In Vivo and in Vitro Phosphorylation of Ribosomal Proteins by Protein Kinases from *Saccharomyces cerevisiae*†

Doris Becker-Ursic and Julian Davies*

ABSTRACT: From the high salt wash of the ribosomes of the yeast *Saccharomyces cerevisiae,* three protein kinases have been isolated and separated by DEAE-cellulose chromatography. The three kinases differ in their abilities to phosphorylate substrates such as histones (calf thymus), casein, and *S. cerevisiae* ribosomes; two of the kinases showed increased activity in the presence of cyclic adenosine 3′,5′-monophosphate when histones and 40S ribosomal subunits were used as substrates. The protein kinases catalyzed phosphorylation of certain proteins of the 40S and 60S ribosomal subunits, and 80S ribosomes in vitro. Nine proteins of the 80S ribosome, seven proteins of the 40S subunit, and eleven of the 60S subunit were phosphorylated; different proteins were modified to various extents when different kinases were used. We have identified several proteins of 40S and 60S ribosomal subunits which are not available to the kinases in the 80S particles. Ribosomes isolated from *S. cerevisiae* cells growing in logarithmic phase of growth were found to contain a number of phosphorylated proteins. Studies by two-dimensional polyacrylamide gel electrophoresis indicated that the ribosomal proteins phosphorylated in vivo correspond with those phosphorylated in vitro. The relationship of in vivo phosphorylation of ribosomes to the growth and physiology of *S. cerevisiae* is not known.

Protein kinases have been isolated from a variety of organisms and key functions have been assigned to these enzymes (e.g., in the regulation of glycogen breakdown; Sutherland, 1971), but by and large their roles in cell metabolism are poorly understood. The protein kinases catalyze the transfer of the terminal phosphoryl group of nucleoside triphosphates to amino acid residues in protein substrates. So far, the only amino acid acceptors for the phosphoryl group in vivo and in vitro have been identified as threonine and serine; the phosphoryl groups can undergo intracellular turnover (Krystosek et al., 1974).

In several different eukaryotes, phosphorylation of ribosomal proteins has been demonstrated to occur in vivo and in vitro catalyzed by cAMP[1] dependent or cAMP independent protein kinases (Loeb and Blat, 1970; Li and Amos, 1971; Walton and Garren, 1970; Yamamura et al., 1972; Fontana et al., 1972; Jergil, 1972; Traugh and Traut, 1974).

Kabat (1970, 1971, 1972) has demonstrated that phosphorylation of the same five ribosomal proteins from rabbit reticulocytes occurs in vivo as in vitro and that the pattern of phosphorylation of ribosomal proteins of Sarcoma 180 tumor cells resembles that of reticulocyte ribosomes (Bitte and Kabat 1972, 1974). Gressner and Wool (1974) have demonstrated the in vivo phosphorylation of one ribosomal protein in rat liver. By contrast, studies of phosphorylation of rat liver ribosomes in vitro indicate at least four 40S and at least ten 60S ribosomal subunit proteins to be modified (Eil and Wool, 1971, 1973a,b).

Ventimiglia and Wool (1974) recently described a protein kinase in rat liver cytosol that transfers the terminal phosphate of GTP to serine and threonine residues of a protein of the small ribosomal subunit. Issinger and Traut (1974) observed phosphorylation of proteins L7 and L12 of the 50S ribosomal subunit with [γ-^{32}P]GTP in the presence of protein kinase from rabbit reticulocytes.

The yeast *S. cerevisiae* is a simple eukaryote with a well-characterized genetic system. As with other eukaryotes, yeasts contain protein kinases (Takai et al., 1974; Grankowski et al., 1974) and we wish to describe a simple isolation of three protein kinases from the high salt wash of *S. cerevisiae* ribosomes and the ability of these kinases to phosphorylate substrates such as histones, casein, and ribosomal proteins. We have found that as many as 18 ribosomal proteins can be phosphorylated in vitro. In addition, ribosomal proteins from cells grown in the presence of [^{32}P]orthophosphate under various conditions were found to be phosphorylated extensively. The patterns of phosphorylation in vivo corresponded closely to those seen with purified kinases in vitro. Although the function of the protein kinases is not known, they may provide useful ways of studying the topology and interrelationships between proteins on the ribosome. By phosphorylating yeast 80S ribosomes and comparing them with separated 40S and 60S subunits, we have been able to identify the ribosomal proteins which are protected from enzymatic phosphorylation in the 80S particles.

Materials and Methods

Materials. Tris(hydroxymethyl)aminomethane, calf thymus histones (IV, VI, VII, VIII), cAMP, and casein were obtained from Sigma. [γ-^{32}P]ATP (0.5 mCi/0.0146 μmol in 0.5 ml of H_2O) and [γ-^{32}P]GTP (250 μCi, 13.7 Ci/mmol) were purchased from New England Nuclear. DEAE-Cellulose DE 52 was obtained from Whatman and RP Royal "X-Omat" Kodirex Autoprocess film from Kodak.

Media. YEP: 1% yeast extract, 2% Bacto-Peptone, 2% glucose adjusted to pH 5.1 with 1 N HCl. Buffer A: 20 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, 1 mM EDTA, 20% glycerol. Buffer 1: 50 mM Tris-HCl, pH 7.8, 12.5 mM $Mg(OAc)_2$, 80 mM KCl, 1 mM dithiothreitol. TMK buffer: 50 mM Tris-HCl, pH 7.8, 50 mM KCl, 5 mM $Mg(OAc)_2$, 1 mM dithiothreitol. Scintillation fluid: 2.85 g of 2,5-diphe-

† From the Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706. *Received December 1, 1975.* This work was supported by a grant from the Institute of Allergy and Infectious Diseases, National Institutes of Health.

[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; EDTA, ethylenediaminetetraacetic acid.

nyloxazole, 95 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 l. toluene. Buffer B: 50 mM Tris-HCl, pH 7.4, 10 mM KCl, 5 mM $Mg(OAc)_2$, 17 mM dithiothreitol.

