Studies on Native Ribosomal Subunits from Rat Liver. Purification and Characterization of Three Eukaryote Binding Factors Specific for Initiator tRNA†

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ABSTRACT: A number of activities have been identified in extracts prepared from native ribosomal subunits of rat liver with solutions containing 0.88 M KCl. Two initiator tRNA-binding factors in the extract have been resolved from a ribosome-dissociation factor and Met-tRNA$_f$ deacylase, and extensively purified. One activity stimulates binding of the natural (Met-tRNA$_f$) and of the analogue (AcPhe-tRNA) initiator tRNA to purified 40S ribosomal subunits. This reaction requires the appropriate template, and magnesium ion concentrations above 2 mM, but is not affected by GTP. Some of its characteristics are similar to those reported previously for a template-dependent cytosol binding factor specific for 40S subunits and initiator tRNA. Another binding activity purified from native subunit extracts reacts with Met-tRNA$_f$ only and GTP, to form a ternary complex. This reaction occurs in the absence of template, Mg^{2+}, or derived 40S ribosomal subunits. If 40S subunits and Mg^{2+} are present at the beginning of the incubation, they tend to inhibit the formation of the ternary complex, but if added to the preformed complex, the Met-tRNA$_f$ is transferred to the ribosomal subunit. Molecular weights of about 50 000 for the two template- and Mg^{2+}-dependent binding factors have been estimated. They are similar with respect to the specificity for initiator tRNAs and the ribosomal subunit, their requirements for magnesium ions and template, and to some extent their affinity for AcPhe-tRNA. They differ, however, in their electrophoretic mobility, polypeptide composition, and thermal stability in the presence and absence of ribosomes. The GTP-dependent, template-independent factor differs from the other two in its specificity for initiator tRNA (it reacts with Met-tRNA$_f$ but not AcPhe-tRNA), thermal stability, requirements for GTP and template, subunit structure, and the effects of magnesium ion. Thus, although the three binding factors can form complexes involving Met-tRNA$_f$, they are distinct entities.

The initiation of protein synthesis in prokaryotic cells involves the interaction between the initiator tRNA molecule fMet-tRNA$_f$,[1] and the small (30S) ribosomal subunit; this reaction is catalyzed by the protein factor IF-2 and requires GTP. (See reviews by Haselkorn and Rothman-Denes (1973) and by Ochoa and Mazumder (1974).) The initiation of protein synthesis in eukaryotic cells also seems to require an initiator tRNA molecule Met-tRNA$_f$, the small (40S) ribosomal subunit, and specific initiation factors (see review by Weissbach and Ochoa, 1976).

Two protein factors have been obtained from a number of eukaryotic cells which direct the binding of initiator Met-tRNA$_f$ to 40S ribosomal subunits. One of these requires GTP, the reaction is template independent, and a ternary complex containing the factor, GTP, and Met-tRNA$_f$ appears to be an intermediate (Chen et al., 1972; Dettman and Stanley, 1972; Gupta et al., 1973; Levin et al., 1973a,b; Schreier and Staehelin, 1973; Cashion and Stanley, 1974; Treadwell and Robinson, 1975; Elson et al., 1975; Safer et al., 1975a,b; Ranu and Wool, 1976). The other factor does not require GTP and is strictly dependent on the presence of initiation codon (Zasloff and Ochoe, 1971, 1973; Gasior and Moldave, 1972; Leader and Wool, 1972; Ilan and Ilan, 1973; Grummt, 1974; Merrick and Anderson, 1975; Cimadevilla and Hardesty, 1975; McCuiston et al., 1976). In addition, the former activity appears to be maximal at relatively low concentrations of magnesium ions while the latter activity requires Mg^{2+}. Both factors can participate in the formation of an 80S initiation complex (Adams et al., 1975; Filipowicz et al., 1975, 1976; McCuiston et al., 1976), both appear to be present in some cells (Adams et al., 1975; Filipowicz et al., 1975, 1976), and both have been obtained from the cytosol as well as from ribosomal washes (Cimadevilla and Hardesty, 1975; Filipowicz et al., 1975; Sadnik et al., 1975).

A protein that catalyzed the template-dependent, GTP-independent binding of initiator tRNA to ribosome-derived 40S subunits was partially purified from rat liver cytosol (McCuiston et al., 1976). Native 40S subunits were also shown to contain a template-dependent binding activity (Sadnik et al., 1975), and subsequent studies suggested that a complex with Met-tRNA$_f$ was formed when incubations contained native 40S subunits and GTP but no $MgCl_2$. These findings suggested the presence of multiple binding factors for initiator tRNA in rat liver. This paper describes the purification and characteristics of three binding factors that are specific for the natural (Met-tRNA$_f$) or the model (AcPhe-tRNA) initiator tRNAs. Two of these factors, one from the cytosol (cIF-1)[2] and one from native subunits (nsIF-1), are template and Mg^{2+} dependent, and GTP independent, but are not identical; the

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[1] Abbreviations used are: fMet-tRNA$_f$, initiator formylmethionyl transfer ribonucleic acid which can be formylated with *Escherichia coli* transformylase; AcPhe-tRNA, acetylphenylalanyl tRNA; Tris, tris(hydroxymethyl)aminomethane; TKGED, 50 mM Tris-HCl buffer, 100 mM KCl, 10% glycerol, 0.1 mM EDTA, and 2 mM dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

[2] The designation of eIF-1B for cIF-1, eIF-1C for nsIF-1, eIF-2 for IF-2, and eIF-3 for IF-3 has been proposed (Anderson et al., 1977).

other factor, from native subunits (IF-2), is GTP dependent, and template and Mg^{2+} independent.

Experimental Procedures

Preparation of Radioactive Aminoacylated tRNAs. Isotopically labeled [^{3}H]Phe-tRNA (Siler and Moldave, 1969), Ac[^{3}H]Phe-tRNA (Haenni and Chapeville, 1966; Siler and Moldave, 1969), and Met-tRNA$_f$ and Met-tRNA$_m$ (Smith and Marcker, 1970; Schroer and Moldave, 1973) were prepared as described previously. The specific activity of the tRNA-bound phenylalanine was 4500 cpm/pmol (2200 cpm/μg of aminoacyl-tRNA) and that of the tRNA-bound methionine was 3400 cpm/pmol (1000–1600 cpm/μg of aminoacyl-tRNA).

Preparation of Derived Ribosomal Subunits. Deoxycholate- and NH_4Cl-extracted rat liver ribosomes (Skogerson and Moldave, 1967, 1968) were stripped of endogenous peptidyl-tRNA with puromycin (Gasior and Moldave, 1972; Sadnik et al., 1975) and dissociated into subunits with 0.88 M KCl in buffered salts (Martin and Wool, 1968; Gasior and Moldave, 1972; Sadnik et al., 1975). The buffered salts solution contained 50 mM Tris-HCl (pH 7.3), 12.5 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, and 0.35 M sucrose. The derived subunits (d40S and d60S) were resolved by zonal gradient ultracentrifugation and sedimented from the appropriate gradient fractions (Sadnik et al., 1975).

Preparation of Native Subunits and Native Subunits Extract. The ribonucleoprotein particles in the postmicrosomal supernatant of rat liver homogenates were sedimented at 80 000g for 16 h, resuspended in the buffered salts media described above, washed once (total native subunits), and resolved by zonal gradient centrifugation (Sadnik et al., 1975). The native 40S (n40S) subunits were sedimented from the appropriate pooled gradient fractions, resuspended in the buffered salts media, and stored frozen at −70 °C. The subunit extract was prepared from the total native subunits or from n40S subunits by the addition of KCl and $MgCl_2$ to the subunit suspension, to a final concentration of 0.88 M and 12.5 mM, respectively, and removal of the ribonucleoprotein particles by ultracentrifugation (Sadnik et al., 1975). When the extract was prepared from the total native subunits fraction, the particles were washed once by centrifugation through a discontinuous sucrose gradient prior to the extraction with high salt (Thompson et al., 1977), to avoid contamination with the cytosol binding factor. The extracts were dialyzed overnight against a solution containing 10 mM Tris-HCl buffer (pH 7.5), 0.05 M KCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol, and then concentrated and stored frozen at −70 °C.

