Proc. Nat. Acad. Sci. USA
Vol. 71, No. 2, pp. 350–354, February 1974

A Kinase That Transfers the γ-Phosphoryl Group of GTP to Proteins of Eukaryotic 40S Ribosomal Subunits

(rat-liver cytosol)

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Communicated by Dwight J. Ingle, October 11, 1973

ABSTRACT **An enzyme in rat-liver cytosol transferred the γ-phosphoryl of GTP to serine and threonine residues of at least four proteins (S6, S10, S14 or S15, and S17) of the small (40S) subunit of rat-liver ribosomes. A number of nonribosomal proteins in the enzyme preparation were also phosphorylated; they were preferentially and tightly bound to the large subunit. The enzyme could be distinguished from protein kinase-ATP (which also phosphorylated ribosomal proteins) by a number of criteria: (1) GTP was the phosphoryl donor; (2) the pattern of phosphorylation of ribosomal proteins by the two enzymes was different; and (3) the protein kinase that used GTP as the phosphoryl donor was not stimulated by cyclic AMP (or by cyclic GMP).**

Eukaryotic ribosomal proteins are phosphorylated *in vivo* and *in vitro* by protein kinases bound to the particle or free in the cytoplasm (1–3); that reaction has been shown, or assumed, to result from the transfer of phosphate from ATP. We report now on a related but novel reaction. There is an enzyme activity in rat-liver cytosol that catalyzes the transfer of the γ-phosphoryl of GTP to several proteins of the 40S subunit of eukaryotic ribosomes.

MATERIALS AND METHODS

Materials. [^{32}P]Orthophosphoric acid (carrier free) was obtained from Tracerlab and used to prepare [γ-^{32}P]ATP and [γ-^{32}P]GTP (4).

Preparation of Ribosomal Subunits. Liver ribosomes were isolated from male Sprague–Dawley rats that weighed 100–120 g (5, 6) and used to prepare ribosomal subunits (5, 7).

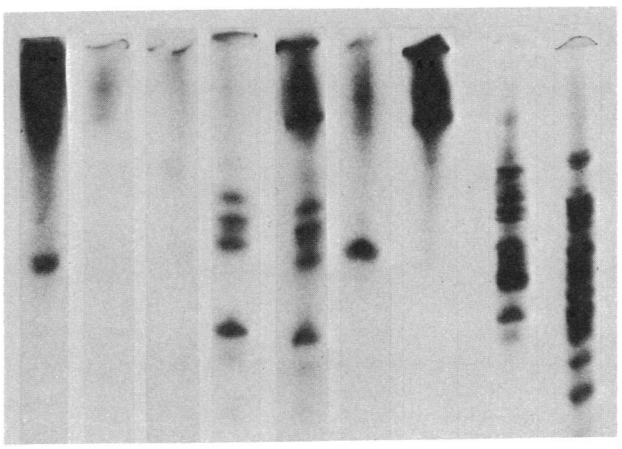
Preparation of Enzymes. The enzyme responsible for the transfer of the γ-phosphoryl of GTP to ribosomal protein was prepared from rat-liver cytosol by hydroxylapatite chromatography, followed by filtration through Sephadex G-200, using the protocol described by Schneir and Moldave (9) for the isolation of elongation factor 1 (EF-1). We refer to the activity as "protein kinase-GTP." A rat-liver protein kinase that transfers the γ-phosphoryl group of ATP to serine and theonine in ribosomal proteins was prepared as described (3, 10); the enzyme is referred to as "protein kinase-ATP."

Assay of the Phosphorylation of Ribosomal Proteins. Ribounits were incubated for 5 min at 37° in 0.2 ml of Medium A (50 mM Tris·HCl, pH 7.8; 80 mM KCl; 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol) containing 50 μM [γ-^{32}P]GTP and the enzyme preparation to be assayed. The reaction was started by addition of the [γ-^{32}P]nucleoside triphosphate. Phosphorylated ribosomes were precipitated by addition of 0.2 volumes of ethanol (11). The precipitated ribosomes were resuspended in Medium A. Two volumes of 10% trichloroacetic acid was added and the sample was heated for 15 min at 90°–95°. The precipitate was collected on glass-fiber filters, washed with 30 ml of 5% trichloroacetic acid, and dried under an infrared lamp. The radioactivity was determined (with an efficiency greater than 95%) as described (6).

