Proc. Nat. Acad. Sci. USA
Vol. 70, No. 1, pp. 41-45, January 1973

Protein Initiation in Eukaryotes: Formation and Function of a Ternary Complex Composed of a Partially Purified Ribosomal Factor, Methionyl Transfer RNA_f, and Guanosine Triphosphate

(binding assay/L-cells/ribosome subunits/protein inhibitors)

DANIEL H. LEVIN, DAVID KYNER, AND GEORGE ACS

Institute for Muscle Disease, Inc., 515 East 71st Street, New York, N.Y. 10021

Communicated by Norton D. Zinder, August 10, 1972

ABSTRACT A protein factor contained in a 1 M KCl extract of L-cell ribosomes and partially purified by chromatography on DEAE-cellulose forms a specific ternary complex with rat-liver Met-$tRNA_f$ and GTP. The complex is measured by its quantitative retention on nitrocellulose membranes. Complex assembly is optimal at 100 mM KCl and 0.2 mM $MgCl_2$, and is independent of mRNA and of ribosomes. The GTP requirement can be replaced over 65% by its methylene analogue $GDPCH_2P$, indicating that GTP hydrolysis is not involved. Complex formation is inhibited by 10 μM aurintricarboxylic acid, but is unaffected by 100 μM pactamycin, 100 μM fusidic acid, or by excess uncharged methionine $tRNA_f$. The ternary complex is relatively stable and appears at the void volume during filtration on Sephadex G-100. At 1-3 mM $MgCl_2$ and in the presence of other factors, the ternary complex is implicated in protein initiation by (*i*) its capacity to bind to the 40S ribosomal subunit to form a 48S complex; and (*ii*) the subsequent association of the 48S complex with a 60S subunit to form a functional "80S complex."

There is considerable evidence to suggest that in eukaryotes, the rate-determining step in protein synthesis occurs during the initiation phase of translation. Such a rate-limiting step has been observed in such diverse tissues as cells in mitosis (1), muscles of diabetic animals (2), livers of fasted animals (3, 4), as well as in cell cultures exposed to aminoacid-deficient medium (5, 6), temperature changes (7), and alterations in sodium-potassium ratios (8). Other studies in both prokaryotes and eukaryotes indicate that the sequence of events leading to the formation of an 80S initiation complex is mediated by the stepwise participation of specific ribosome factors (9-14). Consequently, the first steps in the initiation sequence are of particular importance if it is assumed that they represent logical sites for the operation of regulatory controls. Recent studies in prokaryotic systems have reported that one of the earliest steps in the initiation sequence is the formation of a ternary complex containing the ribosome initiation factor IF-2, fMet-$tRNA_f$, and GTP (15, 16).

In this report, we describe the *in vitro* formation of a eukaryotic ternary complex with similar properties, composed of a partially purified ribosome factor from L-929 mouse fibroblasts (L-cells), rat-liver Met-$tRNA_f$, and GTP. Preliminary data were presented in a previous communication (17). Complex formation is specific for initiator Met-$tRNA_f$ and GTP, and is independent of mRNA and ribosomes. The complex is implicated in the initiation process by its capacity to bind 40S ribosomal subunits, which in turn are incorporated into functional 80S complexes.

METHODS

Chromatography of a Ribosomal Extract on DEAE-Cellulose. A post-mitochondrial extract of L-cells was centrifuged overnight at 0° through a deep cushion of 68% sucrose in TKM buffer (18). The pellet was suspended in 10 mM Tris·HCl (pH 7.5)-250 mM sucrose-0.1 mM EDTA-1 mM dithiothreitol to 5 mg/ml of ribosomes and brought to 1 M KCl. The suspension was slowly stirred for 1 hr at 13°, then centrifuged at 0° for 1 hr at 180,000 × *g*. The top three-fourths of the supernate was dialyzed at 4° for 2 hr against 30 mM Tris·HCl (pH 7.5)-100 mM KCl-1 mM $MgCl_2$-2 mM dithiothreitol (TKMD buffer). Debris was removed by low-speed centrifugation. About 10 mg of dialyzed protein was applied to a DEAE-cellulose column (0.9 cm × 20 cm) previously equilibrated in the same buffer, but containing 50 mM KCl. Four protein fractions were eluted by a discontinuous gradient in TKMD buffer with KCl concentrations of 0.05 M (*A*), 0.1 M (*B*), 0.2 M (*C*), and 0.3 M (*D*). Effluent fractions were monitored at 280 nm to insure separation. Peak fractions were pooled and concentrated by ammonium sulfate precipitation; protein was dissolved in TKMD buffer containing 0.2 M KCl and dialyzed at 4° for 90 min against the same buffer-KCl. Debris was removed as before and the extract was stored at −80° in small aliquots. Under these conditions, fraction *C* maintained its activity for several months without significant loss.

