Protein kinase activity of RNA polymerase I purified from a rat hepatoma: Probable function of M_r 42,000 and 24,600 polypeptides

(phosphorylation/RNA polymerase I subunits/antibodies/diazobenzyloxymethyl-filter technique)

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ABSTRACT   RNA polymerase I was purified to homogeneity from Morris hepatoma 3924A. Purified RNA polymerase I contained a protein kinase activity that comigrated with the polymerase in nondenaturing gels. RNA polymerase II, purified from the same hepatoma, lacked protein kinase activity. Analysis of the subunit composition of the RNA polymerase I showed the presence of eight polypeptides: S1, M_r 190,000; S2, M_r 120,000; S3, M_r 62,000; S4, M_r 42,000; S5, M_r 24,600; S6, M_r 21,000; S7, M_r 19,500; and S8, M_r 17,500. Antibodies prepared against purified polymerase I specifically inhibited RNA synthesis catalyzed by RNA polymerase I. When subunits of the enzyme were covalently linked to diazobenzyloxymethyl paper, complexes between the antibody preparation and S1–S6 were visualized. No immune complexes were formed between RNA polymerase I antibodies and RNA polymerase II subunits. The antibody preparation was able to inhibit both the protein phosphorylation catalyzed by RNA polymerase I and that catalyzed by a nuclear kinase (NII) purified from the same hepatoma. The two polypeptides of the nuclear kinase—M_r 42,000 and 24,600 (identical in size to S4 and S5 of polymerase I)—formed visible complexes with the RNA polymerase I antibodies. Both S4 and S5 of the polymerase contained an ATP binding site, a property associated with protein phosphorylation and also exhibited by the polypeptides of the purified kinase. These data suggest that polypeptides of M_r 42,000 and 24,600 associated with polymerase I are responsible for its kinase activity.

Phosphorylation of nuclear proteins has been correlated with enhanced gene expression in several systems (1). A number of observations suggest that ribosomal gene transcription may be regulated by protein phosphorylation (2–6). We have recently purified (6) a spermine-activated, cyclic nucleotide-independent nuclear protein kinase (classified as NII). This kinase has a high affinity for RNA polymerase I and stimulates RNA synthesis in vitro. We now report that RNA polymerase I itself contains an endogenous protein kinase capable of phosphorylating both exogenous protein acceptors and endogenous RNA polymerase subunits.

METHODS

Preparation of Enzymes. RNA polymerase I, RNA polymerase II, and nuclear protein kinase NII were purified to homogeneity from Morris hepatoma 3924A by chromatography on DEAE-Sephadex, DNA-cellulose, and heparin-Sepharose columns, followed by centrifugation through sucrose gradients (6, 7).

Protein Kinase Assay. The protein kinase activity of RNA polymerase I was measured in 10–30 μM [γ-^{32}P]ATP [Amersham; 2000 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels), final specific activity, 3500 cpm/pmol]/5 mM MgCl$_2$/96 mM Tris chloride (pH 7.5) containing 100 μg of casein and 0.3–1.5 μg of enzyme protein in a total volume of 50 μl. After incubation at 30°C for 30 min, reactions were terminated by addition of 10 μg of unlabeled ATP, and the mixtures were transferred onto Whatman GF/C filters, which were washed batchwise with 5% trichloroacetic acid. One unit refers to 1 pmol of phosphate transferred from the terminal position of ATP to casein in 15 min.

Preparation of Antibodies. Male New Zealand White rabbits were injected biweekly with 50–100 μg of purified RNA polymerase I emulsified with an equal volume (0.5–1.0 ml) of Freund complete adjuvant. An IgG fraction was obtained from the sera (collected 8–20 weeks after the first injection) by (NH$_4$)$_2$SO$_4$ precipitation (50% saturation at 4°C), followed by chromatography on Sephacryl S-200. IgG preparations containing traces of ribonuclease or protein phosphatase were further purified by affinity chromatography on protein A-Sepharose. Nonimmune IgG samples were prepared in the same manner from sera of uninjected rabbits.

