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Phosphorylation of mammalian translation initiation factor 5 (eIF5) *in vitro* and *in vivo*

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ABSTRACT

Eukaryotic translation initiation factor 5 (eIF5) interacts with the 40S initiation complex (40S•eIF3•AUG•Met-tRNA$_f$•eIF2•GTP) and, acting as a GTPase activating protein, promotes the hydrolysis of bound GTP. We isolated a protein kinase from rabbit reticulocyte lysates on the basis of its ability to phosphorylate purified bacterially expressed recombinant rat eIF5. Physical, biochemical and antigenic properties of this kinase identify it as casein kinase II (CK II). Mass spectrometric analysis of maximally *in vitro* phosphorylated eIF5 localized the major phosphorylation sites at Ser-387 and Ser-388 near the C-terminus of eIF5. These serine residues are embedded within a cluster of acidic amino acid residues and account for nearly 90% of the total *in vitro* eIF5 phosphorylation. A minor phosphorylation site at Ser-174 was also observed. Alanine substitution mutagenesis at Ser-387 and Ser-388 of eIF5 abolishes phosphorylation by the purified kinase as well as by crude reticulocyte lysates. The same mutations also abolish phosphorylation of eIF5 when transfected into mammalian cells suggesting that CK II phosphorylates eIF5 at these two serine residues *in vivo* as well.

INTRODUCTION

Eukaryotic translation initiation factor 5 (eIF5), a monomeric protein of ~49 kDa in mammals (1–3) and 46 kDa in the yeast *Saccharomyces cerevisiae* (4,5), plays an essential role in initiation of protein synthesis. *In vitro* biochemical studies using purified translation initiation factors have shown that, following scanning of mRNA by the 40S pre-initiation complex (40S•eIF3•Met-tRNA$_f$•eIF2•GTP) and formation of the 40S initiation complex at the AUG codon of mRNA (40S•eIF3•AUG•Met-tRNA$_f$•eIF2•GTP), eIF5 interacts with the 40S initiation complex to promote hydrolysis of the bound GTP. Hydrolysis of GTP causes the release of eIF2•GDP and eIF3 from the 40S subunit, an event that is essential for the subsequent joining of the 60S ribosomal subunit to the 40S complex to form the functional 80S initiation complex (80S•Met-tRNA$_f$•mRNA) that is active in peptidyl transfer (for reviews see 6–8). However, once GTP hydrolysis has occurred and the 40S subunit-bound initiation factors are released, the subsequent 60S subunit joining reaction does not require the participation of eIF5 (9). It has been reported (10) in the yeast *S.cerevisiae*, that eIF5-promoted GTP hydrolysis plays an important role in the mRNA start site selection by the 40S pre-initiation complex. The mammalian cDNA and the yeast gene, designated *TIF5*, encoding eIF5 have been cloned and expressed as functional proteins in *Escherichia coli* (3,5). The yeast gene *TIF5* is a single-copy essential gene (5) that has been shown to be required for initiation of protein synthesis *in vivo* (11,12) and consequently for cell growth and viability.

A number of proteins involved in the eukaryotic translation system are known to be phosphorylated *in vivo* and *in vitro* (for a review see 13–15). These include the 40S ribosomal protein S6, the α and β subunits of initiation factor eIF2, eIF2B, eIF3, eIF4B and elongation factor eEF2. Phosphorylation of some of these proteins such as eEF2 at Thr^{58} by Ca^{2+}/calmodulin-dependent protein kinase III and the α subunit of eIF2 at Ser^{51} by an eIF2-specific kinase causes a drastic inhibition of the functional activity of these proteins both *in vitro* and *in vivo* (16,17).

We have demonstrated previously that eIF5 also exists in mammalian cells as a phosphoprotein (2). When rat pituitary GH3 cells were labeled with ^{32}Pi and eIF5 was immunoprecipitated from cell-free extracts of ^{32}P-labeled cells, eIF5 was found to be phosphorylated at serine residues. Phosphopeptide mapping revealed two major sites of phosphorylation (2) although neither the protein kinase(s) responsible for phosphorylation at these two *in vivo* sites nor the sites of phosphorylation were identified.

The availability of purified, bacterially expressed, recombinant rat eIF5 (18), which is presumably devoid of phosphate groups, has allowed us to use it as a substrate to identify the protein kinase responsible for phosphorylation of eIF5 *in vitro* and *in vivo*. We have purified the protein kinase from rabbit reticulocyte lysates and identified the serine residues in eIF5 that are phosphorylated by the purified kinase. Evidence is presented that indicates that the protein kinase responsible for phosphorylation of eIF5 *in vitro* and *in vivo* is casein kinase II (CK II).

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MATERIALS AND METHODS

Purified proteins and other reagents

Escherichia coli XLI Blue cells, transformed with recombinant pGEX-KG plasmid containing the rat eIF5 open reading frame [expressing eIF5 as a glutathione-*S*-transferase (GST) fusion protein], were grown as described (19). Untagged eIF5 was purified from bacterial cells and stored at –20°C as described previously (19). Purified recombinant rat eIF5 is presumably devoid of phosphate groups as it has been reported (20) that none of the bacterial protein kinases are able to phosphorylate a wide variety of eukaryotic proteins tested. More importantly, we treated a bacterial culture expressing rat eIF5 with ^{32}P-orthophosphate (25 µCi/ml of bacterial culture) at the same time as eIF5 expression was induced by the addition of isopropyl-β-D-thiogalactoside. Analysis by SDS–polyacrylamide gel electrophoresis (PAGE) showed that while a number of bacterial proteins were phosphorylated under these conditions, the expressed eIF5 was unlabeled (data not shown). It should be noted that for this experiment the bacterial culture was grown in standard low-phosphate medium.

Frozen rabbit reticulocyte lysates were obtained from Green Hectares Company. The preparation of crude ribosomal salt-wash proteins and post-ribosomal supernatant were as described (2). Polyclonal antibodies specific for mammalian (bovine) CK II were a kind gift from Dr Michael Dahmus of the University of California at Davis.

