Phosphorylation of Yeast DNA-dependent RNA Polymerases in Vivo and in Vitro

ISOLATION OF ENZYMES AND IDENTIFICATION OF PHOSPHORYLATED SUBUNITS*

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Yeast DNA-dependent RNA polymerases I, II, and III are phosphorylated in vivo. Yeast cells were grown continuously in 
$^{32}$P, and the RNA polymerases were isolated by a new procedure which allows the simultaneous purification of these enzymes from small quantities (35 to 60 g) of cells. Each of the RNA polymerases was phosphorylated. The following phosphorylated polymerase polypeptides were identified: polymerase I subunits of 185,000, 44,000, 36,000, 24,000, and 20,000 daltons; a polymerase II subunit of 24,000 daltons; and polymerase III subunits of 24,000 and 20,000 daltons. The incorporated $^{32}$P was acid-stable but base-labile. Phosphoserine and phosphothreonine were identified after partial acid hydrolysis of purified $^{32}$P-polymerase I.

A yeast protein kinase that co-purifies with polymerase I during part of the isolation procedure was partially purified and characterized. This protein kinase phosphorylates the subunits of the purified polymerases that are phosphorylated in vivo and, in addition, a polymerase I subunit of 48,000 daltons and a polymerase II subunit of 33,500 daltons. Phosphorylation of the purified enzymes with this protein kinase had no substantial effect on polymerase activity in simple assays using native yeast DNA as a template. Preincubation of purified polymerase I with acid or alkaline phosphatase also had no detectable effect on polymerase activity.

In eukaryotes, transcription is regulated during development and in response to physiological stimuli (reviewed in Refs 1 and 2). In principle, the intracellular function of RNA polymerase can be regulated by altering the enzyme concentration, by chromatin modifications which change the interaction between polymerase and template, or by modifications of the enzyme which alter its specific activity. It has been difficult to measure the relative contribution of each of these mechanisms in regulating transcription but, in a number of cases (3-8), the data have been interpreted to suggest that the increased polymerase activity may be due to a change in the rate of initiation or elongation of the enzyme rather than to a change in the absolute number of enzyme molecules. Several groups have reported that protein kinases stimulate the activity of polymerases I and II in cell-free systems (9-11) and suggest that RNA polymerase activity in vivo might be regulated through phosphorylation by protein kinases. However, these studies did not determine which (if any) of the component proteins were phosphorylated. In a preliminary report (12), we presented data which indicated that polymerase I was labeled when isolated from yeast cells grown in 
$^{32}$P, and that the yeast RNA polymerases I, II, and III could be phosphorylated in vitro by a yeast protein kinase. This paper extends these studies and demonstrates that certain polypeptides of each of the three RNA polymerases are phosphorylated in vivo and also by a purified protein kinase in vitro. However, we have been unable to measure a significant effect of phosphorylation on the activity of these enzymes in conventional assays using native yeast DNA templates.

Experimental Procedures

Yeast Strains

An auxotrophic tetraploid strain of Saccharomyces cerevisiae, 5178 $1C^3 	imes 2B^1$, obtained from L. Hartwell, University of Washington, was used in all in vivo phosphate labeling experiments. Unlabeled RNA polymerases were isolated from a commercial strain of yeast, F1, a gift of the Red Star Yeast Co., Oakland, Calif. There were no apparent differences in the physical properties or the subunit composition of the enzymes isolated from these two sources.

Materials

Phosvitin, histone (calf thymus), and acid phosphatase (potato) were purchased from Calbiochem, La Jolla, Calif. Protamine-Cl, phosphoserine, and phosphothreonine were obtained from Sigma Chemical Co., St. Louis, Mo. Casein (Pentex) was purchased from Miles Laboratories Inc., Kankakee, Ill. and alkaline phosphatase (Escherichia coli) from Worthington Biochemical Corp., Freehold, N. J. $^{32}$P (as orthophosphoric acid in a 0.02 M HCl solution, carrier-free) was obtained from New England Nuclear, Boston, Mass. A 50% solution of Polymin P was obtained from Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N. Y., diluted 10 times to a 5% (w/v) solution, and the pH adjusted to 8.0 with HCl using pH indicator paper. Yeast DNA was isolated from the tetraploid strain grown in phosphate-depleted YEPD medium (13) by the procedure of Cramer et al. (14). This DNA had a double-stranded molecular weight of approximately $35 \times 10^6$ and was about half as efficient as commercial calf thymus DNA as a template for the purified yeast RNA polymerases. All other materials have been described previously (15).

Preparation of Resins and Buffers

Phosphocellulose, DEAE-cellulose, and DEAE-Sephadex A-25 were preincubated and equilibrated with the appropriate buffers as

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described previously (15). Denatured calf thymus DNA-cellulose was prepared by the procedure of Alberts and Herrick (16). The buffers used are those described by Valenczula et al. (15). Buffer A is 0.2 M Tris/ HCl, pH 8.0, 0.01 M 2-mercaptoethanol, 0.5 mM EDTA, and 10% glycerol. Buffer B is 0.01 M K,HPO, pH 8.0, 0.01 M 2-mercaptoethanol, 0.5 mM EDTA, and 25% glycerol.

