Further Characterization of Eukaryotic Initiation Factor 5 from Rabbit Reticulocytes

IMMUNOCHEMICAL CHARACTERIZATION AND PHOSPHORYLATION BY CASEIN KINASE II

(Received for publication, September 28, 1988)

Sankar Ghosh, Jorge Chevesich, and Umadas Maitra
From the Department of Developmental Biology and Cancer, Division of Biology, Albert Einstein College of Medicine, Bronx, New York 10467

Eukaryotic initiation factor 5 (eIF-5), isolated from rabbit reticulocyte lysates, is a monomeric protein of $M_r = 58,000 - 62,000$. Immunochemical methods were employed to identify eIF-5 in crude cell lysates. Antibodies against purified denatured eIF-5 were prepared in rabbits and characterized by immunoblotting and immunoprecipitation techniques using native and denatured eIF-5 as antigens. Monospecific antibodies to denatured eIF-5 were affinity-purified using eIF-5 blotted onto aminophenylthioether paper. Rabbit reticulocytes, HeLa cells and mouse L cells were lysed directly into a denaturing buffer containing 3% sodium dodecyl sulfate. The denatured proteins were analyzed by polyacrylamide gel electrophoresis followed by immunoblotting with anti-eIF-5 antibodies. With each lysate, one major immunoreactive polypeptide was observed whose molecular weight corresponded to that of purified eIF-5 ($M_r = 58,000 - 62,000$). No degradation products or precursor forms of molecular weight higher than 62,000 were detected in any lysate. These results indicate that isolated eIF-5 is the same size as that found in crude lysates. Additional characterization of eIF-5 indicates that purified eIF-5 can be phosphorylated at serine residues in vitro by casein kinase II. Furthermore, in vitro phosphorylated eIF-5 retains full biological activity in catalyzing the joining of 60 S ribosomal subunits to a preformed 40 S ribosomal initiation complex to form an 80 S initiation complex. Based on its specific activity, we demonstrate that 1 pmol of rabbit reticulocyte eIF-5 mediates the formation of approximately 180 pmol of 80 S initiation complex under the conditions of in vitro initiation reactions.

Eukaryotic initiation factor 5 (eIF-5) catalyzes the hydrolysis of GTP bound to a 40 S ribosomal polypeptide chain initiation complex with the simultaneous joining of a 60 S ribosomal subunit to form an 80 S ribosomal polypeptide chain initiation complex (for a review, see Refs. 1–3). In our laboratory, using an assay that directly measures the formation of an 80 S polypeptide chain initiation complex from a preformed 40 S initiation complex, eIF-5 was purified to apparent electrophoretic homogeneity from the ribosomal salt-wash proteins of calf liver and rabbit reticulocyte lysates (4, 5). The homogeneous factor, isolated from these mammalian cell extracts, prepared in the presence of a large number of protease inhibitors, is a monomeric protein of apparent $M_r = 58,000 - 62,000$. These results were in contrast to reports from several laboratories (6–9) suggesting that eIF-5 purified from rabbit reticulocyte lysates is a protein of much higher molecular weight ranging from 120,000 to 168,000 (6–9). In one report (9), eIF-5 was isolated in multiple forms which appeared to be generated by limited proteolysis. In recent review articles, the factor was listed as a protein of $M_r = 150,000$ (10) or $120,000$ (11). We have consistently observed that, although homogeneous, eIF-5 behaves as a monomeric protein of apparent $M_r = 58,000 - 62,000$, less pure preparations of reticulocyte eIF-5 behave in gel filtration columns and in glycerol gradient centrifugation in buffers containing 75–100 mM KCl as a protein of apparent $M_r = 140,000 - 160,000$ (4, 5). Presumably, this is due to association of eIF-5 with other proteins, since eIF-5 activity present in such preparations was shown by (a) glycerol gradient centrifugation in buffers containing 500 mM KCl or (b) elution of protein from gel slices following electrophoresis under denaturing conditions, to be associated with a $58,000 - 62,000$-dalton protein (4, 5).

To obtain further evidence concerning the in vivo form of the initiation factor, we describe here the preparation and characterization of rabbit antisera specific for rabbit reticulocyte eIF-5. We have used affinity-purified anti-eIF-5 antibodies to show that the molecular size of eIF-5 in cells lysed rapidly in denaturing buffers containing high concentrations of SDS is similar to that of homogeneous eIF-5 isolated from rabbit reticulocyte lysates. Furthermore, we have carried out additional characterization of purified eIF-5 with regard to phosphorylation of the protein by purified casein kinase II and the effect of phosphorylation on the activity of eIF-5 in vitro. A preliminary report of this work has been presented (12).