Purification of eIF5 kinase activity

All operations were carried out between 0 and 4°C unless otherwise indicated. The elution of proteins from columns was monitored by measuring the A_{280} of the column eluates. The post-ribosomal supernatant of rabbit reticulocyte lysates (300 ml), prepared as described previously (2), was fractionated with solid ammonium sulfate. The proteins precipitating between 0 and 50% saturation (291 g/l) and between 50 and 80% saturation (additional 194 g/l) were separately dissolved in buffer A [20 mM Tris–HCl pH 7.5, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 10% glycerol] containing 100 mM KCl and dialyzed for ~10 h against 1 l of the same buffer. The dialyzed protein fraction obtained from the 0–50% ammonium sulfate saturation was then applied to a 100-ml bed volume of a phosphocellulose column equilibrated in buffer A + 100 mM KCl. After washing the column with the same buffer, bound proteins were eluted with buffer A + 0.7 M KCl, dialyzed for ~4 h against buffer A + 70 mM KCl, then loaded onto a 25-ml bed volume column of DEAE-cellulose, equilibrated in buffer A + 100 mM KCl. After washing the column with the same buffer, the bound proteins were eluted with buffer A + 300 mM KCl. The eluted proteins were then applied to a 12-ml bed volume column of phosphocellulose equilibrated in buffer A + 300 mM KCl. After washing the column with the same buffer, the bound proteins were eluted with a 96 ml linear gradient of KCl (0.3–1.5 M) in buffer A. Fractions of 3 ml were collected and assayed for eIF5 kinase activity using the assay method B. A single peak of eIF5 kinase activity eluted from the column at a KCl concentration of ~540 mM. Fractions containing the kinase activity were pooled, dialyzed for ~2 h against buffer B (20 mM Tris–HCl pH 7.5, 1 mM DTT, 0.1 mM EDTA and 5% glycerol) containing 100 mM KCl and then applied to a 1-ml bed volume FPLC-Mono Q column (Pharmacia Biotech, Inc.) equilibrated with buffer B + 150 mM KCl. The column was washed with this buffer and absorbed proteins were eluted with a linear gradient (0.5 ml/min) of 25 ml total volume from buffer B + 150 mM KCl to buffer B + 1 M KCl. Fractions of 0.5 ml were collected and assayed for eIF5 kinase activity. A single peak of eIF5 kinase activity emerged from the column at a KCl concentration of ~480 mM. Fractions containing the kinase activity were pooled and concentrated to ~400 µl by Centricon-30 filtration. The concentrated protein sample was layered onto an 11 ml linear 10–30% glycerol gradient containing buffer B + 500 mM KCl and centrifuged in a SW41 rotor for 32 h at 40 000 r.p.m. at 4°C. A set of known molecular weight (M_r) marker proteins was also centrifuged in a parallel tube. Fractions of 0.5 ml were collected from the bottom of the tube and assayed for eIF5 kinase activity. The eIF5 kinase activity sedimented as a single peak of activity corresponding to an M_r position of ~130 000–140 000.

Assay of eIF5 kinase activity

Phosphorylation of eIF5 was carried out in reaction mixtures (20 µl each) containing 20 mM Tris–HCl pH 7.5, 100 mM KCl, 5 mM DTT, 10 mM $MgCl_2$, 2 mM sodium phosphate buffer pH 7.5, 1 mM [γ-^{32}P]ATP (1000 c.p.m./pmol), 100 pmol of purified recombinant rat eIF5 and the indicated amount of eIF5 kinase-containing protein fraction. Following incubation for 10 min at 30°C, the formation of ^{32}P-labeled eIF5 was measured by either assay method A or assay method B as follows. In assay method A, the reactions were terminated by the addition of 20 µl of an electrophoresis loading buffer (250 mM Tris–HCl pH 6.8, 570 mM 2-mercaptoethanol, 2% SDS and 0.01% bromophenol blue) and heating at 100°C for 3 min. The reaction mixtures were subjected to SDS–PAGE (15% gel) and the dried gel was analyzed by autoradiography. A set of marker proteins was run in a parallel lane to determine the molecular weight values of stained and radioactive bands. In assay method B, the reactions were terminated by the addition of 20 µg of bovine serum albumin followed by 5 ml of 5% trichloroacetic acid (TCA) solution containing 1 mM sodium pyrophosphate. After 10 min, the mixtures were filtered through nitrocellulose membrane filters that were pre-soaked in 5% TCA solution containing 1 mM sodium pyrophosphate. The filters were washed with 5% TCA, dried and assayed for radioactivity in a liquid scintillation spectrometer. Control values of ^{32}P bound to filters (<0.1 pmol) in reaction mixtures containing no eIF5 were subtracted to calculate the eIF5 kinase activity. One unit of eIF5 kinase activity was defined as the amount of protein that catalyzes the incorporation of 1 pmol of ^{32}P into eIF5 under the conditions of the assay.

Isolation of maximally phosphorylated eIF5 for mass spectrometric analysis

Two eIF5 kinase reaction mixtures (50 µl each), one containing [γ-^{32}P]ATP (100 c.p.m./pmol) and the other without ATP, were prepared as described above in the 'Assay of eIF5 kinase activity' except that each reaction mixture contained 200 pmol of purified recombinant eIF5 and 0.5 µg of purified kinase. After incubation at 30°C for 30 min, each reaction mixture was loaded onto a 5-ml bed volume of Sephadex G-75 gel filtration column, equilibrated in 100 mM NH_4HCO_3 pH 7.5. The column was developed with the same buffer at room temperature. Fractions of 150 µl were collected. The elution of

eIF5 was monitored by Cerenkov counting and by western blot analysis. Fractions (usually two to three fractions) containing eIF5 were pooled. The yield was >80% of the input eIF5.

Site-directed mutagenesis and purification of recombinant mutant eIF5 proteins

Point mutations within the coding sequence of eIF5 present in the bacterial expression plasmid pGEX-KG-eIF5 were constructed by one-stage PCR using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol. Appropriate 33–36mer mutagenic oligonucleotide primers were designed to change serine at position 174 to an alanine (S174A), serine at position 387 to an alanine (S387A) and serine at position 388 to an alanine (S388A). The above mutations were created while maintaining the reading frame of eIF5. All mutated open reading frames were sequenced to confirm the desired mutations and to ensure error-free DNA synthesis. *Escherichia coli* XLI Blue cells, transformed with recombinant pGEX-KG plasmids containing either the wild-type or mutant eIF5 coding sequences expressing GST–eIF5 fusion proteins, were grown as described previously (19). Untagged wild-type and mutant eIF5 proteins were purified from these cells following the procedures described by Das and Maitra (19).