Assays for Binding Factors. The interaction of initiator tRNAs with specific binding factors, or with 40S ribosomal subunits catalyzed by specific factors, occurs under two sets of conditions. In one case, IF-1 (eIF-2B or eIF-2C), the reaction is dependent on polynucleotide template and magnesium ions; in the other (IF-2 or eIF-2), the reaction requires GTP, is not dependent on template, and occurs at relatively low levels of magnesium ions.

(a) IF-1 Assay. Approximately 16 pmol of d40S subunits was incubated with binding factor preparations and 30 μg of radioactive AcPhe-tRNA (plus 10 μg of polyuridylate) or 10 μg of radioactive Met-tRNA$_f$ (plus 0.1 A_{260} unit of ApUpGp). Unless specified otherwise, incubations in a total volume of 0.1 mL were carried out for 20 min at 37 °C for AcPhe-tRNA, or for 40 min at 10 °C for Met-tRNA$_f$, in solutions containing 30 mM morpholinopropanesulfonate (pH 7.2), 6 mM $MgCl_2$, 60 mM NH_4Cl, and 2 mM dithiothreitol. At the end of the incubation period, 3 mL of cold (2 °C) incubation fluid was added, the solutions were filtered through Millipore membranes and washed 3 times, and the radioactivity on the filters was determined with a scintillation counter.

(b) IF-2 Assay. Binding factor preparations were incubated with 20 or 50 μg of radioactive Met-tRNA$_f$, and 0.05 mM GTP, in solutions containing 20 mM Tris-HCl (pH 7.3), 100 mM KCl, and 1 mM dithiothreitol; in the absence of added $MgCl_2$, less than 0.1 mM Mg^{2+} was contributed by other components. The total volume was 0.5 mL and incubations were at 37 °C for 5 min. At the end of the incubation period, 3 mL of cold (2 °C) incubation fluid containing 10 mM $MgCl_2$ (Safer et al., 1975b) was added and the solutions were filtered through Millipore membranes. The filters were washed 3 times with cold $MgCl_2$-containing incubation fluid, and the radioactivity was determined with a scintillation counter.

Purification Procedures. The IF-1 and IF-2 activities in native subunit extracts were obtained by fractionation with ammonium sulfate (described below), and resolved on cellulose phosphate; the resolved IF-1 (eIF-2C) activity was purified by chromatography on DEAE-cellulose and CM-cellulose, and by glycerol gradient centrifugation; the resolved IF-2 (eIF-2) activity was purified by chromatography on DEAE-cellulose and glycerol gradient centrifugation.

Cellulose Phosphate Chromatography. A 1.6 × 11 cm column of Whatman P11 (Galasinski and Moldave, 1969) was equilibrated with a solution (TKGED) containing 50 mM Tris-HCl buffer (pH 7.5), 100 mM KCl, 10% glycerol, 0.1 mM EDTA, and 2 mM dithiothreitol. After the protein sample was applied, 200 mL of TKGED solution and 400 mL of a linear KCl gradient between 0.1 and 0.65 M in the same buffer were passed through the column. Fractions of 5 mL were collected and analyzed for absorbance at 280 nm. Analyses for IF-1 and IF-2 activities, as described above, were carried out on 0.02- and 0.05-mL aliquots, respectively, of the eluate fractions.

DEAE-Cellulose Chromatography. The column of DEAE-cellulose (0.9 × 11 cm, microgranular DE 52, Whatman) was equilibrated with TKGED solution that did not contain KCl. Five to ten milligrams of protein containing IF-1 or IF-2 individually were placed on top of the column, followed by about 40 mL of the equilibration buffer, and eluted with a linear KCl gradient (50 mL) between 0 and 0.25 M KCl in the same buffer; 1-mL fractions were collected. Eluate fractions were analyzed for absorbance at 280 nm and aliquots of 0.01 mL were analyzed for IF-1 or for IF-2 activities; the results are presented as counts per minute of substrate complexed, per aliquot assayed.

CM-Cellulose Chromatography. The ion exchanger (0.7 × 12 cm, microgranular CM 52, Whatman) was equilibrated with a phosphate buffer solution containing the following: 20 mM potassium phosphate (pH 6.0), 10% glycerol, 0.1 mM EDTA, and 2 mM dithiothreitol. One to two milligrams of protein containing IF-1 from the DEAE column, diluted with phosphate buffer solution to pH 6.0, was applied, followed by 15 mL of the phosphate buffer solution, and eluted with a linear gradient (20 mL) between 0 and 0.25 M KCl in the same buffer; 1-mL fractions were collected. Eluate fractions were analyzed for absorbance at 280 nm and 0.01-mL aliquots were assayed for IF-1 activity.

Glycerol Gradient Centrifugation. Protein samples (0.2–1.0 mg of purified binding factor preparations) in 0.5 mL were layered on 12 mL of linear 15–30% glycerol gradients containing the following buffered salts: 10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 50 mM KCl, and 2 mM dithiothreitol. Centrifugation in an SW 41 (Spinco) rotor was carried out at 40 000 rpm for 24 h. The gradients were analyzed automatically with a scanning recording spectrophotometer (at 254

TABLE I: Effects of GTP, Derived 40S Subunits, and Template on the Interaction of Initiator tRNAs with Native Subunit Extract.[a]

Incubation additions	pmol of [^{3}H]aminoacyl-tRNA retained on Millipore	
	AcPhe-tRNA	Met-tRNA$_f$
None	0.02	0.08
Extract	0.31	0.19
Extract, GTP	0.29	0.79
Extract, d40S subunits	0.24	0.20
Extract, GTP, d40S subunits	0.21	0.35
Extract, 40S subunits, template	1.20	0.13
Extract, GTP, 40S subunits, template	1.14	0.54

[a] Approximately 200 μg of protein extracted from n40S subunits was incubated with and without GTP, d40S subunits, poly(U), ApUpGp, and Ac[^{3}H]Phe-tRNA or [^{3}H]Met-tRNA$_f$. Incubations with AcPhe-tRNA were carried out in 6 mM $MgCl_2$-containing buffered salts, as described under Experimental Procedures (see IF-1 Assay); incubations with Met-tRNA$_f$ were carried out in the absence of $MgCl_2$ (see IF-2 Assay).

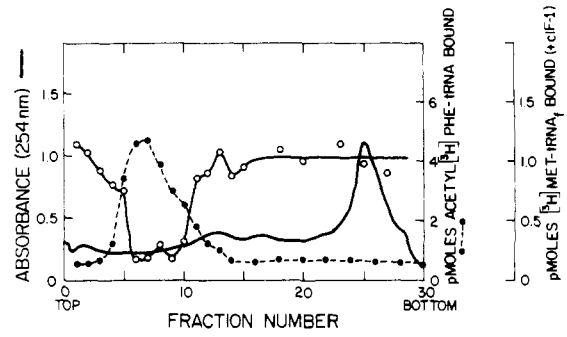

FIGURE 1: Gradient centrifugation of n40S subunit extract. A 0.5-mL sample of dialyzed extract, containing 9 mg of protein, was centrifuged through a linear 15–30% glycerol gradient as described under Experimental Procedures, over a 2-mL cushion of 60% glycerol. The absorbance pattern is presented in the figure in the form of the spectrophotometric scan obtained from the recording apparatus. Aliquots from the gradient fractions were analyzed for IF-1 in the presence of radioactive AcPhe-tRNA (closed circles), as described in the text, and in the presence of purified cIF-1 and radioactive Met-tRNA$_f$ to assay for the inhibitor of Met-tRNA$_f$ binding (Met-tRNA$_f$ deacylase) as shown by the open circles. The results are expressed as picomoles of radioactive aminoacyl-tRNA retained on the Millipore filter, per aliquot assayed.

nm), 0.4-mL fractions were collected, and 0.01-mL aliquots were assayed for various activities. The results are expressed as counts per minute of radioactive aminoacyl-tRNA retained on the Millipore membrane, per aliquot assayed. Sedimentation, in all of the gradient figures, was toward the right.