EXPERIMENTAL PROCEDURES

Materials—Pepstatin and leupeptin were purchased from Boehringer Mannheim and phenylmethylsulfonyl fluoride from Sigma. [35S]Met-tRNA was prepared by aminoacylating total rabbit liver tRNA with [35S]Methionine (20,000–30,000 cpm/pmol) by an Escherichia coli synthetase preparation as described previously (13). Ribosomal subunits were obtained from Amersham, Corp. Goat anti-rabbit reticulocyte eIF-5 antibodies were generously provided by Dr. Rosemary Jagus of the University of Pittsburgh. These antibodies reacted strongly with the $\beta$ and $\alpha$ subunits of eIF-2, but did not react with the $\gamma$ subunit.
Characterization of Eukaryotic Initiation Factor 5

Enzymes and Initiation Factors—Rabbit reticulocyte lysates, obtained from Green Hectares Company, were used for isolation of casein kinase II and initiation factors. These lysates were initially treated with phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.5 µg/ml), and pepstatin (1 µg/ml) prior to use for isolation of the protein factors. In addition, all buffer solutions used for isolation of proteins contained 0.5 mM phenylmethylsulfonyl fluoride. Casein kinase II and initiation factors were isolated from the post-ribosomal supernatant of rabbit reticulocyte lysates as described by Hataway et al. (15) using the assay system described by these workers. The purified preparation exhibited two major polypeptide bands of M, = 43,000 and 25,000 and two minor bands of M, = 38,000 and 35,000 upon SDS-polyacrylamide (15%) gel electrophoresis followed by sensitive silver staining (16). The preparation was stored at −20°C in a buffer containing 20 mM Tris-HCl, pH 7.5, 190 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 55% glycerol. The procedure for isolation of homogeneous eIF-5 from rabbit reticulocyte lysates was described (6). In addition to homogeneous eIF-5, a partially purified “eIF-2/eIF-5 preparation,” designated “CM-Sephadex eIF-5,” was also isolated by chromatography of the 0.5 M KCl-wash proteins of polysomal pellets obtained from rabbit reticulocyte lysates on a DEAE-cellulose column followed by phosphocellulose and CM-Sephadex chromatography as described (5). Analysis of this eIF-2/eIF-5 preparation was performed by elution of the antibody from the Sephadex G-75 gel filtration, casein kinase II and appropriate amounts of the substrate eIF-2 or eIF-5 as indicated. Following incubation at 30°C for 45 min, the reactions were terminated by the addition of 20% of an electrophoresis loading buffer (250 mM Tris-HCl, pH 6.8, 2% SDS, 570 mM β-mercaptoethanol, and 0.01% bromphenol blue) and heating for 3 min at 100°C. After standing at room temperature for 10–20 min, the reaction mixtures were electrophoresed in 15% polyacrylamide, 0.09% bisacrylamide gel at pH 8.8 in the presence of 0.1% SDS at 110 V for 3 h according to Schreier et al. (7). The gels were stained with silver reagents according to Merrill et al. (16), and autoradiograms of the dried gel were obtained. Marker proteins were run in parallel lanes to determine the Mo values of stained and radioactive bands.

Affinity Purification of Antibodies—Antibodies specific for eIF-5 or the β subunit of eIF-2 were purified by elution of the antibody from immunoblots, as described by Olmsted (22). About 60 µg of the CM-Sephadex eIF-2/eIF-5 fraction was phosphorylated by casein kinase II and γ-[32P]ATP and subjected to electrophoresis in a SDS-15% polyacrylamide gel. Casein kinase II phosphorylates both eIF-5 (this paper) and the β subunit of eIF-2 (23–28). Homogeneous eIF-5 and homogeneous eIF-2 (containing the α, β, and γ subunits) were also phosphorylated by casein kinase II and run in parallel lanes on the same gel. Following electrophoresis, the proteins were electrotransferred onto aminoethylthioether paper in transfer buffer containing 25 mM sodium phosphate, pH 6.5. The paper was then incubated in 10% ethanolamine, 1% bovine serum albumin in 10 mM Tris-HCl, pH 8.8, for 2 h at room temperature to block all nonspecific sites and then washed with TBS. To locate the band of interest, the blot was air-dried and exposed to Kodak X-Omat AR film. Using the bands corresponding to eIF-5 and eIF-2 (β) as markers, the regions (2 mm wide) corresponding to eIF-5 and eIF-2 (β) were excised and cut into small pieces (2 × 2 mm). The pieces were then incubated with a 1:10 dilution of the appropriate antisera for 6-12 h at 4°C (for eIF-5 and eIF-2, respectively). Homogeneous eIF-2 containing α, β, and γ subunits was isolated by an adaptation of the procedure of Schreier et al. (7) except that CM-Sephadex was used instead of CM-cellulose. The specific activity of homogeneous eIF-5 is about 3,300 and 130,000 units/mg protein, respectively. Homogeneous eIF-2 containing the α, β, and γ subunits of eIF-2 while a fourth, "CM-Sephadex eIF-5," was also prepared by elution of the antibody from CM-Sephadex G-75. The purified preparation was stored at −20°C in a buffer containing 10% ethanolamine, 1% bovine serum albumin, 10 mM Tris-HCl, 1 mM MgCl2, 5 mM MgCl2, 150 µM [γ-32P]ATP (2,000-20,000 cpm/pmol), 0.5 µg of purified casein kinase II, and appropriate amounts of the substrate eIF-2 or eIF-5 as indicated. Following incubation at 30°C for 45 min, the reactions were terminated by the addition of 20% of an electrophoresis loading buffer (250 mM Tris-HCl, pH 6.8, 2% SDS, 570 mM β-mercaptoethanol, and 0.01% bromphenol blue) and heating for 3 min at 100°C. After standing at room temperature for 10–20 min, the reaction mixtures were electrophoresed in 15% polyacrylamide, 0.09% bisacrylamide gel at pH 8.8 in the presence of 0.1% SDS at 110 V for 3 h according to Schreier et al. (7). The gels were stained with silver reagents according to Merrill et al. (16), and autoradiograms of the dried gel were obtained. Marker proteins were run in parallel lanes to determine the Mo values of stained and radioactive bands.

