Preparation and Characterization of Eukaryotic Initiation Factor EIF-3

FORMATION OF BINARY (EIF-3 MET-tRNA₆) AND TERNARY (EIF-3 MET-tRNA₆-GTP) COMPLEXES*

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The 133,000 x g supernatant fraction prepared from ascites cells in 20 mM KCl (low KCl supernatant) contained the initiation factors EIF-1 and EIF-2 (and the elongation factors EF-1 and EF-2) but lacked EIF-3; thus, low KCl supernatant could be used to assay for EIF-3. EIF-3 was prepared from a crude initiation factor preparation (a 250 mM KCl extract of ascites cell ribosomes precipitated with 70% saturated ammonium sulfate) by chromatography on DEAE-Sephadex A-50 and hydroxylapatite. The EIF-3 had no detectable EIF-1 and little or no EIF-2. Factor EIF-3 was required for translation of encephalomyocarditis virus RNA. The molecular weight of EIF-3 was estimated by Sephadex G-200 filtration to be 139,000; the sedimentation coefficient was calculated to be about 5.8.

EIF-3 formed a binary complex specifically with the initiator tRNA, Met-tRNA₆, and if GTP was present the factor formed a ternary complex (EIF-3.Met-tRNA₆.GTP). The EIF-3 preparation had no methionyl-tRNA synthetase activity to account for binding. Complex formation was with eukaryotic Met-tRNA₆ and no other aminoacyl-tRNA. The binary and ternary complexes were retained quantitatively on Millipore filters (which was the most convenient assay), but they could also be demonstrated by filtration through Sephadex G-100 or by glycerol gradient centrifugation. GTP increased the rate, the amount, and the stability of complex formed; the ratio of GTP to Met-tRNA₆ in the ternary complex appeared to be 1. The binary and the ternary complexes transferred Met-tRNA₆ to 40 S ribosomal subunits, but not to 60 S subparticles. The factor-dependent binding of Met-tRNA₆ to the 40 S subunit did not require mRNA (or GTP). In the presence of 60 S subunits, the initiator tRNA bound to 40 S subunits was not transferred to 80 S ribosomes even if mRNA was added—that reaction may require another initiation factor. Treatment of EIF-3 with N-ethylmaleimide led to loss of its activity in complex formation and in support of the translation of encephalomyocarditis virus RNA. In addition to forming the binary and ternary complexes, and supporting the translation of encephalomyocarditis virus RNA, EIF-3 also increases the number of free ribosomal subunits by either preventing their association or causing dissociation of 80 S couples (Nakaya, K., Ranu, R. S., and Wool, I. G. (1973) Biochem. Biophys. Res. Commun. 54, 246-255).

At least three protein factors, and perhaps several more, are required for the initiation of protein synthesis in eukaryotic cells (1-20). The exact number and identity of the factors as well as the details of their mechanism of action remain to be resolved. One or more of the factors forms a ternary complex with Met-tRNA₆ and GTP and then transfers the initiator aminoacyl-tRNA to 40 S ribosomal subunits (5, 8-13, 17, 18, 20). The factor(s) required for those reactions has been prepared from rabbit reticulocytes (1-5, 8, 11-14, 17, 18) and from L-cells (9-10). While there is agreement about the general nature of the process, the details, once again, are a matter of dispute—for example, whether the reaction requires mRNA or not, and if so at just what stage (cf. for example, Refs. 5 and 14). We have prepared an initiation factor, EIF-3, from mouse ascites cells: the factor has been resolved from two other initiation factors, EIF-1 and EIF-2 (7), and characterized. EIF-3 is required, along with EIF-1 and EIF-2 and elongation factors, for the translation of encephalomyocarditis virus RNA. The same factor forms a binary complex with Met-tRNA₆ and if GTP is present a ternary complex (EIF-3-Met-tRNA₆-GTP). Finally, EIF-3 preparations cause dissociation of 80 S ribosomes, or prevent association of subunits (21).

EXPERIMENTAL PROCEDURES

Materials—The sources of the materials were as follows: [¹H]phenylalanine (5 Ci/mmol), [¹H]valine (1.3 Ci/mmol), and [¹H]methionine (4.3 Ci/mmol) from New England Nuclear Corp.;...
poly(U) and poly(AUG) from Miles Research Laboratories; human γ-globulin from Calbiochem; bovine serum albumin from Armour Laboratories; NADPH from Sigma Chemical Co.; DDEA- Sephadex A-50, Sephadex G-25, Sephadex G-100 and Sephadex G-200 from Pharmacia Fine Chemicals; BD-cellulose from Schwartz Bioresearch, Inc.; and hydroxyapatite from Clarkson Chemical Co.