One-Dimensional Polyacrylamide Gel Electrophoresis and Radioautography of Ribosomal Proteins. Phosphorylated ribosomal subunits (see legend of Fig. 1) were precipitated with ethanol (11), resuspended in Medium B (50 mM Tris·HCl, pH 7.8; 500 mM KCl; 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol), and centrifuged through 20% sucrose in Medium B. The salt-washed ribosomal subunits were suspended in 10 mM Tris·HCl (pH 7.7) and 100 mM magnesium acetate, and the protein was extracted with 66% acetic acid (12). The radioactivity and the concentration of protein (13) was determined after dialysis overnight against 7.5% acetic acid. Samples (containing 150–200 μg of protein) were lyophilized; the protein was dissolved in 8 M urea and made 0.1 M in 2-mercaptoethanol; the pH was adjusted to 8 with solid Tris before the sample was incubated for 3 hr at 37° to assure that the proteins were reduced (14). Ribosomal proteins were separated by electrophoresis at pH 4.5 on discontinuous polyacrylamide gels (15, 16). The destained gels were sliced into longitudinal sections and dried on filter paper on a Büchner funnel (3). The dried gels were put on x-ray film (Kodak, NS-54T) and exposed 5–7 days.

Two-Dimensional Polyacrylamide Gel Electrophoresis and Radioautography of Ribosomal Proteins. The ^{32}P-labeled ribosomal proteins were separated by two-dimensional polyacrylamide gel electrophoresis by a modification (17) of the procedure of Kaltschmidt and Wittmann (18), except that the gel slabs for the second dimension were reduced in size (10 cm × 13 cm and 2 mm thick). The conditions of electrophoresis were: 8% acrylamide gel, pH 8.6, for 6 hr at 2.0 mA per tube (19 cm × 2.5 mm) in the first dimension; and 18% acrylamide gel, pH 4.2, for 18 hr at 12 mA in the second dimension. Generally 400–700 μg of ribosomal protein dissolved in 50 μl of sample gel containing 8 M urea was analyzed. The gels were stained for 20 min in 1% Amido Black in 7.5%

Abbreviations: rRNA, ribosomal RNA; EF, elongation factor.



	a	b	c	d	e	f	g	h	i
SUBSTRATE									
40S RIBOSOMAL SUBUNIT	+		+	+	+	+		+	
60S RIBOSOMAL SUBUNIT	+		+	+	+		+		+
ENZYME									
PROTEIN KINASE - GTP	+	+			+	+	+		
PROTEIN KINASE - ATP			+	+					
PHOSPHORYL DONOR									
[γ-^{32}P] GTP	+	+	+			+	+		
[γ-^{32}P] ATP				+	+				

FIG. 1. Radioautographs (*a–g*) and electropheretograms (*h* and *i*) of phosphorylated ribsomal proteins. Ribosomal subunits (either 1125 μg of 40S or 60S rRNA when they were present alone, or 320 μg of 40S and 800 μg of 60S rRNA when both were present) were incubated for 5 min at 37° in 1 ml of Medium A containing 50 μM [γ-^{32}P]GTP (0.3 to 0.9 $\times$ 10^3 cpm/pmol) or 50 μM [γ-^{32}P]ATP (2×10^3 cpm/pmol). The amount of enzyme when present was: rat-liver protein kinase-GTP, 474 μg; rat-liver protein kinase-ATP, 245 μg. The ribosomal proteins were extracted and separated by polyacrylamide gel electrophoresis; radioautographs were made. Stained gels (*h* and *i*) are included for comparisons.

acetic acid, and destained by diffusion in 7.5% acetic acid. The destained gels were dried (19, 20), placed on x-ray film (Kodak, NS-54T) and exposed for 5–10 days.