RESULTS

Purity, Specific Activity, and Molecular Weight of eIF-5—Purified eIF-5 was isolated from rabbit reticulocyte lysates by procedures similar to those described previously in this laboratory (5). Analysis of the final eIF-5 preparation by polyacrylamide gel electrophoresis in the presence of SDS followed by staining with silver reagents according to Merrill et al. (16) displayed a single band of M, = 58,000 (Fig. 1, Panel A, lane α; see also Ref. 5). When the eIF-5 preparation was subjected to (a) glycerol gradient centrifugation and (b) Sephadex G-75 gel filtration, eIF-5 activity was found to be associated with a protein of apparent Mo, of about 58,000–62,000 as described previously (5). Furthermore, in agreement with our earlier results (5), when purified eIF-5 was subjected to SDS-polyacrylamide gel electrophoresis and the separated proteins reconstituted, all the factor activity (approximately 10% yield) was found to be associated with a polypeptide of M, = 58,000–62,000 (data not shown in this paper; see Ref. 5 for earlier work). These results confirm earlier reports from this laboratory (5) that homogeneous eIF-5, isolated from rabbit reticulocyte lysates is a monomeric protein of M, = 58,000–62,000.

The specific activity of the homogeneous eIF-5 preparation was determined using an assay (5) that measured its ability to...
Following electrophoresis, one-half of the gel was incubated at 30 °C for 45 min with 0.5 μg (90 units) of casein kinase 11, the reactions were terminated and subjected to SDS-gel electrophoresis as described under "Experimental Procedures." All kinase 11, the reactions were terminated and subjected to SDS-gel electrophoresis as described under "Experimental Procedures." All samples were run in duplicate in two separate panels in the same gel. Following electrophoresis, one-half of the gel (Panel A) was stained with silver reagents (16) while the other half (Panel B) was dried and subjected to autoradiography. A set of marker proteins was run in a parallel gel lane (not shown) to determine the Mr values of the stained and radioactive bands.

to mediate joining of a 60 S ribosomal subunit to a preformed 40 S initiation complex to yield an 80 S initiation complex (Fig. 2). Under the conditions of the in vitro assay described in Fig. 2, 0.44 ng of purified eIF-5 mediated the formation of nearly 1.4 pmol of 80 S initiation complex, indicating that the specific activity of homogeneous eIF-5 is about 2.6 × 10⁶ units/mg protein. (One unit of eIF-5 was defined as that amount of protein that promoted the binding of 1 pmol of [35S]Met-tRNAᵦ to 80 S ribosomes to form an 80 S initiation complex.) It can be calculated, based on a Mr = 58,000 for native eIF-5 and results presented in Fig. 2, that 1 pmol of eIF-5 mediated the formation of nearly 180 pmol of an 80 S initiation complex under in vitro initiation reaction conditions. These results indicate highly catalytic reutilization of eIF-5 in initiation reactions.

Phosphorylation of eIF-5 by Casein Kinase II—A number of eukaryotic translation factors have been shown to be phosphorylated in vivo (reviewed in Refs. 10, 29, and 30; also see Ref. 31). Examples are phosphorylation of the α and β subunits of eIF-2, eIF-3, eIF-4B, eIF-4E, and eIF-4E, and a number of subunits of eIF-2B (GEF). Studies on in vitro phosphorylation of initiation factors by protein kinases have shown that casein kinase II, a cyclic AMP-independent protein kinase isolated from rabbit reticulocyte lysates, specifically phosphorylated the β subunit of eIF-2, eIF-4B, and eIF-3 (23-30).

It was therefore of interest to determine whether our homogeneous eIF-5 preparation (Mr = 58,000-62,000) can be phosphorylated by purified casein kinase II. For this purpose, purified eIF-5 preparations were incubated with [γ-32P]ATP and casein kinase II, and the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Fig. 1). An intense 32P-labeled band which comigrated with homogeneous eIF-5 preparation (Mr = 58,000-62,000) was observed (Fig. 1, Panel B, lane a). In addition to this band, another 32P-labeled band corresponding to the 25,000-dalton subunit of casein kinase II was also observed due to autophosphorylation of this kinase subunit (Fig. 1, Panel B, lane d). When a homogeneous eIF-2 preparation, isolated from rabbit reticulocyte lysates, was used as a substrate for casein kinase II, only the β-subunit of eIF-2 was phosphorylated (Fig. 1, Panel B, lane b). Incubation of a partially purified eIF-2/eIF-5 preparation (CM-Sephadex eIF-2/eIF-5) by casein kinase II and [γ-32P]ATP phosphorylated both the eIF-2α and eIF-5 (Fig. 1, Panel B, lane c). In addition to eIF-2β and eIF-5, a number of other polypeptides, present in this partially purified eIF-2/eIF-5 preparation, were also phosphorylated by casein kinase II (Panel B, lane c).

The amino acid residues of eIF-5 phosphorylated by casein kinase II were identified by subjecting the 32P-labeled eIF-5 band to acid hydrolysis in 6 N HCl, followed by high voltage electrophoresis at pH 3.5, using unlabeled phosphoserine, phosphothreonine, and phosphotyrosine as internal standards. Incorporation of 32P occurred only into serine residues of eIF-5 (Fig. 3).