**Growth of Ascites Cells**—Krebs II ascites cells were originally obtained from Dr. A. T. H. Burchmore (Glasgow-Retiring Research Laboratory). They were propagated by intraperitoneal injection of 0.1 ml of ascites fluid per animal in HA/ICR male Swiss mice weighing 20 to 25 g (22).

**Growth of EMC Virus and Preparation of EMC Virus RNA**—The K2 strain of EMC virus was grown in Krebs II ascites cells (23). The virus was purified by equilibrium centrifugation in cesium chloride (24), and the RNA was extracted as described by Kerr et al. (25). The EMC virus RNA had an A260/A280 ratio of 2.0, and the RNA sedimented as a single peak with a coefficient of 33 in 10 to 30 mg sucrose density gradients both with and without 0.5% sodium dodecyl sulfate. One microgram of EMC virus RNA had an absorbance of 0.025 at 260 nm (25).

**Preparation of Ascites Cell Ribosomes and Supernatant Fraction**—The ascites cells were washed and an homogenate was prepared as described by Mathews and Korner (22) except that the cells were suspended in an equal volume of buffer; this and the subsequent procedures were carried out at 4°. The ascites cell extract was diluted with 4/5 volume of 10× Medium K (0.2 M Tris-HCl, pH 7.6/1.25 M KC1/50 mm magnesium acetate/60 mm β-mercaptoethanol). Ribosomes were sedimented from the homogenate by centrifugation for 3 hours at 45,000 rpm in a Spinco Ti 50 rotor. The supernatant was carefully aspirated and passed through a column of Sephadex G-25 equilibrated with Medium K (20 mm Tris-HCl, pH 7.6/125 mm KC1/5 mm magnesium acetate/6 mm β-mercaptoethanol); the eluate was stored in small aliquots at −70°.

Ribosomes were suspended in Medium K and centrifuged at 45,000 rpm, 4°, in a Spinco Ti 50 rotor, through 1 mm sucrose in Medium K. The cell fractions prepared in this way are referred to as high KC1 (125 mm KC1) ribosomes and supernatant. Low KC1 (20 mm KCl) ribosomes and supernatant were prepared in the same way except that the ascites cell extract was diluted with 4/5 volume of Medium C (0.1× Medium Tris-HCl pH 7.6/0.1 M KC1/16 mm magnesium acetate) rather than 10× Medium K.

Before the ribosomes (prepared in low KCl or high KCl) were used to conduct the experiments, they were dialyzed against 0.1 M KCl and the fines were removed. The DEAE-Sephadex was equilibrated with starting buffer (50 mm Tris-HCl, pH 7.6/10 mm KC1/0.1 mm EDTA/5 mm β-mercaptoethanol) and packed in a column (1.5 × 27 cm); starting buffer was allowed to run through the column for 10 to 20 hours at 4° at a rate of 17.5 ml/hour before a sample was applied.

**Hydroxylapatite** was washed three times with buffer (20 mm Tris-HCl, pH 7.6/100 mm KC1/5 mm β-mercaptoethanol/1 mm potassium phosphate/5% glycerol) and packed in a column (0.9 × 23 cm). Starting buffer was run through the column for 24 to 30 hours at 4° at a rate of 0.6 ml/hour before a sample was applied.

**Translation of EMC Virus RNA**—Ribosomes (8 μg of RNA or the amount indicated in the legends to the figures) were incubated for 45 min at 37° in 50 μl of medium containing: Tris-HCl (pH 7.6), 20 mm; KC1, 100 mm; magnesium acetate, 4.5 mm; β-mercaptoethanol, 6 mm; ATP, 1 mm; GTP, 0.1 mm; creatine phosphate, 5 mm; 19 unlabeled amino acids, 0.00 mm each; [H3]phenylalanine, 1 μCi; creatine phosphokinase, 10 μg; EMC virus RNA, 5 μg; and supernatant enzyme preparation (high KCl or low KCl), 250 μg. The low KCl supernatant will not support translation of EMC virus RNA; when it was used it was necessary to add various amounts of ribosomal salt wash or EIF-3.

A reaction mixture containing 1 ml of 10% trichloroacetic acid and kept on ice for 10 min. The sample was heated to 90° to 10 min, cooled, and the precipitate was collected on Whatman GFC glass fiber filter discs, and washed three times with 3 ml of 5% trichloroacetic acid. The filters were dried and placed in glass vials containing 5 ml of scintillation fluid (30). Radioactivity was determined in a Packard Tri-Carb spectrometer; the efficiency of the determination of the radioactivity was 16%.