Results

Evidence for Two Initiator tRNA-Binding Activities in Extracts of Native Ribosomal Subunits. The activity dependent on template and Mg^{2+} but not GTP (IF-1) and the activity dependent on GTP but not template or Mg^{2+} (IF-2) were detected in high salt extracts prepared from n40S subunits or from the total native subunits fraction (Table I). IF-1 activity was demonstrated when the extracts were incubated in buffered salts solutions containing 6 mM $MgCl_2$, d40S subunits, and template, with Ac[^{3}H]Phe-tRNA. Labeled AcPhe-tRNA was used in these experiments because Met-tRNA$_f$ binding to 40S subunits was negligible with crude extracts (Sadnik et al., 1975); inhibition appeared to be due to the presence of a Met-tRNA$_f$ hydrolase activity, also present in the cytosol (McCuiston et al., 1976). AcPhe-tRNA formed a complex that was retained on Millipore filters, in reaction mixtures containing d40S subunits, poly(U), and extract (line 6); GTP had no effect on the reaction. When incubations were carried out with [^{3}H]Met-tRNA$_f$, in the absence of added $MgCl_2$, the extract formed a complex with GTP which was retained on the Millipore membrane (line 3). Derived 40S subunits appeared to inhibit the binding reaction (line 5), but this effect was variable. These data suggested the formation of a ternary complex containing IF-2, Met-tRNA$_f$, and GTP.

Purification of Binding Factors Specific for Initiator tRNAs. In addition to the two initiator tRNA-binding activities described above, a high molecular weight ribosome dissociation factor (IF-3 or eIF-3) was also found in extracts of native subunits (Sadnik et al., 1975; Thompson et al., 1977). A partial resolution of these activities was obtained by glycerol gradient centrifugation (Figure 1). Most of the protein in the extract was recovered toward the bottom of the gradient. Analyses for IF-1 (closed circles) indicated that most of the activity recovered was present in fractions numbered 5 to 12, well resolved from the bulk of the protein and dissociation factor IF-3. Analyses for Met-tRNA$_f$ deacylase, carried out with [^{3}H]Met-tRNA$_f$, d40S subunits, ApUpGp, and purified cytosol IF-1 (open circles), revealed that the activity sedimented in the same region of the gradient as the IF-1 binding factor. Analyses for IF-2 activity (not shown here) indicated that most of the GTP-dependent binding to protein occurred in the fractions toward the bottom of the gradient, corresponding to the bulk of the protein and IF-3 activity. Cashion and Stanley (1974) found that a factor equivalent to IF-2, in reticulocytes, was also found initially in association with a high molecular weight complex.

Complete resolution of IF-3 from the other activities was achieved by ammonium sulfate fractionation. About 85–90% of the protein and IF-3 activity was recovered in the precipitate obtained when the total native subunits extract was made 40% saturated with respect to $(NH_4)_2SO_4$; about 75% of the IF-1 and IF-2 activities was recovered in the 40% ammonium sulfate supernatant, and was precipitated by increasing the level of saturation with $(NH_4)_2SO_4$ to 70%. Purification was about threefold for the two factors, IF-1 and IF-2.

The 40–70% ammonium sulfate fraction from n40S extract or total native subunits extract was used for the resolution and purification of the two initiator tRNA binding activities (IF-1 and IF-2). The template-dependent activity in native subunits is referred to as nsIF-1 (eIF-2C), to distinguish it from that in the cytosol (cIF-1 or eIF-2B). Resolution of nsIF-1 and IF-2 was obtained by chromatography on cellulose phosphate of the 40–70% ammonium sulfate fraction. As shown in Figure 2, most of the protein (open circles) emerged in the first 30 fractions. The nsIF-1, eluted from the column with KCl concentrations near 0.25 M, was recovered in fractions 80 to 100 (triangles); the IF-2, eluted with KCl concentrations near 0.5 M, was recovered in fractions 102 to 115 (closed circles). Both fractions were well resolved from each other. Between 75 and 95% of the nsIF-1 and 25 to 50% of the IF-2 activities chromatographed on cellulose phosphate were recovered in the eluate; purification with this step was 25-fold for nsIF-1 and 20-fold for IF-2. The individual activities were pooled and concentrated by vacuum dialysis at 2 °C against TKGED solution containing 10 mM KCl.

The nsIF-1 obtained was further purified by chromatography on DEAE-cellulose and CM-cellulose, and by glycerol gradient centrifugation, as shown in Figure 3. Analyses of the eluate fractions from the DEAE column (A) for nsIF-1 indicated that the factor was eluted with about 20 mM KCl. About

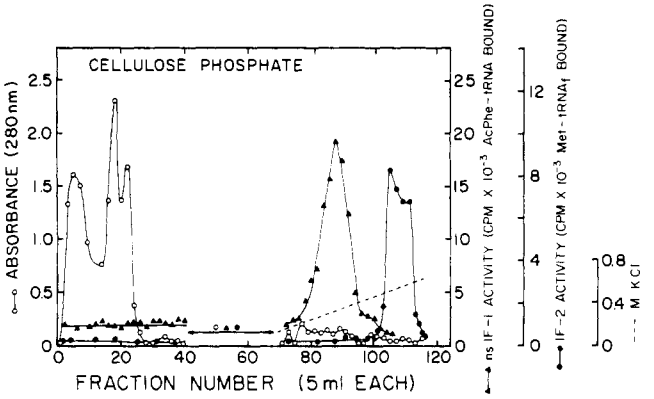

FIGURE 2: Column chromatography of the 40–70% ammonium sulfate fraction of total native subunits extract on cellulose phosphate. The 70% $(NH_4)_2SO_4$ precipitate was resuspended in sufficient TKGED buffer (without KCl) to obtain a salt concentration of about 0.1 M, as determined by conductivity measurements. Approximately 80 mg of protein was applied to the column. The chromatographic procedures and the analyses of eluate fractions for protein at 280 nm (open circles), for IF-1 (closed triangles), and for IF-2 (closed circles) were as described in the text. Fractions 40–70 were combined into several pools and analyzed together. The data presented in this figure indicate the counts per minute of initiator tRNA in the complex, per aliquot assayed.

80% of the activity applied was recovered in the active fractions (closed triangles); purification with this step was about fivefold. The nsIF-1 activity from the DEAE-cellulose column, in phosphate buffer (pH 6.0), was chromatographed directly on CM-cellulose (B). Most of the activity recovered from the column was obtained in fractions containing about 100 mM KCl (closed triangles). Purification by CM-cellulose chromatography was about threefold and the yield was 80–85%. An additional twofold purification was obtained by centrifuging the active fractions from the CM-cellulose column through a glycerol gradient (C). Approximately 35% of the initial activity was recovered in the top third of the gradient (closed triangles). The final specific activity of nsIF-1 was approximately 22 000 pmol of AcPhe-tRNA and 900 pmol of Met-tRNA$_f$ bound per mg of protein.

The IF-2 activity resolved from nsIF-1 on the cellulose phosphate column (Figure 2) was further purified by chromatography on DEAE-cellulose and by centrifugation on glycerol gradients (Figure 4). Analyses of the DEAE column eluate fractions revealed that most of the IF-2 activity (70% yield) emerged at KCl concentrations near 0.1 M (closed circles); purification with this step was about threefold. On linear glycerol gradient centrifugation (B), approximately 35% of the IF-2 activity was recovered in the upper one-third of the tube (closed circles). The final specific activity of IF-2 was 620 pmol of Met-tRNA$_f$ bound per mg of protein.