The number of sites in eIF-5 phosphorylated by casein kinase II was determined by analyzing tryptic phosphopep-
Characterization of Eukaryotic Initiation Factor 5

FIG. 3. Phosphoamino acid analysis of \(^{32}\)P-labeled eIF-5. Purified eIF-5 (250 ng) was phosphorylated with 0.3 mM \(\gamma\text{-}^{32}\)P[ATP (10,400 cpm/pmol) and 90 units of casein kinase II in a 20-\(\mu\)l reaction mixture ("Experimental Procedures"). Following incubation for 45 min at 30 °C, the reaction was terminated and subjected to SDS-polyacrylamide (15%) gel electrophoresis. Following electrophoresis, the gel was silver-stained (16), dried, and the strip of gel corresponding to eIF-5 was excised. After swelling the gel in 10% methanol, the dried gel was mixed with 25 \(\mu\)g each of unlabeled phosphoserine, phosphothreonine, and phosphotyrosine and subjected to acid hydrolysis in 6 N HCl in a sealed ampula for 1.5 h at 110 °C. Following hydrolysis, the solution was lyophilized three times, dissolved in 20 \(\mu\)l of water, spotted on a Whatman 3MM paper, and subjected to electrophoresis for 2 h at 4,000 volts at 10 °C in pyridine acetate buffer (0.5% pyridine, 5% acetic acid) at pH 3.5. The dried filter paper was first developed with 0.5% ninhydrin in acetone containing 30% glacial acetic acid to determine the position of each unlabeled phosphoamino acid. Subsequently, the filter paper was subjected to autoradiography.

Phosphopeptides ranging in composition from "P-eIF-5 on SDS-18% polyacrylamide gels also indicated the presence of phosphoamino acid

Purified eIF-5 (250 ng) was phosphorylated with 0.3 mM \(\gamma\text{-}^{32}\)P[ATP (20,000 cpm/pmol) in a reaction mixture (40) similar to that described under "Experimental Procedures." Following incubation for 45 min at 30 °C, the protein was reduced and carboxymethylated by first adding 13 \(\mu\)l of a buffer containing 1.12 M 2-mercaptoethanol, 500 mM Tris-HCl, pH 8.8, 4% SDS, 40% glycerol, and 0.01% bromphenol blue and heating for 3 min at 100 °C. Subsequently, the cooled reaction mixture was treated with sodium iodoacetate (140 mM, final concentration) and incubated at 37 °C for 30 min in dark. The resulting protein sample was then resolved in a SDS-15% polyacrylamide gel and subjected to autoradiography.

Acid hydrolysis, the solution was lyophilized three times, dissolved in 20 \(\mu\)l of water, spotted on a Whatman 3MM paper, and subjected to electrophoresis for 2 h at 4,000 volts at 10 °C in pyridine acetate buffer (0.5% pyridine, 5% acetic acid) at pH 3.5. The dried filter paper was first developed with 0.5% ninhydrin in acetone containing 30% glacial acetic acid to determine the position of each unlabeled phosphoamino acid. Subsequently, the filter paper was subjected to autoradiography.

Complete digestion of \(^{32}\)P-labeled eIF-5 by trypsin yielded two major and two minor phosphopeptides by reverse-phase HPLC column chromatography. It should be noted that if the HPLC column was developed with 0.1% trifluoroacetic acid and increasing concentrations of acetonitrile, the two major peaks II and III were not resolved and eluted as a single peak (data not shown). Analysis of the tryptic peptides derived from \(^{32}\)P-eIF-5 on SDS-18% polyacrylamide gels also indicated the presence of 2 major phosphopeptides and 2 minor phosphopeptides ranging in \(M_e\) values from 3,000 to 1,500 (data not shown). These results indicated that casein kinase II phosphorylated multiple sites in the eIF-5 molecule.

We also determined whether phosphorylation of eIF-5 by casein kinase II affected the function of eIF-5 in mediating joining of a 60 S ribosomal subunit to a preformed 40 S initiation complex. For this purpose, purified eIF-5 was phosphorylated with \(\gamma\text{-}^{32}\)P[ATP and casein kinase II, and the reaction mixture was subjected to Sephadex G-75 gel filtration (Fig. 5). Aliquots of each eluted fraction were assayed for \(^{32}\)P radioactivity as well as for eIF-5 activity. In addition, fractions containing \(^{32}\)P radioactivity were subjected to SDS-polyacrylamide gel electrophoresis to identify the phosphoryproteins eluted. As expected, both \(^{32}\)P-labeled autophosphorylated casein kinase II and \(^{32}\)P-labeled eIF-5 eluted from the column in agreement with the \(M_e\) value of each protein (Fig. 5). Furthermore, \(^{32}\)P-labeled eIF-5 retained biological activity (Fig. 5). The total recovery of eIF-5 activity from the column was about 51%. Similar recovery of eIF-5 activity was observed when 600 units of eIF-5, without treatment with ATP and casein kinase II, was subjected to gel filtration in the same column (data not shown). These results demonstrate that phosphorylation of eIF-5 by casein kinase II does not affect its ability to catalyze joining of a 60 S ribosomal subunit to a preformed 40 S initiation complex.

Preparation and Characterization of Anti-eIF-5 Antibodies—A major problem for preparation of antibodies against eIF-5 was the relatively low yield of homogeneous eIF-5 obtained by purification from either calf liver extracts (4) or from rabbit reticulocyte lysates (5). Furthermore, the possi-
with the same buffer. All operations were carried out at about 5 °C, buffered in 20 mM Tris-HCl, pH 7.5, 100 mM KCl.