Identification of [^{32}P]Phosphoserine and [^{32}P]Phosphothreonine. Protein was extracted from ribosomal subunits—about 1.8 mg of 40S or of 60S ribosomal RNA (rRNA)—with 66% acetic acid (12, 17), lyophilized, and dissolved in 0.5 ml of 6 N HCl in a Pyrex tube. The phosphorylated proteins were hydrolyzed (21) and separated by paper electrophoresis (3). Radioautographs were made (3), and samples were compared with authentic markers (22).

RESULTS AND DISCUSSION

Phosphoryl Transfer from [γ-^{32}P]GTP to Ribosomal Proteins. An enzyme in rat-liver cytosol catalyzed the transfer of the γ-phosphoryl group of GTP to ribosomal proteins (Table 1). Phosphorylation did not require protein synthesis since it was not dependent on the presence of template, aminoacyl-tRNA, or elongation factors (results not shown); the enzyme preparation contains EF-1 but not EF-2. It is clear that phosphorylation of the ribosome is uncoupled from protein synthesis.

The transfer of radioactivity from [γ-^{32}P]GTP by the kinase was to a single ribosomal protein band; in addition there were a number of phosphoproteins that migrated a shorter distance from the anode than even the least basic of the ribosomal proteins (Fig. 1*a*)—determined by comparison with stained slices of the same gels (Fig. 1*h* and *i*). We assume the latter are proteins in the enzyme preparation; labeled proteins with similar electrophoretic mobility were observed in radioautographs after polyacrylamide gel electrophoresis of protein kinase-GTP preparations that had been incubated with [γ-^{32}P]GTP but without ribosomes (Fig. 1*b*). Thus, the phosphoproteins at the top of the radioautographs are not likely to be ribosomal proteins whose mobility has been altered by phosphorylation.

It might appear that the phosphorylation of the proteins in the enzyme preparation required the presence of ribosomal subunits since the amount of radioactivity at the top of the gel was greater when they were present (compare Fig. 1*a* and *b*). However, we found (see below; Fig. 1*f* and *g*) that those proteins bind to the subparticles, especially to the 60S subunit. Thus, the greater radioactivity may be apparent

TABLE 1. *Phosphoryl transfer from [γ^{32}-P]GTP to ribosomal proteins*

(*pmol of ^{32}P per pmol of ribosome*)

Addition	Radioactivity in ribosomal proteins
None	0.009
Protein kinase-GTP preparation	0.170

Ribosome subunits—64 μg (rRNA) of 40S and 160 μg (rRNA) of 60S subunits—were incubated with 50 μM [γ-^{32}P]GTP (10^3 cpm/pmol) and, where indicated, 70 μg of rat-liver protein kinase-GTP preparation. For calculation of the specific radioactivity, the molecular weight of 80S ribosomes was assumed to be 4.5×10^6. The calculation includes radioactivity in nonribosomal proteins.