**Growth Conditions and Preparation of Spheroplasts**

Stationary phase cells were inoculated at a 1:100 dilution into phosphate-depleted TEPD medium containing 4 to 30 ml/culture of TEP, and grown overnight at 30°. The generation time at midlog phase of growth was approximately 150 min. The cells were harvested when the density reached approximately 8 x 10^6 cells/ml and washed once with cold distilled water. Spheroplasts were prepared at 30° from the washed cells by the two-step procedure of Cabib (17) as modified by Wintersberger et al. (18).

**RNA Polymerase Assay**

The standard incubation mixture of 0.050 ml contained 60 mM Tris/HCl, pH 7.0, 1.6 mM MnCl_2, 0.6 mM concentration each of ATP, CTP, and GTP, 0.01 mM ['^32P]UTP (500 to 4000 dpm/pmol), 10 mM 2-mercaptoethanol, and 0.2 mg of phosphatase. After incubation for 10 min at 30°, a 0.040-mg aliquot was applied to a Whatman DE81 filter disc. The filter discs were washed and counted as described previously (15).

**Protein Kinase Assay**

The standard incubation mixture of 0.050 ml contained 60 mM Tris/HCl, pH 7.9, 0.5 M MgCl_2, 0.01 mM ['gamma]ATP (500 to 8000 dpm/pmol), 10 mM 2-mercaptoethanol, and 0.2 mg of phosphatase. After incubation for 10 min at 30°, a 0.040-mg aliquot was applied to a Whatman 3MM filter disc. The discs were washed batchwise at 4° (six times, 10 min each) in 10% trichloroacetic acid, 0.01 mM sodium pyrophosphate, and 0.01 M K,HPO, to remove unincorporated isotope. The discs were then washed in ethanol, ether-dried, and counted in Omnifluor/ toluene scintillant (New England Nuclear). When the specific activity of the different protein kinase fractions was measured, the ATP concentration was raised to 0.1 mM.

**Polyacrylamide Gel Electrophoresis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in slabs as described previously (15). Buffers and solutions were prepared according to Lecomml (16). The polyacrylamide concentration of the resolving gel was either 10% or 11% as indicated.

**Partial Acid Hydrolysis and Analysis of Hydrolysate for Phosphoserine and Phosphothreonine**

Thirty micrograms of purified [y-^32P]polymerase I (1500 dpm/µg) was precipitated from 5% trichloroacetic acid for 30 min at 0°. The precipitate was adjusted to 0.15 M with Buffer A. After washing with 0.2 M ammonium sulfate in Buffer A, the polymerase fractions were concentrated to approximately 8 x 10^6 cells/ml and washed once with cold distilled water. Spheroplasts were prepared at 30° from the washed cells by the two-step procedure of Cabib (17) as modified by Wintersberger et al. (18).

**Enzyme Purification**

**DNA-dependent RNA Polymerases** - Unlabeled yeast RNA polymerases I, II, and III were prepared as described elsewhere (15, 22). ['gamma]P-labeled polymerases I, II, and III were purified simultaneously from spheroplasts by modifying the procedures described in detail by Valenczula et al. (16, 22). These modified procedures are presented below and describe the isolation of the three RNA polymerases from 35 to 60 g (wet weight) of yeast. All steps were carried out at 0-4°.

**Preparation of Cell Extract - Yeast spheroplasts were collected from the spheroplast buffer (1 ml sorbitol) by centrifugation (10 min at 27,000 x g). The pellet was suspended with a glass rod and finally sonicated for 10 min. Fifteen percent of the supernatant was centrifuged at 40,000 rpm for 30 min, 48,000 x g (Sorvall SS34, 20,000 rpm) and the supernatant containing the RNA polymerase activity was treated as follows.

**Purification of Polymerase I - RNA polymerase I was purified from the extract batchwise with phospho- and DEAE-cellulose to Fraction 3 by the procedure of Valenczula et al. (16). This procedure was reduced proportionately to accommodate the small amount of cells used. RNA polymerases II and III were purified from the protein not absorbed by phosphocellulose at 0.15 M ammonium sulfate in Buffer A. The polymerase I in Fraction 3 was precipitated by dialysis against Buffer A saturated with ammonium sulfate. The precipitate was collected by centrifugation (27,000 x g, 30 min, Sorvall H14), dissolved in 0.7 ml of 0.2 M KCl in Buffer A and layered onto a linear, 11.8 ml, 5 to 20% (w/v) sucrose gradient in 15% glycerol, 0.05 M Tris/HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM KCl, and 10 mM 2-mercaptoethanol. The gradient was centrifuged in a Beckman SW 41 rotor at 40,000 rpm for 28 h. The gradient fractions were assayed for RNA polymerase activity, protein concentration, and ['gamma]P-labeled protein. In measurements of ['gamma]P-protein, nucleic acids were destroyed by heating the filters containing the sample in 5% trichloroacetic acid at 100° for 15 min (20). The peptide composition of the fractions was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Purification of Polymerase II - RNA polymerases II and III in the 0.15 M ammonium sulfate/Buffer A phosphocellulose filtrate were separated from the bulk of the nucleic acid and some of the protein by precipitation with and selective extraction from Polymer P. Eight milliliters of a 5% (w/v) solution of Polymyxin P was added dropwise per 100 ml of filtrate with stirring. After equilibration for 5 min, the suspension was centrifuged (27,000 x g, 20 min), the supernatant was discarded, and polymerases II and III were extracted from the pellet with 0.3 M ammonium sulfate in Buffer A containing 1 mM phenylmethylsulfonyl fluoride and 1% dimethylsulfoxide (0.5 volumes relative to volume of original filtrate) by hand homogenization. The suspension was centrifuged (27,000 x g, 20 min) and the Polymyxin P precipitate was discarded. Polymerases II and III were precipitated from the supernatant by the addition of solid ammonium sulfate at 35° to 60° (wet weight) of yeast. All steps were carried out at O-4°. The pellet was suspended with a glass rod and finally sonicated for 10 min. Fifteen percent of the supernatant was centrifuged at 40,000 rpm for 30 min, 48,000 x g (Sorvall SS34, 20,000 rpm) and the supernatant containing the RNA polymerase activity was treated as follows.