In addition, the void volume problem of possible conservation in different species, as the presence of anti-eIF-5 antibodies in the serum was assayed using "Immunoblot Analysis of Initiation Factors" under "Experimental Procedures." Using this procedure, sufficient quantities of pure anti-eIF-5 antibodies were obtained (1 ml of immune serum at 1:100 titer yielded 8 ml of affinity-pure antibodies of the same 1:100 titer). Furthermore, the signal

FIG. 5. Effect of phosphorylation of eIF-5 with casein kinase II on the activity of the initiation factor. Purified reticulocyte eIF-5 (5 μg) was incubated with 180 units of purified casein kinase II and 62 μM [γ-32P]ATP (12,000 cpm/pmol) in a reaction mixture (80 μl) containing 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM MgCl2, 5 mM dithiothreitol and 20% glycerol for 2 h at 5 °C. Subsequently, the reaction mixture was loaded onto Sephadex G-75 (Superfine) column (12-ml bed volume) that was previously equilibrated in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, and 10% glycerol. The column was developed with the same buffer. All operations were carried out at about 5 °C, and fractions of 0.3 ml were collected. Panel A, aliquots of eluted fractions were assayed for eIF-5 activity by the joining of 60 S ribosomal subunits to a preformed 40 S initiation complex as described in legend to Fig. 2. In addition, the 32P content of each eluted fraction was measured by determining the Cerenkov radiation. Panel B, aliquots (25 μl) from fractions 14 to 22 were subjected to SDS-polyacrylamide (15% polyacrylamide and 0.09% bisacrylamide) gel electrophoresis at pH 8.8 according to the method of Scherier et al. (7). The dried gel was subjected to autoradiography. In a separate experiment, the positions of elution of peak activity of several marker proteins, muscle aldolase, bacterial alkaline phosphatase, human hemoglobin type IV, and ovalbumin, were determined in the same column. In addition, the void volume (Vv) was determined using blue dextran. From these results, it was calculated that eIF-5 eluted from the column as a protein of apparent Mr of 55,000-60,000.

bility exists that a protein synthesis initiation factor like eIF-5 may be highly conserved and hence might be poorly antigenic. Attempts were therefore made to raise antibodies against relatively large amounts of denatured eIF-5. We reasoned that denaturation of eIF-5 would help to overcome the problem of possible conservation in different species, as the denatured form would not resemble any self protein. We therefore followed the procedure of Knudsen (18) to raise anti-eIF-5 antibodies in rabbits ("Experimental Procedures"). In our case, approximately 16 μg of eIF-5, phosphorylated with casein kinase II, was injected intradermally into four sites of each of two rabbits (approximately 8 μg/rabbit) ("Experimental Procedures"). Following booster injections, the presence of anti-eIF-5 antibodies in the serum was assayed by performing immunoblotting analysis using purified eIF-5 as an antigen. A dilution of 1:100 of the serum from one rabbit gave a strong signal of the presence of antibodies against eIF-5 (data not shown) and was used as the working dilution in subsequent experiments. Unexpectedly, there was a background staining which was most likely due to the anti-nitrocellulose antibodies which were also generated in the rabbit. To circumvent this problem, we affinity-purified the anti-eIF-5 antibodies from the serum as described under "Experimental Procedures." Using this procedure, sufficient quantities of pure anti-eIF-5 antibodies were obtained (1 ml of immune serum at 1:100 titer yielded 8 ml of affinity-pure antibodies of the same 1:100 titer). Furthermore, the signal

FIG. 6. Immunoblot analysis of eIF-5 preparations. Immunoblot analysis was performed as described under "Immunoblot Analysis of Initiation Factors" using homogeneous eIF-5 (lane a), CM-Sephadex eIF-2/eIF-5 (lane b), and homogeneous eIF-2 containing α, β, and γ subunits (lane c) as protein samples. All samples were run in duplicate in two separate panels in the same gel. A set of prestained markers (myosin H chain (Mr = 200,000), phosphorylase b (Mr = 97,000), bovine serum albumin (Mr = 67,000), ovalbumin (Mr = 43,000), and lysozyme (Mr = 14,300)) was run in separate lanes in the separate gel to also electrotransferred onto the PVDF membrane (not shown). Following electrophoresis, one-half of the gel (Panel A) was stained with silver reagents according to Merrill et al. (16), while the other half (Panel B) was electrotransferred onto the PVDF membrane and probed with 1:100 dilution of the anti-eIF-5 antibodies as described under "Experimental Procedures."

FIG. 7. Immunoprecipitation of eIF-5 by affinity-purified anti-eIF-5 antibodies. Purified reticulocyte eIF-5 (0.6 μg) was phosphorylated with 90 units of purified casein kinase II (CKII) in the presence of 0.25 mM of [γ-32P]ATP (7000 cpm/pmol) in a reaction mixture (40 μl) containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl2, 5 mM dithiothreitol at 30 °C for 45 min. Following incubation, one-half of the reaction mixture was heated at 65 °C for 10 min to denature the proteins. Both the heated and the unheated fractions were then incubated with affinity-purified anti-eIF-5 antibodies (1:25 final dilution) for 12 h at 4 °C. Subsequently, the antigenantibody complex formed was incubated with 20 μl of 50% protein A-Sepharose CF-4B (Pharmacia) suspended in Blotto buffer (1% BSA, 0.1% Tween 20, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0), and finally three times with 0.8 ml of low salt buffer (2 M NaCl, 5 mM EDTA, 0.2% Triton X-100, 10 mM Tris-HCl, pH 8.0). Finally, the pellet was boiled in 40 μl of a SDS buffer (125 mM Tris-HCl pH 6.8, 280 mM 2-mercaptoethanol, 1% SDS, 1% glycerol, and 0.04% bromphenol blue) for 3 min and subjected to SDS-polyacrylamide gel electrophoresis (15% polyacrylamide and 0.09% bisacrylamide) at pH 8.8 according to the method of Scherier et al. (7). In parallel gel lanes, eIF-5 labeled with 32P by casein kinase II (lane c), and casein kinase II autophosphorylated with [γ-32P]ATP (lane d) were run as marker proteins. The dried gel was analyzed by autoradiography. Lane a, heated 32P-labeled eIF-5 as antigen; lane b, unheated 32P-eIF-5 as antigen; lane c, 32P-labeled eIF-5 as marker; lane d, 32P-labeled (autophosphorylated) casein kinase II as marker.
Characterization of Eukaryotic Initiation Factor 5