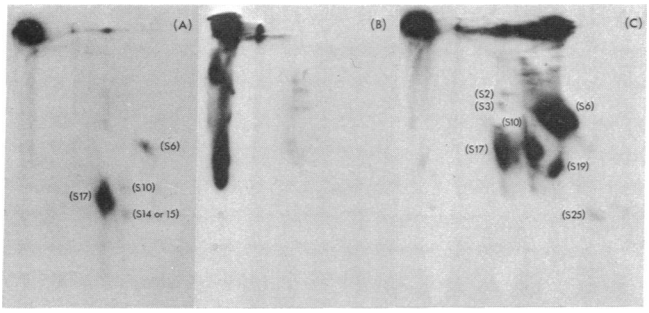


FIG. 2. Radioautographs of phosphorylated 40S and 60S ribosomal proteins separated by two-dimensional polyacrylamide gel electrophoresis. 40S (*a*) or 60S (*b*) ribosomal subunits (1.35 mg of rRNA) were incubated in 1 ml of Medium A for 20 min at 37° with 450 μg of a protein kinase-GTP preparation and 50 μM [γ-^{32}P]GTP (1.2 $\times$ 10^3 cpm/pmol). In (*c*), 40S ribosomal subunits (1.35 mg of rRNA) were incubated as in ref. 3 with 50 μM [γ-^{32}P]ATP (2 $\times$ 10^3 cpm/pmol), 10^{-5} M cyclic AMP, and 250 μg of protein kinase-ATP. The ribosomes were extracted and separated by two-dimensional polyacrylamide gel electrophoresis; radioautographs were made.

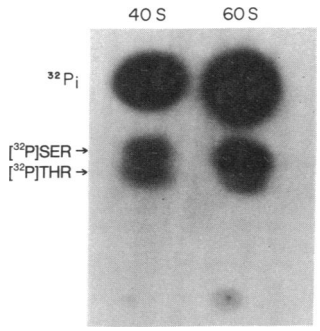


FIG. 4. Radioautographs of hydrolyzed phosphorylated ribosomal proteins. Ribosomal subunits (1.8 mg of either 40S or 60S rRNA) were incubated for 5 min at 37° in 1 ml of Medium A containing 50 μM [γ-^{32}P]GTP (4 $\times$ 10^3 cpm/pmol) and 275 μg of rat-liver protein kinase-GTP preparation. The phosphorylated proteins were hydrolyzed and separated by paper electrophoresis; radioautographs were made.

rather than real; the subparticles would be expected to bind the phosphoproteins in the enzyme preparation and thereby facilitate their recovery from the reaction mixture.

Nucleoside Triphosphate Specificity of Phosphoryl Transfer to Ribosomal Proteins. Rat-liver cytosol has two protein kinases which catalyze the transfer of the terminal phosphate of ATP to serine and threonine residues in ribosomal proteins (3, 10). We shall refer to those enzymes for convenience as protein kinase-ATP. We wished to contrast that reaction with the transfer of the γ-phosphoryl of GTP to ribosomal proteins catalyzed by the protein kinase-GTP preparation. Protein kinase-ATP did not catalyze the phosphorylation of ribosomal proteins if GTP was the phosphate donor (Fig. 1*c*). If the nucleoside triphosphate was ATP, then protein kinase produced a characteristic pattern of phosphorylated proteins (Fig. 1*d*); the pattern was distinct from that caused by protein kinase-GTP preparations incubated with [γ-^{32}P]GTP (Fig. 1*a*). Protein kinase-GTP preparations do contain protein kinase-ATP, for they caused the typical pattern of phosphorylation of ribosomal proteins when [γ-^{32}P]ATP was the phosphoryl donor (Fig. 1*e*). It is noteworthy that nonribosomal proteins in the protein kinase-GTP preparation were phosphorylated when the radioactive nucleoside triphosphate was ATP (Fig. 1*e*—note the radioactive material at the top of the gel) just as had occurred with GTP (Fig. 1*a*). There was no phosphorylation of enzyme proteins, however, when phosphoryl transfer was catalyzed by purified protein kinase-ATP (compare Fig. 1*d* with Fig. 1*e*). Thus the phosphoryl acceptor proteins are present in the protein kinase-GTP preparation but not in purified protein kinase-ATP. We con-