**Purification of Polymerase III** - The polymerase III in the flow through from the DEAE-cellulose column was absorbed onto a DEAE-Sephadex A-25 column (15-ml bed volume) equilibrated with 0.1 M ammonium sulfate in Buffer A. After washing with 0.2 M ammonium sulfate in Buffer A, the polymerase III was eluted with 0.5 M ammonium sulfate in Buffer A. The polymerase fractions were pooled and the enzyme precipitated by dialysis against Buffer A containing 0.35 g/ml of ammonium sulfate. The precipitate was centrifuged (27,000 x g, 30 min), dissolved in 0.25 ml of 0.2 M KCl in Buffer A, and layered onto a linear 5 to 30% (w/v) sucrose gradient that was prepared, sedimented, and analyzed as described for polymerase I.

**Phosphorylation of Yeast RNA Polymerases 3083**

DNA-dependent RNA polymerase activity was assayed by a method that was modified from that described by Alberts and Herrick (16). The buffers used were those described by Valenczula et al. (15). Buffer A is 0.2 M Tris/HCl, pH 8.0, 0.01 M 2-mercaptoethanol, and 0.5 mM EDTA, and 25% glycerol.
collected by centrifugation (21,000 × g, 30 min) and dissolved in sufficient Buffer C so that the conductivity was equivalent to 0.10 M KCl in Buffer C. This solution was absorbed to a denatured calf thymus DNA-cellulose column (5-ml bed volume, 100 μg DNA/ml of packed cellulose) equilibrated with 0.10 M KCl in Buffer C. The column was washed with 0.15 M KCl in Buffer C and then the polymerase III was eluted with 0.70 M KCl in Buffer C. The polymerase III was precipitated by dialysis against Buffer A containing 0.35 g/ml of ammonium sulfate. The precipitate was collected by centrifugation (27,000 × g, 60 min), dissolved in 0.2 ml of 0.2 M KCl in Buffer A and layered on a linear, 4.8-ml, 5 to 20% (w/v) sucrose gradient in 0.2 M KCl in Buffer C. After sedimentation for 14 h at 55,000 rpm (Beckman SW 60), the gradient was analyzed as described for polymerase I.

**Protein Kinase – RNA polymerase I, purified to Fraction 4 by the procedure of Valenzuela et al. (15),** was subjected to sucrose gradient sedimentation in 0.5 M KCl as described above. At salt concentrations less than 0.5 M KCl, the protein kinase activity is distributed throughout the sucrose gradient and contaminates polymerase I. The gradient was fractionated and assayed for protein kinase and RNA polymerase activity and protein concentration (Fig. 1). The protein kinase fractions were pooled, diluted with Buffer C to 0.2 M KCl, and then absorbed to a phosphocellulose column (0.5 mg of protein/ml of bed volume) equilibrated with 0.2 M KCl in Buffer C. The column was washed with 0.2 M KCl in Buffer C and then developed with a linear gradient from 0.2 M to 0.6 M KCl in Buffer C (10 column volumes) (Fig. 2). The protein kinase eluted at 0.38 M KCl. The enzyme fractions were pooled, dialyzed against 0.2 M KCl in Buffer C and then concentrated by absorption to a phosphocellulose column (0.5 mg of protein/ml of bed volume). The column was washed with 0.05 M KCl in Buffer C and then developed with a linear (10 column volumes) gradient of 0.05 M to 0.35 M KCl in Buffer C (Fig. 3). The protein kinase, eluting at 0.25 M KCl, was pooled and stored at −76°C.

The protein kinase activity has been purified approximately 100-fold (Table II) from polymerase I, Fraction 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolved the proteins in the DEAE-Sephadex pool into four major polypeptides with apparent molecular weights of 41,000, 38,000, 35,000, and 31,000 as well as several higher molecular weight polypeptides present in much lower amounts. The relationship of these polypeptides to the protein kinase activity has not been determined. The protein kinase (ion filtration pool) has a sedimentation coefficient (s20,w) in 0.5 to 2.0 M KCl, as calculated by the procedure of Martin and Ames (23), of 6.8 relative to RNA polymerase I which has an s20,w of 16.2 (15). This suggests that the native protein kinase, if globular, has a molecular weight of approximately 135,000 and so could be composed of one or more of the four major polypeptides present in the DEAE-Sephadex pool. The specific activity of the protein kinase after DEAE-Sephadex chromatography is comparable to similar protein kinases purified by others (11, 24) but is only 10% that reported for a yeast protein kinase purified by Lerch et al. (25). This latter enzyme exists as a monomer of 42,000 daltons, whereas the enzyme described here appears to be larger. The subcellular localization of this protein kinase remains to be determined but it has general enzymatic properties similar to those described by others for nuclear protein kinases (26-28). For example, it is not stimulated by CAMP, can use either ATP or GTP as a phosphate donor, and is more active with acidic protein phosphate acceptors like phosvitin and casein than basic proteins like histones or protamine. Under standard assay conditions and an acceptor concentration of 4 mg/ml, initial velocities of 6.34, 3.11, 1.01, and 0.02 pmol of phosphate incorporated/min were obtained, respectively. Some of the other enzymatic properties of this