Strain Used: S. cerevisiae Y 166 *αtrp5 his4 MAL1*. This was obtained from the collection of H. Halvorson.

Protein Determination. The protein concentration was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

Preparation of 80S Ribosomes and Their Subunits. Ribosomes were prepared from *S. cerevisiae* as described previously (Jimenez et al., 1972). The crude ribosomes were stirred overnight at 4 °C in TMK buffer containing 500 mM KCl and 30% glycerol and then centrifuged for 20 h in a Spinco 42 rotor at 30 000 rpm. The supernatant fraction, which contains the protein kinase activity, was removed and the pelleted 80S ribosomes were resuspended in TMK buffer. Preparation of the kinase fractions is shown in Figure 1. Ribosome subunits were prepared by taking advantage of the fact that sodium azide prevents initiation of protein synthesis and causes the accumulation of free ribosomes in the cells (Van der Zeijst et al., 1972). *S. cerevisiae* Y 166 was grown in YEP media to late log phase at 28 °C. Sodium azide (1 mM) was added and incubation continued for 15 min; the cells were harvested and disrupted in a French press, and a crude ribosome preparation was obtained (Jimenez et al., 1972).

Subunits were prepared from these ribosomes as follows: 100–150 A_{260} units/ml crude ribosomes was layered onto a linear gradient of 10–30% sucrose in buffer 1 containing 500 mM KCl and centrifuged for 6.5 h at 27 000 rpm in a Spinco SW 27 rotor at 10 °C. The gradient was fractionated and absorption at 260 nm was monitored in an Isco flow cell. Fractions containing the 40S and 60S subunits were pooled and concentrated by centrifugation at 30 000 rpm for 20 h at 10 °C. The pellets were dissolved in TMK buffer and stored at −70 °C.

Protein Kinase Assay. Phosphorylation catalyzed by protein kinases was measured as follows. Ribosomes (1.7 A_{260} units), histones or casein (0.1 mg) were incubated at 37 °C in a mixture containing 20 mM Tris-HCl, pH 7.8, 13 mM $Mg(OAc)_2$, 5 μl of [γ-^{32}P]ATP (specific activity 2.6×10^5 cpm/nmol; 4.6 nmol of ATP) or 5 μl of [γ-^{32}P]GTP (specific activity 2.5×10^6 cpm/nmol; 16 nmol of GTP), and, when present, cAMP at 1×10^{-5} M. The total volume of each incubation was 0.1 ml. Final enzyme concentrations in the incubations were as follows: enzyme I, 1.1 μg; enzyme II, 5.6 μg; and enzyme III, 3.2 μg. After 15 min of incubation, 1 ml of 10% Cl_3CCOOH was added and the mixture heated at 90 °C for 15 min. The acid-insoluble material was collected by filtration on Whatman glass fiber paper GF/C filters and rinsed with 5% Cl_3CCOOH. The filters were dried under a heat lamp and placed in toluene scintillation fluor and the incorporated radioactivity was counted in a Packard liquid scintillation counter.

To demonstrate that phosphorylation of proteins and not RNA was being detected, the reaction mixture was divided into two parts after incubation: one part was precipitated with Cl_3CCOOH at 90 °C, and the other was treated with a mixture of T1 RNase (9 μg/ml) and pancreatic RNase (9 μg/ml) in the presence of 10^{-2} M EDTA, pH 7.0. The latter mixture was incubated for 20 min at 37 °C. After cooling, the samples were precipitated with Cl_3CCOOH at 90 °C and the precipitates treated as described above. Less than ±5% difference was found between the two samples.

Analysis of Phosphorylation of Ribosomal Proteins by One-Dimensional Polyacrylamide Gel Electrophoresis and Autoradiography. The 80S ribosomes, 40S and 60S ribosomal subunits (100 μg), were incubated in the standard kinase assay with [γ-^{32}P]ATP of specific activity of 2.5×10^7 cpm/nmol (10 μl). After incubation at 37 °C for 15 min, samples (30 μl) were removed and precipitated with Cl_3CCOOH and radioactivity was determined to assess the degree of phosphorylation. The ribosomal proteins were extracted by RNase digestion (pancreatic RNase plus T1 RNase each 9 μg/ml, EDTA (0.03 M), 37 °C for 20 min) and dialyzed against unpolymerized spacer gel; the proteins were subjected to polyacrylamide gel electrophoresis; 10% acrylamide cylindrical gels (pH 4.5) of size 3.5×0.5 cm were prepared by the method of Reisfeld et al. (1962). Electrophoresis was carried out at room temperature, applying a current of 4 mA/tube for about 3 h; the cathode was at the bottom. One drop of a 0.1% solution of pyronine red was applied to the top of the sample gel after it had been polymerized to provide a tracking dye to indicate the solvent front. After electrophoresis the gels were removed from the glass tubes and stained for more than 1 h in a 1% solution of Amido Schwarz in 7.5% acetic acid.

For autoradiography the gels were sliced longitudinally and dried under suction; drying was assisted by heating with an infrared lamp. The dried gels were placed on x-ray film and exposed for 1–7 days.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Ribosomes were phosphorylated under conditions of the standard kinase assay. Extraction of the proteins was carried out with acetic acid and $MgCl_2$ (Hardy et al., 1969). The precipitated RNA was removed by low-speed centrifugation and the supernatant protein was dialyzed against 67% acetic acid for 5 h to remove Mg^{2+} to decrease the ionic strength of the sample. The proteins were lyophilized and dissolved in 8 M urea containing 1 mM dithiothreitol. For each separation 0.1–0.2 mg of protein was added.

Ribosomal proteins were separated in the first dimension on the basis of their mobility in buffer at pH 4.5 in 8 M urea and 4% polyacrylamide and separated in the second dimension by molecular weight difference in the presence of sodium dodecyl sulfate according to the method of Mets and Bogorad (1974).

Slight modifications were made in that the first dimension was run at pH 4.5 rather than 5.0 to allow separation of acidic proteins. The slab gel apparatus designed by Traut et al. (1974) was used. The 0.5×10 cm long 4% polyacrylamide gel (first dimension) was layered directly onto the separation gel slabs (8.5×9.5 cm) without stacking gel; no impairment of the separation was observed and the process was shortened.