**Poly(U)-directed Polyphenylalanyl Synthesis**—Ribosomes (the amount is indicated in the legends to the figures) were incubated for 30 min at 37° in 50 μl of medium containing: Tris-HCl (pH 7.6), 20 mm; KC1, 125 mm; magnesium acetate, 5 mm; β-mercaptoethanol, 6 mm; ATP, 1 mm; GTP, 0.1 mm; creatine phosphate, 5 mm; creatine phosphokinase, 10 μg; Escherichia coli tRNA that had been aminoacylated (31) with [3H]phenylalanine, 44 ng containing 25,000 to 45,000 cpm; poly(U), 5 μg; and supernatant enzyme preparation (the kind and the amount are given in the figure legends). The radioactivity incorporated into protein was determined as described (see under "Translation of EMC Virus RNA")

Assay of Binding of N-Acetyl-[3H]phenylalanyl-tRNA to 40 S Ribosomal Subunits—N-acetyl-[3H]phenylalanyl-tRNA was prepared as described by Haenni and Chappeville (39). Ribosomal subunits, 40 S, 60 S, and 80 S, which were made from ascites cell RNA were incubated for 30 min at 30° in 50 μl of Medium K containing: 20 mm Tris-HCl, pH 7.6; 80 mm KC1; 3.5 mm magnesium acetate; 5 μg of poly(U), 7.4 μg of N acetyl-[3H]phenylalanyl tRNA (6,300 cpm); and the reaction was stopped by adding 1 ml of 10% trichloroacetic acid.
Characterization of EIF-3

various amounts of EIF-3 preparation. The reaction was stopped by adding 3 ml of ice-cold wash buffer (20 mM Tris-HCl, pH 7.6/80 mM KC1/5 mM magnesium acetate), and the samples were collected on nitrocellulose filters (Millipore filter; HAWP, 25 mm diameter, 0.45 μm pore size) and washed three times with 1 ml of cold wash buffer (39). The filters were dried and the radioactivity was determined (30).

Determination of Molecular Weight by Gel Filtration—The molecular weight of EIF-3 was estimated by gel filtration (33). A column (0.9 x 30 cm) of Sephadex G-200 was equilibrated with buffer (20 mM Tris-HCl, pH 7.6/155 mM KC1/0.1 mM EDTA/0.5 mM dithiothreitol and samples (0.5 mg of protein in 200 μl) containing EIF-3 preparations or standards were applied. The standards and their molecular weights (34) were: human γ-globulin, 150,000; bovine serum albumin, 67,000; ovalbumin, 44,000. Protein was filtered in the same buffer at a rate of 1.8 ml/hour and 0.5-ml fractions were collected. The concentration of protein standards in the fractions was calculated from the absorbance at 280 nm. The EIF-3 in the filtrate was determined from the ability of fractions to support the translation of EMC virus RNA. Kav, for the proteins was calculated (35) by Vc - Vn/V, where Vc is the elution volume of the protein, Vn is the void volume (obtained from passing dextran blue through the column), and V, is the column volume.

Glycerol Density Gradient Centrifugation—The sedimentation coefficient of EIF-3 was determined by centrifugation in 10 to 30% glycerol density gradients in buffer (20 mM Tris-HCl, pH 7.6/125 mM KC1/0.1 mM EDTA/0.5 mM dithiothreitol) at 2 ml/hour and 0.5-ml fractions were collected. EIF-3 preparations or standards were applied to the gradients and centrifuged at 48,000 × g for 14.5 hours at 4°C in a Spinco SW-50.1 rotor. The standards and their sedimentation coefficients (34) were: catalase, 11.3 S; bovine serum albumin, 4.1 S; equine myoglobin, 1.97 S. The sedimentation coefficient of human γ-globulin (6.6 S) is from Eisen (36). The bottom of the centrifuge tube was pumiced and 0.2-ml fractions were collected. The concentration of protein and of EIF-3 in the fractions was estimated as described under “Determination of Molecular Weight by Gel Filtration.”

Preparation of Aminoacyl-tRNA Synthetases from Escherichia coli and Mouse Ascites Cells—The procedures were carried out at 4°C. The E. coli or ascites cell ribosome-free supernatant was treated with streptomycin and prolamide (31); the fraction that was precipitated with 35 to 70% saturated ammonium sulfate was collected. The precipitate from the ascites cell supernatant fraction was dissolved in 5 to 10 ml of buffer (0.02 M Tris-HCl, pH 7.6/0.1 M KC1/0.1 mM EDTA/0.01 M β-mercaptoethanol) and dialyzed for 6 hours against the buffer (20 mM Tris-HCl, pH 7.6/155 mM KC1/0.1 mM EDTA/0.5 mM dithiothreitol). Samples (300 μl) containing EIF-3 preparations or standards were applied. The standards and their molecular weights (34) were: human γ-globulin, 150,000; bovine serum albumin, 67,000; ovalbumin, 44,000. Protein was filtered in the same buffer at a rate of 1.8 ml/hour and 0.5-ml fractions were collected. The concentration of protein standards in the fractions was calculated from the absorbance at 280 nm. The EIF-3 in the filtrate was determined from the ability of fractions to support the translation of EMC virus RNA. Kav, for the proteins was calculated (35) by Vc - Vn/V, where Vc is the elution volume of the protein, Vn is the void volume (obtained from passing dextran blue through the column), and V, is the column volume.