Analysis of Antibody Binding to Polypeptides on Diazobenzyloxymethyl-Paper. After polyacrylamide gel electrophoresis under denaturing conditions (8), the purified enzyme subunits were transferred to diazobenzyloxymethyl (DBM)-paper (9) by horizontal electrophoresis for 1 hr at 24 V. Complete (>90%) transfer of polypeptides to the DBM-paper was confirmed by staining gels after horizontal electrophoresis. After transfer, the DBM-paper was incubated first with unfractionated antiserum and then with ^{125}I-labeled protein A (10).

RESULTS

Phosphorylation of Exogenous Protein by Purified RNA Polymerase I. RNA polymerase I and protein kinase NII copurified until the sucrose gradient fractionation step. This step separated RNA polymerase I (16S) from the bulk of the NII protein kinase (7S) (6). Even after removal of the major kinase fraction, the purified polymerase I was able to transfer the terminal phosphate of ATP to exogenously added proteins. The ratio of kinase activity to RNA polymerase I activity remained unchanged after a subsequent sucrose gradient fractionation. Due to the inhibitory effects of high salt concentrations, the kinase activity of RNA polymerase I was detectable in the gradient fractions only after appropriate dilution or dialysis.

In the presence of 5 mM MgCl$_2$, casein (apparent K_m^{ATP} = 12 μM) and phosvitin, but not histone and serum albumin, were able to serve as protein acceptors for the RNA polymerase I-catalyzed kinase reaction. Phosphorylation of casein was stimulated 37% by 5 mM spermine and inhibited by heparin (1% of control at 1 μg/ml). RNA polymerase I was also able to use GTP (20% as efficiently as ATP) as nucleotide substrate. RNA polymerase II, purified from the same hepatoma, had no sig-

Abbreviation: DBM, diazobenzyloxymethyl.

nificant kinase activity when casein or histone were used as acceptor (data not shown).

The covalent attachment of radioactive phosphate to amino acids was confirmed by hydrolyzing the reaction product and subjecting the hydrolysate to chromatography on Dowex 50W-X8 as described (11). RNA polymerase I could effectively phosphorylate both the serine and the threonine moieties of casein (threonine/serine = 1.5). The phosphoester linkage was sensitive to alkaline hydrolysis. Coupled with the use of phosvitin (which contains no tyrosine) as phosphate acceptor, these data indicate that the kinase associated with polymerase I is not tyrosine specific. The characteristics of the protein kinase activity associated with RNA polymerase I are similar to those of nuclear kinase NII obtained during the purification of the polymerase. In particular, the sensitivity to heparin, the stimulation by spermine, the ability to use GTP as phosphoryl donor, and the preference for threonine as amino acid acceptor are distinguishing characteristics of nuclear protein kinase NII (6).

Migration of RNA Polymerase I and Protein Kinase in Polyacrylamide Gels. As shown in Fig. 1 *Left*, the RNA polymerase I preparation contained only two visible proteins. Both visible bands contained RNA polymerase activity and, hence, correspond to RNA polymerase IA (the faster migrating band) and IB (the slower migrating band), the two subclasses of polymerase I known to exist in mammalian tissues (12, 15, 16). Analysis of the protein kinase activity in the gel showed that those fractions that contained RNA polymerase activity could also catalyze the transfer of the terminal phosphate from ATP to casein. Further, no kinase activity was detected in regions of the gel that did not possess RNA polymerase activity (data not shown). Autophosphorylation of the RNA polymerase I preparation prior to electrophoresis demonstrated that the only proteins capable

of accepting phosphate corresponded to RNA polymerase IA and IB (Fig. 1 *Right*). RNA polymerase IB appeared more highly phosphorylated than IA.