Immediately after electrophoresis the slabs were placed in Coomassie brilliant blue in glacial acetic acid–methanol for at least 1 h at 37 °C. The gels were destained in glacial acetic acid–methanol, dried, and exposed to x-ray film exactly as described for the one-dimensional gels.

Poly(U)-Dependent Polypeptide Synthesis. The incubation mixture contained: 50 mM Tris-HCl, pH 7.7; 15 mM $Mg(OAc)_2$; 80 mM KCl; 12 mM phosphoenolpyruvate; 1 mM spermidine-HCl; 1 mM ATP; 0.05 mM GTP; 1 mM dithiothreitol; 5 μl/ml phosphoenolpyruvate kinase; 500 μg/ml yeast tRNA; 50 nCi of [^{14}C]phenylalanine, specific activity 69 mCi/mmol; 300 μg/ml of poly(U); 17 A_{260} units/ml ribosomes; and 10 μl of supernatant enzyme. The final volume of the incubation mixture was 100 μl. Incubations were carried out at 30 °C for 30 min and terminated with 1 ml of 10% Cl_3CCOOH. After heating at 80 °C for 20 min, the acid-insoluble material was collected on glass fiber filters, which were washed with 5% Cl_3CCOOH, dried, and counted after addition of scintillation fluor.

TABLE I: Phosphorylation of Various Substrates Catalyzed by Kinase I, II, or III.

Protein Kinases from *S. cerevisiae*	Incorporation[a] Conditions: Absence (A) or Presence (P) of 10^{-5} M cAMP	Substrates: Ribosomal Particles 80S	60S	40S	Casein	Calf Thymus Histones IV (Arginine Rich)	VI (Slightly Lysine Rich)	VII (Lysine Rich)	VIII (Arginine Rich)
Kinase I	A	1.2	1.0	6.2×10^{-2}	7.0	7.4×10^{-3}	1.7×10^{-3}	6.0×10^{-3}	2.4×10^{-3}
	P	1.3	1.1	9.1×10^{-2}	7.0	2.2×10^{-2}	8.4×10^{-3}	9.6×10^{-3}	5.4×10^{-3}
Kinase II	A	7.0×10^{-1}	8.5×10^{-1}	1.2×10^{-2}	3.0	5.0×10^{-2}	1.8×10^{-2}	1.3×10^{-3}	1.7×10^{-3}
	P	7.2×10^{-1}	8.8×10^{-1}	3.4×10^{-2}	3.0	8.4×10^{-2}	5.4×10^{-2}	3.4×10^{-3}	4.2×10^{-3}
Kinase III	A	7.0×10^{-2}	1.8	1.3	1.7	1.3×10^{-2}	1.7×10^{-2}	None	None
	P	7.0×10^{-2}	1.8	1.3	1.7	1.3×10^{-2}	1.7×10^{-2}	None	None

[a] The incorporation of phosphate from [γ-^{32}P]ATP into proteins of the 80S ribosome, 60S and 40S ribosomal subunits, casein, and histone was analyzed in the standard kinase assay in absence or presence of cAMP. Values are given as moles of ^{32}P incorporated/mole of substrate.

The supernatant enzyme was prepared the same way as described by Jimenez et al. (1972).

Alkaline Phosphatase Treatment of Ribosomes. Dephosphorylation of 80S ribosomes was carried out according to the procedure described by Bitte and Kabat (1974). Ribosomes (200 A_{260}) were incubated for 15 min at 37 °C in presence of 0.4 mg/ml bacterial alkaline phosphatase, 250 mM KCl, 10 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.8. Dephosphorylated ribosomes were obtained by applying the incubation mixture to a Sepharose B-6 column (13 × 1 cm) and eluting with buffer B.

Fractions were assayed for A_{260} and alkaline phosphatase activity (using *p*-nitrophenyl phosphate as chromogenic substrate). The ribosome fractions, free of alkaline phosphatase, were pooled and used for subsequent studies.

Results

Isolation of Protein Kinases. During studies on the components of the soluble fraction obtained by washing *S. cerevisiae* ribosomes with buffer containing high concentrations of KCl ("high salt wash"), we detected the presence of substantial protein kinase activity, using *S. cerevisiae* ribosomes as substrate. The high salt wash was submitted to DEAE-cellulose chromatography and three fractions of kinase activity were separated (Figure 1). Further purification was not attempted and we cannot rule out the possibility that the three fractions may contain multiple protein kinases.

Other reports of the association of protein kinases with ribosomes have been made, and in each case the enzyme was separated from the ribosomes with high ionic strength buffers (0.5–1.0 M KCl) (Kabat, 1971; Eil and Wool, 1971; Li and Amos, 1971; Jergil, 1972; Traugh and Traut, 1974)

Properties of Protein Kinases. The kinases isolated from the yeast ribosome high salt wash were numbered I, II, and III according to their order of elution during DEAE column chromatography. It is not known how the kinases that we have isolated correspond to kinases extracted from yeast or mammalian systems by other workers.

Not all protein kinases are stimulated by cAMP. Enzyme I and enzyme II (Table I) preferentially phosphorylate casein but also modify substrates such as histones, and the proteins of 40S and 60S subunits and 80S ribosomes. Both enzymes I and II are stimulated by cAMP when histones and 40S ribosomal subunits are used as substrates. Enzyme III (Table I) differs in that it catalyzes the phosphorylation of casein, 60S and 40S subunits equally well, and is not stimulated by cAMP. The phosphorylation of casein using enzyme I, II, or III is not affected by cAMP, and the degree of phosphorylation is about 100 times higher with casein than with histones as substrate. The lack of cAMP dependence for phosphorylation of casein has been described previously (Lerch et al., 1975; Takai et al., 1974).

