. The radioactive phosphopeptides were then analyzed by polyacrylamide gel electrophoresis as described in the legend to Figure 2. The electrophoresis time was extended in order to be able to clearly distinguish the M_r 25000 polypeptide of the polymerase from the smaller peptides. After removal of the protein kinase phosphopeptide, it was readily apparent that the M_r 21000 polypeptide of RNA polymerase II was also phosphorylated by the kinase (not shown). It should be noted that all radioactive phosphate present in the gels was released by alkaline hydrolysis under the conditions described by Cooper & Hunter (1981), indicating that tyrosine was not the phosphoryl acceptor (Fischer et al., 1959).

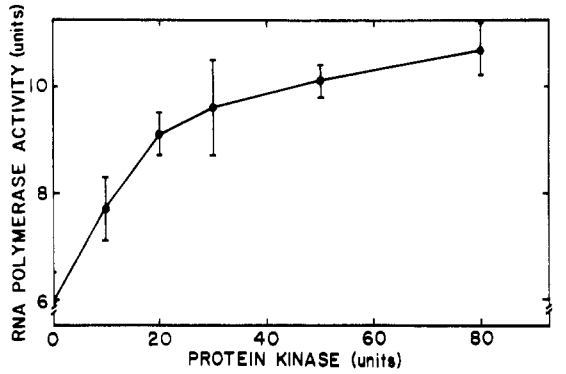

FIGURE 3: Effect of protein kinase NII on RNA polymerase II activity. Purified RNA polymerase II was assayed in the presence of increasing quantities of NII protein kinase as described under Materials and Methods. Results are the mean of triplicate samples ± SEM.

Table II: Effect of Protein Kinase on Transcription of Various Templates[a]

addition	template	RNA polymerase activity (units)
none	double-stranded DNA	2.3 ± 0.4
plus actinomycin D	double-stranded DNA	0.0 ± 0.1
plus kinase NII	double-stranded DNA	8.1 ± 0.8
plus kinase NII, plus actinomycin D	double-stranded DNA	0.0 ± 0.1
none	denatured DNA	15.3 ± 1.0
plus kinase NII	denatured DNA	24.9 ± 1.9
none	poly[d(A-T)]	2.2 ± 0.1
plus kinase NII	poly[d(A-T)]	16.5 ± 0.6

[a] RNA polymerase II was assayed under standard conditions as described under Materials and Methods in the absence or presence of protein kinase NII (10 units), double-stranded or heat-denatured calf thymus DNA (60 μg/mL), poly[d(A-T)] (42 μg/mL), and actinomycin D (50 μg/mL) as indicated. Results are the mean of triplicate samples ± SEM.

For demonstration of the specificity of the NII kinase catalyzed phosphorylation of polymerase II, protein kinase NI, obtained during the purification of nuclear poly(A) polymerase (Rose & Jacob, 1979), was employed. Incubation of this protein kinase alone resulted in the phosphorylation of two polypeptides (M_r 40000 and 25000; Figure 2, panel D). Although the NI kinase was capable of phosphorylating poly(A) polymerase (M_r 48000; Figure 2, panel E), none of the RNA polymerase II polypeptides were modified by this kinase (panel F).

Effect of Protein Kinase on RNA Polymerase II Activity. Addition of protein kinase NII to the polymerase II reaction mixture resulted in a stimulation of RNA synthesis by the purified polymerase (Figure 3). This increase in RNA synthesis was proportional to the amount (units) of protein kinase NII present. Maximal stimulation by protein kinase was observed after 30 min of incubation. RNA synthesis in the presence or absence of kinase was linear during this time. Enhanced RNA synthesis was also observed when [^{3}H]GTP replaced [^{3}H]UTP.

To ascertain whether activation of polymerase depended on the nature of the template, we monitored the effect of kinase on the transcription of denatured DNA and a synthetic polynucleotide. As shown in Table II, the kinase was capable of stimulating the transcription of denatured DNA; upon kinase addition, an increase in RNA synthesis from 15 to 25 units was observed. An even greater enhancement (up to

Table III: Effect of Protein Kinase on RNA Synthesis in the Presence of Mg^{2+} and Mn^{2+} [a]

addition	metal ion	RNA synthesis (units)
none	Mg^{2+}	32 ± 2.1
plus kinase NII	Mg^{2+}	56 ± 3.2
plus kinase NI	Mg^{2+}	32 ± 1.7
none	Mn^{2+}	88 ± 3.9
plus kinase NII	Mn^{2+}	72 ± 0.8
none	Mn^{2+}, Mg^{2+}	92 ± 2.7
plus kinase NII	Mn^{2+}, Mg^{2+}	134 ± 3.7

[a] RNA polymerase II was assayed as described under Materials and Methods in the absence or presence of 45 units of protein kinase as indicated. Mg^{2+} (5 mM) and/or Mn^{2+} (1.25 mM) were present as divalent ions. Results are the mean of triplicate samples ± SEM.