The cytosol IF-1 was prepared from the pH 5 supernatant of rat liver homogenates by a modification of the procedure described previously (McCuiston et al., 1976). The activity obtained after ammonium sulfate fractionation and chromatography on calcium phosphate (McCuiston et al., 1976) was chromatographed on DEAE-cellulose, CM-cellulose, and cellulose phosphate as described above. Elution from the 3 columns, with salt gradients, released cIF-1 at concentrations of KCl near 20, 100, and 300 mM, respectively; comparison of the chromatographic behavior of nsIF-1 and cIF-1 revealed that they were eluted from the three columns at identical salt concentrations. In some cases, the final step involved centrifugation on 15–30% glycerol gradients. The final specific activity of cIF-1 was 9000 pmol of AcPhe-tRNA and 735 pmol of Met-tRNA$_f$ bound per mg of protein.

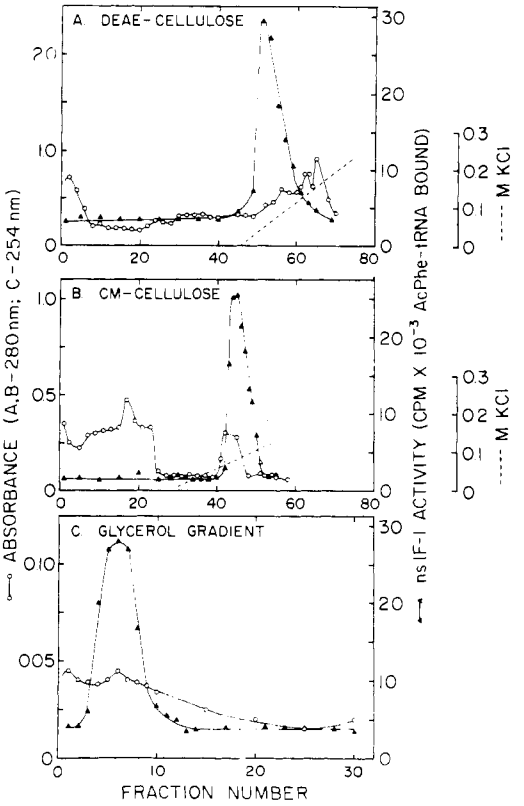

FIGURE 3: Purification of nsIF-1. (A) Chromatography of the cellulose phosphate eluate fractions containing IF-1 (shown above, Figure 2), on DEAE-cellulose columns. (B) Chromatography of the IF-1 eluted from DEAE-cellulose (pooled fractions 49–60), on CM-cellulose columns. (C) Centrifugation of the IF-1 eluted from CM-cellulose (pooled fractions 40–52), on glycerol gradients. The procedures for chromatography, centrifugation, and analyses for protein (open circles) and for IF-1 activity (closed triangles), were as described in the text.

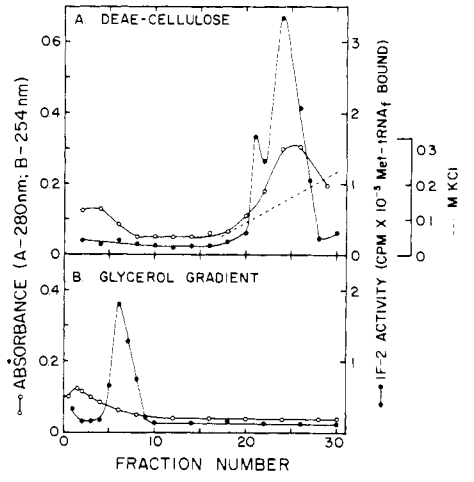

FIGURE 4: Purification of IF-2. (A) Chromatography of the cellulose phosphate eluate fractions containing IF-2 (shown above, Figure 2), on DEAE-cellulose columns. (B) Centrifugation of the IF-2 eluted from DEAE-cellulose (pooled fractions 21–28), on glycerol gradients. The procedures for chromatography, centrifugation, and analyses for protein (open circles) and for IF-2 activity (closed circles) were as described in the text.

Comparison of the Three Binding Factors Specific for Initiator tRNAs. The sedimentation characteristics of the three factors were examined on 10–30% glycerol gradients (Figure 5). The peaks of activity for cif-1 (squares) and nsIF-1 (triangles) coincided, with *s* values between 2.5 and 3.1 S in

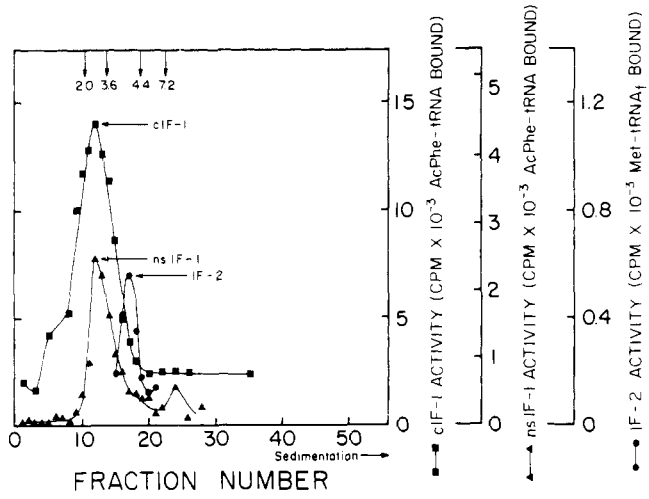

FIGURE 5: Glycerol gradient centrifugation of purified binding factors. Approximately 77 μg of cIF-1 (squares), 18 μg of nsIF-1 (triangles), and 50 μg of IF-2 (circles) were centrifuged in individual tubes, and the results obtained by analyzing 0.01-mL aliquots (cIF-1 and nsIF-1) or 0.03-mL aliquots (IF-2) of the 0.2-mL fractions are plotted together. Myoglobin, ovalbumin, albumin, and γ-globulin were centrifuged in the same rotor; the *s* values (2.0–7.2 S) and the distances sedimented by the protein standards are noted at the top of the figure.

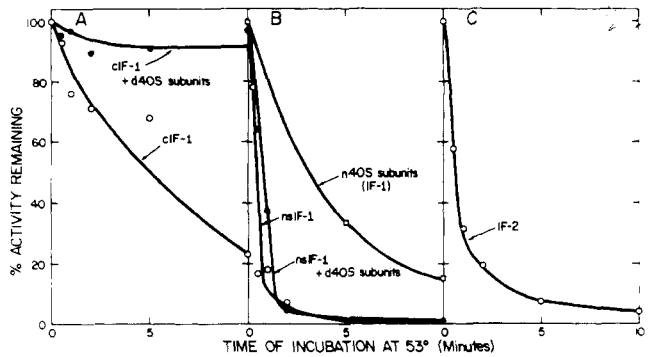

FIGURE 6: Effect of time of incubation at 53 °C, and of 40S subunits, on the activities of the binding factors. Purified preparations of cIF-1 (A), nsIF-1 (B), and IF-2 (C) were maintained at 53 °C in the buffered salts solutions used for subsequent incubations, for varying periods of time up to 10 min. Some incubations also contained about 16 pmol of d40S subunits per μg of factor. Aliquots containing about 1 μg of cIF-1 or nsIF-1, with (closed circles) and without (open circles) d40S subunits, were removed at different times and incubated at 37 °C with poly(U), Ac[^{3}H]Phe-tRNA, d40S subunits if not present, etc., as described above (Experimental Procedures; see IF-1 Assay). The amount of radioactive AcPhe-tRNA bound to d40S subunits was determined by the Millipore filtration technique, and compared to the activity (100%) in samples not preincubated at 53 °C. The rate of inactivation of the nsIF-1 activity not resolved from n40S subunits (B, squares) was determined by preincubating n40S subunits at 53 °C, and assaying for IF-1 activity at 37 °C without additional 40S subunits. Part C presents the data obtained with IF-2 preincubated at the elevated temperature, then assayed with [^{3}H]Met-tRNA$_f$, GTP, etc., as described above (Experimental Procedures; see IF-2 Assay).

several analyses; the peak of IF-2 activity (circles) sedimented in a region of the gradient corresponding to 4.1–4.7 S in several runs.