![Fig. 1. Sucrose density gradient centrifugation of yeast RNA polymerase I, Fraction 4. Gradients were prepared, loaded, and centrifuged as described in the text. Fractions were assayed for protein, RNA polymerase, and protein kinase activity.](image1)

![Fig. 2. Phosphocellulose column chromatography of yeast protein kinase. The active fractions from the sucrose gradient were pooled (~14 mg of protein), diluted to 0.2 M KCl with Buffer C, and applied to a 28-ml phosphocellulose column (2.5 × 6.8 cm) equilibrated with 0.2 M KCl in Buffer C. The column was washed with 75 ml of 0.2 M KCl in Buffer C and then developed with a 300-ml linear gradient of 0.2 to 0.6 M KCl in Buffer C. Fractions of 3.9 ml were collected.](image2)

![Fig. 3. DEAE-Sephadex A-25 chromatography of yeast protein kinase. The concentrated protein kinase (~200 μg) after phosphocellulose chromatography was dialyzed against 0.05 M KCl in Buffer C and then applied to 2.2-ml DEAE-Sephadex A-25 column (0.6 × 7.7 cm) equilibrated with 0.05 M KCl in Buffer C. After washing with 7 ml of 0.05 M KCl in Buffer C, the column was developed with a 22-ml linear gradient of 0.05 to 0.35 M KCl in Buffer C. Fractions of 0.5 ml were collected.](image3)

**Table I**

**Summary of purification of yeast protein kinase**

The data are taken from one representative experiment. Of wet weight of yeast cells, 2,000 g were used as starting material. One unit corresponds to 1 nmol of ATP incorporated in 10 min at 30°C under the conditions described under "Experimental Procedures."

| Purification step | Volume | Total protein | Activity | Specific activity | Yield |
|------------------|--------|--------------|----------|------------------|-------|
| Ion filtration   | 106    | 70           | 7,150    | 102              | 100   |
| DEAE-Sephadex    | 22     | 13.6         | 6,852    | 504              | 96    |
| Sucrose gradient | 34     | 0.68         | 2,431    | 3,574            | 34    |
| Phosphocellulose | 2      | 0.40         | 2,700    | 6,749            | 38    |
| Concentration    | DEAE-Sephadex A-25 | 0.09 | 926 | 10,288 | 13 |
kinase are summarized in Fig. 4. The protein kinase had a broad pH optimum, is inhibited by KCl concentrations greater than 0.2 M, and requires a divalent metal ion (Mg\(^{2+}\) or Mn\(^{2+}\)) for activity.

RESULTS

Simultaneous purification of RNA polymerases I, II, and III from small quantities of cells, whether yeast or higher eukaryotes, has been difficult. We have been able to purify the three yeast RNA polymerases from 35 g of cells. A batchwise purification procedure (15) originally developed for the large scale purification of yeast polymerase I was adapted to accommodate small amounts of cells by reducing the amount of resin and buffer volumes used proportionately to the amount of yeast cells. The ion filtration column described in the original procedure was eliminated. Polymerase I was purified by batchwise absorption and elution from phosphocellulose and then DEAE-cellulose, and finally by high salt sucrose density gradient sedimentation. RNA polymerases II and III were not absorbed by phosphocellulose under the same conditions as polymerase I and were purified from the filtrate. We have utilized the synthetic polyanion, Polymyxin F, first used by Zillig et al. (29) in the purification of Escherichia coli RNA polymerase and subsequently by Burgess and Jendrisak (30).

RNA Polymerase I—The RNA polymerase I purified by this procedure is similar to the enzyme purified by other procedures (15, 32). Figs. 5 and 6 show the activity, protein concentration, and polypeptide composition of fractions of the sucrose gradient, the final purification step. Sodium dodecyl sulfate-polyacrylamide gel analysis resolved the protein in the region of the polymerase activity (Fig. 6a, Fractions 11 to 13) into 10 polypeptides (185,000, 137,000, 48,000, 44,000, 41,000, 36,000, 28,000, 24,000, 20,000, and 14,500 daltons (subunit 12,300 is not resolved under these conditions)) which have been identified by Valenzuela et al. (15) and Buhler et al. (32) as components of the enzyme. Thus the enzyme is essentially pure. When polymerase I was purified by this procedure from yeast cells grown in \(^{32}P\), a significant fraction of the phosphate-labeled protein sediments with the polymerase activity. This suggested that polymerase I contained phosphate (Fig. 6a). The relationship of the phosphate-labeled polypeptides to polymerase subunits was determined by autoradiography of the dried sodium dodecyl sulfate-polyacrylamide gel. The majority of the radioactivity migrated with five polymerase I polypeptides (185,000, 44,000, 36,000, 24,000, and 20,000 daltons (Figs. 6b and 7)). Besides these radioactive polypeptides, there is a small amount of radioactivity at the dye front. The majority of the label at the dye front probably represents contamination of the enzyme fractions with the material from the region of Fractions 15 to 20.