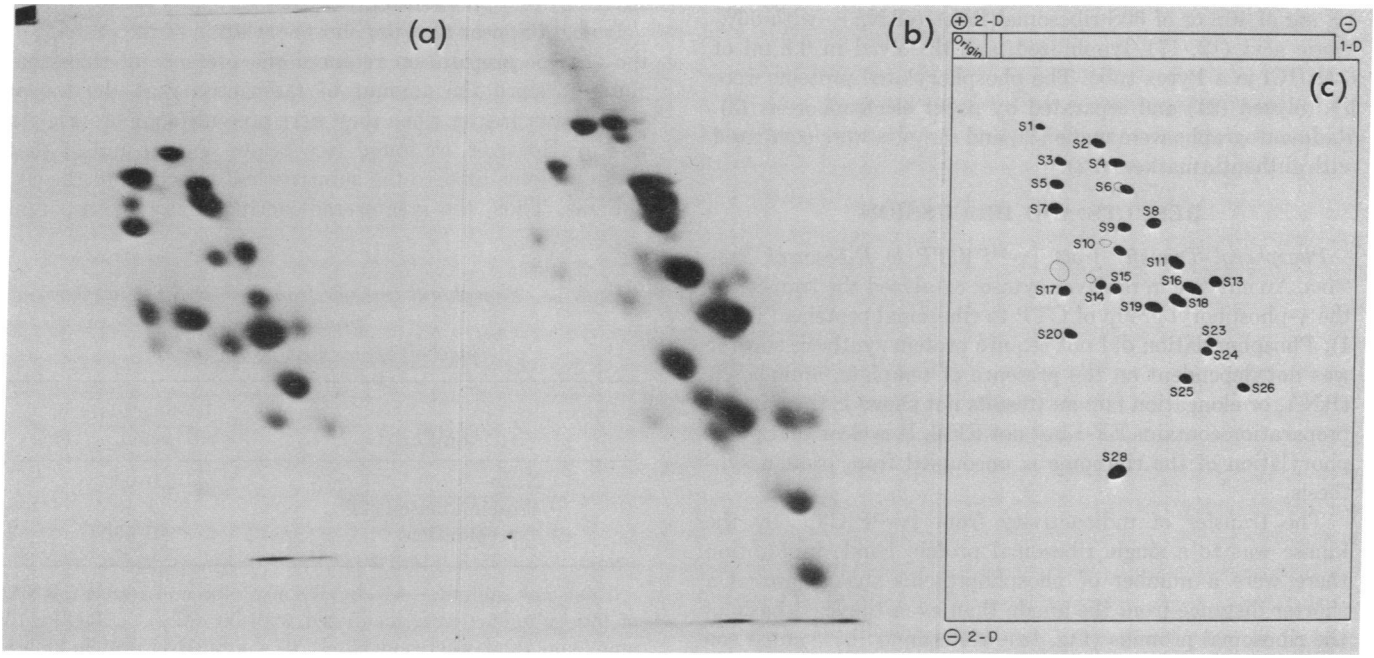


FIG. 3. Two-dimensional electropherogram and diagram of ribosomal proteins. (*a*) Electropherogram of 40S ribosomal proteins. (*b*) Electropherogram of 60S ribosomal proteins. (*c*) Composite diagram of the electropherogram and the radioautograph of 40S ribosomal proteins: *solid spots* indicate the zones seen on the electropherogram; *interrupted lines* indicate radioactive zones seen on the radioautograph.

clude, then, that our preparations are contaminated with protein kinase-ATP, but contain, in addition, a unique enzyme that transfers the γ-phosphoryl group of GTP to ribosomal proteins. We suggest the trivial name, protein kinase-GTP, for the latter enzyme.

Identification of the Phosphorylated Ribosomal Protein. The single ribosomal protein band that was phosphorylated by protein kinase-GTP preparations is in the 40S subunit (Fig. 1*f*); moreover, phosphorylation of that protein did not require the 60S subparticle, for the same band was phosphorylated in the presence and absence of the large subunit (compare Fig. 1*a* and 1*f*). The proteins in the enzyme preparation that were phosphorylated associated preferentially with the 60S subunit (Fig. 1*g*); they were not removed by centrifugation through 20% sucrose containing 0.5 M KCl.