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**RESULTS AND DISCUSSION**

**Influence of Potassium Chloride on Distribution of Initiation Factors between Ribosomes and Supernatant Fraction**—The 133,000 x g supernatant fraction from ascites cell extracts prepared in high concentrations of KCl (125 mM) sustains the translation of EMC virus RNA by ribosomes (Ref. 22 and Table I). We considered whether the presence of all the initiation factors in the supernatant might not be the result of the use of relatively high concentrations of KCl in the preparation of cell extracts. We found, in fact, that if the extract was prepared in low concentrations of KCl (20 mM) the supernatant was no longer able to support translation of EMC virus RNA (Table I). It seemed likely then that the low KCl supernatant lacked one or more of the initiation factors, and that possibly the missing factor or factors was bound to the ribosomes. To test that possibility we assessed the capacity of ribosomal salt wash fraction to supplement low KCl supernatant. Ribosomal salt wash fraction restored the ability of low KCl supernatant to support translation of EMC virus RNA (results not shown): 19 μg of ribosomal salt wash protein gave maximal translation; greater amounts were slightly inhibitory.

We next undertook to determine which initiation factor or factors were absent from low KCl supernatant. Shafritz and Anderson (3) have shown that in the presence of EIF-1 and EIF-2 poly(U)-directed synthesis of polyphenylalanine is maximal at low concentrations (4 to 6 mM) of magnesium, whereas in the absence of one or both factors synthesis is greatest at a higher concentration of magnesium (8 to 10 mM). Moreover, Leader et al. (7) have shown that EIF-2 is precipitated from supernatant at pH 5. We assayed then the ability of high and low KCl supernatant to sustain polyphenylalanine synthesis as a function of the magnesium concentration before and after treatment at pH 5 in an attempt to determine whether they contained EIF-1 or EIF-2 or both. The synthesis of polyphenylalanine was considerable at low concentrations of magnesium (5 mM) with untreated high KCl supernatant (results not shown; they are similar to those in Fig. 1) and with untreated low KCl supernatant (Fig. 1); however, after treatment at pH 5, synthesis with both high KCl and low KCl supernatant (Fig. 1) was greater at higher concentrations of magnesium (8 mM). It needs to be noted that the synthesis of polyphenylalanine with untreated supernatant was biphasic and nearly equal at 5 mM and 8 mM magnesium; the second increase in polyphenylalanine synthesis at 8 mM magnesium may be the consequence of increased binding of phenylalanyl-tRNA due to greater concentration of the cation. The results indicate that both high and low KCl supernatant contain EIF-1 and EIF-2. Since low KCl supernatant does not sustain the translation of EMC virus RNA it is likely to lack EIF-3.

**Table I**

| Supernatant enzyme fraction (250 μg) | Translation of EMC virus RNA
<table>
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<tr>
<td>Prep 125 mM KCl</td>
<td>pmol [3H]Phe incorporated into protein</td>
</tr>
<tr>
<td>Prepared in 250 mM KCl</td>
<td>3.79</td>
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<tr>
<td>Prepared in 20 mM KCl</td>
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![Fig. 1. Polyphenylalanine synthesis with low KCl (20 mM) supernatant: effect of magnesium concentration. Ribosomes (4.62 μg of tRNA) were incubated in 50 μl of medium containing various concentrations of magnesium acetate, and either 90 μg of untreated 20 mM KCl supernatant (O-O), or 87 μg of pH 5-treated 20 mM KCl supernatant (O-O).](http://www.jbc.org/)