Treatment of Ribosomal Subunits with 1 M KCl. The isolation and separation of L-cell 40S and 60S subunits in 0.5 M KCl have been described (18). For treatment of these particles with 1 M KCl, 20 A_{260} units of each subunit were diluted to 1 ml in 50 mM triethanolamine·HCl (pH 7.6)-1 M KCl-1.8 mM $MgCl_2$ (increased to 3 mM for 60S subunits). The 40S suspension was incubated at 27° for 40 min and the 60S suspension at 18° for 5 min. Each suspension was then centrifuged at 18° through separate 30-ml 10-30% sucrose density gradients in 50 mM triethanolamine·HCl (pH 7.6)-500 mM KCl-5 mM $MgCl_2$ for 5 hr at 64,000 × *g* (18, 19). The subunit peaks were collected and stored as described for the 0.5 M KCl subunits (18).

Preparation of Labeled Aminoacyl-tRNAs. Rat-liver $tRNA_f^{Met}$ and $tRNA_m^{Met}$ were separated by chromatography of bulk tRNA on benzoylated DEAE (BD)-cellulose (18, 20). $tRNA_f^{Met}$ was charged with [^{3}H]methionine (4 Ci/mmol) or [^{35}S]methionine (8 Ci/mmol) with S100 extracts free of

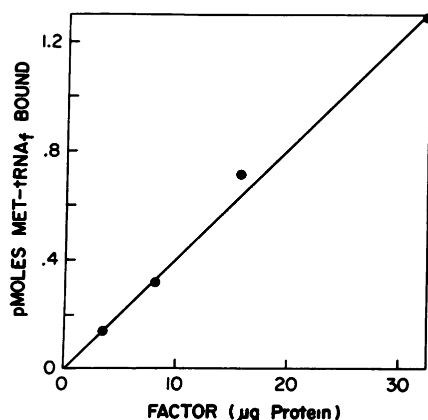


FIG. 1. Dependence of ternary complex formation on factor C concentration. Reaction mixtures and assay conditions are as described in Table 1, except that 7 pmol of [^{3}H]Met-tRNA$_f$ (1200 cpm/pmol) was present in all incubations. Factor C was added as indicated. Each value on the curve is an average of two determinations, and is corrected for the amount of label (0.03 pmol) bound in the absence of C.

endogenous tRNA from *Escherichia coli* strain MRE; tRNA$_m^{Met}$ and purified rat-liver tRNA^{Phe} were charged with [^{3}H]methionine (4 Ci/mmol) and [^{3}H]phenylalanine (11 Ci/mmol), respectively, by crude L-cell synthetases free of endogenous tRNA.

MATERIALS

Reticulocyte supernatant factors EF-T$_1$ and EF-T$_2$ were gifts of Drs. I. Krisco and D. Richter, Rockefeller University (New York, N.Y.). Fusidic acid was a gift of Drs. A. L. Haenni and F. Lipmann of Rockefeller University. Bulk rat-liver tRNA and purified rat-liver tRNA^{Phe} were gifts of Drs. D. Grünberger and I. B. Weinstein of Columbia University (New York, N.Y.). Pactamycin was donated by the National Products Section of the National Cancer Institute (Bethesda, Md.). Aurintricarboxylic acid was obtained from Sigma Chemical Co. (St. Louis, Mo.). [^{3}H]Methionine and [^{3}H]-phenylalanine were purchased from New England Nuclear Corp. (Boston, Mass.), and [^{35}S]methionine was from Amersham/Searle (London).