Subunit Composition of RNA Polymerase I and Autophosphorylation of Individual Subunits. The subunit composition of purified RNA polymerase I was determined by electrophoresis on polyacrylamide gels under denaturing conditions (Fig. 2). Analysis by densitometry indicated that the following polypeptides were present: S1, M_r 190,000; S2, M_r 120,000; S3, M_r 62,000–65,000; S4, M_r 40,000–42,000; S5, M_r 24,600; S6, M_r 21,000; S7, M_r 19,500; S8, M_r 17,500. The molar ratios of these subunits were 0.9, 1.0, 2.1, 1.2, 1.6, 1.0, 1.5, and 1.2, respec-
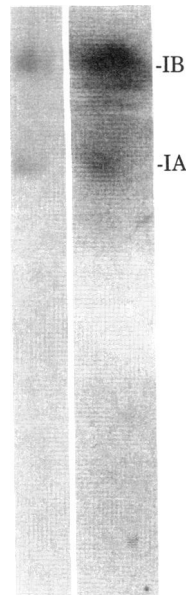

FIG. 1. Electrophoresis of RNA polymerase I under nondenaturing conditions. RNA polymerase I was prepared for electrophoresis by dialysis under reduced pressure against 50 mM Tris chloride, pH 7.9/ 25% glycerol/5 mM MgCl₂/0.01 mM EDTA/0.5 mM dithiothreitol/ 0.15 M KCl. Electrophoresis was carried out on linear polyacrylamide (2–16%) slab gels at 70 V for 6 hr at 4°C in 50 mM Tris/0.2 M glycine/ 10% glycerol/0.1 mM dithiothreitol/1 mM thioglycerol. (*Left*) Coomassie blue stained aliquot (6 μg) of RNA polymerase applied directly to the gel. (*Right*) Autoradiogram of the phosphorylation pattern produced by incubation of RNA polymerase I for 30 min at 30°C with 3.5 μCi of [γ-³²P]ATP (2000 Ci/mmol) in 20 μl of 10 mM MgCl₂/5 mM spermine/0.5 mM dithiothreitol/50 mM Tris chloride, pH 7.5.
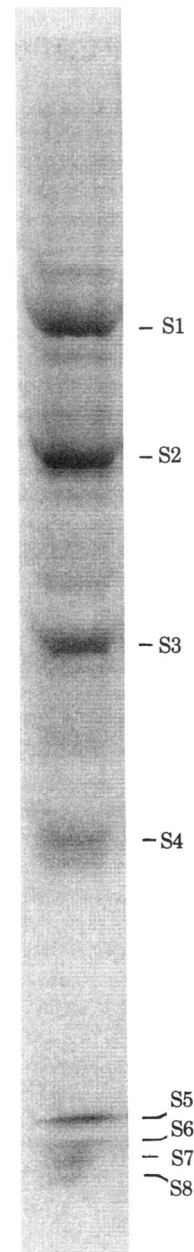

FIG. 2. Electrophoresis of RNA polymerase I under denaturing conditions. Purified RNA polymerase I was precipitated with trichloroacetic acid, washed with acetone, and denatured as described (11). The polypeptides were separated on linear polyacrylamide gradient (2–16%) slab gels using conditions described by Laemmli (8). Molecular weights were estimated from markers run in parallel gel tracks. The gel was analyzed by densitometry and polypeptides were present (assuming equal staining of all polypeptides) as indicated.

tively. The subunit composition and purity of hepatoma RNA polymerase I are comparable with those obtained in other laboratories (12, 13, 15).

It should be pointed out that, depending on the method and speed of purification, the content of S4 and S5 could vary as much as 2-fold. In all cases, S4 and S5 varied in the same direction. Of particular interest was the observation that those preparations with high proportions of these two polypeptides always possessed more kinase activity than those with lesser quantities. Further, the specific activity of RNA polymerase I was always positively correlated with the content of S4 and S5.