![Fig. 2. Chromatography of ribosomal extract (salt wash) on DEAE-Sephadex A-50. 0.25 m KCl extract of ribosomes (145 mg) in buffer (20 mM Tris-HCl, pH 7.6, 200 mM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol) were applied to a column (1.5 x 27 cm) of DEAE-Sephadex A-50. Elution was with 0.3 M KCl. EIF-3 activity in fractions was determined by assaying 5 μl samples for their ability to support the translation of EMC virus RNA in the presence of low KCl supernatant.](http://www.jbc.org/)
KCl/(0.1 mM EDTA/0.25 mM dithiothreitol) was applied to a column containing DEAE-Sephadex A-50, and the column was washed with the same buffer. In preliminary experiments we established that no EIF-3 activity was eluted when the concentration of KCl was 0.2 M or less. The test for EIF-3 activity was the supplementation of low KCl supernatant in supporting the translation of EMC virus RNA.) After the absorbance of the eluate at 280 nm was below 0.3, the buffer was changed to one containing 0.3 M KCl (Fig. 2). Stepwise elution was found to be necessary because EIF-3 was slowly inactivated and this procedure decreased the amount of time required for chromatography. Moreover, gradient elution led to excessive dilution of the factor and probably contributed further to the loss of activity.

The fraction eluted from DEAE-Sephadex A-50 with 0.3 M KCl was applied to a column of hydroxylapatite: all of the protein applied to the column was absorbed. (Chromatography on hydroxylapatite was within 3 to 4 hours of elution of the fraction from DEAE-Sephadex.) In preliminary experiments we found that no EIF-3 activity was eluted from hydroxylapatite when the concentration of potassium phosphate buffer (pH 7.4) was less than 0.17 M; most of the activity was eluted at about 0.28 M. Elution, therefore, was stepwise: first, with 0.17 M potassium phosphate and when there was little or no absorbance at 280 nm with 0.35 M potassium phosphate buffer (Fig. 3). Because potassium phosphate in concentrations greater than 15 mM inhibited the translation of EMC virus RNA, fractions from hydroxylapatite chromatography were dialyzed for 6 hours against 1 liter of buffer (20 mM Tris-HCl, pH 7.6/125 mM KCl/O.1 mM EDTA/6 mM β-mercaptoethanol) before they were assayed for EIF-3 activity. The fractions with EIF-3 activity were pooled, concentrated to 2 mg/ml by dialysis for 6 hours against 50% glycerol in 500 ml of buffer (20 mM Tris-HCl/100 mM KCl/0.1 mM EDTA/4 mM β-mercaptoethanol), and stored in liquid nitrogen. EIF-3 lost activity when the protein concentration was less than 2 mg/ml. Freezing and thawing also reduced the activity of the factor. The purification achieved by the procedure described here was 50- to 80-fold when assessed by the ability of the factor to participate in ternary complex formation (see below).

Resolution of EIF-3 from EIF-1 and EIF-2—Before we proceeded to characterize EIF-3, we wished to determine whether it had been resolved from the other initiation factors, EIF-1 and EIF-2. EIF-1 stimulates the binding of phenylalanyl-tRNA and N-acetylphenylalanyl-tRNA to 40 S ribosomal subunits (2, 6, 15), and that reaction can be used to detect the presence of the initiation factor. Preparations of EIF-3 (as much as 30 μg) did not stimulate the binding of N-acetyl[3H]phenylalanyl-tRNA to 40 S subunits (results not shown); hence EIF-3 preparations do not have detectable amounts of EIF-1.

We tested for the presence of EIF-2 in EIF-3 preparations by assessing the capacity of the latter to supplement pH 5 treated ascites cell supernatant (which lacks EIF-2) in supporting the synthesis of polyphenylalanine at 5 mM magnesium. EIF-3 preparations gave at most a small stimulation of polyphenylalanine synthesis (results not shown). We conclude that EIF-3 preparations contain little or no EIF-2.

Characteristics of EIF-3-dependent Translation of EMC Virus RNA—The optimum concentration of KCl (results not shown) for the EIF-3-dependent translation of EMC virus RNA was between 80 and 110 mM (when the magnesium concentration was 5 mM). The narrow range of KCl concentrations at which translation was maximal is striking. The optimum concentration of magnesium (when the KCl concentration was 100 mM) was 4.5 mM (results not shown), and again small changes from the optimum decreased translation of EMC virus RNA significantly. The translation of EMC virus RNA increased with increasing concentration of EIF-3 up to 10 μg (Fig. 4); greater amounts of EIF-3 were inhibitory. We do not know the basis of the inhibitory effect, which is more marked than that which occurs with large amounts of crude ribosomal salt wash: the inhibition is not the result of a change in concentration of cation or of pH.

Physical Properties of EIF-3—The molecular weight of the
EIF-3 preparation was estimated from Sephadex G-200 filtration to be 139,000 (results not shown); the sedimentation coefficient was calculated from centrifugation in a glycerol gradient to be about 5.8 (Fig. 6).