Phosphorylation of eIF5 in human U_2OS cells

The human U_2OS cells obtained from ATCC were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a 7% CO_2 atmosphere. The coding sequence(s) of the wild-type and mutated eIF5 cloned in pGEM7Z vector were amplified by PCR with *Pyrococcus* DNA polymerase using appropriate primer sequences. Each PCR product was sequenced to ensure error-free DNA synthesis, digested with *Xho*I and *Eco*RI and cloned into the same sites of pcDNA 3.1(+)/*Myc*-HisB-eIF5 expression plasmid. U_2OS cells were transfected with each expression plasmid using Lipofectamine Plus reagent (Gibco BRL). Briefly, the cells were seeded 12 h before transfection at 5×10^6/10 cm diameter plate (60% confluency). Before transfection, the culture medium was changed to serum-free DMEM. Transfections were carried out in five such plates with 20 µg of purified expression plasmid (containing either wild-type or mutated eIF5 coding sequences) per plate. Another set of five plates was transfected with 20 µg of the expression vector, pcDNA 3.1(+)*Myc*-HisB plasmid not containing the eIF5 coding sequence. Five hours post-transfection, the transfection medium was replaced by DMEM containing 10% FBS. Thirty-six hours post-transfection, the cells were washed three times with phosphate-free DMEM medium. The cells were then incubated at 37°C for 30 min in phosphate-free medium, following which 0.5 mCi of ^{32}Pi was added per plate. Cells were harvested 42 h post-transfection and lyzed in a lysis buffer (20 mM Tris–HCl pH 8.3, 0.5 M NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100 and 30 mM ATP) containing a cocktail of protease inhibitors (Complete, Mini EDTA-free, Roche Diagnostic GmbH) and phosphatase inhibitors (10 mM sodium pyrophosphate, 1 mM sodium vanadate, 100 mM sodium fluoride and 50 mM calyculin). The lyzed cells were incubated for 90 min at 4°C with pre-equilibrated anti-*Myc* 9E10 antibody coupled to agarose beads (Mouse Monoclonal, Santa Cruz Biotech). Each immunocomplex was centrifuged at 6000 r.p.m. for 1 min, washed three times with the lysis buffer containing 0.1% bovine serum albumin, boiled in electrophoresis loading buffer and then subjected to SDS–PAGE (15% gel). The resolved polypeptides were then electrophoretically transferred to a Immobilon-P membrane (Millipore). The blot was dried and analyzed by autoradiography. The same membrane blot was used to determine the levels of the immunoprecipitated eIF5 (wild-type and mutant) proteins by western blot analysis. The rabbit polyclonal anti-*Myc* antibody (Santa Cruz Biotech) was used as the primary antibody, while peroxidase-conjugated goat polyclonal anti-rabbit IgG was used as the secondary antibody.

Figure 1. Phosphorylation of eIF5 by protein fractions derived from rabbit reticulocyte lysates. Purified, bacterially expressed eIF5 (5 pmol) was incubated with [γ-^{32}P]ATP and the protein fractions derived from rabbit reticulocyte lysates in assay mixtures similar to that described in the Materials and Methods [Assay of eIF5-kinase activity (method A)]. The protein fractions used as the source of kinase were as follows. (**A**) Lanes: b, ribosomal 0.5 M KCl-wash protein fraction (15 µg); c, 0–50% ammonium sulfate fraction of post-ribosomal supernatant (16 µg); d, 50–80% ammonium sulfate fraction of post-ribosomal supernatant (76 µg). In lane a, eIF5 phosphorylated with ^{32}P by recombinant CK II was used as a marker. (**B**) Phosphorylation of eIF5 with protein fractions obtained by chromatography of the 0–50% ammonium sulfate fraction of the post-ribosomal supernatant as described in the Materials and Methods (Purification of eIF5 kinase activity) and in the text. Lanes: a, flow-through fraction of first phosphocellulose chromatography (1.2 µg); b, 0.7 M phosphocellulose eluate (0.88 µg); c, DEAE-cellulose flow-through fraction of the phosphocellulose eluate (0.6 µg); d, 0.3 M DEAE-cellulose eluate (0.6 µg). Following incubation, the reaction mixtures were subjected to SDS–PAGE followed by autoradiography.

RESULTS

Isolation and initial characterization of an eIF5 protein kinase from rabbit reticulocyte lysates

Rabbit reticulocyte lysates contain a potent protein kinase activity that phosphorylates bacterially expressed recombinant rat eIF5 using ATP as the phosphoryl donor (Fig. 1). Fractionation of the crude lysates into ribosomes and post-ribosomal supernatant showed that eIF5 kinase activity was associated with both ribosomes (Fig. 1A, lane b) and a 0–50% ammonium sulfate fraction of the post-ribosomal supernatant (Fig. 1A, lane c). The 50–80% ammonium sulfate fraction was virtually devoid of any eIF5 kinase activity (Fig. 1A, lane d). We initially purified the eIF5 kinase activity from the 0–50% ammonium sulfate fraction of the post-ribosomal supernatant as this fraction had a much higher total kinase activity than the ribosomal 0.5 M KCl-wash protein fraction. The procedure of purification is outlined in the Materials and Methods. We observed that when the total eIF5 kinase activity present in the 0–50% ammonium sulfate fraction was purified by stepwise elution from successive phosphocellulose and DEAE-cellulose columns using buffers containing 0.7 and 0.3 M KCl, respectively, both the initial phosphocellulose eluate and the subsequent DEAE-cellulose eluate had the eIF5 kinase activity. However, no eIF5 kinase activity was detected in either the phosphocellulose flow-through fractions or the DEAE-cellulose flow-through fractions