TABLE 1. *Specific binding of Met-tRNA$_f$ to a ribosomal protein fraction*

	pmol of aminoacyl-tRNA bound		
Fraction	Met-tRNA$_f$	Met-tRNA$_m$	Phe-tRNA
A	0.03	0.03	1.31
B	0.05	0.09	0.33
C	0.92	0.17	0.26
D	0.10	0.04	0.19
Unfractionated	0.22	0.45	4.51

Incubations contained the following components in a final volume of 50 μl: 20 mM Tris·HCl (pH 7.5), 100 mM KCl, 0.2 mM $MgCl_2$, 0.1 mM GTP, 1 mM dithiothreitol, and—where indicated—5 pmol of [^{3}H]Met-tRNA$_f$ (1200 cpm/pmol), [^{3}H]-Met-tRNA$_m$ (1200 cpm/pmol), or 5 pmol of [^{3}H]Phe-tRNA (2900 cpm/pmol). Ribosomal protein fractions obtained by DEAE-cellulose fractionation (*Methods*) were added (where indicated) as follows: 14 μg of fraction *A*, 12 μg of fraction *B*, 17 μg of fraction *C*, 14 μg of fraction *D*, and 27 μg of unfractionated extract. Incubation was at 37° for 5 min. The reaction was stopped by dilution with 3 ml of cold 20 mM Tris·HCl (pH 7.5)–100 mM KCl–0.2 mM $MgCl_2$, and immediately collected on a nitrocellulose membrane (0.45 μm, Millipore Filter Corp.) followed by three rinses of 3 ml each; only the binding complexes were retained. The membrane was dried and counted in Liquiflor (New England Nuclear Corp.) in a Nuclear–Chicago scintillation counter.

RESULTS

Specific Binding of Met-tRNA$_f$ to a Ribosomal Protein Factor. A crude 1 M KCl extract of L-cell ribosomes (see *Methods*) was fractionated on DEAE-cellulose into four protein fractions by a discontinuous gradient with different concentrations of KCl (Table 1). Each protein fraction was tested for its ability to bind rat-liver Met-tRNA$_f$, Met-tRNA$_m$, and Phe-tRNA. The extent of binding was measured by the quantitative retention of the binding complex on nitrocellulose membranes. Fraction C, which eluted at 0.2 M KCl, displayed a selective affinity for Met-tRNA$_f$ compared to Met-tRNA$_m$ and Phe-tRNA (Table 1). Assembly of the complex was dependent upon GTP; on that basis, it was designated a ternary complex with the tentative composition [C–Met-tRNA$_f$–GTP]. Optimal binding of Met-tRNA$_f$ was obtained at 150 mM KCl and 0.2 mM $MgCl_2$, a concentration that contrasts with the high $MgCl_2$ concentration (5 mM) used for the homologous prokaryotic ternary complex (15, 16). The efficient binding of Phe-tRNA by the unfractionated extract may have been due to Phe-tRNA synthetase, which is present in high concentration in the preparation and is eluted primarily in fraction A (Table 1). In this regard, it should be noted that fraction C, which binds Met-tRNA$_f$, was free of Met-tRNA synthetase activity. The amount of ternary complex formed was proportional to the concentration of fraction C (Fig. 1). At limiting Met-tRNA$_f$ concentrations, all of the substrate was bound.

Effect of Inhibitors and Specificity of GTP. The addition of excess uncharged tRNA$_f^{Met}$ or tRNA$_m^{Met}$ did not interfere with the binding of Met-tRNA$_f$, indicating that the methionyl moiety is required for recognition by fraction C (Table 2). Complex formation was essentially unaffected by 0.1 mM fusidic acid or 0.1 mM pactamycin, but was 80%

TABLE 2. *Effect of uncharged tRNA and inhibitors on ternary complex formation*

Additions	pmol Met-tRNA$_f$ bound
—	1.71
tRNA$_f^{Met}$	1.69
tRNA$_m^{Met}$	1.65
ATA (0.1 mM)	0.06
ATA (0.01 mM)	0.32
Pactamycin (0.1 mM)	1.80
Fusidic acid (0.1 mM)	1.58

Incubation conditions and components are as described in Table 1, except that 7 pmol of [^{3}H]Met-tRNA$_f$ (1200 cpm/pmol) and 27 μg of fraction C were present in all incubations. In addition, uncharged tRNA$_f^{Met}$ (0.1 A_{260} units), uncharged tRNA$_m^{Met}$ (0.1 A_{260} units), aurintricarboxylic acid (*ATA*), pactamycin, and fusidic acid were added as indicated.

inhibited by 0.01 mM aurintricarboxylic acid emphasizing the selectivity of aurintricarboxylate inhibition in this step (Table 2). Examination of the GTP requirement revealed that neither ATP nor GDP could substitute for GTP; however, the methylene analogue of GTP, $GDPCH_2P$, was 65% effective in replacing GTP, indicating that complex assembly did not involve GTP hydrolysis (Table 3).