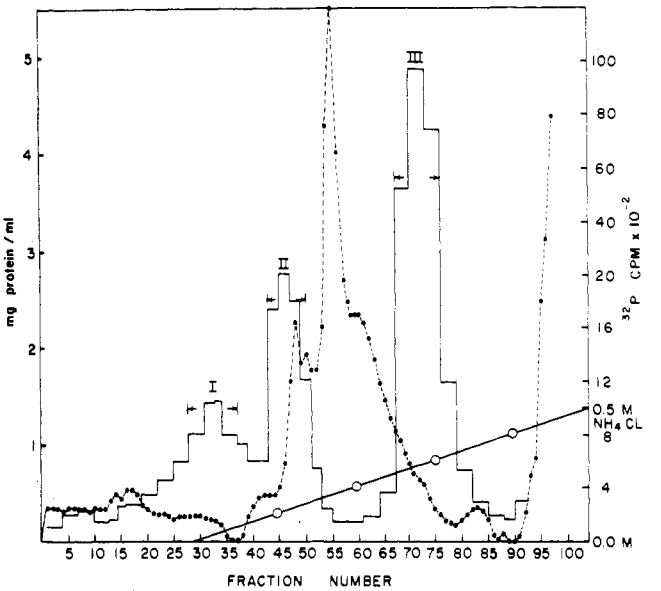

FIGURE 1: DEAE-Cellulose chromatography of the crude protein kinase. The high salt wash of the ribosomes was fractionated on DEAE-cellulose, equilibrated with buffer A. A column (1.8 × 30 cm) was loaded with 300 mg of protein and washed with 100 ml of buffer A. The protein kinases eluted with a 400-ml linear gradient containing 0.0–0.4 M NH_4Cl in buffer A at 24 ml/h (O—O). Fractions of 6 ml were collected and the protein concentration of the eluate was determined (●- -●). The closed line represents the enzymatic activity of the eluate using 80S ribosomes from *S. cerevisiae* as substrate in the standard kinase assay. The arrows indicate the pooled fractions for each kinase, which were concentrated through Diaflo ultrafilter UM 2. Enzymatic activity was eluted in the run off fraction (enzyme I) at 0.12 M NH_4Cl (enzyme II) and at 0.28 M NH_4Cl (enzyme III).

Magnesium ion (10–13 mM) was required for the phos-

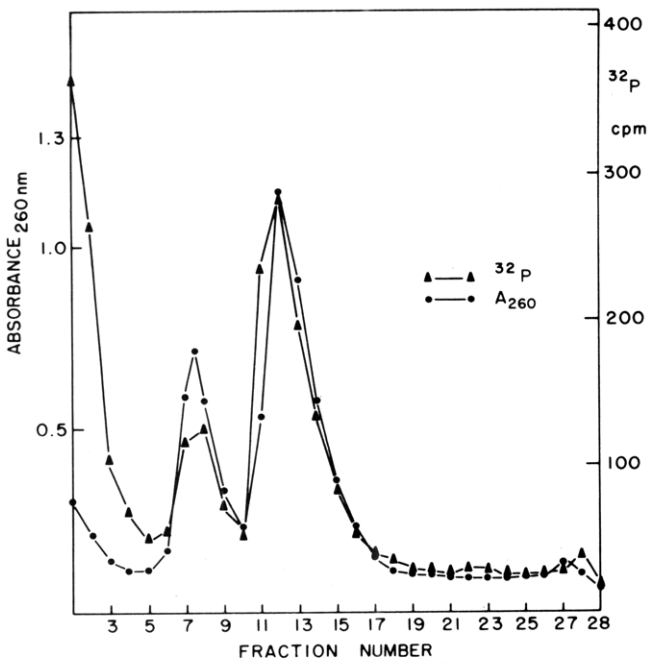

FIGURE 2: Sucrose density centrifugation analysis of phosphorylated 80S ribosomes on dissociation to 40S and 60S subunits. *S. cerevisiae* 80S ribosomes (10 A_{260} units/ml) were phosphorylated under conditions of the standard kinase assay using enzyme I followed by their dissociation into subunits. This was obtained after treatment with 1 M KCl (final concentration) for 15 min at 37 °C, layered onto a 10–30% sucrose gradient in 880 mM KCl, buffer I, and centrifuged in a Spinco SW 50.1 rotor for 2 h at 38 000 rpm. Fractions were collected in an Isco flow cell eluting the top fractions first. Each fraction was treated with RNase and EDTA for 20 min at 37 °C (see Methods) and the incorporation of label from [γ-^{32}P]ATP into Cl_3CCOOH-precipitable counts determined by filtration and scintillation counting.

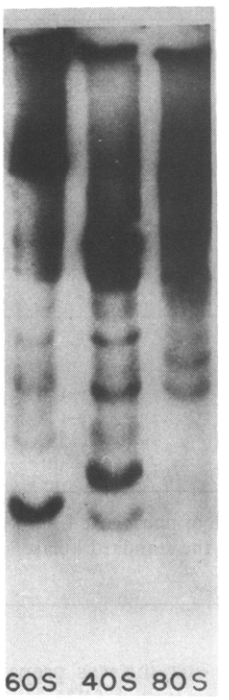

FIGURE 3: Autoradiogram of a one-dimensional gel electrophoretogram of phosphorylated 80S, 60S, and 40S ribosomes. Proteins of the 40S and 60S subunits and 80S ribosomes were phosphorylated with enzyme I in the standard kinase assay. After completion of the reaction, ribosomal proteins were extracted and separated by one-dimensional polyacrylamide gel electrophoresis, which was photographed, and the gels were exposed to x-ray film as described in Methods.

phorylation of 80S ribosomes catalyzed by kinase I; higher concentrations of magnesium ions caused inhibition of the reaction.

Phosphorylation of Ribosomal Proteins in Vitro. When 80S ribosomes were phosphorylated in the presence of kinase I, a clear distribution of ^{32}P labeling over the 80S ribosome peak was shown on analysis by sucrose density gradient centrifugation. If the ribosomes were centrifuged under conditions sufficient to dissociate the 80S ribosomes into 40S and 60S subunits, it could be seen that both ribosomal subunits were labeled with radioactive phosphate (Figure 2).