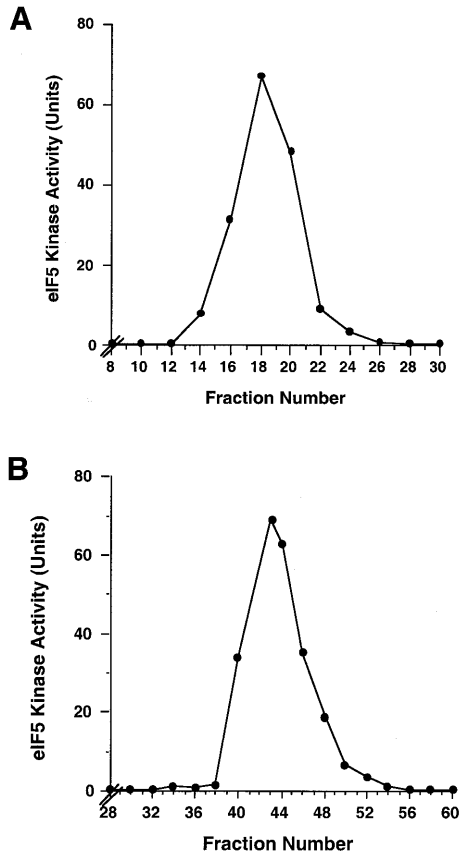

Figure 2. Elution of eIF5 kinase activity from phosphocellulose and FPLC-Mono Q columns. (**A**) Phosphocellulose chromatography of the 0.3 M DEAE-cellulose eluate. The pooled DEAE-cellulose 0.3 M eluate (11.3 mg protein), containing all the eIF5-kinase activity, was loaded onto a phosphocellulose column (12-ml bed volume) as described in the Materials and Methods (Purification of eIF5 kinase activity). Fractions of 3 ml were collected and assayed for eIF5 kinase activity using the assay method B as described in the Materials and Methods. (**B**) FPLC-Mono Q chromatography of the phosphocellulose protein fraction. The phosphocellulose eIF5 kinase fraction (150 U) was loaded onto a 1-ml bed volume of FPLC-Mono Q column as described in the Materials and Methods. Fractions of 0.5 ml were collected and assayed for eIF5 kinase activity.

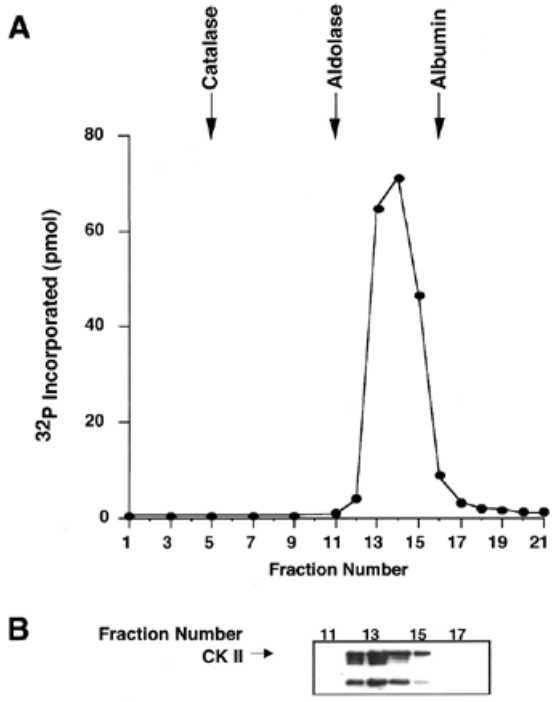

Figure 3. Glycerol gradient centrifugation of eIF5 kinase activity. (**A**) The eIF5 kinase activity recovered from the FPLC-Mono Q column was concentrated to 400 µl by Centricon-30 filtration and layered onto a 10–30% (v/v) linear glycerol gradient (11 ml) as described in the Materials and Methods (Purification of eIF5 kinase activity). A marker gradient containing catalase, aldolase and albumin (2 mg each) was run in a parallel tube. The gradients were centrifuged in a SW41 rotor for 32 h at 40 000 r.p.m. at 4°C. Fractions (0.5 ml) were collected from the bottom of the tube and assayed for eIF5 kinase activity using the assay method B as described in the Materials and Methods. The sedimentation positions of the marker proteins are indicated. (**B**) The glycerol gradient fractions 11 through to 17 corresponding to eIF5 kinase activity (20 µl each) were analyzed by SDS–PAGE followed by immunoblotting with anti-CK II antibody.

(Fig. 1B, compare lanes a and b and lanes c and d). Further purification of the DEAE 0.3 M KCl eluate by gradient elution from a phosphocellulose column chromatography showed that eIF5 kinase activity eluted from the column in a single symmetrical peak (Fig. 2A). When the pooled phosphocellulose eluate was purified by chromatography on an FPLC-Mono Q column, all the detectable eIF5 kinase activity again eluted from the column in a single peak of activity (Fig. 2B). Such FPLC-Mono-Q-purified eIF5 kinase preparations sedimented in glycerol gradient centrifugation as a protein of ~130–140 kDa (Fig. 3A).

When the 0.5 M KCl-wash protein fraction of ribosomes was subjected to a similar purification procedure as described for the 0–50% ammonium sulfate fraction, similar elution profiles of the eIF5 kinase activity were observed at all stages of purification (data not shown). Furthermore, the sedimentation profile of the protein kinase, purified from the ribosomal salt-wash proteins, in glycerol gradient centrifugation was similar to the eIF5 kinase activity purified from the post-ribosomal supernatant fraction (data not shown). The results suggest that the eIF5 kinase activity present in the post-ribosomal supernatant and that associated with ribosomes is the same protein.

The purified eIF5 kinase is CK II

When the pooled glycerol gradient fractions containing a major fraction of eIF5 kinase activity (Fig. 3) were subjected to SDS–PAGE (15% gel) and stained by Coomasie Blue, we observed two major polypeptides of apparent M_r = 43 000 and 25 000 (data not shown). The apparent molecular weight of the purified kinase (M_r = ~140 000) and the presence of two polypeptides of M_r = 43 000 and 25 000 comprising the native protein are similar to those reported previously (21–23) for purified mammalian CK II. To confirm that the purified eIF5 kinase was indeed CK II, we first carried out western blot analysis of the glycerol gradient fractions shown in Figure 3 using polyclonal anti-bovine CK II antibodies as probes. Strong immunoreactive polypeptide bands corresponding to the subunits of mammalian CK II were found to co-migrate with eIF5 kinase activity (Fig. 3B). Additional evidence (data not shown) that the purified eIF5 kinase is CK II came from the following observations: (i) eIF5 kinase activity was nearly totally inhibited if 1 nM heparin was included in the kinase reaction. (ii) The purified kinase utilized either ATP or GTP as the phosphoryl donor in