The exact number and the identity of the phosphorylated proteins is best determined by two-dimensional polyacrylamide gel electrophoresis. 40S ribosomal proteins, phosphorylated in the reaction with [^{32}P]GTP and the rat-liver enzyme, were separated by two-dimensional polyacrylamide gel electrophoresis and radioautographs were made: there were four radioactive zones (Fig. 2*a*). When we compared the radioactive zones with the ribosomal proteins on the stained, dried gel slabs (Fig. 3*a*), it was apparent that the phosphoproteins were shifted in a north-westerly direction, e.g., toward the origin or anode of the gel (see Fig. 3*c*). That is the change in electrophoretic mobility to be expected of proteins that have gained negative charge(s) as a result of phosphorylation. Similar changes in the electrophoresis of ribosomal proteins has been demonstrated before. Affinity labeling of *Escherichia coli* ribosomes with radioactive *p*-nitrophenyl-carbamyl-phenylalanyl-tRNA causes acylation of the strongly basic ε-amino groups of lysine in some proteins (24). The loss of positive charge leads to a decrease in migration toward the cathode on two-dimensional polyacrylamide gel electrophoresis. Although the modified ribosomal proteins cannot be detected on the stained gel, since only 0.4% of the particles are acylated, the shifted proteins have been positively identified by crossreaction with antibodies to the normal proteins (24). A difference of one positive charge (acetylation of the NH_2-terminal serine) in two otherwise identical *E. coli* ribosomal proteins (L7 and L12) cause a similar change in position (25). Thus, one can predict with some confidence the behavior of chemically modified proteins.

The phosphorylated 40S ribosomal proteins were not detected on the stained electropherogram since, at most, only 2% of the particles were affected by protein kinase-GTP (see below). However, when [^{32}P]orthophosphate is administered to hepatectomized rats, a number of phosphorylated derivatives of S6 are seen on the stained gel and on the radioautograph (Gressner and Wool, unpublished observation). Each of the phosphorylated forms of S6 appears to the north-west of that protein on the electrophoretogram: if the phosphorylated ribosomal proteins are treated with phosphatase, the extra zones (i.e., S6 derivatives) disappear and the normal S6 is generated. Once again the observations support our interpretation of the behavior of the phosphorylated ribosomal proteins.

Because the radioactive zones and the stained proteins are not congruent, their identification can only be tentative. Keeping the reservations in mind, the 40S ribosomal proteins that were phosphorylated by protein kinase-GTP were: S6, S10, S14 or 15, and S17 (Fig. 2*a* and 3). Several additional proteins, of which S19 was the most prominent, were phosphorylated by protein kinase-ATP (Fig. 2*c*); thus, the number and variety of proteins phosphorylated was distinct.

60S subunits were incubated with [^{32}P]GTP and the rat-liver enzyme preparation: the proteins were separated once again by two-dimensional polyacrylamide gel electrophoresis (Fig. 3*b*), and radioautographs of the gels were made (Fig. 2*b*). The radioactive phosphoproteins hardly migrated in the first dimension, indicating they are far less basic than the bulk of 60S ribosomal proteins. No radioactive zone corresponded to an authentic ribosomal protein, even if allowance was made for change in charge due to phosphorylation. The finding decisively distinguishes protein kinase-GTP from protein kinase-ATP, since the latter catalyzes the phosphorylation of a number of 60S ribosomal proteins (10).

Determination of the Formation of o-Phosphoserine and o-Phosphothreonine. The radioactive phosphorylated proteins formed in the reaction with the protein kinase-GTP preparation were hydrolyzed and analyzed by paper electrophoresis and radioautography. The hydrolysate contained radioactivity that migrated the same as authentic *o*-phosphoserine and *o*-phosphothreonine during electrophoresis; there was also a good deal of radioactive inorganic phosphate (Fig. 4)—the latter probably arose from hydrolysis of phosphoester bonds (21). Phosphorylated 40S subunits were also treated with bacterial alkaline phosphatase: approximately 45% of the radioactive phosphate was removed by treatment with the enzyme for 2 hr (results not shown). The results confirm that the radioactivity was in phosphate monoesters. We cannot definitely exclude that the covalently bound phosphorylated amino acids were present only in proteins in the enzyme preparation (and that the ribosomal proteins were phosphorylated in some other way). However, the [^{32}P]-phosphate associated with ribosomal proteins must have been covalently bound since they migrated together after electrophoresis in urea in two dimensions at pH 8.6 and 4.2, and after electrophoresis in the second dimension in sodium dodecyl sulfate (results not shown). We conclude then that the phosphoryl groups transferred to ribosomal proteins were covalently bound to serine and threonine.