Purified NII protein kinase contains two polypeptides (M_r 42,000 and 24,600). As RNA polymerase I and the purified NII protein kinase contain polypeptides of similar size, it was of interest to determine which polypeptides were autophosphorylated by the respective enzymes. As shown in Fig. 3 (track A), RNA polymerase I subunits S2 (120,000), S3 (65,000), and S5 (24,600) were phosphorylated by the endogenous kinase. Purified protein kinase could also phosphorylate one of its subunits, transferring radioactive phosphate to the M_r 24,600 polypeptide (Fig. 3, track B). When protein kinase was combined with RNA polymerase I and the phosphorylation reaction allowed to proceed, the amount of phosphate transferred to the peptides was increased (Fig. 3, track C). However, the pattern of phosphorylated RNA polymerase I products was identical with that observed with RNA polymerase I alone, indicating (*i*) that no additional RNA polymerase I subunits served as substrates for the pure protein kinase and (*ii*) that the M_r 24,600 polypeptide may be a common subunit acceptor of RNA polymerase I and purified protein kinase. As autophosphorylation of RNA polymerase II could not be demonstrated, the associ-

ation of the protein kinase activity with polymerase I appears to be selective.

Interaction of RNA Polymerase I Antibodies with RNA Polymerase I and Nuclear Protein Kinase. In an attempt to demonstrate a relationship between RNA polymerase I and the purified protein kinase, antibodies were raised against purified RNA polymerase I. Double immunodiffusion analysis of the antibody preparations with partially purified polymerase I resulted in a single band of immunoprecipitation. Typically, 70 μg of IgG from RNA polymerase I-injected animals resulted in 50% inhibition of 130 units of RNA polymerase I activity. In contrast, the same concentration of antibodies had no effect on the activity of either RNA polymerase II, purified from the same hepatoma, or the *Escherichia coli* enzyme (data not shown).

The effect of IgG preparation on protein phosphorylation was also measured. The endogenous kinase activity of RNA polymerase I (12 kinase units) was reduced by the IgG fraction to a similar extent (50% inhibition at 100 μg) as RNA synthesis catalyzed by an identical amount of polymerase protein. Purified hepatoma nuclear kinase NII was also sensitive to inhibition by the antibody preparation; a 50% reduction in activity (12 kinase units) was observed at 40 μg of IgG. In contrast, cyclic AMP-dependent cytoplasmic kinase (bovine heart) was only moderately affected by the antipolymerase immunoglobulins (30% inhibition of 9 units of activity at 270 μg of IgG). [In these experiments, total immunoglobulin concentration (immune plus nonimmune) in all reactions was kept constant to minimize nonspecific effects.]

To ascertain which polypeptides of RNA polymerase I and protein kinase NII were interacting with the antibody preparation, the subunits were transferred to DBM paper and treated with RNA polymerase I antiserum (see *Methods*). As shown in Fig. 4 (lane A), the antiserum reacted with RNA polymerase subunits S1 (190,000), S2 (120,000), S3 (62,000), S4 (42,000), S5 (24,600), and S6 (21,000). Only the subunits of M_r 19,500 (S7) and 17,500 (S8) lacked a corresponding antibody–protein A band on the autoradiograph. In agreement with the results of the inhibition studies, no antigen–antibody complexes were formed with immobilized RNA polymerase II subunits (Fig. 4, lane B). The antiserum to purified RNA polymerase I reacted with both subunits (M_r 42,000 and 24,600) of the purified protein kinase (Fig. 4, lane C). However, because only microgram quantities of homogeneous NII protein kinase are recovered
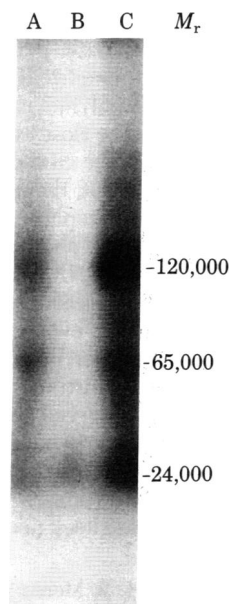