7.5-fold) of polymerase activity was obtained when poly[d-(A-T)] was the template. Although some variation in the extent of stimulation of RNA polymerase II by protein kinase NII was observed with different enzyme preparations, 1.5–3.5-fold increases in RNA synthesis were generally obtained when double-stranded calf thymus DNA served as template. RNA polymerase activity, even in the presence of kinase, was completely sensitive to actinomycin D (Table II).

Several approaches were taken to establish that protein kinase activity was responsible for the observed stimulation of RNA polymerase. First, because protein kinase NII is not active in the absence of Mg^{2+} (Rose et al., 1981a), the requirement for this divalent cation was tested (Table III). RNA polymerase activity was not stimulated by the kinase when Mg^{2+} was replaced by Mn^{2+}. Specifically, in the presence of Mg^{2+}, RNA synthesis was stimulated 1.75-fold by protein kinase NII (Table III). When Mn^{2+} was substituted for Mg^{2+}, addition of protein kinase NII actually inhibited RNA polymerase; the activity was 82% of that obtained in the absence of the phosphorylating enzyme. When both divalent ions were present, RNA synthesis was enhanced 1.45-fold by addition of protein kinase NII. Activation of RNA polymerase II appeared specific for the NII protein kinase since comparable units of nuclear protein kinase NI, purified from the same hepatoma (Rose & Jacob, 1979), did not alter enzyme activity (Table III).

Next, the requirement for the presence of ATP during activation was determined. In one experiment, the sensitivity of protein kinase NII to $(NH_4)_2SO_4$ was exploited. The kinase has been shown to be inhibited by $(NH_4)_2SO_4$ concentrations higher than 12 mM, and activity is completely abolished at 200 mM (Rose et al., 1981a). Preincubation of polymerase II and the protein kinase in the presence of ATP and $MgCl_2$, followed by RNA synthesis in the presence of 200 mM $(NH_4)_2SO_4$ to inhibit further phosphorylation, resulted in a 2.4-fold stimulation of the polymerase activity (Table IV, experiment A). In contrast, when the polymerase was preincubated with the kinase in the absence of ATP, no activation was observed. In another experiment, an ATP analogue, adenylyl 5′-imidodiphosphate (AMP-PNP), was substituted for ATP (Table IV, experiment B). The terminal phosphate of AMP-PNP cannot be cleaved by protein kinases (Yount et al., 1971), but RNA polymerase II is capable of utilizing this analogue for RNA synthesis (Kranias & Jungmann, 1978). Because protein kinase NII can utilize GTP as well as ATP as nucleotide substrate (Rose et al., 1981a), poly[d(A-T)] was used as template for the polymerase reaction so that GTP could be deleted from the reaction mixture. In this experiment, protein kinase stimulated RNA polymerase II 1.8-fold in the presence of ATP. When AMP-PNP was substituted for ATP, the addition of protein kinase had no significant effect on RNA synthesis (Table IV, experiment B). Since ATP is needed for protein phosphorylation and since protein kinase NII has an absolute requirement for Mg^{2+} (Rose et al., 1981a), the lack of stimulation in the absence of the nucleotide and this divalent cation indicated that the activity of the protein kinase was required for the observed increase in RNA synthesis.

Table IV: ATP Dependence of Protein Kinase Activation of RNA Polymerase II[a]

addition	nucleotide	RNA synthesis (units)
experiment A		
none	ATP	2.6 ± 0.2
plus kinase NII	ATP	6.3 ± 0.4
none		2.1 ± 0.2
plus kinase NII		2.0 ± 0.3
experiment B		
none	AMP-PNP	5.9 ± 0.4
plus kinase NII	AMP-PNP	5.6 ± 0.5

[a] RNA polymerase II [14 units in the standard assay with 50 mM $(NH_4)_2SO_4$ and double-stranded DNA] was incubated in the presence or absence of protein kinase (15 units) in a reaction mixture containing 12.5 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, and 0.33 mg/mL bovine serum albumin (BSA) with or without 40 μM ATP or adenylyl 5′-imidodiphosphate (AMP-PNP) as indicated. After 15 min at 30 °C, the $(NH_4)_2SO_4$ concentration was raised to 200 mM (experiment A) or 50 mM (experiment B). Cofactors for RNA synthesis were then added as described under Materials and Methods for experiment A. In experiment B, GTP and CTP were excluded, 100 μg/mL poly[d(A-T)] was substituted for DNA, and AMP-PNP replaced ATP throughout. Incubation at 30 °C was continued for an additional 30 min. Results are the mean of triplicate samples ± SEM.