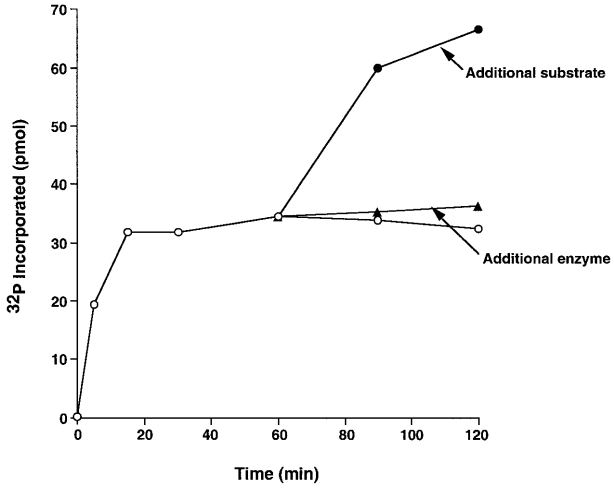

Figure 4. Time course of eIF5 kinase activity. Reaction mixtures (20 ml) were prepared as described in the Materials and Methods except that 200 pmol of eIF5 and 0.5 µg of purified eIF5 kinase were added. Incubation was at 30°C. At the indicated times, aliquots (2 µl) were withdrawn and assayed for ^{32}P incorporation into eIF5 using the assay method B. After 30 min of incubation, one series of tubes (indicated by 'additional enzyme') received an additional 0.5 µg of purified CK II, while another series (indicated by 'additional substrate') received an additional 200 pmol of eIF5 and incubation at 30°C was continued. At indicated times, 2 µl aliquots were withdrawn and assayed for ^{32}P incorporation into eIF5 by assay method B. Open circles denote no further addition at 30 min, closed triangles denote additional 0.5 µg of CK II added at 30 min and closed circles denote additional 200 pmol of eIF5 added at 30 min. It should be noted that the results shown in the figure represent ^{32}P incorporated into 2 µl reaction aliquot containing 20 pmol of eIF5.

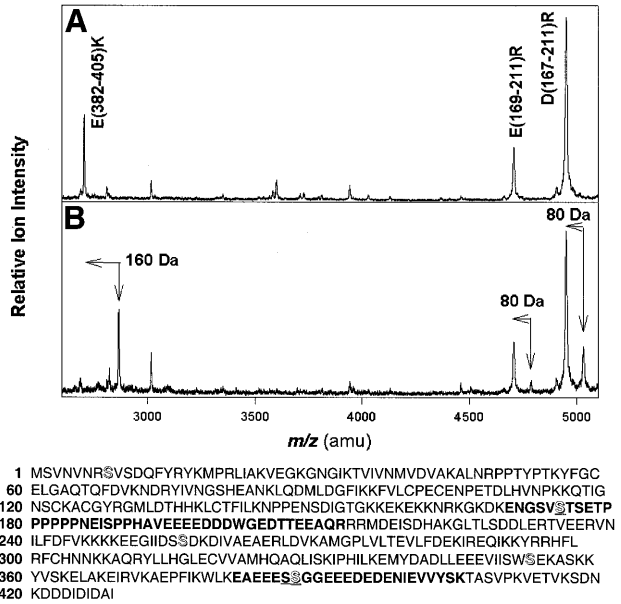

Figure 5. MALDI-TOF mass spectra of tryptic peptides of unphosphorylated and phosphorylated eIF5. Unphosphorylated eIF5 (**A**) and phosphorylated eIF5 (**B**) were digested in solution with trypsin and pepsin and analyzed by MALDI-TOF without further separation. The tryptic peptides were labeled in (A) and their respective phosphorylated products were observed in (B) with mass differences of 80 or 160 Da. The vertical arrows represent the mass of the phosphorylated peptides while the horizontal arrows represent the mass shift from the unphosphorylated peptide. (**C**) The derived amino acid sequence of rat eIF5 taken from Si *et al.* (26). All the potential serine phosphorylation sites are shadowed. The serine residues at positions 174, 387 and 388 that are phosphorylated *in vitro* are underlined.

phosphorylating eIF5. (iii) Incubation of purified eIF5 kinase with [γ-^{32}P]ATP resulted in the autophosphorylation of the 25 kDa subunit of the purified kinase. These properties of purified eIF5 kinase are similar to those reported previously for purified mammalian CK II (23) and suggest that the eIF5 kinase purified from the post-ribosomal supernatant is CK II.

Identification of the serine residues of eIF5 phosphorylated *in vitro* by eIF5 kinase (CK II)

Phosphoamino acid analysis of eIF5 phosphorylated by purified eIF5 kinase showed that serine was the only amino acid residue phosphorylated in eIF5 (data not shown). The time course for phosphate incorporation into purified eIF5 is shown in Figure 4. Under the conditions of the assay system used, the maximum level of phosphate incorporation (1.6 pmol of ^{32}P/pmol of eIF5 protein) was achieved in ~15 min. When incorporation of ^{32}P ceased, the addition of more enzyme had no effect. However, the addition of more eIF5 substrate resulted in a resumption of ^{32}P incorporation until an additional incorporation of 1.5 pmol/pmol of eIF5 protein was achieved (Fig. 4). The results suggest that there are two major phosphorylation sites in eIF5.

To identify the serine residues in eIF5 that are phosphorylated *in vitro* by purified eIF5 kinase (CK II), eIF5 was maximally phosphorylated *in vitro* by the purified kinase, and ^{32}P-labeled eIF5 was isolated free of unreacted reaction components by Sephadex gel filtration as described in the Materials and Methods. Both the maximally phosphorylated and the unphosphorylated eIF5 (20 pmol each) were digested completely with trypsin and pepsin following the established protocol. The digestion products were analyzed by MALDI-TOF to identify phosphopeptides with a PerSeptive MALDI-TOF DE-STR mass spectrometer. Nano-electrospray tandem mass spectrometry was used to probe phosphorylation sites in the identified phosphopeptide with a Finnigan LCQ mass spectrometer. Three distinct peptide masses were found at *m/z* 2864, 4784 and 5027 by MALDI-TOF analysis of the tryptic digestion of phosphorylated eIF5 versus unphosphorylated eIF5 (Fig. 5). The mass at *m/z* 2864 corresponds to the addition of 160 Da to peptide E382-K405 of eIF5 at *m/z* 2704 Da. The MALDI-PSD (post-source decay) analysis of this peptide at *m/z* 2864 shows two consecutive losses of 98 Da that indicate the presence of two phosphorous groups in this peptide. The possible phosphorylation sites are Ser-387 and Ser-388 as predicted from the known phosphorylation motif SXX(E/D) for CK II. However, the peptide ion E382-K405 was not observed in the mass spectrum from digestion of phosphorylated eIF5, which suggested that there was complete conversion of the E382-K405 peptide of eIF5 to its diphosphorylated counterpart after 30 min of incubation of eIF5 with CK II. The masses at *m/z* 4784 and 5027 correspond to the addition of a phosphorous group onto peptides E169-R211 and D167-R211, respectively. The intensity ratio of *m/z* 4704 to 4784 is about 4 to 1. The MS/MS analysis of the phosphorylated peptide E169-R211 using nano-ESI ion trap mass spectrometer indicated that residue Ser-174 was the phosphorylated site in this peptide, which is predicted from the known phosphorylation motif SXX(E/D) for CK II. However, unlike the peptide E382-K405,