![Fig. 4](http://www.jbc.org/)

![Fig. 5](http://www.jbc.org/)
Phosphorylation of Yeast RNA Polymerases

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein from sucrose gradient of yeast RNA polymerase I described in Fig. 5. The enzyme subunits are designated by their molecular weights. The fraction numbers correspond to those of the gradient in Fig. 5. The gel concentration was 10%. a, stained polypeptides. The lane designated S is yeast RNA polymerase I reference, b, autoradiogram of gel.

Following partial acid hydrolysis of [32P]polymerase I and analysis of the hydrolysate by high voltage paper electrophoresis, approximately 7% of the radioactivity migrated with phosphothreonine, 22% with phosphoserine, 70% with inorganic phosphate, and the remaining 1% remained at the origin. These data suggest that these amino acids are present in RNA polymerase I, but do not exclude the possibility that other amino acids might be phosphorylated. 32Pi is produced under these hydrolysis conditions by decomposition of the phosphate ester of phosphoserine or phosphothreonine (21). Since the rate of decomposition of phosphoserine is approximately 4 times that of phosphothreonine, the fraction of the radioactivity in phosphoserine and, to a lesser extent, phosphothreonine is underestimated. N-Phosphoamino acids also decompose under acidic conditions (33). The incorporated 32P was acid-stable (6% was released as 32Pi in 15 min in 0.5 N HCl at 60°) and base-labile (69% was released as 32Pi in 15 min in 0.5 N NaOH at 60°) which suggests that most of the phosphate is attached to serine and threonine residues and not to lysine, arginine, or histidine residues which are acid-labile and base-stable (33).

Of the incorporated 32P, 85% was sensitive to potato acid phosphatase and 65% was sensitive to bacterial alkaline phosphatase. It is possible that a small fraction of the phosphate label could be incorporated by adenylylation or ADP ribosylation of enzyme polypeptides. We consider the latter unlikely since we have been unable to detect any NAD+:protein ADP-ribosyltransferase activity in isolated yeast nuclei.

The 185,000- and 44,000-dalton polypeptides were more highly labeled than the others (Fig. 7). The 20,000-dalton polypeptide was only lightly labeled. The relative extent of phosphorylation of this subunit appears to be sensitive to the phosphate content of the culture medium since it is more highly labeled when the enzyme was isolated from cells grown in complete rather than phosphate-depleted medium (Fig. 7).

RNA Polymerase II—The RNA polymerase II purified as described under "Experimental Procedures" was 90 to 95% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figs. 8 and 9a, Fractions 15 to 15) and has the following polypeptide composition: 205,000, 145,000,
Phosphorylation of Yeast RNA Polymerases

FIG. 8. Sucrose density gradient centrifugation of phosphorylated yeast RNA polymerase II. The gradient was prepared, loaded, and centrifuged as described in the text. Fractions were assayed for protein, RNA polymerase activity, and 32P-protein.

FIG. 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of protein from sucrose gradient of yeast RNA polymerase II described in Fig. 8. The enzyme subunits are designated by their molecular weights. The fraction numbers correspond to those of the gradient in Fig. 8. The gel concentration was 11%. a, stained polypeptides. The lane designated M is myosin; the lane designated S is yeast RNA polymerase II reference. b, autoradiogram of gel.

46,000, 33,500, 28,000, 24,000, 18,000, 14,500, and 12,500 daltons (the 12,500-dalton subunit is not resolved in Fig. 8 but is evident in Fig. 12). The polypeptide composition of this enzyme preparation is similar to those described by Hager et al. (34) and Buhler et al. (32) except that the molecular weight of the largest subunit in these previous preparations was approximately 175,000 (Fig. 8, Column S). However, the apparent molecular weight of the largest subunit when polymerase II was isolated by this procedure and in a preparation recently described by Dezelee et al. (35) is similar to that of the large subunit of myosin, i.e., approximately 220,000 (36) (Fig. 8a, Columns M and S and Fraction 14). This subunit has an apparent molecular weight of 205,000 as determined by electrophoresis in a 6.5% sodium dodecyl sulfate-polyacrylamide gel which resolves high molecular weights polypeptides better than an 11% gel (data not shown). There is little of the 175,000-dalton polypeptide evident in this preparation. This is consistent with an origin by proteolysis during isolation as proposed by Dezelee et al. (35). Thus, when proteolysis is efficiently prevented, the only form of polymerase II isolated is one possessing the 205,000-dalton subunit. There is no apparent difference in molecular weight in the other subunits in the polymerase II preparation (Fig. 8a, Column S and Fraction 14). These subunits do not appear to be as sensitive to proteolysis. Dezelee et al. (35) have found no significant differences in α-amanitin sensitivity or template preference between the two forms. The contaminants in this polymerase II preparation are several polypeptides with molecular weights between 46,000 and 145,000 that are relatively tightly associated with the polymerase since they sediment with the enzyme in 0.5 M KCl.