FIG. 3. Phosphorylation of RNA polymerase and protein kinase subunits. Homogeneous RNA polymerase I and protein kinase were incubated for 30 min at 30°C with 5 mM MgCl₂/50 mM Tris chloride, pH 7.5/0.5 mM dithiothreitol/[γ-³²P]ATP (13 μCi, 1500 pmol) in total vol of 150 μl. Reactions were terminated by the addition of trichloroacetic acid, and the products were denatured and subjected to electrophoresis as described in the legend to Fig. 2. Autoradiograms were prepared by exposure to XR-5 x-ray film. Track A, Autophosphorylation of hepatoma RNA polymerase I; track B, autophosphorylation of hepatoma 3924A protein kinase; track C, phosphorylation pattern produced by a mixture of RNA polymerase I and protein kinase. Molecular weights were calculated from marker proteins run on parallel tracks.
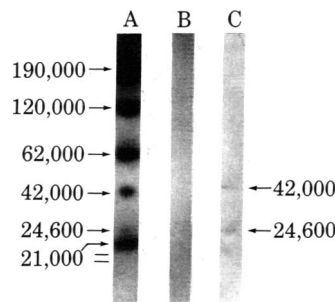
FIG. 4. Interaction of anti-RNA polymerase I serum with individual enzyme subunits. Hepatoma RNA polymerase I and II and the nuclear protein kinase, purified to homogeneity, were subjected to polyacrylamide gel electrophoresis under denaturing conditions and transferred to DBM paper; antigens detected as described in *Methods*. Arrows indicate corresponding subunit M_r and positions of antigen–antibody complex bands. Bars indicate RNA polymerase I subunits observed in stained, parallel gel tracks to which no antibodies could be detected. RNA polymerase II contained subunits of M_r 200,000, 140,000, 33,000, 25,000, 21,000, 19,000, 18,000, and 17,000 (not shown). Lane A, RNA polymerase I (50 μg); lane B, RNA polymerase II (50 μg); lane C, homogeneous hepatoma protein kinase (1 μg).

from kilogram amounts of hepatoma, we have been unable to prepare sufficient quantities of pure kinase to test the interaction between kinase-specific antibodies and RNA polymerase I.

The presence of two antigens of similar M_r (42,000 and 24,600) as components of both polymerase I and hepatoma nuclear kinase NII suggested that these polypeptides are common to both enzymes and are responsible for the kinase activity of the polymerase. If so, S4 and S5 and the kinase should exhibit similar binding properties. As indicated in Table 1, ATP, a protein kinase substrate, could compete with antibodies for binding sites on S4 and S5. None of the other polymerase subunits could bind ATP in a manner that affected antibody binding. The nucleotide also decreased antibody binding to both kinase subunits. As only S4 and S5 exhibited binding sites in common with the protein kinase subunits, the possibility that other polymerase polypeptides are antigenically related to the purified kinase is remote.

DISCUSSION

We have shown that purified RNA polymerase I contains a protein kinase activity. Several observations prompt us to conclude that the 42,000 (S4) and 24,600 (S5) M_r polypeptides associated with RNA polymerase I may be responsible for the kinase activity. (i) Protein kinase NII separated during purification of RNA polymerase I has a number of *in vitro* reaction characteristics in common with the endogenous kinase of polymerase I. The purified protein kinase contains two polypeptides—M_r 42,000 and 24,600—that are present in RNA polymerase I (S4 and S5) in molar proportions. (ii) Antibodies specific for purified RNA polymerase I inhibit the polymerase-associated kinase activity as well as the activity of the purified NII kinase. Further, the antibodies bind to S4 and S5 and the two kinase polypeptides. (iii) S4 and S5 bind ATP in a similar manner as the kinase components of the same M_r. Our data do not comment on whether all of the protein content of S4 and S5 is accounted for by kinase antigens. Direct proof of the identification of S4 and S5 as the kinase component(s) of the polymerase must await peptide analyses. Unfortunately, the minute quantities of these enzymes in higher eukaryotes render such studies difficult.