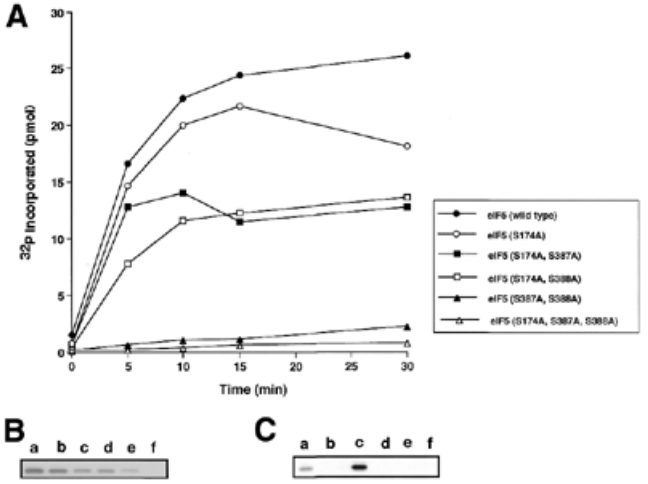

Figure 6. Analysis of eIF5 point mutations (Ser → Ala) for their ability to be phosphorylated *in vitro*. Reaction mixtures (80 µl each) containing 200 pmol of purified wild-type or mutant eIF5 proteins and 0.5 µg of purified eIF5 kinase were incubated at 30°C. At the indicated times, aliquots (5 µl) were withdrawn and assayed for ^{32}P incorporation using either assay method B (**A**) or assay method A (**B**) as described in the Materials and Methods. It should be noted that the results shown in (A) represent ^{32}P incorporated into 5 µl reaction aliquots containing 12.5 pmol of eIF5. (B) Lanes: a, wild-type eIF5; b, mutant eIF5 (S174A); c, mutant eIF5 (S174A, S387A); d, mutant eIF5 (S174A, S388A); e, mutant eIF5 (S387A, S388A); f, mutant eIF5 (S174A, S387A, S388A). (**C**) Wild-type or mutant (S174A, S387A, S388A) eIF5 proteins were incubated at 30°C with ribosomal 0.5 M KCl-wash fractions (lanes a and b, respectively) or with 0–50% ammonium sulfate fraction of the post-ribosomal supernatant (lanes c and d, respectively) or 50–80% ammonium sulfate fraction of the post-ribosomal supernatant (lanes e and f, respectively) under the conditions of eIF5 kinase assay as described in the Materials and Methods. After incubation at 30°C for 10 min, a 10 µl aliquot of each reaction mixture was subjected to SDS–PAGE followed by autoradiography of the dried gel.

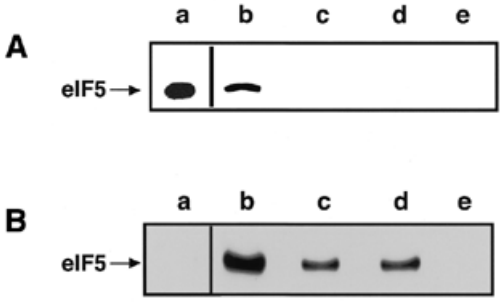

Figure 7. Phosphorylation of eIF5 in mammalian cells. Human U_2OS cells were transfected with eIF5 expression plasmids, pc DNA 3.1(+)*Myc*-HisB, containing either the wild-type eIF5 coding sequence or the mutant eIF5 coding sequence or with empty control vector. Following vector transfection, the cells were metabolically labeled with ^{32}Pi as described in the Materials and Methods. eIF5 present in each cell lysate was immunoprecipitated with anti-Myc antibody and the washed immunocomplex was subjected to SDS–PAGE followed by electrophoretic transfer to a PVDF membrane. (**A**) The washed membrane blot was subjected to autoradiography. (**B**) The same membrane blot was subjected to immunoblot analysis using anti-Myc antibodies. The eIF5 expression plasmids used were as follows. Lanes: b, wild-type eIF5; c, mutant eIF5 (S387A, S388A); d, mutant eIF5 (S174A, S387A, S388A); e, control vector not containing the eIF5 coding sequence. In lane a, the wild-type, purified rat eIF5 protein (untagged), phosphorylated *in vitro* by purified CK II, was electrophoresed as a marker.

only ~20% of the E169-R211 peptide was phosphorylated at Ser-174.

To verify that Ser-387, Ser-388 and Ser-174 are indeed sites of *in vitro* eIF5 phosphorylation, we carried out site-directed mutagenesis in the coding sequence of eIF5-cDNA such that each of these serine residues, either singly or in combination, was converted to alanine in the bacterially expressed eIF5 protein. The purified mutant eIF5 proteins were then used as substrates for *in vitro* phosphorylation by purified eIF5 kinase (CK II). As shown in Figure 6, mutation of Ser-174 to Ala-174 reduced the incorporation of ^{32}P into eIF5 by only ~15–20%. However, when this mutation was combined with mutation at either Ser-387 or Ser-388, the extent of incorporation of ^{32}P into each double mutant, eIF5 (S174A, S387A) or eIF5 (S174A, S388A), was reduced by ~50% as compared with wild-type eIF5 protein (Fig. 6). In contrast, when the eIF5 double mutant (S387A, S388A) was used as a substrate, there was nearly a 90% reduction in the extent of *in vitro* phosphorylation of eIF5 (Fig. 6). When all three serine residues were mutated to alanine there was virtually no incorporation of ^{32}P into eIF5 (Fig. 6). The results were confirmed by subjecting aliquots of each *in vitro* phosphorylation reaction mixture to SDS–PAGE followed by autoradiography of the dried gel (Fig. 6B). Taken together, the results confirm the mass spectrometric analysis and indicate that Ser-387 and Ser-388 are the major *in vitro* phosphorylation sites in eIF5 whereas Ser-174 is a relatively minor phosphorylation site.