Table V: Effect of Protein Kinase NII on the Size of RNA Chains Synthesized by RNA Polymerases[a]

enzymes	incorpn (pmol/30 min) UMP	incorpn (pmol/30 min) Urd	estimated av chain length [(UMP + Urd)/Urd]
RNA polymerase II	24.9 (24.9)	0.55 (0.51)	46 (50)
RNA polymerase II plus kinase NII	41.3 (55.0)	0.94 (0.70)	45 (49)
RNA polymerase I	40.1	0.54	75
RNA polymerase I plus kinase NII	54.5	0.51	108

[a] RNA was synthesized in vitro by purified RNA polymerase I or II in the absence or presence of protein kinase NII (80 units). The conditions were as described under Materials and Methods for the standard assay except that the specific radioactivity of the [^{3}H]UTP was increased to 800 cpm/pmol. The reaction product was hydrolyzed with KOH and analyzed as indicated under Materials and Methods. Numbers in parentheses are values obtained from a separate experiment.

Mechanism of Enhanced RNA Synthesis. To determine whether the NII kinase affected the initiation or elongation of RNA chains by polymerase II, we estimated the number of 3′-OH termini and the average chain length. As shown in Table V, addition of protein kinase NII did not alter the average length of RNA chains synthesized by polymerase II under standard assay conditions. In particular, average chain lengths of 46 and 45 nucleotides were obtained in the absence and presence of the kinase, respectively. However, the protein kinase caused a 1.7-fold increase in the number of 3′ termini (uridine) synthesized by polymerase II. These results were in contrast to the 1.4-fold increase in chain length observed when RNA polymerase I was activated with the kinase (Table

Table VI: Effect of Protein Kinase NII on RNA Synthesis in the Absence of Reinitiation[a]

kinase (units)	RNA polymerase activity (units)
none	2.0 ± 0.17
5.0	2.9 ± 0.14
8.0	3.4 ± 0.24
16.0	3.9 ± 0.18

[a] RNA polymerase II (4.1 ± 0.23 units in the standard assay) was incubated with increasing quantities of protein kinase NII under conditions which allowed the initiation of RNA chains (see Materials and Methods). After 15 min at 30 °C, $(NH_4)_2SO_4$ was added to a final concentration of 275 mM along with the remaining cofactors required for RNA synthesis. Incubation at 30 °C was then continued for an additional 30 min.

V). Addition of protein kinase did not significantly affect the number of RNA chains (reflected by the number of chains ending in uridine) synthesized by RNA polymerase I.

There are several possible explanations for the increased number of RNA chains synthesized by RNA polymerase II in the presence of protein kinase NII. First, the kinase could enhance the initiation of RNA chains by a variety of mechanisms. Alternatively, a more rapid elongation reaction and/or release of the enzyme from the transcription complex could make more polymerase available for reinitiation. Reinitiation was blocked by a high salt concentration in order to differentiate between these possibilities. As shown in Table VI, RNA polymerase II was stimulated by protein kinase even under conditions which prevented reinitiation. For example, RNA synthesis in the presence of 16 units of protein kinase NII was approximately twice that obtained in the absence of the phosphorylating enzyme. As when reinitiation was allowed, the addition of protein kinase NII under these conditions had no significant effect on the average length of RNA chains (Table VII, experiment A). Rather, there was an increase (1.6-fold) in the number of RNA chains synthesized in the presence of the kinase. In order to eliminate the possibility that protein kinase NII promoted preferential termination with uridine residues, we repeated the experiment with [^{3}H]GTP as the labeled nucleotide in place of [^{3}H]UTP. As observed when [^{3}H]UTP was the radioactive substrate, protein kinase NII had no significant effect on the average length of RNA chains but did cause an increase in the number of chains initiated (Table VI, experiment B). The stimulation in the number of RNA chains observed with [^{3}H]GTP was not as great as that obtained with [^{3}H]UTP. This was most likely due to a suboptimal concentration of GTP during chain initiation. It should be noted that the salt concentration required to block reinitiation completely [275 mM $(NH_4)_2SO_4$] also inhibited elongation by approximately 50%. For example, RNA polymerase II activity in the absence of protein kinase under the high salt conditions was 2.0 units compared to the 4.1 units obtained with the same enzyme in the standard assay (see legend to Table VI). Because of this inhibition of elongation, the average length of RNA chains synthesized by RNA polymerase II in the absence of reinitiation (Table VI) was approximately 50% of that observed with the standard assay (Table V).