To determine if specific ribosomal proteins were modified by the kinases, 40S and 60S subunits and 80S ribosomes were incubated, in turn, with the three kinases in presence of [γ-^{32}P]ATP. The phosphorylated ribosomal proteins were separated by one-dimensional polyacrylamide gel electrophoresis as described in Methods and analyzed by autoradiography. Figure 3 shows the autoradiogram of the modified ribosomal proteins.

Six ribosomal proteins of the 80S subunit, seven proteins of the 40S subunit, and eleven 60S ribosomal proteins accept phosphate residues from ATP in the presence of the kinases. Since the separation of the proteins by one-dimensional gel electrophoresis is not complete, other proteins may have been modified and not detected. The three kinases show differences with respect to extent of phosphorylation as well as in the number of proteins being modified.

To identify all the proteins phosphorylated in the complete 80S protein for comparison with the 40S and 60S subunits, two-dimensional polyacrylamide gel electrophoresis was carried out, as described in Methods. The stained gels of the 80S, 60S, and 40S ribosomal proteins can be seen in Figures 5, 6, and 7, respectively. 80S, 60S, and 40S ribosomes were phosphorylated in the standard kinase assay and the proteins extracted and applied directly onto two-dimensional gels (Figure 4). In addition 80S ribosomes were phosphorylated, dissociated to 40S and 60S subunits, and analyzed in the same way.

Table II indicates the ribosomal proteins being phosphorylated under various conditions in vitro. The separated proteins and their numbering are indicated in Figures 8A–C. Radioactively labeled proteins are marked by bars. Six proteins of the 80S ribosome were strongly phosphorylated; on occasion phosphorylation of three other proteins occurred, but to a much smaller extent. Matching the autoradiograms to the stained polyacrylamide gels showed that the phosphorylated 80S ribosomal proteins P1 and P2 as well as protein spot L1 and L2 of the 60S subunit migrate from the anode more slowly than the unmodified proteins (not shown). The shift of the radioactive spots to the left with respect to the stained spot corresponding to these proteins could be attributed to the addition of phosphate groups, which would increase the mobility of the more acidic proteins toward the anode. However, the positions of the other radioactively labeled proteins corresponded with the positions of the unphosphorylated proteins on 2-D gel electrophoresis.

In 80S ribosomes at least two proteins from the small subunit and four of the large are phosphorylated. On dissociation of the phosphorylated 80S particles the radioactive spots associated with 60S proteins L1 and L2 disappear, but the unmodified proteins are still present to the same extent. The loss of phosphate labeled proteins was observed in in vitro studies only. It could be attributed to either weak or labile phosphorylation of these two proteins.

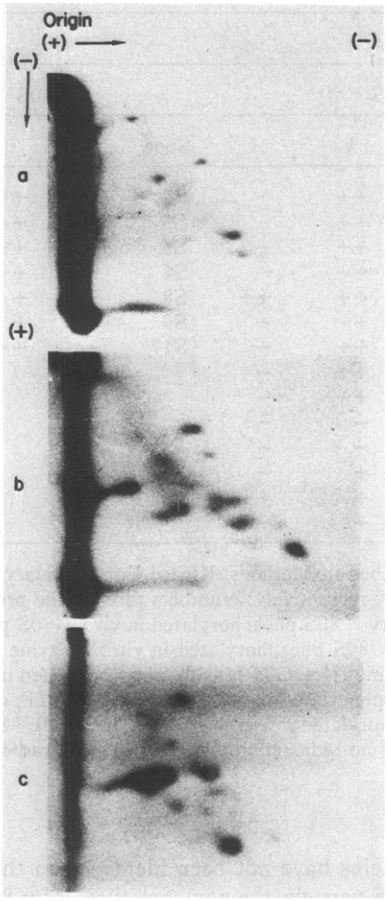

FIGURE 4: Autoradiogram of two-dimensional gel electrophoretograms of in vitro (a) phosphorylated 80S ribosomal proteins, (b) 60S and (c) 40S ribosomal subunit proteins, respectively. The phosphorylated proteins were separated by polyacrylamide gel electrophoresis and the gels exposed to x-ray film as described in Methods.

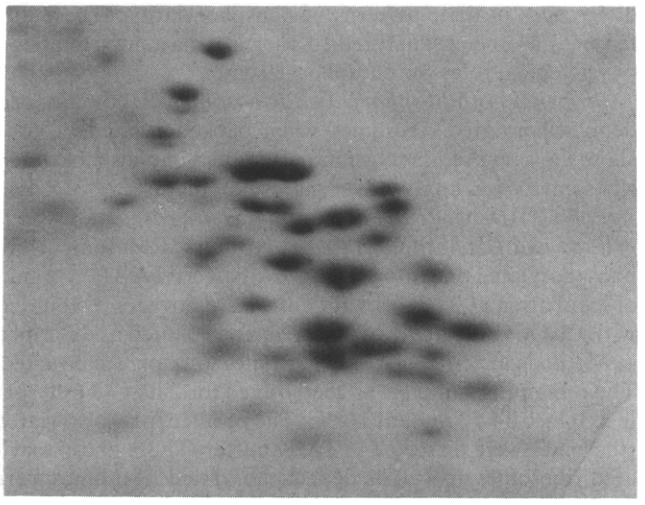

FIGURE 5: Two-dimensional gel electrophoretogram of 80S ribosomal proteins. Proteins of the 80S ribosome of *S. cerevisiae* were separated on two-dimensional polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue as described in Methods.