We also investigated whether, in addition to these three CK II phosphorylation sites, there are any other sites in eIF5 that are phosphorylated by any other protein kinase present in crude reticulocyte lysates. For this purpose, both the wild-type eIF5 and the triple eIF5 mutant (S174A, S387A, S388A) were incubated with either crude reticulocyte lysates or the protein fractions derived from the crude lysates as the source of protein kinase using [γ-^{32}P]ATP as the phosphoryl donor. As shown in Figure 6C, while the wild-type eIF5 was readily phosphorylated by the rabbit reticulocyte 0–50% ammonium sulfate fraction of the post-ribosomal supernatant, the mutant eIF5 was not phosphorylated by this protein fraction (Fig. 6C, compare lanes c and d). Additionally, we observed that mutant eIF5 was also not phosphorylated by the eIF5 kinase present in the ribosomal 0.5 M KCl-wash proteins (Fig. 6C, compare lanes a and b) indicating that the kinase responsible for phosphorylating eIF5 in the ribosomal salt-wash protein fraction is also CK II. The 50–80% ammonium sulfate fraction did not contain any eIF5 kinase activity (Fig. 6C, lanes e and f).

Phosphorylation of eIF5 *in vivo*

To examine whether eIF5 is phosphorylated *in vivo* in mammalian cells at the same serine residues that are phosphorylated *in vitro* by CK II, we metabolically ^{32}P-labeled both the wild-type and the mutant eIF5, tagged with Myc at the C-terminus, in human osteosarcoma (U_2OS) cells as described in the Materials and Methods. eIF5 present in each cell lysate was immunoprecipitated with anti-Myc antibodies and the washed immunocomplex was subjected to SDS–PAGE followed by autoradiography (Fig. 7A). In the lysates of cells where wild-type eIF5 was expressed, a ^{32}P-labeled polypeptide band was observed that migrated with the same mobility as purified eIF5 labeled with ^{32}P *in vitro* by CK II (Fig. 7A, compare lane b with

a). In contrast, in lysates of cells where the eIF5 mutant (S387A, S388A) or the mutant (S387A, S388A, S174A) was expressed, no ^{32}P-labeled eIF5 was immunoprecipitated (Fig. 7, lanes c and d). When U_2OS cells were transfected with vectors not containing the eIF5 coding sequence and the cell lysates were treated the same way, no ^{32}P-labeled polypeptide band was detected in the autoradiogram (Fig. 7A, lane e). A western blot analysis of the same blot using anti-Myc antibody revealed that both the wild-type eIF5 and the mutant eIF5 proteins were expressed in the transfected cells (Fig. 7B). It may be noted that although the expression of the mutant eIF5 proteins was somewhat lower than that of the wild-type protein, this cannot account for the total lack of phosphorylation of the mutant proteins. No phosphorylation of the mutant proteins could be detected even after long periods of exposure. Furthermore, the relative intensities of the bands in Figure 7A and B were quantified using NIH Image 1.61 and ImageQuant 5.0 software, respectively. We observed that the ratio of the mutant proteins, eIF5 (S387A, S388A) and eIF5 (S174A, S387A, S388A), to wild-type eIF5 in Figure 7B was 0.29 and 0.35, respectively. In contrast, in Figure 7A, the intensity of each band of mutant forms of eIF5 was only ~0.06% of that of wild-type eIF5. Taken together, the results show that eIF5 is phosphorylated *in vivo* in mammalian cells at the same serine residues that are also phosphorylated *in vitro* by CK II.

Effect of phosphorylation of recombinant eIF5 on its *in vitro* activity

In functional studies we have analyzed the effect of the phosphorylation of recombinant eIF5 on its *in vitro* activity in promoting the joining of the 60S ribosomal subunit to the 40S initiation complex formed with AUG codon as mRNA (40S•AUG•Met-tRNA$_f$•eIF2•GTP). It is well established (8) that in such a simplified model *in vitro* translation initiation system, the sole function of eIF5 is to interact with the 40S initiation complex and to act as a GTPase activating protein (GAP) to promote the hydrolysis of bound GTP. However, once GTP hydrolysis has occurred and the 40S subunit-bound eIF2 is released, subsequent joining of the 60S ribosomal subunit to the 40S complex does not require the participation of eIF5 (9). It has been shown that eIF5 acts catalytically in this overall reaction (8).

In agreement with these previous reports, we observed that when a preformed 40S initiation complex (40S•AUG•Met-tRNA$_f$•eIF2•GTP) (2.2 pmol) was incubated with 1.2 A_{260} units of 60S ribosomal subunits and only 0.1 pmol (5 ng) of unphosphorylated recombinant eIF5 under conditions described in legend to Figure 8, the entire 40S initiation complex was nearly quantitatively converted to the 80S initiation complex (data not shown). Phosphorylation of eIF5 had no effect on its activity when tested under similar assay conditions (data not shown). In contrast, when we used a very low concentration of eIF5 in the reaction mixture (0.01 pmol), such that only ~25% of the added 40S initiation complex was converted to the 80S initiation complex, phosphorylation of eIF5 stimulated formation of the 80S initiation complex by ~20 ± 5% (Fig. 8). The results suggest that phosphorylation of eIF5 may have a role in stimulating the rate of eIF5-promoted GTP hydrolysis. However, additional work using an efficient mRNA-dependent *in vitro* translation initiation system will be necessary to address this issue in greater detail.