A number of eukaryotic translational initiation factors have been shown to be phosphorylated in vivo and in vitro (23-31). Examples are phosphorylation of the α and β subunits of eIF-2, eIF-4B, eIF-4F, eIF-4E, eIF-3 and a number of subunits of eIF-2B (also designated as GEF). Hershey and his colleagues (34-37) have demonstrated changes in covalent modification (phosphorylation status) of these initiation factors in response to physiological signals, e.g. heat shock, serum depletion, and serum stimulation of growing cells. The extents of phosphorylation of some of these proteins correlate with protein synthesis activity levels. However, utilizing existing initiation factor assays, it has not been possible to demonstrate that phosphorylation causes a change in functional activity of any of these proteins with the exception of phosphorylation of the α subunit of eIF-2. In this case, phosphorylation of the α subunit of eIF-2 affects the ability of this initiation factor to be catalytically re-utilized in initiation reactions (29,30).

Earlier reports from several laboratories (6-9) suggested that eIF-5, isolated from rabbit reticulocyte lysates, is a monomeric protein of Mr, of 120,000-168,000. Thus, when in vitro phosphorylation of eIF-5 was examined, a 120,000-168,000 dalton polypeptide was shown to be phosphorylated by casein kinase II (15, 24, 25, 38). However, results published previously from this laboratory (4, 5) as well as those presented in this communication show that eIF-5 is a monomeric protein of Mr, of 58,000-62,000 indicating that the original eIF-5 preparations of reported Mr, values of >120,000 were not homogeneous. It is likely therefore that phosphorylation of contaminating proteins in eIF-5 preparations was observed.

Results presented in this communication demonstrate that our homogeneous eIF-5 preparations (Mr, of 58,000-62,000) isolated from rabbit reticulocyte lysates can be phosphorylated at multiple serine residues by highly purified casein kinase II. However, phosphorylation of eIF-5 by casein kinase II had no apparent effect on the activity of eIF-5 in joining 60 S ribosomal subunits to a 40 S initiation complex to form an 80 S initiation complex. However, the ability of casein kinase II to specifically label eIF-5 with 32P without loss of biological activity of the initiation factor will allow us to prepare labeled factor for mechanistic studies. It will also be of considerable interest to determine whether eIF-5 as isolated from cell extracts is endogenously phosphorylated in vivo at sites other than casein kinase II and if such phosphorylation/dephosphorylations play a role in biological activity of the factor.

Availability of homogeneous eIF-5 in our laboratory has now allowed us to raise antibodies against eIF-5. Characterization of these antibodies has shown that antibodies were under these conditions, freshly grown HeLa cells, mouse L cells, were lysed directly into denaturing buffers. In addition, crude rat liver and calf liver extracts were also treated with SDS denaturing buffers. Each of the freshly prepared denatured lysates were analyzed immediately by SDS-polyacrylamide gel electrophoresis and immunoblotting (Fig. 8). In all the species examined, a polypeptide (Mr, of 58,000) reacted with the antibody and was the major band in rabbit reticulocytes, rat and calf livers and mouse L cells. Both HeLa and mouse L cells lysates exhibited an additional immunoreactive polypeptide (Mr, of 62,000) which was the major species in HeLa extracts. No precursor or degradation forms of eIF-5 were detectable under these conditions (Fig. 8). These data indicated that the molecular weight of eIF-5 is unaltered during the isolation procedure.

**DISCUSSION**

Immunoblotting is an essential technique in the study of initiation factors. To determine specificity of anti-eIF-5 antibodies, both native and heat-denatured eIF-5 were separately treated with anti-eIF-5 antibodies (Fig. 7). Only the heat-denatured form of eIF-5 was immunoprecipitated with anti-eIF-5 antibodies indicating that denatured conformation of eIF-5 had the antigenic determinants (Fig. 7, compare lanes a and b). Consistent with this observation, prior incubation of a homogeneous heat-denatured eIF-5 preparation with various dilutions of the affinity-purified anti-eIF-5 antibodies had no effect on the ability of the factor to mediate 80 S initiation complex formation (data not shown).