When polymerase II was purified by this procedure from yeast grown in 32P, analysis of the sucrose gradient showed a small peak of radioactivity sedimenting with the enzyme. The identity of the radioactive polypeptides was determined after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins in the gradient fractions by autoradiography. Most of the radioactivity which sedimented with polymerase migrated with the 24,000-dalton subunit (Fig. 9, Fractions 14 and 15). In addition, there were radioactive polypeptides with apparent molecular weights of 225,000, 165,000, and 140,000 present in minor amounts which do not migrate with any polymerase II subunits. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of [32P]polymerase II in a 6.5% resolving gel and subsequent autoradiography also indicated that the three large phosphorylated polypeptides are not polymerase II subunits (data not shown). These large phosphorylated polypeptides do not sediment exactly with the enzyme which also suggests that they are contaminating polypeptides. Sucrose gradient centrifugation resolved polymerase II from a 33,500-dalton radioactive polypeptide. The enzyme subunit of this molecular weight is not labeled.

RNA Polymerase III—This enzyme has generally been difficult to purify; the procedure described here has been used to purify polymerase III from variable quantities of cells. After affinity chromatography on denatured DNA-cellulose, polymerase III was approximately 80% pure (data not shown) and sucrose gradient sedimentation removed most of the remaining protein contaminants (Figs. 10 and 11a). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolved the protein in the active fractions into 10 polypeptides (160,000, 128,000, 82,000, 41,000, 37,000, 34,000, 28,000, 24,000, 20,000, and 14,500 daltons) (Fig. 11a, Fractions 14 and 15) which Valenzuela et al. (37) have previously designated as components of the enzyme.
When polymerase III was purified by this procedure from yeast grown in $^{32}$P, a large fraction of the radioactivity loaded on the sucrose gradient sedimented with the polymerase activity. The 24,000- and 20,000-dalton subunits of polymerase III were phosphorylated (Fig. 11a, Fractions 14 and 15). In addition, there were several phosphorylated polypeptides in the range of 47,000 to 53,000 that presumably are contaminants.

In Vitro Phosphorylation of Yeast RNA Polymerases—The purification and some properties of a yeast protein kinase which purifies with polymerase I are discussed under "Experimental Procedures." This protein kinase, when incubated with purified RNA polymerases I, II, and III and [γ-$^{32}$P]ATP, phosphorylates the polymerase subunits that are phosphorylate in vivo ($I_{120,000}$, $I_{14,000}$, $I_{13,500}$, $I_{14,000}$, $I_{20,000}$, $I_{24,000}$, $III_{24,000}$, and $III_{83,000}$) and also $I_{45,000}$ and $II_{38,000}$ daltons (Fig. 12). The protein kinase also phosphorylated contaminating polypeptides particularly in polymerase III. Since several groups have reported...
that protein kinases stimulate RNA polymerase I and II activity in a cell-free system presumably by phosphorylating the polymerase (9-11), we tested the effect of phosphorylation of polymerase on activity using purified yeast RNA polymerases I, II, and III and protein kinase. Polymerase activity was measured with native yeast DNA as a template, conditions similar to those used by the other groups. As shown in Table II, the phosphorylation of purified polymerase I (0.4 mol of phosphorus were incorporated per mol of enzyme) resulted in a modest (14%) decrease in enzyme activity. Phosphorylation of yeast polymerase II (0.2 to 0.3 mol of phosphorus were incorporated per mol of enzyme) had no effect on enzyme activity as measured under these conditions with either MgCl₂ or MnCl₂. However, a heat-insensitive component of the protein kinase preparation caused a small inhibition of polymerase II activity. Phosphorylation of polymerase III (1.8 mol of phosphorus were incorporated per mol of enzyme) had no effect on enzyme activity under these conditions. The results presented in Table II suggest that phosphorylation of purified yeast RNA polymerase has little effect on activity. Since the enzymes are already phosphorylated when isolated, the effect of additional phosphorylation on activity might be reduced. We therefore tested the effect of acid and alkaline phosphatase on polymerase activity. There was no significant difference (±10%) in the activity of polymerase I in the presence of acid or alkaline phosphatase. Polymerase I, dephosphorylated by alkaline phosphatase, incorporated 3 times as much phosphate on a molar basis as the control enzyme. Thus, the ability to accept phosphate varies, as expected, with the degree of phosphorylation.

**DISCUSSION**

Phosphorylation of chromosomal proteins has been suggested as one mechanism for the regulation of gene expression in eukaryotes. This hypothesis is based on the fact that there is a correlation between phosphorylation of certain non-histone chromosomal proteins and gene activation (38). The observation that bacterial RNA polymerase is phosphorylated upon bacteriophage infection (39), as well as reports which suggested that eukaryotic RNA polymerases might be phosphorylated in cell-free systems (9-11), caused us to examine whether phosphorylation regulates RNA polymerase activity in yeast which is a more experimentally tractable system. We have shown that all three polymerases are phosphorylated, have identified the phosphorylated subunits, and have isolated a protein kinase which can catalyze this modification.

In order to determine if yeast RNA polymerases are phosphorylated in vivo, it was necessary to develop procedures for the purification of these enzymes from small quantities of cells. Procedures were developed for the simultaneous purification of the three enzymes. These procedures are highly reproducible, can be scaled to any limits, and we now believe them to be the method of choice for the isolation of the three RNA polymerases from yeast. They may be of general utility in the isolation of these enzymes from other eukaryotic sources as well.