Inactivity of Supernatant Elongation Factors. Fraction *C* also contained traces of the supernatant elongation factors, $EF-T_1$ and $EF-T_2$, which were contaminants of the 1 M KCl extract of the ribosomes. In order to eliminate the possibility that complex formation was due to the elongation factors, we examined the effect of highly purified $EF-T_1$ and $EF-T_2$ on $Met-tRNA_f$ binding to fraction *C* (Table 4). Alone or in combination, the eukaryotic supernatant factors did not replace fraction *C*, nor did they interfere with ternary complex formation (Table 4). Consequently, the complex is unrelated to the ternary complexes containing supernatant elongation factors derived from bacteria (21–23) and calf brain (24).

Molecular Sieve Filtration of the Ternary Complex. Analysis by gel filtration on Sephadex G-100 of the ternary complex prepared with $[^3H]Met-tRNA_f$ and $[\gamma-^{32}P]GTP$ revealed a profile with a peak at the void volume that contained both labeled components (Fig. 2). The position of the complex indicated a molecular weight of at least 100,000. In the absence of either GTP or fraction *C*, no complex was formed. The impurity of fraction *C* and a low level binding of $[\gamma-^{32}P]$-GTP in the absence of $Met-tRNA_f$ (not shown) does not permit a stoichiometric analysis of the reaction components. The appearance of $[\gamma-^{32}P]GTP$ in the complex (Fig. 2) is in accord with the observation that GTP is not hydrolyzed during complex formation (see Table 3). Similar results were reported for the prokaryotic ternary complex (15, 16).

Binding of Ternary Complex to Ribosomes. In the course of studies on a possible functional role for the ternary complex during protein initiation, we found that at 1.5 mM $MgCl_2$, the labeled complex binds to 40S ribosomal subunits previously stripped of initiation factors with 1 M KCl; the new complex sediments to the 48S region in sucrose density gradients (Fig. 3*b*). Binding does not occur with similarly-treated 60S subunits alone (Fig. 3*c*). However, addition of 60S ribosomal subunits to the 48S assembly at 3 mM $MgCl_2$ produces a further shift of label to the 80S region (Fig. 3*d*). No binding of label or subsequent shifts take place if the first incubation for ternary complex formation is omitted. The two stepwise shifts are dependent upon the presence of additional ribosomal factors, but are independent of mRNA. This phenomenon resembles an mRNA-independent binding of Met-

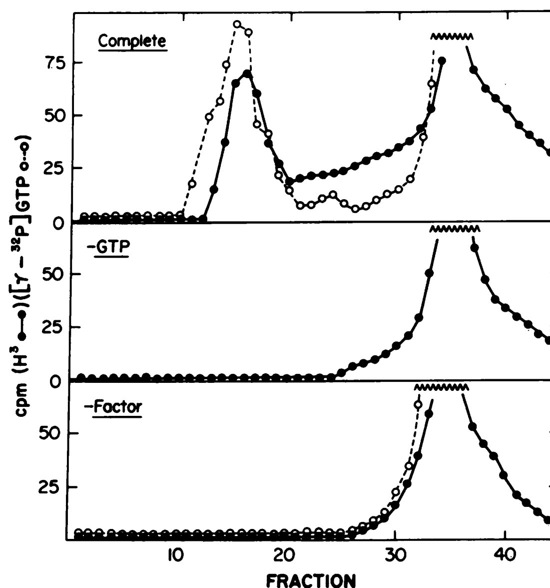


FIG. 2. Profile of ternary complex on Sephadex G-100. Three 50-µl reaction mixtures were prepared as described in Table 1, except that all three contained 5 pmol of $[^3H]Met-tRNA_f$ (1200 cpm/pmol). In addition, two incubations contained 0.1 mM $[\gamma-^{32}P]GTP$ (1800 cpm/pmol) (*top, bottom* panels), and in one incubation GTP was omitted (*middle*); 27 µg of fraction *C* was present in two incubations (*top, middle*) and omitted in one incubation (*bottom*). After 5 min at 37°, the incubations were diluted to 1 ml with cold 20 mM Tris·HCl (pH 7.5)–100 mM KCl–0.2 mM $MgCl_2$, loaded on separate columns of Sephadex G-100 (0.9 cm × 24 cm), and collected in 0.4-ml fractions during filtration in the same buffer. Aliquots were counted directly in Bray's scintillation solution (27) in a Nuclear-Chicago scintillation counter with voltage settings adjusted for double-label counting $[^3H/^{32}P]$ where required (*top* and *bottom*). The intact ternary complex eluted at the void volume (*top*). $[^3H]Met-tRNA_f$ (●——●); $[\gamma-^{32}P]GTP$ (O- - -O).