The rates of thermal inactivation were compared by incubating the purified factors at 53 °C, for varying periods at time, before assay (Figure 6). The cIF-1 (A, open circles) lost 50% of its activity in about 5 min, while the nsIF-1 (B, open circles) and the IF-2 (C, open circles) lost 50% of their activity within 0.5 min. The cIF-1 activity appeared to be markedly protected against inactivation by d40S subunits (A, closed circles), but nsIF-1 did not seem to be significantly affected (B). When

TABLE II: Effects of Various Translation Components and Conditions on the Interaction of [^{3}H]Met-tRNA$_f$ with Purified Binding Factors.

Incubation additions[a]	pmol of [^{3}H]-Met-tRNA$_f$ retained on Millipore	
	A[b]	B[c]
None	0.06	0.05
cIF-1	0.16	0.07
cIF-1, GTP	0.12	0.08
cIF-1, d40S subunits, AUG	1.13	0.06
cIF-1, d40S subunits, AUG, GTP	0.96	0.07
nsIF-1	0.36	0.18
nsIF-1, GTP	0.28	0.17
nsIF-1, d40S subunits, AUG	1.01	0.23
nsIF-1, d40S subunits, AUG, GTP	1.07	0.21
IF-2	0.20	0.06
IF-2, GTP	0.40	1.07
IF-2, d40S subunits, AUG	0.25	0.24
IF-2, d40S subunits, AUG, GTP	0.37	0.51

[a] Incubations contained 20 μg of [^{3}H]Met-tRNA$_f$, approximately 1 μg of purified cIF-1, nsIF-1, or IF-2, and GTP, d40S subunits, and ApUpGp, where noted. [b] A, incubation conditions and components for IF-1 activity, as described in Experimental Procedures (see If-1 Assay). Radioactive Met-tRNA$_f$ was incubated at 10 °C for 40 min with the purified factors, plus the additional components noted, in buffered salts containing 6 mM $MgCl_2$. [c] B, incubation conditions and components for IF-2 activity, as described in Experimental Procedures (see IF-2 Assay). Radioactive Met-tRNA$_f$ was incubated at 37 °C for 5 min with purified factors, plus the additional components noted, in buffered salts without $MgCl_2$.

cIF-1 and nsIF-1 were incubated together at 53 °C, to determine whether one of the preparations contained a component that affected heat lability, the experimental rate curve obtained was the same as that calculated for preparations that were inactivated independently. Also, the finding that all of the IF-1 activity in crude extracts of native 40S subunits or total native subunits was inactivated within 30 s at 53 °C indicated that native ribosomal subunits were not contaminated with the more stable cIF-1. The rate of inactivation of the endogenous nsIF-1 activity associated with n40S subunits is shown for comparison (B, open squares). The half-life of the IF-1 on n40S subunits was approximately 2.5 min; purified d40S subunits were not appreciably inactivated at 53 °C for 10 min.

The three binding activities exhibited markedly different requirements and incubation conditions (Table II). With the two IF-1 preparations, significant formation of a complex containing [^{3}H]Met-tRNA$_f$ occurred only when the incubations contained $MgCl_2$ (A), d40S subunits, and template; GTP did not seem to affect these binding reactions. In contrast, the reaction between Met-tRNA$_f$ and IF-2 was markedly stimulated by GTP, in the absence of $MgCl_2$ (B). Although not shown here, elongation factor EF-1, which is specific for 80S ribosomes, did not react with Met-tRNA$_f$ under any of the conditions or in the presence of any of the components described in this table.

Polyacrylamide gel electrophoresis of purified cIF-1 and nsIF-1 under nondenaturing conditions, not shown here, revealed primarily one component in each preparation, which accounted for at least 85% of the protein. The mobility of the major band of cIF-1, relative to that of nsIF-1, was 0.61 in basic (pH 8.9) gels and 0.85 in acidic (pH 4.3) gels. The optical density patterns obtained on electrophoresis of the purified factors in the presence of sodium dodecyl sulfate are shown in Figure 7. The cIF-1 preparation (A) contained two polypep-

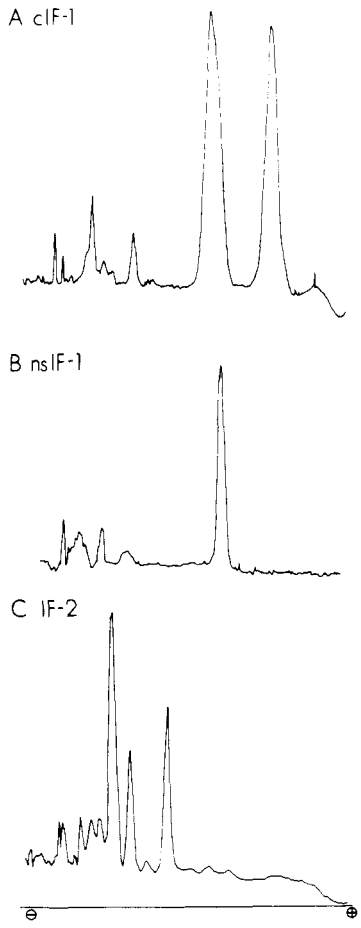

FIGURE 7: Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate, of purified binding factors. Approximately 10 μg of cIF-1 (A), nsIF-1 (B), or IF-2 (C) was maintained at room temperature for 6 h in 0.1% sodium dodecyl sulfate, then submitted to gel electrophoresis (in sodium dodecyl sulfate containing buffers) and staining with Coomassie blue as described by Weber and Osborne (1969). The stained gels were scanned spectrophotometrically at 600 nm.

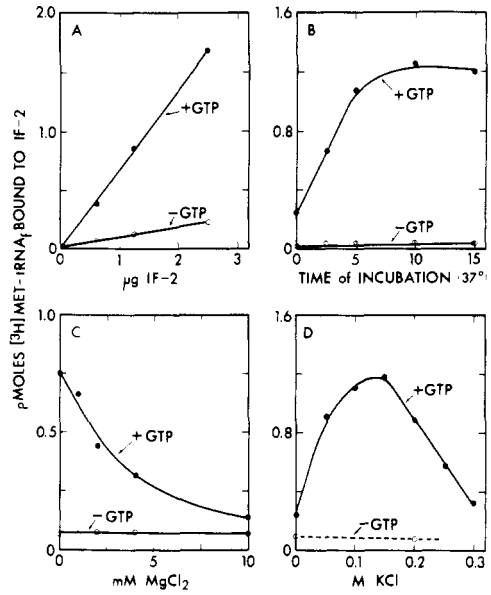

FIGURE 8: Characterization of the reaction between purified IF-2, Met-tRNA$_f$, and GTP. Incubations containing radioactive Met-tRNA$_f$, in the absence of $MgCl_2$, with (closed circles) and without (open circles) GTP, were carried out as described under Experimental Procedures (see IF-2 Assay): (A) incubations with varying amounts of IF-2, for 5 min; (B) incubations for varying periods of time, with about 1.2 μg of purified IF-2; incubations with varying concentrations of $MgCl_2$ (C) and of KCl (D).