The three enzymes were isolated from yeast cells grown continuously in ³²P. The purified RNA polymerases I, II, and III contained ³²P. Chemical analysis of ³²P-polymerase I indicated that the incorporation of phosphorus by the enzymes is primarily due to the phosphorylation of serine and, to a lesser extent, threonine residues. The phosphorylation of *E. coli* RNA polymerase on T₇ bacteriophage infection occurs primarily on threonine residues (39). The phosphorylated enzyme subunits were identified according to the following criteria. First, the phosphorylated polypeptide must sediment with the purified RNA polymerase in the sucrose gradient step. Secondly, the phosphorylated polypeptide must

**Table II**

Effect of in vitro phosphorylation of purified RNA polymerases on activity

| Enzyme     | Divalent metal ion | Addition                      | U incorp. | [³²P]UTP incorp. | Control polymerase ac. | Moles of phosphorus incorp. per mole of polymerase |
|------------|--------------------|-------------------------------|-----------|-----------------|------------------------|--------------------------------------------------|
| Polymerase I | MgCl₂              | None                          | 0.80      | 4.86            | 86                     | 0.40                                             |
| Polymerase II | MgCl₂             | None                          | 1.30      | 14.68           | 100                    | 0.29                                             |
| Polymerase II | MnCl₂             | None                          | 0.04      | 17.48           | 105                    | 0.22                                             |
| Polymerase III | MnCl₂            | None                          | 0.03      | 15.09           | 100                    | 1.77                                             |

Purified RNA polymerases I, II, and III, 1.16 µg (2 pmol), 2.25 µg (4.5 pmol), and 1.52 µg (2.5 pmol), respectively, were incubated with yeast protein kinase (0.05 µg, DEAE-Sephadex A-25 pool), heat-inactivated (5 min, 100°C) protein kinase (0.05 µg, DEAE-Sephadex A-25 pool), or without additions in an incubation mixture of 0.050 ml which contained 50 mM Tris/HCl, pH 7.9, either 10 mM MgCl₂ or 3.2 mM MnCl₂. The phosphorylation was allowed to proceed for 15 min at 30°C and then the polymerase activity was assayed. The final incubation mixture contained in 0.10 ml: 60 mM Tris/HCl, pH 7.9, either 5 mM MgCl₂ or 1.6 mM MnCl₂, 0.60 mM MnCl₂, 0.01 mM [γ-³²P]ATP (1475 cpm/pmol), 10 mM 2-mercaptoethanol, 0.1 M KCl, and 10% glycerol. After incubation for 10 min at 30°C, 0.090-ml aliquots were applied to Whatman 3MM filter discs which were washed as described for the protein kinase assay and counted as described elsewhere (15). In these reactions, the incorporation of ³²P into acid-insoluble material reflects the amount of polymerase phosphorylation, whereas the incorporation of [³²P]UTP into acid-insoluble material indicates the amount of RNA synthesis. There was no incorporation of ³²P into acid-insoluble material in the presence of protein kinase alone under these conditions.
migrate with the stained polymerase subunit in a sodium dodecyl sulfate-polyacrylamide gel and thirdly, it should be possible to demonstrate in vitro phosphorylation of the subunits phosphorylated in vivo by an enzyme present in the cell extract. It is necessary to use the first two criteria since our data indicate that the purified enzymes are contaminated with phosphorylated polypeptides which sediment with the enzyme activity in the sucrose gradient but do not migrate with enzyme subunits in a polyacrylamide gel. These polypeptides may be minor contaminants but can be very highly labeled. The third criterion is insufficient of itself to establish in vivo phosphorylation of the enzymes since protein kinases can phosphorylate subunits in vitro which are not phosphorylated in vivo. Using these criteria, the following polymerase subunits were phosphorylated in vitro: I_{18,000}, I_{44,000}, I_{38,000}, I_{14,000}, I_{20,000}, I_{16,000}, III_{24,000}, and III_{20,000}.

The stoichiometry of the in vivo phosphorylation of the RNA polymerase is difficult to determine precisely but it is possible to obtain approximate values. The enzymes were purified from yeast cells grown in inorganic phosphate depleted medium containing ^32P. Under these conditions, the inorganic phosphate content of the medium is no higher than 10^{-4} M (13). Our determinations suggest that it may be approximately 2 \times 10^{-3} M. Using these values for the inorganic phosphate concentration and neglecting the contribution of the organic phosphate in the medium which enters the pool of inorganic phosphate after hydrolysis by an inducible phosphomonoesterase (14), the stoichiometry of the phosphorylation reaction can be estimated from the amount of ^32P incorporated into the purified enzymes. These estimations indicate that there are 2.5 to 12 mol of phosphorus incorporated/mol of polymerase I, 0.2 to 1.0 mol of phosphorus/mol of polymerase II, and 0.4 to 2.0 mol of phosphorus/mol of polymerase III. This range of values is compatible with the thesis that all enzyme molecules are phosphorylated. Also, the relative disparity in the labeling of individual subunits (especially polymerase I (Fig. 7)) suggests that some subunits may be phosphorylated at more than one site.