Discussion

In this report, we have described the purification of RNA polymerase II from a rat tumor, Morris hepatoma 3924A, and have defined the conditions necessary for its phosphorylation and activation by an extensively purified homologous cyclic nucleotide independent nuclear protein kinase (NII). Phosphorylation of RNA polymerase II by homologous protein

Table VII: Effect of Protein Kinase NII on the Length of RNA Chains Synthesized in the Absence of Reinitiation[a]

addition	incorpn (pmol/30 min) UMP	Urd	estimated av chain length, (UMP + Urd)/Urd
experiment A			
none	16.4	0.72	24
plus protein kinase	22.8	1.14	21
addition	**incorpn (pmol/30 min) GMP**	**Guo**	**estimated av chain length, (GMP + Guo)/Guo**
experiment B			
none	22.0	1.02	22
plus protein kinase	26.8	1.26	22

[a] RNA was synthesized in vitro by RNA polymerase II (30 units in the standard assay) in the absence or presence of protein kinase NII (80 units) as described in the legend to Table V. The specific radioactivities of the [^{3}H]UTP (experiment A) and [^{3}H]GTP (experiment B) were increased to 800 cpm/pmol. Following synthesis, the RNA was hydrolyzed with KOH and analyzed as indicated under Materials and Methods.

kinases with concomitant stimulation of activity has been reported in other systems (Dahmus, 1976; Kranias et al., 1977; Kranias & Jungmann, 1978). However, this is the first report describing activation of highly purified polymerase II by a well-defined nuclear protein kinase. Several conditions were required to obtain consistently stimulation of hepatoma RNA polymerase II by protein kinase NII. First, because the kinase is sensitive to high $(NH_4)_2SO_4$ concentrations (Rose et al., 1981a), it was necessary to keep the ionic strength relatively low compared to the optimum for polymerase II (Rose et al., 1976). This could be achieved either by conducting RNA synthesis at 12.5 mM $(NH_4)_2SO_4$ (not shown) or by activating the polymerase under optimal conditions for the protein kinase followed by the addition of polymerase cofactors and $(NH_4)_2SO_4$ to 50 mM (Figure 3). Second, because ATP and Mg^{2+} are required for the activity of the protein kinase (Rose et al., 1981a), the presence of the nucleotide and this divalent metal ion during activation was critical (Tables III and IV). Mn^{2+} could not replace Mg^{2+} and actually decreased the stimulation of RNA polymerase II by the kinase in the presence of Mg^{2+} (Table III). Third, the quantity (units) of protein kinase required to stimulate the polymerase varied among different kinase and polymerase preparations. This was most likely because of variability in the phosphorylated state of the enzymes resulting from in vivo reactions. The stoichiometry of the in vitro phosphorylation of polymerase II by protein kinase NII was rather low (0.1 mol of phosphate/mol of enzyme; calculated from data in Figure 2), suggesting that the polymerase may have retained significant phosphate. It is also feasible that only a small fraction of the polymerase molecules were capable of being phosphorylated and participating in the transcription reaction. It should be pointed out that in vitro phosphorylation was conducted for limiting times to avoid cleavage of RNA polymerase II polypeptides. Hence, the computed in vitro added phosphate is most likely an underestimate. Regardless, it was necessary to perform preliminary experiments with each preparation of both enzymes to determine the appropriate ratio for stimulation. Finally, although partially purified preparations of polymerase II could be activated by the kinase, maximal stimulation was achieved with highly purified enzyme. Partially purified RNA polymerase II preparations contain significant protein kinase activity (D. A. Stetler and K. M. Rose, unpublished results). The

relatively poor response of partially purified polymerase to activation was probably due to a greater phosphate content of the polymerase either because of the presence of NII kinase (which could phosphorylate the polymerase in vitro) or because of a greater retention of in vivo attached phosphate.