Immunoblot Analysis of Cell Lysates—The availability of anti-eIF-5 antibodies allowed us to examine eIF-5 in different species and to determine if the size of purified rabbit reticulocyte and calf liver eIF-5 is the same as in vivo. Lysis of cells directly into protein denaturing buffers containing high amounts of SDS is expected to minimize possible proteolytic degradation of initiation factor proteins. Such lysis procedures are thus considered to freeze the proteins in forms present in vivo (32,33). For analysis of size of eIF-5 in crude lysates to noise ratios of the affinity-purified antibodies were far higher than whole sera, thereby allowing the detection of trace levels of eIF-5. Fig. 6 shows that affinity-purified anti-eIF-5 antibodies reacted strongly with homogeneous eIF-5 (Panel B, lane a). When anti-eIF-5 antibodies were tested for cross-reaction with eIF-2 using either a partially purified eIF-2 or trace levels of eIF-5. Fig. 6 shows that affinity-purified anti-eIF-5 antibodies reacted strongly with homogeneous eIF-5 and were run in a parallel lane of the same gel, and their migration positions following transfer to the membrane is shown: myosin H chain (Mr, of 200,000); phosphorylase b (Mr, of 97,000); bovine serum albumin (Mr, of 67,000); ovalbumin (Mr, of 43,000); chymotrypsigen (Mr, of 18,400); lysozyme (Mr, of 14,300). Lysates of mouse L cells and HeLa cells used in this experiment were prepared by sonicating freshly grown packed cells into 2 volumes of a denaturing buffer containing 3% SDS, 125 mM Tris-HCl, pH 6.8, 280 mM 2-mercaptoethanol, and 10% glycerol as described by Meyer et al. (32). Other eIF-5 preparations were used as indicated below. Lane a, 0.5 µg of a homogeneous eIF-5 preparation from rabbit reticulocytes; lane b, 50 µg of calf liver 40-60% ammonium sulfate II fraction prepared as described (4); lane c, 90 µg of ammonium sulfate II fraction prepared from fresh rat liver by procedures similar to that described previously (4); lane d, 50 µg of mouse L cell lysates; lane e, 50 µg of HeLa cell lysate.

FIG. 8. Immunoblot analysis of cell lysates. Immunoblotting of samples transferred to a PVDF membrane was as described under "Immunoblotting of Initiation Factors" under "Experimental Procedures." Affinity-purified rabbit anti-eIF-5 antibodies were used at 1:100 dilution as the primary antibody, while an anti-rabbit IgG coupled to alkaline phosphatase was used to detect the binding of the primary antibody to eIF-5 in the blots. The following preestained molecular weight marker proteins were run in a parallel lane of the same gel, and their migration positions following transfer to the membrane is shown: myosin H chain (Mr, of 200,000); phosphorylase b (Mr, of 97,000); bovine serum albumin (Mr, of 67,000); ovalbumin (Mr, of 43,000); chymotrypsigen (Mr, of 18,400); lysozyme (Mr, of 14,300). Lysates of mouse L cells and HeLa cells used in this experiment were prepared by sonicating freshly grown packed cells into 2 volumes of a denaturing buffer containing 3% SDS, 125 mM Tris-HCl, pH 6.8, 280 mM 2-mercaptoethanol, and 10% glycerol as described by Meyer et al. (32). Other eIF-5 preparations were used as indicated below. Lane a, 0.5 µg of a homogeneous eIF-5 preparation from rabbit reticulocytes; lane b, 50 µg of calf liver 40-60% ammonium sulfate II fraction prepared as described (4); lane c, 90 µg of ammonium sulfate II fraction prepared from fresh rat liver by procedures similar to that described previously (4); lane d, 50 µg of mouse L cell lysates; lane e, 50 µg of HeLa cell lysate.
directed against denatured conformation of eIF-5 and did not recognize the native eIF-5 protein. However, such antibodies are an important tool for structural studies, e.g. as probes for (a) screening mammalian λgt11 libraries, and (b) immunoblot analysis of eIF-5 protein to investigate relative concentration and covalent modification of eIF-5 in growing cells in response to a variety of growth regulatory physiological signals. For example, in the present work, we have used these anti-eIF-5 antibodies to show that the size of eIF-5 in fresh mammalian liver extracts or in cell lysates, prepared by lysing growing mouse L cells or HeLa cells directly in 3% SDS containing denaturing buffers, is similar to the size of the factor purified in our laboratory from calf liver extracts and rabbit reticulocyte lysates. In all the species examined, a 58,000-dalton polypeptide band reacted with the anti-eIF-5 antibodies. However, HeLa cell extracts exhibited a 62,000-dalton polypeptide which is due to proteolytic processing of the factor purified in our laboratory from calf liver extracts and rabbit reticulocyte lysates. The possibility exists therefore that the 62,000-dalton polypeptide may be a major species in these cell lysates (Fig. 8). The possibility exists therefore that the 62,000-dalton polypeptide may be a form present in vivo which is very labile and proteolytically processed to the 58,000-dalton form. Further work is clearly necessary to answer this question. However, the immunoblot analysis of freshly prepared cell lysates in 3% SDS-buffers (Fig. 8) clearly indicates that eIF-5 is a protein of M, = 58,000–62,000 which is not due to proteolytic processing of a 120,000–160,000 dalton protein during isolation of the initiation factor from cell extracts. This experiment therefore provides additional evidence that earlier reports from other laboratories (6–9) that eIF-5 was of apparent M, = 120,000–160,000, probably represents the presence of proteins unrelated to eIF-5 activity. However, the most convincing proof for the molecular size of eIF-5 will come from cloning and sequencing of the cDNA corresponding to the coding sequence of eIF-5. Using anti-eIF-5 antibodies, we are currently screening several mammalian λgt11 libraries to identify cDNA clones which encode mRNA for eIF-5.