It is probable then that ascites cell EIF-3 is different than reticulocyte IF-M1, since the latter has been estimated by Merrick et al. (45) to have a molecular weight of >600,000; ascites cell EIF-3 must also be different than the initiation factor (IF\textsubscript{EMC}) specifically required for translation of EMC virus RNA, which was calculated to have a molecular weight of 53,000 (19). The activities (see below) and the physical properties of ascites cell EIF-3 suggest it is similar to or identical with the factor called IF-1 (\(M_r = 150,000\)) described by Dettman and Stanley (17) and Cashon and Stanley (18); with IF-1 (\(M_r = 160,000\)) of Chen et al. (11) and Gupta et al. (13); with IF-E\textsubscript{L} of Schreier and Staehelin (8); with IF-L-3 of Levin et al. (9); or as well as with IF-MP (\(M_r = 140,000\)) of Elson et al. (5).

Preparations of EIF-3 Lack Methionyl-tRNA Synthetase Activity—In experiments designed for another purpose we had found that \[^{3}H\]Met-tRNA\textsubscript{E} was retained on Millipore filters in the presence of EIF-3, suggesting that the two formed a complex. Before proceeding to determine the characteristics of the putative complex and assessing its significance for the initiation of protein synthesis, it was important to know if preparations of EIF-3 were contaminated with methionyl-tRNA synthetase, since that enzyme forms a specific complex with Met-tRNA\textsubscript{E} and Met-tRNA\textsubscript{M}. Preparations of EIF-3 were assayed for methionyl-tRNA synthetase activity: none could be detected (results not shown) even when 28.5 \(\mu\)g of EIF-3 were tested, whereas 16 \(\mu\)g of a synthetase preparation charged 2.5 pmol of tRNA with methionine. That amount (28.5 \(\mu\)g) of EIF-3 is far more than is generally used in an assay of EIF-3 were tested, whereas 16 \(\mu\)g of a synthetase preparation charged 2.5 pmol of tRNA with methionine. That amount (28.5 \(\mu\)g) of EIF-3 was far more than is generally used in an assay of EIF-3. We conclude that the initiation factor is not contaminated with significant amounts of methionyl-tRNA synthetase.

Characteristics of Formation of Binary and Ternary Complexes—The retention of the EIF-3-Met-tRNA\textsubscript{E} complex on Millipore filters was absolutely dependent on the presence of GTP, although the nucleoside triphosphate caused a consistent, if small, increase in complex formation (Table II). It should be noted that in this experiment complex formation was at 37\(^\circ\); the effect of GTP was greater when the reaction occurred at 30\(^\circ\) (see below). Retention of \[^{3}H\]Met-tRNA\textsubscript{E} on Millipore filters was absolutely dependent on the presence of EIF-3. The initiation factor did not bind appreciable amounts of other aminoacyl-tRNA species (Table II). Perhaps the most important discrimination is that it did not bind Met-tRNA\textsubscript{M}, nor, for that matter, did it associate with appreciable quantities of N-acetylphenylalanyl-tRNA. Factors with similar activity have been isolated from mouse fibroblasts and rabbit reticulocytes (5, 8, 11, 12, 17).

EIF-3-Met-tRNA\textsubscript{E} was also separated by filtration on Sephadex G-100 (Fig. 5). The void volume eluate contained \[^{3}H\]Met-tRNA\textsubscript{E} when EIF-3 was present, whereas it eluted later when the factor was omitted (results not shown). Only about 19\% of the amount of complex applied to the column (estimated from the Millipore filter assay) was in the void volume eluate. \[^{3}H\]Met-tRNA\textsubscript{E} and \[^{3}H\]methionine (presumably due to the deacylation of the former) were eluted later and were clearly separated from the complex (Fig. 5a). The results suggest the complex is relatively unstable, and that a portion is dissociated during filtration.

If the reaction mixture contained \[^{\gamma,32P}\]GTP in addition to EIF-3 and Met-tRNA\textsubscript{E}, then the nucleoside triphosphate was also in the void volume eluate (Fig. 5b), indicating the formation of a ternary complex, EIF-3-Met-tRNA\textsubscript{E}-GTP. A greater amount of complex was recovered if GTP was added.
Characterization of EIF-3

to the reaction mixture, suggesting that it increased the stability of the complex.

Analysis of the complex on glycerol gradients (Fig. 6) indicated that it sedimented faster (7.8 S) than EIF-3 (5.8 S). The activity that supports translation of EMC virus RNA sedimented at 5.8 S with EIF-3 (Fig. 6). If [γ-32P]GTP was included in the reaction mixture with [3H]Met-tRNA, both 32P and 3H radioactivity sedimented at 7.8 S with the complex. Once again only a fraction of the complexes (binary and ternary) survived centrifugation for 15 hours without dissociation.