When the ribosomal subunits were used as substrates for the kinases more proteins were found to be modified than when the 80S ribosomes were used. Eight 60S proteins were phosphorylated strongly and three weakly. Five of the 40S proteins were strongly labeled and two others were weakly labeled. These results indicate that at least nine proteins are protected

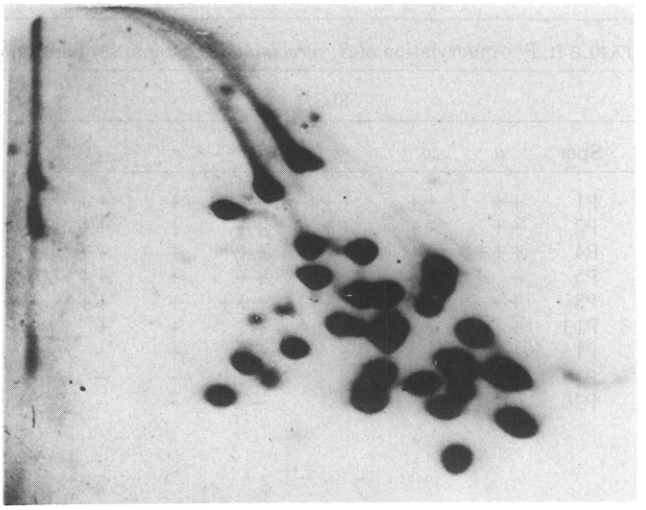

FIGURE 6: Two-dimensional gel electrophoretogram of 60S ribosomal subunit proteins. The method used was the same as in Figure 5 for 80S ribosomal protein.

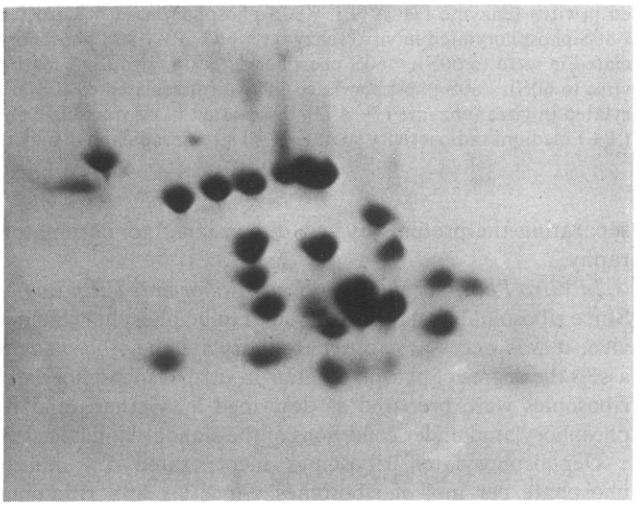

FIGURE 7: Two-dimensional gel electrophoretogram of 40S ribosomal subunit proteins. Same technique was used as in Figure 5 for 80S ribosomal protein.

from phosphorylation in the undissociated ribosome. These proteins could be present in the interface between the two subunits or "buried" in the 80S monosome, and subsequently exposed on subunit formation.

In Vitro Phosphorylation of Ribosomal Proteins with GTP as Phosphate Donor. Since GTP has been shown to be a phosphate donor in kinase reactions using ribosomes of rat liver and rabbit reticulocytes (Ventimiglia and Wool, 1974; Issinger and Traut, 1974), we have studied the specificity of the kinases using [γ-^{32}P]GTP as phosphate donor instead of [γ-^{32}P]ATP. GTP was shown to be 20-fold less efficient than ATP using enzyme I. In reactions catalyzed by kinase III, ATP and GTP were utilized to the same extent; no difference in the pattern of phosphorylation of ribosomal proteins was observed. Thus, we were not able to detect specific phosphorylation of a unique protein using GTP, as has been reported by Ventimiglia's and Issinger's groups. It was not possible to correlate the proteins phosphorylated from yeast ribosomes with those of other eukaryotes since none of the yeast proteins have been purified to homogeneity and compared directly with pure rat liver ribosomal proteins. Furthermore, we used a different method of

TABLE II: Phosphorylation of *S. cerevisiae* Ribosomal Proteins in Vivo and in Vitro.

80S							60S				40S			
Spot	*a*	*b*	*c*	*d*	*e*	*f*	Spot	*g*	*h*	*i*	Spot	*j*	*k*	*l*
P1	++	++	++	++	+	++	L1	++	++	−	S2	+++	+++	++
P2	++	++	++	++	+	++	L2	++	++	−	S3	++	++	−
P4	+++	++	++	++	+	++	L4	−	++	−	S5	−	++	−
P5	+	++	++	++	+	++	L5	+	++	−	S6	++	++	++
P8	++	++	++	+++	+	+++	L6	++	++	++	S8	−	+	−
P13	+	+	+	+	+	+	L7	+	++	−	S9	++	+	−
P15	++	+	+	−	+	+	L8	−	++	−	S17	−	++	−
P18	++	±	−	−	−	−	L10	+	++	−				
P20	+	±	−	−	−	−	L12	+	++	−				
							L13	+	−	−				
							L15	+	−	−				
							L23	+	+	−				
							L24	+	+	++				
							L27	−	+	−				

Kinase I, II, or III was used to catalyze in vitro reactions utilizing ATP as described in Methods. Kinase I or III catalyzed reactions under standard conditions with GTP as phosphate donor are indicated under *e* and *f*, respectively. Numbers refer to the protein spots in Figures 8A–C. Spot numbers not appearing on the table did not show any radioactivity. [a] 80S phosphorylated in vivo. [b] 80S phosphorylated in vitro (enzyme I + ATP). [c] 80S phosphorylated in vitro (enzyme II + ATP). [d] 80S phosphorylated in vitro (enzyme III + ATP). [e] 80S phosphorylated in vitro (enzyme I + GTP). [f] 80S phosphorylated in vitro (enzyme III + GTP). [g] 80S phosphorylated in vivo (dissociated in vitro to 60S). [h] 60S phosphorylated in vitro (enzyme I + ATP). [i] 80S phosphorylated in vitro (enzyme I + ATP, dissociated in vitro to 60S). [j] 80S phosphorylated in vivo (dissociated to 40S in vitro). [k] 40S phosphorylated in vitro (enzyme I + ATP). [l] 80S phosphorylated in vitro (enzyme I + ATP, dissociated in vitro to 40S). Symbols used are: (−) no radioactivity found; (+) weak radioactivity spot; (++) medium radioactivity spot; (+++) strong radioactivity spot.

separating the proteins by two-dimensional gel chromatography.