TABLE III: Effects of GTP and Various Aminoacyl-tRNAs on the Formation of a Ternary Complex with IF-2.[a]

Incubation additions	pmol of [^{3}H]aminoacyl-tRNA retained on Millipore			
	Met-tRNA$_f$	Met-tRNA$_m$	AcPhe-tRNA	Phe-tRNA
None	0.05	0.07	0.07	
IF-2	0.05	0.12	0.09	0.09
GTP	0.04	0.04	0.08	
IF-2 + GTP	0.38	0.07	0.09	0.11

[a] Where noted, incubations without $MgCl_2$ as described in Experimental Procedures (see IF-2 Assay) contained GTP, 1.2 μg of purified IF-2, 50 μg of [^{3}H]Met-tRNA$_f$ (62 000 cpm, 18 pmol of labeled methionine), 21.2 μg of [^{3}H]Met-tRNA$_m$ (64 000 cpm, 18 pmol of labeled methionine), 28.6 μg of acetyl[^{3}H]Phe-tRNA (64 000 cpm, 14 pmol of labeled phenylalanine), and 30 μg of [^{3}H]Phe-tRNA (63 000 cpm, 14 pmol of labeled phenylalanine). The experiments with radioactive Phe-tRNA were carried out separately, with a 35-fold purified IF-2 preparation.

tides, with molecular weights of about 28 500 and 18 500, in agreement with Cimadevilla and Hardesty (1975). Analysis of the most purified preparations of nsIF-1 (B) revealed a main component corresponding to 24 500 daltons, although some purified samples and material obtained at various steps in the purification procedure contained significant quantities of two additional polypeptides, with molecular weights of about 73 000 and 55 500. Sodium dodecyl sulfate gel electrophoresis of samples containing equivalent amounts of binding activity, from different steps in the nsIF-1 purification procedure, indicated that the material giving rise to the 24 500-dalton polypeptide was the most constant one. The electrophoretic pattern in sodium dodecyl sulfate obtained with IF-2 (Figure 7C) indicated that it was composed of 3 subunits whose molecular weights were approximately 57 000, 49 500, and 38 000. Coelectrophoresis of the factors in sodium dodecyl sulfate cylindrical gels, in a variety of combinations, or in adjacent wells in sodium dodecyl sulfate slab gels revealed that there were no common subunits among the three preparations; for example, the 24 500-dalton subunit of nsIF-1 migrated about 10.2 cm in the slab gel, while the 18 500- and the 28 500-dalton subunits of cIF-1 migrated 11.8 and 9.1 cm, respectively.

Characterization of the IF-2 Dependent Binding Reaction. The behavior of nsIF-1 was very similar, in most respects, to that of cIF-1 (McCuiston et al., 1976); that is, both factors required template and Mg^{2+} but not GTP. Some of the characteristics of the IF-2 catalyzed reaction with purified factor are described below. Figure 8 shows that the formation of the ternary complex containing IF-2, GTP, and Met-tRNA$_f$ (closed circles) was dependent on the concentration of IF-2 protein (A) and on the time of incubation at 37 °C (B); very little binding of Met-tRNA$_f$ occurred without GTP (open circles), and the reaction at 20 °C, not shown here, was 30% of that observed at 37 °C. Increasing concentrations of $MgCl_2$ (C) inhibited the formation of the ternary complex, as reported from other laboratories. When 2 mM $MgCl_2$ was added after 5 min (to allow formation of the ternary complex), and then incubated for an additional 5 min, the amount of radioactive Met-tRNA$_f$ recovered on the Millipore filters was decreased 30–50%. The K^+ optimum (D) was between 100 and 150 mM, and higher concentrations of KCl severely inhibited. Table III summarizes experiments on the substrate specificity of the

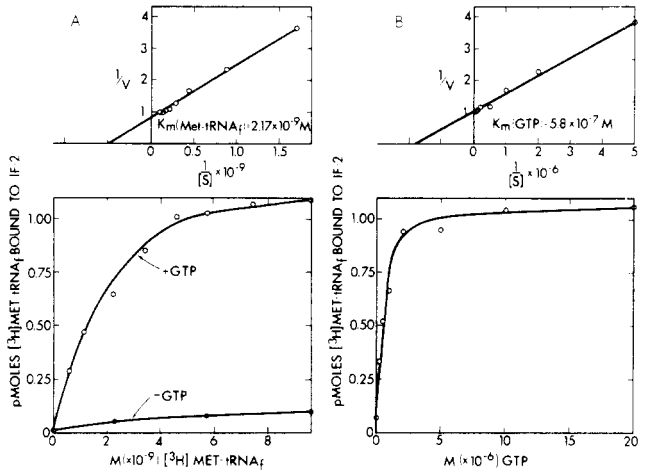

FIGURE 9: Effects of [^{3}H]Met-tRNA$_f$ and GTP concentrations on the formation of ternary complex with IF-2. The lower parts of this figure show the results of experiments in which approximately 1.2 μg of purified IF-2 was incubated with varying concentrations of [^{3}H]Met-tRNA$_f$ (A) and GTP (B) as described under Experimental Procedures (IF-2 Assay). Double reciprocal plots of velocity vs. concentration are presented in the upper part of the respective figures.

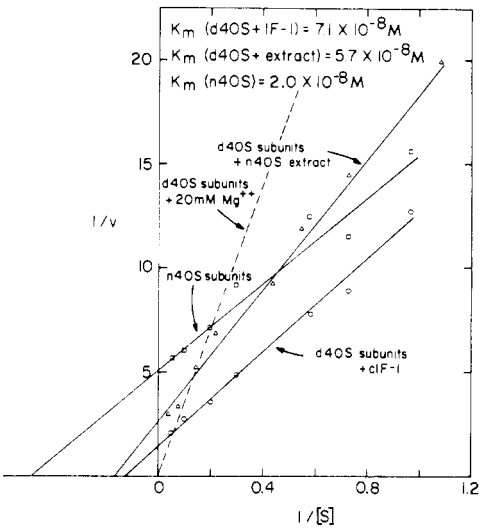

FIGURE 10: Double reciprocal plots of velocity vs. Ac[^{3}H]Phe-tRNA concentrations, from data obtained from incubations containing cIF-1 plus d40S subunits (circles), n40S extract (nsIF-1) plus d40S subunits (triangles), n40S subunits (squares), and d40S subunits without factors but in the presence of 20 mM $MgCl_2$ (broken lines).

reaction with IF-2. Met-tRNA$_f$ was the only active aminoacyl-tRNA; the interaction of Met-tRNA$_m$, AcPhe-tRNA, or Phe-tRNA, with or without GTP, was negligible.

The effects of Met-tRNA$_f$ and GTP concentrations on the initial rate of formation of ternary complex with IF-2 are shown in Figure 9. Maximal rate of binding was obtained with about 4×10^{-9} M [^{3}H]Met-tRNA$_f$ (A, open circles) and 5×10^{-6} M GTP (B); very little binding was detected in the absence of GTP, even at the higher concentrations of Met-tRNA$_f$. The upper parts of this figure show the double reciprocal (Lineweaver–Burk) plots of velocity vs. Met-tRNA$_f$ (A) and GTP (B) concentrations. The apparent Michaelis constants calculated from these data were about 2.2×10^{-9} M for the former and 5.8×10^{-7} M for the latter.

The effect of varying substrate concentrations, on the initial rates of the IF-1 catalyzed binding of AcPhe-tRNA to 40S subunits, was also investigated (Figure 10). Double reciprocal plots are presented of velocity vs. substrate concentration with n40S subunits (squares, endogenous nsIF-1), d40S subunits plus exogenous cIF-1 (circles), d40S subunits plus n40S extract (triangles, exogenous nsIF-1), and d40S subunits plus 20 mM $MgCl_2$ (broken line, factor-independent binding). For the three factor-dependent reactions, the apparent K_m values calculated from these data were of the same order of magnitude, although slightly different. The data obtained with the high Mg^{2+}-stimulated, factor-independent reaction revealed that the reciprocal of the velocity and the substrate concentration approached zero value simultaneously, and the reaction did not appear to be catalytic.