Kinetics of Complex Formation and Influence of EIF-3, Met-tRNA<sub>e</sub>, and GTP Concentrations—The rate and the extent of complex formation was increased by GTP (Fig. 7).

The effect was especially marked when the reaction was at 30° rather than 37°. In the presence of GTP the formation of a ternary complex (EIF-3·Met-tRNA<sub>e</sub>·GTP) was very rapid, particularly at 37° (Fig. 7). ATP did not reproduce the effect of GTP (results not shown).

The amount of binary or ternary (i.e. with GTP) complex formed was proportional to the concentration of EIF-3, at least over the range tested (Fig. 8); the reaction appeared stoichiometric. If the amount of Met-tRNA<sub>e</sub> was limiting, essentially all was bound to the complex (Fig. 9).

It should be noted that the specific activity of EIF-3 varied from one preparation to the next (at least three separate preparations were used for the several experiments). Moreover, activity was reduced by freezing and thawing, and sometimes merely during storage. Finally, the apparent activity was affected by the concentration of the factor in the reaction mixture. For those reasons the results in different experiments cannot be compared directly.

GTP did not bind to EIF-3 unless Met-tRNA<sub>e</sub> was present (results not shown). Moreover, [γ-32P]GTP and [3H]Met-tRNA<sub>e</sub> appeared to be present in the complex in stoichiometric amounts (Fig. 10). The results suggest there was little hydrolysis of GTP as a result of the formation of the ternary complex.

It would appear that EIF-3 can form a binary complex with Met-tRNA<sub>e</sub>, but that in the presence of GTP the ternary complex (EIF-3·Met-tRNA<sub>e</sub>·GTP) is formed. The formation of the ternary complex is more rapid, a greater amount is made, and it is more stable than the binary complex, suggesting that the former is the physiologically significant intermediate.

The formation of a complex containing initiation factor and Met-tRNA usually has been found to be dependent on GTP (5, 8, 9, 12, 17). However, there is now a report that Escherichia coli initiation factor IF-2 (46) can bind formyl-Met-tRNA<sub>e</sub> to form a binary complex similar to that described here; and

![Fig. 6](http://www.jbc.org/)

**Fig. 6.** EIF-3-dependent formation of binary and ternary complexes: sedimentation on glycerol gradients. (a), EIF-3 (31.5 µg) was analyzed alone (fractions were collected and assayed for their ability to support the incorporation of [3H]phenylalanine into protein in the EMC virus RNA-dependent reaction); (b) had the EIF-3·[3H]Met-tRNA<sub>e</sub> binary complex; (c) had the EIF-3·[3H]Met-tRNA<sub>e</sub>·GTP ternary complex.

![Fig. 7](http://www.jbc.org/)

**Fig. 7 (left).** Kinetics of complex formation. The reaction mixture contained in 250 µl of Buffer B: 38 µg of EIF-3; 200 µg of tRNA with 75,000 cpm of [3H]Met-tRNA<sub>e</sub>; and 0.2 mM GTP where indicated. Incubation was at 30° or 37°; 40 µl samples were removed at intervals and analyzed for complex formation. A——A, without GTP at 30°; O——O, without GTP at 37°; △——△, with GTP at 30°; O——O, with GTP at 37°.

**Fig. 8 (right).** Complex formation: effect of EIF-3 concentration. The reaction mixture contained in 25 µl of Buffer B: 70 µg of tRNA having 27,000 cpm of [3H]Met-tRNA<sub>e</sub>; and the amount of EIF-3 indicated. Incubation was at 30° for 5 min in the presence of 0.2 mM GTP (O——O) or for 15 min without GTP (O——O).
Smith and Henshaw (20) report formation of a complex of initiator tRNA and a eukaryotic initiation factor from Ehrlich ascites cells that does not require GTP. The initiation factor IF-MP from rabbit reticulocytes also will form a binary complex with Met-tRNA, and transfer the complex to 40 S subunits (47).

Inactivation of EIF-3 by N-Ethylmaleimide—Treatment of EIF-3 with N-ethylmaleimide led to a loss of about 90% in the activity of the initiation factor in complex formation and a 70% reduction in its capacity to support EMC virus RNA translation (results not shown).