A consideration of the function of the phosphorylated polymerase subunits is relevant to the physiological role of phosphorylation. Polymerase I is more extensively phosphorylated than the other two enzymes suggesting that phosphorylation may be involved in the control of ribosomal RNA precursor synthesis. Moreover, the phosphorylated subunits present in polymerases II and III are not unique to these enzymes but are also present in polymerase I. In particular, the 24,000-dalton subunit of each enzyme is phosphorylated. This subunit by peptide mapping and two-dimensional gel electrophoretic analysis appears to be identical (32, 40) in all enzymes. Both biochemical (41) and genetic evidence (42) suggests that it may be necessary for catalytic activity. The phosphorylated subunits I_{20,000} and III_{20,000} also appear to be identical polypeptides (40). Subunits I_{44,000} and I_{38,000} dissociate readily from polymerase I and may be necessary (possibly in conjunction with subunit I_{14,000}) for activity on native DNA templates (15, 34, 43, 44). Subunit I_{14,000} was the only large subunit of the three enzymes that was phosphorylated. The role of the large subunits in the yeast enzymes has not been established but in bacterial RNA polymerases they contain the active center for the polymerization reaction (45). It is evident that phosphorylation of subunits could be one way of controlling transcripive activity; however, the physiological role of this modification is not established.

We have been unable to demonstrate a significant effect of phosphorylation on RNA polymerase activity using conventional assay procedures. Other groups have reported that protein kinases stimulate the activity of partially purified RNA polymerases I and II and have proposed positive regulation by this means (9-11, 46). However, none of these previous studies have demonstrated phosphorylation of the enzyme protein according to the criteria used in this study. Therefore, their conclusions are subject to question. For example, the observed stimulation of polymerase activity may not be due to phosphorylation of the enzyme itself but to the phosphorylation of contaminating proteins (47, 48).

We believe it is necessary to develop more incisive experimental means to test the physiological role of phosphorylation. Since RNA polymerase is a complex molecule which interacts with DNA, RNA, and presumably other chromosomal proteins in vivo, phosphorylation of serine and threonine residues which introduces negative charge onto the molecule could alter both inter- and intramolecular interactions. Therefore it will be necessary to measure parameters of polymerase function besides activity on DNA; for example, affinity for DNA, correct initiation and termination of transcription of specific genes using DNA and chromatin templates, and enzyme stability. Future experiments will be designed to measure these more specific aspects of polymerase function. In vivo phosphorylation experiments should also be continued in order to develop physiological correlates between patterns of polymerase phosphorylation and alterations in the synthesis of specific classes of RNA and so help to establish the role of phosphorylation in the regulation of transcription.

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J. Biol. Chem. 1977, 252:3082-3091.

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Additions and Corrections

Vol. 251 (1976) 7816-7820

Transformation of arachidonic acid and homo-γ-linolenic acid by rabbit polymorphonuclear leukocytes. Monoxygenases from novel lipoxygenases.

Pierre Borgent, Mats Hamberg, and Bengt Samuelsson

The two monohydroxy acids isolated after incubation of arachidonic acid and homo-γ-linolenic acid should both have the "D" configuration, i.e. 5-n-hydroxy-6,8,11,14-eicosatetraenoic acid and 8-n-9,11,14-eicosatrienoic acid, respectively.

Oxidative ozonolysis of the methoxycarbonyl derivatives of the methyl esters afforded mainly the methoxycarbonyl derivatives of dimethyl 2-L-hydroxyadipate and of dimethyl 2-L-hydroxyazelate as described on p. 7818. Since the carbomethoxy group of these dioates that should be oriented upwards in the Fischer projection formulas (C-1) does not correspond to the carboxyl group of the parent unsaturated hydroxy acids, it follows that the latter acids should have the "D" configuration at C-5 (5-n-hydroxy-6,8,11,14-eicosatetraenoic acid) and at C-8 (8-n-hydroxy-9,11,14-eicosatrienoic acid).

Vol. 252 (1977) 1990-1997

Active amino acid transport in plasma membrane vesicles from simian virus 40-transformed mouse fibroblasts. Characteristics of electrochemical Na+ gradient-stimulated uptake.

Julia E. Lever

Page 1991, Right hand column, Legend to Fig. 1.

The concentration of [14C]iso-Abu was written as 1.69 mM instead of 0.17 mM. The correct line should be:

transformed cells, 0.17 mM [14C]iso-Abu (61 fmol/cpm) and 50 mM

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Vol. 252 (1977) 3035-3043

Sidedness of (sodium, potassium)-adenosine triphosphatase of inside-out red cell membrane vesicles. Interactions with potassium.

Rhoda Blostein and Lily Chu

Page 3038, Table V

In the title, $K_{cat}$ should be $K_{cat}$.

The heading should read

Effects of Na+, on $K_{cat}$ inhibition at 37°

Under 0.05 µM ATP concentration, the value for E-P at 5.0 mM Na should be 0.168 pmol/mg.

The line should read

5.0 mM Na 0.168 23.8 142

Vol. 252 (1977) 3082-3091

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Page 3083, Left hand column, paragraph headed RNA Polymerase Assay, Line 4.

The following sentence should be added:

The concentration of KCl in the assay was 0.10 M.

Page 3083, Right hand column, paragraph headed Preparation of Cell Extract, Line 3

The extraction buffer also contained 0.3 M ammonium sulfate. The sentence should read:

The pellet was suspended with a glass rod and finally a glass

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