Stimulation of calf thymus RNA polymerase II by a homologous nuclear cyclic AMP dependent protein kinase was observed by Kranias et al. (1977). Although a M_r 25 000 subunit polypeptide was the principal phosphate acceptor, in some experiments a large (M_r 180 000) polypeptide was also phosphorylated. Kranias & Jungmann (1978) also observed the stimulation and phosphorylation (M_r 25 000 polypeptide) of calf thymus polymerase II by a homologous cyclic nucleotide independent nuclear protein kinase. The state of purity of the calf thymus enzyme was comparable to that of the hepatoma polymerase II purified through the DNA–cellulose column (Table I). We have observed that the M_r 21 000 polypeptide of hepatoma polymerase II is the predominant phosphate acceptor in partially purified preparations (data not shown) but that the larger polypeptides are the major acceptors in the gradient-purified enzyme (Figure 2). Thus, the failure to detect transfer of labeled phosphate to the larger calf thymus polymerase peptides might have been related to the purification stage of the enzyme.

RNA polymerase II from Novikoff ascites cells has been reported to be stimulated by a homologous cyclic nucleotide independent protein kinase (KI) obtained from whole cells (Dahmus, 1976). Another cellular protein kinase (KII) was unable to activate the polymerase (Dahmus & Natzle, 1977). Unfortunately, the polymerase phosphopeptides were not examined in these studies. Subsequent work from the same laboratory indicated that polymerase II from calf thymus was phosphorylated (M_r 214 000 and 20 500 polypeptides) by both KI and KII protein kinases (Dahmus, 1981b), but not activated by either enzyme. In the present report, phosphorylation and activation were observed only with nuclear kinase NII. Because the hepatoma polymerase II polypeptides of M_r 214 000, 140 000, and 21 000 were modified by protein kinase NII when stimulation occurred (Figure 2) while only the M_r 214 000 and 20 000 subunits were phosphorylated in the calf thymus system (Dahmus, 1981b), it is tempting to speculate that phosphorylation of the 140 000-dalton polypeptide is essential for activation. However, the possibility that the NII kinase modifies unique sites, requisite for activation, on the M_r 214 000 or 21 000 polypeptides cannot be eliminated at the present time. The apparent lack of specificity of the cellular calf thymus protein kinase KI and KII extended to RNA polymerase I. The same polymerase I polypeptides were phosphorylated by both kinases (Dahmus, 1981b). However, neither of these cellular kinases activated RNA polymerase I nor phosphorylated the M_r 120 000 subunit of that polymerase. Studies from this laboratory have implicated the 120 000-dalton subunit of polymerase I in RNA chain elongation (D. A. Stetler and K. M. Rose, unpublished results), and phosphorylation of the M_r 120 000 polypeptide was concomitant with enhanced chain elongation in vitro (Duceman et al., 1981; Rose et al., 1981b,c). Although protein kinases KII (Dahmus, 1981a) and NII (Thornburg & Lindell, 1977; Rose et al., 1981a) appear to have similar reaction characteristics and subunit structure, they are most likely not analogous enzymes since they phosphorylate different subunits of the RNA polymerases. Hence, the choice of the protein kinase for use in experiments with RNA polymerases appears to be crucial. To date, those kinases that have been purified from the cell nuclei have been capable of stimulating RNA polymerase activity (Kranias et al., 1977; Kranias & Jungmann, 1978; Duceman et al., 1981; Rose et al., 1981b,c) while those purified from whole cells generally have not had this ability (Dahmus & Natzle, 1977; Dahmus, 1981b).

Finally, the different response of RNA polymerases I and II to modification by protein kinases deserves comment. Phosphorylation of RNA polymerase I enhanced the rate of RNA chain elongation (Duceman et al., 1981), whereas modification of polymerase II resulted in an elevation of the number of RNA chains synthesized (Table V). Whether this was due to stabilization of polymerase II, to activation of previously inactive enzyme, or to stimulation of initiation by already active enzyme molecules was not determined. Nevertheless, it is interesting to note that differential expression of genes transcribed by polymerase II is most likely mediated via selective initiation of transcription while enhanced production of ribosomal RNA is thought to result from an increased RNA polymerase I elongation rate in vivo (Dauphinais, 1981). The presence of protein kinase NII in nuclear extracts, its close association with RNA polymerases, its increased activity in neoplasia, and its ability to activate both RNA polymerases I and II suggest that this kinase plays a key role in gene expression.

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