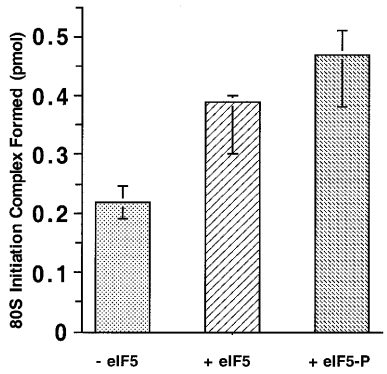

Figure 8. Effect of phosphorylation of eIF5 on its ability to form the 80S initiation complex. Reaction mixtures (50 µl) containing 20 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 100 mM KCl and 1 mM DTT (buffer R), 3 pmol of preformed [^{35}S] Met-tRNA$_f$•eIF2•GTP ternary complex (42 000 c.p.m./pmol), 1.2 A_{260} units of 40S ribosomal subunits and 0.1 A_{260} unit of the AUG codon were incubated for 5 min at 37°C to form the 40S initiation complex (40S•AUG•Met-tRNA$_f$•eIF2•GTP). The chilled reaction mixtures were supplemented with 1.2 A_{260} units of 60S ribosomal subunits and 0.01 pmol of purified unphosphorylated or completely phosphorylated recombinant rat eIF5 as indicated. Following incubation at 37°C for 5 min, the chilled reaction mixtures were sedimented through a 5 ml linear 7.5–30% (w/v) sucrose density gradient in buffer R for 105 min at 48 000 r.p.m. at 4°C in a Beckman SW 50.1 rotor. Fractions (0.4 ml) collected from the bottom of each tube were counted for ^{35}S radioactivity to quantify the formation of the 80S initiation complex.

eIF5 is also known to form a complex with eIF2 by interacting with the β subunit of eIF2 (24,25). This interaction has been shown to play an essential role in the GAP function of eIF5 (19). We observed that in a binding assay similar to that described previously (19,26), both phosphorylated and unphosphorylated eIF5-bound eIF2β with nearly the same efficiency (data not shown).

DISCUSSION

Evidence presented in a previous report from this laboratory showed that eIF5, an essential protein in translation initiation in eukaryotic cells (8), is constitutively phosphorylated at serine residues in mammalian cells (2). Phosphopeptide mapping of ^{32}P-labeled eIF5 isolated from rat pituitary GH3 cells showed two major sites of phosphorylation (2). However, the protein kinase responsible for phosphorylation of eIF5 was not identified.

In the work presented in this paper, we report the isolation of a protein kinase from rabbit reticulocyte lysates that phosphorylates purified, bacterially expressed, recombinant rat eIF5. Physical, biochemical and antigenic properties of the purified kinase identify it as CK II.

Analysis of the amino acid sequence of rat eIF5 (26) indicates that there are potentially five CK II phosphorylation sites located at Ser-8, Ser-174, Ser-257, Ser-353 and Ser-388. However, mass spectrometric analysis of maximally phosphorylated eIF5 shows that Ser-387 and Ser-388 are the predominant sites of phosphorylation, accounting for >90% of the total phosphorylation of eIF5 *in vitro*. These sites are presumably also phosphorylated *in vivo* in mammalian cells as mutation of Ser-387 and Ser-388 to alanine residues abolishes phosphorylation of eIF5 not only *in vitro* but also *in vivo*.

It should, however, be noted that while the amino acid sequence surrounding Ser-388 conforms to the general features of a CK II consensus sequence [(S/T)XX(D/E)], the Ser-387 residue does not conform precisely with the CK II consensus sequence as it lacks an acidic amino acid residue at the Ser-387$_{n+3}$ position (see Fig. 5C). However, both Ser-387 and Ser-388 are embedded in a cluster of acidic amino acid residues at both the N-terminal and the C-terminal side of the serine residues (the sequence of the peptide containing these serine residues is ^{378}KWLKEAEEES*S*GGEEEDEDENIEVVYSK^{405}, with serine at positions 387 and 388 marked with asterisks) presumably making these serine residues efficient targets for phosphorylation by CK II. Furthermore, while inefficient phosphorylation at Ser-174 was also observed *in vitro*, this site may not be utilized *in vivo* since mutation of eIF5 at Ser-387 and Ser-388 to alanine residues without altering Ser-174 abolishes eIF5 phosphorylation in mammalian cells. Additionally, mutant eIF5 (S387A, S388A) is not phosphorylated *in vitro* when crude reticulocyte lysate protein fractions were used as the source of protein kinase (data not shown).

eIF5 acts at a critical step in translation initiation. Following selection of the AUG codon of mRNA by the 40S pre-initiation complex, eIF5-promoted hydrolysis of GTP bound to the 40S initiation complex is a stringent prerequisite for the subsequent joining of the 60S ribosomal subunit to the 40S complex to form an elongation-competent 80S initiation complex (6–8). If GTP hydrolysis is prevented the 60S ribosomal subunit cannot join the 40S initiation complex, thus inhibiting the conversion of the ribosomal initiation complex from the initiation to the elongation mode of protein synthesis. The possibility exists, therefore, that modulation of eIF5 activity by phosphorylation of the initiation factor may be utilized by the cellular translation machinery to regulate the conversion of the 40S initiation complex to the 80S initiation complex. Although we have observed that phosphorylation of eIF5 had only a marginal stimulatory effect on the function of eIF5 *in vitro* when tested in a model simplified AUG-dependent assay system generally used to assay for eIF5 activity in our laboratory (Fig. 8), it is possible that eIF5 phosphorylation has some significant role in translation initiation and that we have not identified conditions where phosphorylation of eIF5 at these sites is important. In this context it is interesting to note that phosphorylation of eIF5 occurs near the C-terminus of the protein, which is the region that has been shown to be involved in making contact with eIF2 (19,25). Such an interaction between eIF5 and eIF2β has been shown to be essential for eIF5-promoted GTPase activity (19). Furthermore, in addition to its interaction with eIF2, eIF5 also interacts with eIF3 as well as with the eIF4G subunit of eIF4F through its C-terminus (27). Additionally, recent work in the yeast *S.cerevisiae* has shown that eIF5 is a component of the multi-factorial protein complex (28) that may be important for coordinating eIF5-promoted GTP hydrolysis to the AUG selection process (10). Thus, the possibility exists that the eIF5-promoted hydrolysis of GTP coupled to the AUG selection process may be modulated by phosphorylation of the initiation factor. Further work using an mRNA-dependent total *in vitro* translation initiation system is necessary to answer this question. Pestova *et al.* (29) have recently described an eIF5-dependent complete translation initiation system. It will be necessary to analyze the system with respect to both the efficiency of the 80S initiation complex formation as well as the absence of protein phosphatases in the initiation factor preparations before the system can be used to investigate the effect of phosphorylation of eIF5 on its activity. It should also be noted that the yeast *S.cerevisiae* eIF5 also has five potential CK II phosphorylation sites (26), three of which are at similar positions to rat eIF5 including Ser-388. It will be of considerable interest to investigate whether yeast eIF5 is phosphorylated *in vivo* at any of these CK II phosphorylation sites and whether phosphorylation of eIF5 has any obvious growth defects.

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