In Vitro Phosphorylation of Dephosphorylated Ribosomes. Since ribosomal proteins were found to be phosphorylated in vivo, it was necessary to dephosphorylate the ribosomes to assess the degree of phosphorylation in vitro. Dephosphorylated ribosomes were prepared as described in Methods and rephosphorylated under conditions of the standard kinase assay.

Dephosphorylated ribosomes incorporated 5.4 mol of phosphate per mol of ribosomes. Since six 80S ribosomal proteins are phosphorylated, this indicated almost stoichiometric amounts of phosphate present per ribosomal protein, based on the assumption that each ribosomal protein contains only one site which can be phosphorylated.

Phosphorylation of Yeast Ribosomal Proteins in Vivo. Since ribosomal proteins are phosphorylated in vitro and protein kinase can be isolated in association with ribosomes, it might be expected that phosphorylated *S. cerevisiae* ribosomal proteins would be present in vivo as has been reported by Kabat (1970, 1971, 1972) for reticulocytes, and by Loeb and Blat (1970) and Eil and Wool (1971, 1973a,b) for rat liver. *S. cerevisiae* cultures (300 ml) were grown in low phosphate medium (Rubin, 1973) containing 3 mCi of ^{32}P-labeled orthophosphate. The cells were harvested, extracted, and ribosomal proteins analyzed on two-dimensional gels for radioactivity associated with these proteins. As seen in Table II, eight proteins of the 80S ribosome were phosphorylated in vivo; these are the same found to be phosphorylated in vitro. On dissociation into subunits, we found that two 40S proteins were modified in addition to those modified in vitro. Eleven 60S ribosomal proteins were phosphorylated, three of them, L1, L2, and L6, were equally strongly phosphorylated, and seven other faint radioactive spots could be observed in the autoradiogram. These modified ribosomal proteins correspond to the protein spots found in vitro when 60S subunits were used as substrate, using enzyme I. Since most of the 40S and 60S subunit proteins have not been identified in the gel of the complete 80S particle, the nomenclature, P for 80S proteins, L for 60S, and S for 40S proteins, was used for easier identification. We are unable to relate our nomenclature to that of other laboratories since pure proteins have not been compared.

Furthermore, ribosomal proteins obtained from cells which were grown in media containing various carbon sources showed differences in their patterns of phosphorylation. These differences are being analyzed further in an attempt to assign physiological roles for phosphorylation.

Properties of Phosphorylated Ribosomes. Ribosomes and dephosphorylated ribosomes when phosphorylated in vitro show the same sedimentation pattern as nonphosphorylated ribosomes when analyzed on sucrose gradients.

Poly(U)-Dependent Polypeptide Synthesis of Phosphorylated and Dephosphorylated Ribosomes. Ribosomes were phosphorylated under the standard kinase assay. After 15 min of incubation at 37 °C, an aliquot was removed, precipitated with Cl_3CCOOH at 90 °C, filtered, and counted as described in Methods to verify that the ribosomes were phosphorylated. The remaining sample was centrifuged for 2 h at 49 000 rpm in a Spinco TI 50 rotor at 10 °C. The pelleted, phosphorylated ribosomes were dissolved in TMK buffer. These phosphorylated ribosomes as well as dephosphorylated ribosomes were analyzed for poly(U)-directed polyphenylalanine synthesis as described above. We found no consistent differences in the activity of phosphorylated and dephosphorylated compared with nonphosphorylated ribosomes.

Natural messenger RNA translation was not tested; since such a system is not available for yeast ribosomes, it may be that phosphorylated or dephosphorylated ribosomes can or can not be distinguished under these circumstances.

Discussion

We have found that the protein kinases of *S. cerevisiae* may be conveniently concentrated and purified from the ribosome

high salt wash by chromatography on DEAE-cellulose. Three active protein kinase fractions (I, II, III) were obtained in this way; these enzymes phosphorylate ribosomes as well as calf thymus histones and casein. Two of the kinases (I, II) were slightly activated by cAMP, but not to the same extent as has been reported for kinases from reticulocytes and rat liver. These results are in agreement with studies by Takai et al. (1974) on the effect of cAMP on yeast protein kinase and liver protein kinase catalyzed phosphorylation of various nonribosomal proteins.

Issinger and Traut (1974) isolated a protein kinase from rabbit reticulocytes which preferentially utilizes GTP as phosphate donor. By two-dimensional gel electrophoresis of proteins of the 50S subunit of *Escherichia coli,* labeled with [γ-^{32}P]GTP, it was shown that only two proteins, L7 and L12, were phosphorylated by the kinase, whereas two additional ribosomal proteins were modified by the same kinase when [γ-^{32}P]ATP was used.

Ventimiglia and Wool (1974) recently described a protein kinase found in rat liver cytosol that transfers the terminal phosphoryl group of GTP to serine and threonine residues of proteins of the small ribosomal subunit. The availability of a variety of such protein kinases with different specificities could provide valuable tools for studies of the topography of ribosomal proteins.

We have studied the specificities of the yeast protein kinases using [γ-^{32}P]GTP as phosphate donor. In reactions catalyzed by kinase III, ATP and GTP were utilized to the same extent. We were not able to detect phosphorylation of a unique protein as was found by Ventimiglia and Issinger. However, we found that one of the 80S ribosomal proteins was more strongly phosphorylated than any other protein in the presence of GTP. This increase in radioactivity of protein P8 was only seen in in vitro experiments catalyzed by kinase III. The failure to observe a specific modified protein when GTP is the phosphate donor could be due to contamination of our kinase III preparation with ATP-kinase.

We have examined the in vitro phosphorylation of yeast ribosomal proteins catalyzed by these kinases and find that nine proteins of 80S ribosome are phosphorylated, three only weakly. Four of these can be attributed to the 40S subunit and four to the 60S subunit. Thus, by radioactively marking proteins we were able to identify some 80S ribosomal proteins that correspond to either proteins of the 40S or 60S subunits. When isolated ribosomal subunits were phosphorylated, 7 ribosomal proteins of the 40S and 11 of the 60S subunit were modified.