Discussion

A factor which requires template and magnesium ions but not GTP, and binds Met-tRNA$_f$ to 40S subunits, has been purified from the cytosol of a number of cells (Zasloff and Ochoa, 1973; Cimadevilla and Hardesty, 1975; McCuiston et al., 1976) and from ribosomal washes (Merrick and Anderson, 1975; Cimadevilla and Hardesty, 1975). This factor has been reported to have a molecular weight of 145 000 and two subunits of 74 000 molecular weight each in *Artemia salina* (Zasloff and Ochoa, 1973); in reticulocytes, 50 000 molecular weight with two subunits of 30 000 and 20 000 each (Cimadevilla and Hardesty, 1975), or a single polypeptide chain of 65 000 molecular weight and a sedimentation coefficient of 3.35 (Merrick and Anderson, 1975), have been obtained. The rat liver factor from the cytosol described here (cIF-1 or eIF-2B) appears to have a sedimentation coefficient between 2.4 and 3.1 and a molecular weight of about 47 000 based on the molecular weight of two unequal subunits of 18 500 and 28 500 daltons.

The other protein factor, which requires GTP but not template or magnesium ions, and forms a ternary complex with Met-tRNA$_f$ and GTP, has been detected in or purified from the cytosol of *A. salina* (Filipowicz et al., 1975) and wheat germ (Treadwell and Robinson, 1975; 1976; Giesen et al., 1976), ribosomal washes of reticulocytes (Cashion and Stanley, 1974; Safer et al., 1975b; Staehelin et al., 1975; Traugh et al., 1976; Issinger et al., 1976; Benne et al., 1976), and native subunits (Smith and Henshaw, 1975); it has also been purified from the combined cytosol–ribosomal wash of pig liver (Harbitz and Hauge, 1976). The molecular weights obtained for the factor purified from reticulocyte ribosomal washes appear to be 140 000–160 000, although in one report a value of 90 000 was obtained by analytical ultracentrifugation and a value of 150 000 was obtained for the same preparation by a gel electrophoresis procedure (Safer et al., 1975b); a sedimentation coefficient of 4.7 S was estimated. The pig liver factor (Harbitz and Hauge, 1976) had a molecular weight of about 122 000 and a sedimentation of 6.5. Subunit analyses by sodium dodecyl sulfate gel electrophoresis in several laboratories indicate that this protein factor is composed of three subunits with molecular weights of 48 000–57 000, 40 000–53 000, and 32 000–38 000. The GTP-dependent factor described here, obtained from native ribosomal subunits of rat liver (IF-2 or eIF-2), has a sedimentation coefficient of about 4.1–4.7 and a minimal molecular weight of about 145 000 based on the molecular weights of 57 000, 49 500, and 38 000 for the three polypeptides.

In addition to the two binding factors described above, another protein factor that binds Met-tRNA$_f$ to 40S ribosomal subunits, described above, has been isolated from extracts of native subunits; this template-dependent, GTP-independent factor (nsIF-1 or eIF-2C) has not been reported previously. The *s* value obtained for this factor is 2.4–3.1, and sodium

dodecyl sulfate gel electrophoresis reveals a polypeptide with a molecular weight of about 24 500. The molecular weight of nsIF-1, calculated (Siegel and Monty, 1966) from the sedimentation value on glycerol gradient centrifugation and the Stokes radius on Ultragel AcA 44, is estimated to be between 50 000 and 55 000. These data are compatible with the possibility that nsIF-1 may be composed of two identical 24 500-dalton subunits. Although in wheat germ, some of the factors normally found in ribosomal washes are obtained in the cell supernatant (Treadwell and Robinson, 1975; Giesen et al., 1976), a factor with the structural characteristics of nsIF-1 has not been described.

The data presented here indicate that nsIF-1 and cIF-1 are similar in the following respects: they catalyze the binding of AcPhe-tRNA and Met-tRNA$_f$ to derived 40S subunits; binding requires template [poly(U) or ApUpGp] and magnesium ion concentrations greater than 2 mM; GTP has no effect on the binding of initiator tRNAs to 40S subunits; the factors do not stimulate binding to 80S ribosomes; the apparent K_m values for binding of AcPhe-tRNA to 40S subunits are quite similar; the chromatographic behavior of the two factors on DEAE-cellulose and CM-cellulose is also similar. The nsIF-1 and cIF-1, however, differ in several important respects: the subunit components of nsIF-1 and cIF-1, as revealed by sodium dodecyl sulfate gel electrophoresis, are quite different; the rates of inactivation at 53 °C vary markedly; d40S ribosomal subunits protect cIF-1 effectively against thermal inactivation at 53 °C, whereas they have no effect on nsIF-1 inactivation.

The IF-1 factors and IF-2 are similar in that they are specific for initiator tRNAs, and the reactions that they catalyze lead to the binding of the initiator tRNAs to 40S subunits. The transfer of Met-tRNA$_f$ to d40S subunits, catalyzed by the three binding factors, has been obtained and will be described in detail subsequently. The factors differ in the following: the IF-1s interact with AcPhe-tRNA and Met-tRNA$_f$, while IF-2 reacts only with Met-tRNA$_f$; the IF-1s require template, and IF-2 does not; the IF-1s are dependent on Mg^{2+}, but IF-2 is inhibited by Mg^{2+}; IF-2 requires GTP, the If-1s do not; IF-1s require 40S subunits in order to form a demonstrable complex, but the IF-2 reaction with Met-tRNA$_f$ and GTP to form a ternary complex is inhibited when d40S subunits are present, although 40S·Met-tRNA is formed if 40S subunits are added to the preformed ternary complex.

The subunit structure of the three factors is considerably different; there do not appear to be common subunits on sodium dodecyl sulfate gel electrophoresis. It is of interest that Treadwell and Robinson (1976) found that the two binding factors that they obtained from wheat germ supernatant appeared to have one common subunit on the basis of sodium dodecyl sulfate gel electrophoretic mobility, and immunodiffusion. Filipowicz et al. (1976), however, reported that an antibody prepared against IF-1 from *A. salina* cytosol did not inhibit the synthesis of globin in a reticulocyte–*A. salina* system containing reticulocyte IF-2.

The existence in a cell of several distinct binding factors that are specific for initiator tRNA is not easily interpretable. All three rat liver factors interact with Met-tRNA$_f$, and as shown previously (Sadnik et al., 1975; McCuiston et al., 1976) and in data to be presented subsequently, they lead to the formation of a 40S·Met-tRNA$_f$ complex. A role that would involve recycling between the ribosomal particles and the cytosol would require some common features in the structure of the three factors, which is not apparent from these studies. Other alternatives include the possibility that the three factors are functional and that individual ones may be required for translation of different classes or groups of mRNA molecules, or that only one is functional and the others represent evolutionary remnants or altered forms (Filipowicz et al., 1976). The presence of multiple, apparently well-defined initiator-tRNA binding factors, particularly in association with native ribosomal subunits, indicates that considerably more information will be required before the exact roles in the initiation of protein synthesis are accurately defined.

Acknowledgments

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References

Adams, S. L., Safer, B., Anderson, W. F., and Merrick, W. C. (1975), *J. Biol. Chem. 250,* 9083–9089.

Anderson, W. F., Bosch, L., Cohn, W. E., Lodish, H., Merrick, W. C., Weissbach, H., Wittmann, H. G., and Wool, I. G. (1977), *FEBS Lett. 76,* 1–10.

Benne, R., Wong, C., Leudi, M., and Hershey, J. W. B. (1976), *J. Biol. Chem. 251,* 7675–7681.

Cashion, L. M., and Stanley, W. M. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 436–440.