The Enzyme That Transfers the γ-Phosphoryl of GTP to Ribosomal Proteins Is Not EF-1. Rat-liver protein kinase-GTP, prepared as we have (9), contains EF-1. However, a purified preparation of rabbit reticulocyte EF-1 (23), provided by Dr. Boyd Hardesty, had good elongation factor activity but did not catalyze phosphoryl transfer from GTP to ribosomal protein (results not shown). Thus, the latter activity is not a property of EF-1 but of a separate and distinct enzyme.

The transfer of the γ-phosphate of GTP to ribosomal proteins was not affected by cyclic AMP (Table 2) or by cyclic GMP (results not shown). The enzyme was not sensitive to inhibition by *N*-ethylmaleimide (Table 2). Thus the activity is not to be attributed to EF-2, which is inhibited by *N*-ethylmaleimide (23); moreover, protein kinase-GTP does not have a sulfhydryl group that is essential for its activity.

Only a small proportion of the ribosomes were phosphorylated by protein kinase-GTP. Even if we assume that a single mole of phosphate was added to each mole of particles, then only about 2% of the 40S subunits were phosphorylated

TABLE 2. *Phosphoryl transfer from [γ-^{32}P]GTP to the proteins of 40S ribosomal subunits*
(pmol of ^{32}P per pmol of 40S subunit)

Exp	Enzyme preparation	Cyclic AMP	Radioactivity in 40S ribosomal proteins
1	None	−	0.004
	Protein kinase-GTP preparation (55 μg)	−	0.023
	Protein kinase-GTP preparation (55 μg)	+	0.023
2	Protein kinase-GTP preparation (55 μg)	−	0.019
	NEM-treated protein kinase-GTP preparation (55 μg)	−	0.014

40S ribosomal subunits (225 μg of rRNA) were incubated with 50 μM [γ-^{32}P]GTP (1.6 × 10^3 cpm/pmol). The concentration of cyclic AMP, when present, was 10^{-5} M. For one experiment, rat-liver protein kinase-GTP preparation (which was in 10 mM 2-mercaptoethanol) was treated at 0° with 20 mM *N*-ethylmaleimide (*NEM*); after 10 min the concentration of 2-mercaptoethanol was made 30 mM and the enzyme was used in the assay. For calculation of the specific radioactivity, the molecular weight of the 40S subunit was assumed to be 1.3 × 10^6.

(calculated from the data in Table 2). However, we do not know that the conditions were optimal; that will require purification of the enzyme and further characterization of the reaction.

There is then an enzyme activity in rat-liver cytosol that transfers the γ-phosphoryl group of GTP to serine and threonine residues of several proteins of the 40S ribosomal subunit. The enzyme also catalyzes the phosphorylation of a number of proteins in the enzyme preparation that bind to the 60S subunit. A large number of ribosomal proteins (perhaps as many as 19) are phosphorylated when the enzyme is protein kinase-ATP (Lin, Ventimiglia, and Wool, unpublished observation); a far smaller number are phosphorylated in the reaction catalyzed by protein kinase-GTP. The results suggest protein kinase-GTP has some specificity and, perhaps, that the reaction is important for ribosome function.

We thank Dr. Boyd Hardesty for a gift of purified rabbit reticulocyte EF-1, and to Charles Eil for purified rat-liver protein kinase-ATP. The expenses of the research were met by grants from the John A. Hartford Foundation and the National Institutes of Health (AM-04842). F.A.V. was supported by Training Grant GM-424 from the National Institute of General Medical Studies.

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