This suggests that the nine extra proteins phosphorylated in isolated subunits must be shielded from the action of protein kinases in the intact ribosome. They may be protected at the subunit interface or, more likely, exposed as a result of conformational changes that occur when the two subunits associate or come apart. It seems unlikely that all nine of these proteins (plus others not susceptible to phosphorylation) would be involved directly in the interface reaction between the two ribosome subunits.

Continued studies of phosphorylation of ribosomal proteins in concert with cross-linking (review by Traut et al., 1974) and immunological studies (Stoeffler, 1974) will be useful in establishing features in the topography of ribosomes and the changes in shape that may accompany dissociation into subunits, or the formation of polysomes.

By growing *S. cerevisiae* in the presence of [^{32}P]orthophosphate, 80S ribosomal proteins were phosphorylated in vivo to a similar extent as found in vitro. In no other system described so far, has such extensive phosphorylation of ribosomal

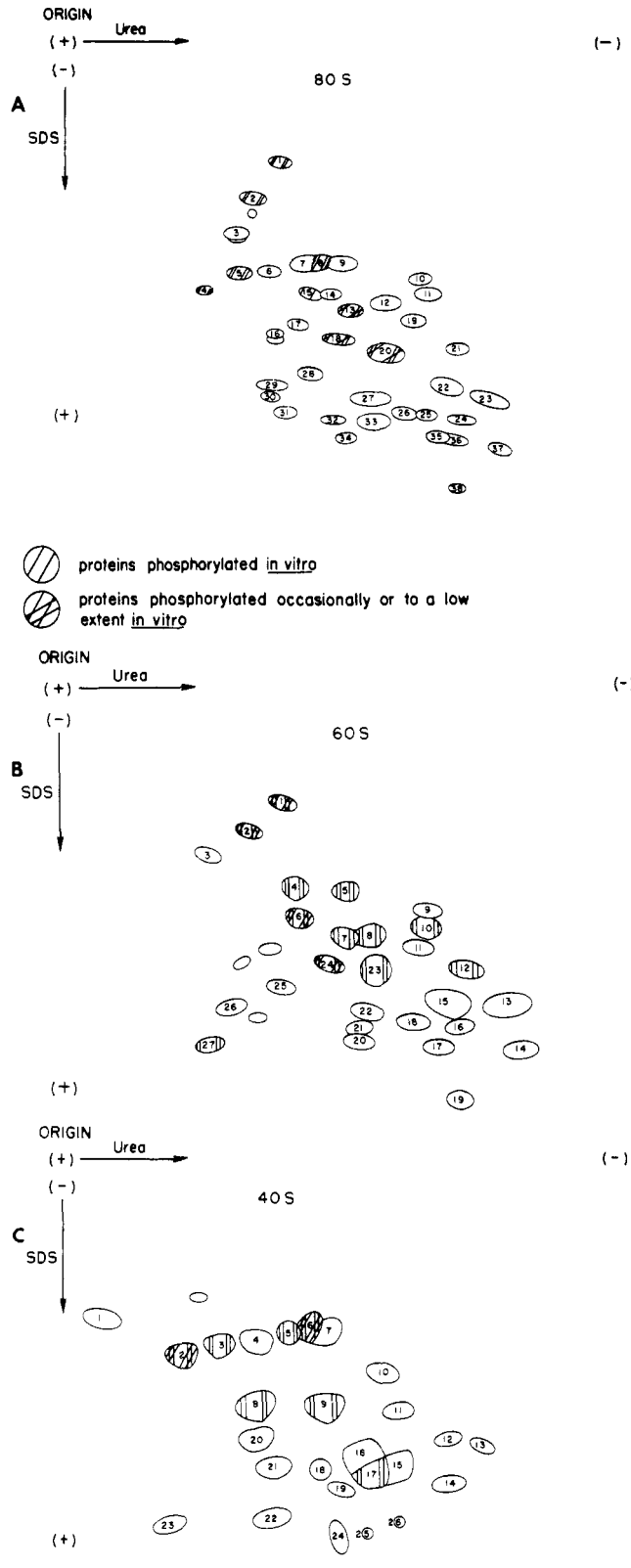

FIGURE 8: Schematic diagram and numbering of ribosomal proteins. The two-dimensional gel Figures 5, 6, and 7, corresponding to proteins of the (A) 80S ribosome and (B) 60S and (C) 40S ribosomal subunits, respectively, with the spots numbered for identification. Most of the 40S and 60S subunits proteins have not been identified in the gel of the complete 80S particle. Proteins phosphorylated under various conditions are indicated by shading.

proteins been reported. The question is: do these modifications have any function in the physiology of the organism? It is likely that ribosomal phosphoryl groups, such as those found in S180 tumor cells and rabbit reticulocyte ribosomes, have an essential function since they have been conserved during eukaryotic evolution. In addition, the pattern of ribosomal protein phosphorylation seems to be dependent on physiological growth conditions and on the nature of the organism since growth on different carbohydrate sources led to different patterns. We have also found different patterns of phosphorylation in yeast cells starved for nitrogen or amino acids.

Many researchers have analyzed a number of protein synthetic functions and found no consistent difference in the activity of phosphorylated compared with nonphosphorylated and dephosphorylated ribosomes. This was true also for poly(U)-directed polyphenylalanine synthesis studies with yeast ribosomes described here. It is possible that ribosomal phosphorylation might be unrelated to protein synthesis per se; for example, the phosphorylation may have an effect on the maturation of ribosomes as proposed by Olson et al. (1974). We have observed that cells grown under starvation conditions showed increased amounts of phosphate uptake in protein S2. This may correspond to rat liver S6 protein observed to be phosphorylated in vivo. The phosphorylation of this protein S6 was stimulated in the presence of cycloheximide and puromycin (Gressner and Wool, 1974) and also in diabetic rats (Gressner and Wool, 1976). At the moment, however, the role of ribosomal protein phosphorylation remains unknown.

Acknowledgments

We thank Daniel Schindler for helpful discussion and criticism.

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