Chen, Y. C., Woodley, C. L., Bose, K. K., and Gupta, N. K. (1972), *Biochem. Biophys. Res. Commun. 48,* 1–9.

Cimadevilla, J. M., and Hardesty, B. (1975), *J. Biol. Chem. 250,* 4389–4397.

Dettman, G., and Stanley, W. M. (1972), *Biochim. Biophys. Acta 287,* 124–133.

Elson, N. A., Adams, S. L., Merrick, W. C., Safer, B., and Anderson, W. F. (1975), *J. Biol. Chem. 250,* 3074–3079.

Filipowicz, W., Sierra, J., Nombela, C., Ochoa, S., Merrick, W. C., and Anderson, W. F. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 44–48.

Filipowicz, W., Sierra, J., and Ochoa, S. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3947–3951.

Galasinski, W., and Moldave, K. (1969), *J. Biol. Chem. 244,* 6527–6532.

Gasior, E., and Moldave, K. (1972), *J. Mol. Biol. 66,* 391–402.

Giesen, M., Roman, R., Seal, S. N., and Marcus, A. (1976), *J. Biol. Chem. 251,* 6075–6081.

Grummt, F. (1974), *Eur. J. Biochem. 43,* 337–342.

Gupta, N. K., Woodley, C. L., Chen, Y. C., and Bose, K. K. (1973), *J. Biol. Chem. 248,* 4500–4511.

Haenni, A.-L., and Chapeville, F. (1966), *Biochim. Biophys. Acta 114,* 135–148.

Harbitz, I., and Hauge, J. G. (1976), *Arch. Biochem. Biophys. 176,* 766–778.

Haselkorn, R., and Rothman-Denes, L. B. (1973), *Annu. Rev. Biochem. 42,* 397–438.

Ilan, J., and Ilan, J. (1973), *Nature (London), New Biol. 241,* 176–180.

Issinger, O.-G., Benne, R., Hershey, J. W. B., and Traut, R. R. (1976), *J. Biol. Chem. 251,* 6471–6474.

Leader, D. P., and Wool, I. G. (1972), *Biochim. Biophys. Acta 262,* 360–370.

Levin, D. H., Kyner, D., and Acs, G. (1973a), *J. Biol. Chem. 248,* 6416–6425.

Levin, D. H., Kyner, D., and Acs, G. (1973b), *Proc. Natl. Acad. Sci. U.S.A. 70,* 41–45.

Martin, T. E., and Wool, I. G. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 569–574.

McCuiston, J., Parker, R., and Moldave, K. (1976), *Arch. Biochem. Biophys. 172,* 387–398.

Merrick, W. C., and Anderson, W. F. (1975), *J. Biol. Chem. 250,* 1197–1206.
Ochoa, S., and Mazumder, R. (1974), *Enzymes, 3rd Ed., 10,* 1–51.
Ranu, R. S., and Wool, I. G. (1976), *J. Biol. Chem. 251,* 1926–1935.
Sadnik, I., Herrera, F., McCuiston, J., Thompson, H. A., and Moldave, K. (1975), *Biochemistry 14,* 5328–5335.
Safer, B., Adams, S. L., Anderson, W. F., and Merrick, W. C. (1975a), *J. Biol. Chem. 250,* 9076–9082.
Safer, B., Anderson, W. F., and Merrick, W. C. (1975b), *J. Biol. Chem. 250,* 9067–9075.
Schreier, M. H., and Staehelin, T. (1973), *Nature (London) 242,* 35–38.
Schroer, R. A., and Moldave, K. (1973), *Arch. Biochem. Biophys. 154,* 422–430.
Siegel, L. M., and Monty, K. J. (1966), *Biochim. Biophys. Acta 112,* 346–362.
Siler, J., and Moldave, K. (1969), *Biochim. Biophys. Acta 195,* 123–129.
Skogerson, K., and Moldave, K. (1967), *Biochem. Biophys. Res. Commun. 27,* 568–572.
Skogerson, L., and Moldave, K. (1968), *Arch. Biochem. Biophys. 125,* 497–505.
Smith, K. E., and Henshaw, E. C. (1975), *Biochemistry 14,* 1060–1067.
Smith, A. E., and Marcker, K. A. (1970), *Nature (London) 226,* 607–610.
Staehelin, T., Trachsel, H., Erni, B., Boschetti, A., and Schreier, M. H. (1975), *Fed. Eur. Biochem. Soc. Meet. [Proc.] 10,* 309–323.
Thompson, H. A., Sadnik, I., and Moldave, K. (1977), *Biochemistry 16,* 2221–2230.
Traugh, J. A., Tahara, S. M., Sharp, S. B., Safer, B., and Merrick, W. C. (1976), *Nature (London) 263,* 163–165.
Treadwell, B. V., and Robinson, W. G. (1975), *Biochem. Biophys. Res. Commun. 65,* 176–183.
Treadwell, B. V., and Robinson, W. G. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1515.
Weber, K., and Osborne, M. (1969), *J. Biol. Chem. 244,* 4406–4412.
Weissbach, H., and Ochoa, S. (1976), *Annu. Rev. Biochem. 45,* 191–216.
Zasloff, M., and Ochoa, S. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 3059–3063.
Zasloff, M., and Ochoa, S. (1973), *J. Mol. Biol. 73,* 65–76.

Yeast Mitochondrial DNA Specifies tRNA for 19 Amino Acids. Deletion Mapping of the tRNA Genes†

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ABSTRACT: We have previously identified 14 aminoacyl-tRNAs that are specified by yeast mitochondrial DNA (mtDNA). We now report four more amino acids (Arg, Cys, Trp, Thr) that acylate tRNAs which hybridize with mtDNA. Furthermore one of the two mitochondrial tRNAs that we had earlier demonstrated to be directly charged with glutamic acid responds to glutamine but not to glutamic acid codons. Thus Gln-tRNA^{Gln} appears to be formed by transamidation of a missense intermediate Glu-tRNA^{Gln}. This brings to 19 the number of amino acids which have corresponding tRNAs specified by mtDNA. Only tRNA^{Asn} has not yet been shown to be a mtDNA transcript. We have also mapped the genes for the newly identified mitochondrial tRNAs, as well as several others that were previously identified but unmapped, by hybridization to the mtDNA of a series of petite deletion mutants. We now have ordered 20 mitochondrial tRNA genes (including two methionyl-tRNAs) with respect to the antibiotic resistance markers chloramphenicol (C^R), erythromycin (E^R), paromomycin (P^R), and oligomycin I and II (O^R_I, O^R_{II}). Eighteen tRNA genes map between the C and E resistance markers. Only the serinyl-tRNA and glutamyl-tRNA genes are localized near the O_I and O_{II} resistance markers.

Mitochondrial assembly depends upon the coordination of nuclear and mitochondrial genetic and protein-synthetic systems. The respiratory elements responsible for electron transport and coupled phosphorylation, i.e., cytochrome oxidase, coenzyme Q–cytochrome *c* reductase, and oligomycin-sensitive ATPase, are oligomeric complexes consisting of as many as 9 peptides. In each complex, some peptides are synthesized on cytoplasmic and others on mitochondrial ribosomes (Schatz and Mason, 1974). The mitochondrial ribosome itself has a dual origin; most of its components are synthesized extramitochondrially and imported into the organelle. The only presently established transcription products of mitochondrial DNA are tRNAs, the two subunits of mitochondrial rRNA, and 9–10 presumptive mRNA species which probably code for three peptides of cytochrome oxidase, one of cytochrome *b*, four of the oligomycin-sensitive ATPase, and probably one ribosomal protein (Locker and Rabinowitz, 1977). It is not yet known whether the tRNA complement of mitochondria has a dual origin, but the possibility of the import of cytoplasmic tRNAs into the mitochondria has been raised (Chiu et al., 1975).

This possibility was suggested by RNA–mtDNA hybrid-

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