Table 1. Effect of ATP on antibody binding to RNA polymerase I and protein kinase subunits

Enzyme	Subunit (M_r)	Antibody binding, % of control
RNA polymerase	S1 (190,000)	108
	S2 (120,000)	104
	S3 (62,000)	93
	S4 (42,000)	47
	S5 (24,600)	35
	S6 (21,000)	99
Protein kinase	(42,000)	27
	(24,600)	23

DBM-papers containing the subunits of RNA polymerase I (50 μg) or hepatoma protein kinase (1 μg) were incubated with anti-RNA polymerase I antiserum and ^{125}I-labeled protein A as described in *Methods*, except that gelatin was excluded from all buffers. Autoradiograms were scanned at 570 nm using a Transidyne 2955 scanning densitometer, and the area under each peak was determined (control). After removal of antibodies and ^{125}I-labeled protein A with urea (10), the papers were reincubated with antiserum in the presence of ATP (1 mM) and processed as before. Results (area under each peak in the presence of ATP ×100 divided by that in the absence of ATP) are the average of duplicate experiments. Immune complexes formed in the presence of ATP were removed with urea, and the papers were reincubated with antibody in the absence of the nucleotide. The values thus obtained were identical to the original control values.

Although it is not yet known whether RNA polymerase I from all sources contains protein kinase activity, the polypeptide composition of the hepatoma RNA polymerase I is similar to that of the same enzyme purified from a number of sources (16). Further, other investigators have noted protein kinase activity with purified polymerase I preparations (13). The positive correlation between polymerase activity and content of S4 and S5 suggests a regulatory role for these peptides in RNA synthesis. The association of these proteins with RNA polymerase I could be analogous to that observed for regulatory polypeptides that copurify with other RNA polymerases—e.g., σ factor with *E. coli* enzyme (14) and ribonuclease H with yeast RNA polymerase I (17). Also, addition of excess protein kinase to purified polymerase I preparations, particularly those from liver, that contain limiting endogenous kinase, results in a marked increase (up to 40-fold) in RNA polymerase activity (ref. 6; unpublished observations), demonstrating a direct role for the kinase in regulation of RNA synthesis *in vitro*.

Hepatoma 3924A, as well as a number of other tumors, contain an increased proportion of RNA polymerase IB to IA, compared with resting liver (5, 7, 16, 18). As RNA polymerase IB is generally increased in tissues with augmented rRNA synthesis and this enzyme contains the phosphopeptide S3 (see Fig. 3) absent in IA, phosphorylation of this subunit might play a key role in regulating rRNA production *in vivo*. Further, if the polypeptides of M_r 42,000 (S4) and 24,600 (S5) regulate the activity of the polymerase via phosphorylation, RNA polymerase I from the hepatoma (or other rapidly growing tissues) would be expected to contain a greater complement of these two polypeptides than enzyme from resting tissues. Indeed, comparison of the subunit composition of RNA polymerase I purified from normal rat liver, using a similar protocol, with that of hepatoma 3924A, showed that the rat liver enzyme contains less than molar quantities of S4 and S5 as well as significantly less kinase activity per microgram of protein (5). In addition, the liver enzyme contains less than stoichiometric levels of S3, consistent with the relatively low level of polymerase IB in normal tissue. The mechanism that regulates the close association of the kinase with RNA polymerase I, as in the case of rapidly growing tumors, is not yet clear. In any event, the implications of an RNA polymerase complex capable of autophosphorylation (activation) are immediately evident. Such a phenomenon would permit rapid alterations in the rate of rRNA synthesis in response to physiological variations without recourse to synthesis of new RNA polymerase I. For example, even in the presence of protein synthesis inhibitors (19), liver RNA polymerase I can be activated by corticosteroid administration (20, 21). Hence, the augmented rRNA synthesis observed on treatment with this class of hormones may well be due to increased phosphorylation of RNA polymerase I. Whether this phosphorylation results from an enhanced association of kinase with polymerase I or from activation of protein kinase itself needs to be investigated.

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