Association of Binary and Ternary Complexes with 40 S Ribosomal Subunits—The factor-dependent formation of the complexes suggested that EIF-3 was the carrier of Met-tRNA,
in the formation of a 40 S ribosomal initiation complex. We tested the possibility by forming the binary or ternary complex in an initial reaction (in the usual way) and then adding the 40 S ribosomal subunits. The subunits had to be incubated in buffer (20 mM Tris-HCl, pH 7.6/100 mM KCl/5 mM magnesium acetate/0.1 mM dithiothreitol) at 37°C for 10 to 15 min or they would not accept the complexes. Incubation may activate the initiation factor in complex formation and a 70% reduction in its capacity to support EMC virus RNA translation (Fig. 11). Unfortunately, in the conditions of the analysis, a large and variable amount of 40 S subunits sedimented as 55 S dimers (49), there was no binding of [3H]Met-tRNA to 40 S subunits. Both the binary and ternary complexes transferred.
[\textsuperscript{4}H]Met-tRNA, to 40 S ribosomal subunits, but not to 60 S subparticles (Fig. 11); the transfer required EIF-3 (Fig. 11), but not a template (results not shown). Since the original observations of Darnbrough et al. (50) and of Schreier and Staehelin (8), it has been often confirmed (10, 18) that the initiation factor-dependent binding of Met-tRNA, to 40 S ribosomal subunits does not require mRNA; there is, however, a demur: Gupta et al. (14) find the reaction entirely dependent on the presence of an AUG codon.

The transfer of the complexes to 40 S subunits was rapid, appearing complete in 1 min (results not shown); however, it needs to be noted that the analysis (sedimentation in sucrose gradients), although at 4\textdegree C, took a long time.

We proceeded next to test whether the 40 S initiation complex could be converted to an 80 S initiation complex by addition of 60 S subunits in the presence of synthetic mRNA (poly(AUG)). The attempt was without success (results not shown). It may be that another factor is required for addition of the 60 S subparticle and formation of an 80 S couple (8, 10, 16, 18, 51).

The EIF-3 preparation forms a binary or ternary complex specifically with Met-tRNA, and no other aminoacyl-tRNA; most critically it discriminates against Met-tRNA,\textsubscript{m}. Binding of Met-tRNA,\textsubscript{m} was not due to contamination of the EIF-3 preparation with methionyl-tRNA synthetase, since that enzyme activity could not be detected; moreover, the synthetase does not distinguish between Met-tRNA, and Met-tRNA,\textsubscript{m} in the way the initiation factor did. EIF-3 also discriminates between formylated and nonformylated and eukaryotic and prokaryotic initiator tRNA, preferentially forming a ternary complex with nonformylated eukaryotic Met-tRNA, (52).

The results suggest that Met-tRNA, and GTP occur in the ternary complex in a ratio of 1:1 and that GTP is not hydrolyzed during complex formation. While it is not possible to calculate the stoichiometry for EIF-3, since it is not pure, and we do not know what fraction of the EIF-3 molecules are still active, nonetheless, the observation that complex formation is linear with EIF-3 concentration is consistent with the complex having one copy of the factor too.

We have considered the possibility that complex formation with the ascites cell EIF-3 preparation also requires GTP, and that the putative binary complex formed when it is not added is the result of contamination of the EIF-3 preparation or of the Met-tRNA, with the nucleoside triphosphate. While we cannot be certain that is not the case, we are inclined to believe it unlikely for the following reasons. The kinetics of binding of Met-tRNA, to EIF-3 are different in the presence and absence of added GTP. EIF-3 does not bind [\gamma-\textsuperscript{32}P]GTP and no exchange of [\gamma-\textsuperscript{32}P]GTP with EIF-3 occurred (i.e. the specific activity of GTP was not changed). The Met-tRNA\textsubscript{m} preparations were passed through Sephadex G-25 and exhaustively dialyzed, procedures that would be expected to remove GTP if it was present.

The binary and ternary complexes transfer Met-tRNA, to the 40 S ribosomal subunit with equal efficiency; the transfer does not require mRNA and is exclusively to the small subunit. The results suggest that GTP acts to stimulate complex formation and is not required for binding to the small ribosomal subunit. The IF-2 dependent association of formyl-Met-tRNA, to E. coli ribosomes apparently does not require GTP for the binding step (53, 54). GTP binding or hydrolysis or both may be necessary for the proper positioning of Met-tRNA, in the peptidyl site (i.e. accommodation) or for the recycling of EIF-3 or for both processes.

The ascites cell EIF-3 preparation catalyzes several of the partial reactions required for the initiation of peptide synthesis. It forms a complex with Met-tRNA, and GTP and then transfers the initiator tRNA to 40 S ribosomal subunits. The same preparation increases the availability of 40 S subparticles, either by preventing association of subunits or causing dissociation of 80 S couples (21). Presumably it is the activity of EIF-3 in those several reactions that accounts for its requirement in the translation of E. coli virus RNA. Whether all of the activities are mediated by a single protein will only be decided when the factor is pure.

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