Acknowledgments—We are indebted to Dr. Matthew D. Scharff of this institution for many helpful discussions and to Ms. Joanne Trojanacki for expert technical assistance in the preparation of anti-eIF-5 antibodies.

REFERENCES

1. Maitra, U., Stringer, E. A., and Chaudhuri, A. (1982) Annu. Rev. Biochem. 51, 809–900
2. Kozak, M. (1983) Microbiol. Rev. 47, 1–45
3. Moldave, M. (1985) Annu. Rev. Biochem. 54, 1109–1149
4. Raychaudhuri, P., Chaudhuri, A., and Maitra, U. (1985) J. Biol. Chem. 260, 2132–2139
5. Raychaudhuri, P., Chevesich, J., Ghosh, S., and Maitra, U. (1987) J. Biol. Chem. 262, 1422–1427
6. Merrick, W. C., Kemper, W. M., and Anderson, W. F. (1975) J. Biol. Chem. 250, 5556–5562
7. Schreier, M. H., Erni, B., and Staehelin, T. (1977) J. Mol. Biol. 116, 727–753
8. Benne, R., Brown-Leudi, M. L., Corbett, S., Tolan, D. R., and Hershey, J. W. B. (1981) J. Biol. Chem. 256, 3070–3077
9. Meyer, L. T., Brown-Leudi, M. L., Corbett, S., Tolan, D. R., and Hershey, J. W. B. (1981) J. Biol. Chem. 256, 351–356
10. Hershey, J. W. B., Duncan, R., and Matthews, M. B. (1985) in Current Communications in Molecular Biology: Translational Control (Matthews, M. B., ed) pp. 1–18, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Abstracts of EMBO Workshop on Regulation of Protein Synthesis in Eukaryotes, Patras, Greece, September 17–19, 1986, pp. 37–38
12. Ghosh, S., Chevesich, J., and Maitra, U. (1988) Fed. Proc. 37, 6180
13. Stringer, E. A., Chaudhuri, A., and Maitra, U. (1979) J. Biol. Chem. 254, 6845–6848
14. Zaaloff, M., and Ochoa, S. (1974) Methods Enzymol. 30, 197–206
15. Hathaway, G. M., Lundak, T. S., Tahara, S. M., and Traugh, J. A. (1979) Methods Enzymol. 60, 495–511
16. Merrill, C. R., Goldman, D., Sedman, S. A., and Ebert, M. H. (1981) Science 211, 1437–1458
17. Chaudhuri, A., Stringer, E. A., Valenzuela, D., and Maitra, U. (1981) J. Biol. Chem. 256, 3988–3994
18. Knudsen, K. A. (1985) Anal. Biochem. 147, 285–288
19. Laemmli, U. K. (1970) Nature 227, 680–688
20. Burnette, W. N. (1981) Anal. Biochem. 112, 195–203
21. Johnson, D. A., Gauchet, J. W., Sportsman, J. R., and Eldred, J. H. (1984) Gene Anal. Tech. 1, 3–8
22. Olmsted, J. B. (1981) J. Biol. Chem. 256, 11955–11957
23. Issinger, O. G., Benne, R., Hershey, J. W. B., and Traut, R. T. (1976) J. Biol. Chem. 251, 6471–6474
24. Benne, R., Edman, J., Traut, R. R., and Hershey, J. W. B. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 108–112
25. Floyd, G. A., Merrick, W. C., and Traugh, J. A. (1979) Eur. J. Biochem. 96, 277–286
26. Tuxon, P. T., Merrick, W. C., and Traugh, J. A. (1980) J. Biol. Chem. 255, 10954–10958
27. DePaoli-Roach, A. A., Roach, P. J., Pham, K., Kramer, G., and Hardesty, B. (1981) J. Biol. Chem. 256, 8871–8874
28. Clark, S. J., Colthurst, D. R., and Proud, C. G. (1988) Biochem. Biophys. Acta 968, 211–219
29. Pain, V. M. (1986) Biochem. J. 235, 625–637
30. Proud, C. G. (1986) Trends Biochem. Sci. 11, 73–77
31. Rychlik, W., Russ, M. A., and Rhoads, R. E. (1977) J. Biol. Chem. 262, 10454–10457
32. Meyer, L. J., Milburn, S. C., and Hershey, J. W. B. (1982) Biochemistry 21, 4204–4212
33. Brown-Leudi, M. L., Meyer, L. J., Milburn, S. C., Yau, P. M. P., Corbett, S., and Hershey, J. W. B. (1982) Biochemistry 21, 4204–4206
34. Ernst, H., Duncan, R. F., and Hershey, J. W. B. (1987) J. Biol. Chem. 262, 1206–1212
35. Duncan, R., and Hershey, J. W. B. (1985) J. Biol. Chem. 260, 5493–5497
36. Duncan, R., and Hershey, J. W. B. (1984) J. Biol. Chem. 259, 11892–11899
37. Duncan, R., and Hershey, J. W. B. (1983) J. Biol. Chem. 258, 7228–7235
38. Traugh, J. A., Tahara, S. M., Sharp, S. B., Safer, B., and Merrick, W. C. (1976) Nature 263, 163–165
39. Albersold, R. H., Lasvitt, J., Saavedra, R. A., Hood, L. E., and Kent, S. B. H. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 6970–6974
40. Bennett, H. P. J., Browne, C. A., and Solomon, S. (1981) Biochemistry 20, 4530–4538
41. Bennett, H. P. J., Browne, C. A., and Solomon, S. (1980) J. Liquid Chromatogr. 3, 1363–1365