TABLE 3. *Dependence of ternary complex formation on GTP*

Nucleotide	pmol $Met-tRNA_f$ bound
—	0.04
GTP	0.97
GDP	0.04
$GDPCH_2P$*	0.63
ATP	0.12
GTP + $GDPCH_2P$	0.98

Incubation conditions and components are as described in Table 1, except that 5 pmol of $[^3H]Met-tRNA_f$ (1200 cpm/pmol) and 17 µg of fraction *C* were present in all reaction mixtures. In addition, 0.3 mM GDP, 0.3 mM $GDPCH_2P$, and 0.5 mM ATP were added where indicated.

* β,γ-methylene guanosine triphosphate.

TABLE 4. *Comparison of ribosome factor with $EF-T_1$ and $EF-T_2$*

Enzyme fraction	pmol $Met-tRNA_f$ bound
C	2.01
$EF-T_1$	0.03
$EF-T_2$	0.13
$EF-T_1$ + $EF-T_2$	0.12
C + $EF-T_1$	2.03
C + $EF-T_2$	1.96
C + $EF-T_1$ + $EF-T_2$	2.00

Incubation conditions and components are as described in Table 1, except that 7 pmol of $[^3H]Met-tRNA_f$ (1200 cpm/pmol) was present in all of the incubations. In addition, 27 µg of fraction *C*, 2.1 µg of reticulocyte $EF-T_1$, and 3.2 µg of reticulocyte $EF-T_2$ were added as indicated.

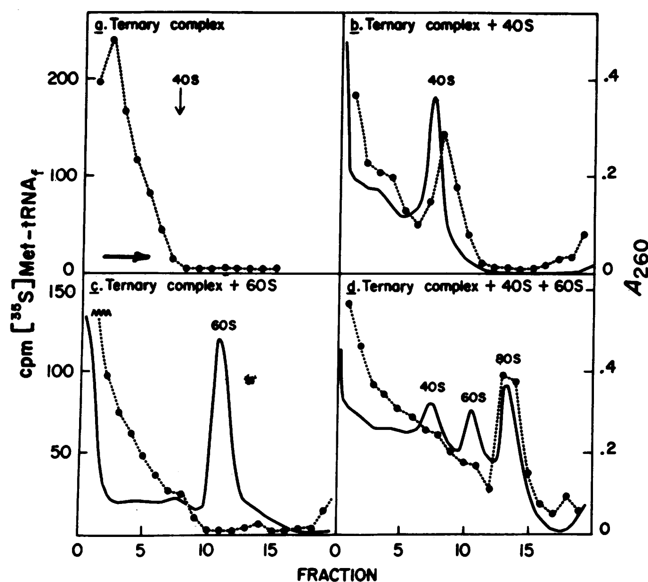


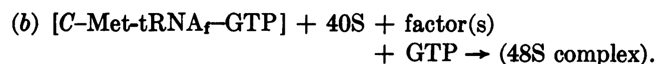
FIG. 3. Sucrose density gradient profiles of initiation complexes. Formation of the ternary complex and binding to ribosomes were performed in three consecutive incubation steps; all contained 20 mM Tris·HCl (pH 7.5), 0.4 mM GTP, and 1 mM dithiothreitol throughout. *Step 1:* additions for ternary complex formation in 25 μl were 4 pmol of [^{35}S]Met-tRNA$_f$ (5900 cpm/pmol), 100 mM KCl, 12 μg of fraction *C*, incubation was at 37° for 5 min (*a*). *Step 2:* additions and changes were 0.25 A_{260} units of 40S subunits (or 0.5 units of 60S subunits), 1.5 mM $MgCl_2$, 80 mM KCl, and 15 μg of unfractionated ribosomal extract in a final volume of 35 μl, incubation was 5 min at 37° (*b*, *c*). *Step 3:* additions and changes were 0.5 A_{260} units of 60S subunits, 3 mM $MgCl_2$, 52 mM KCl in a final volume of 50 μl, incubation was 5 min at 37°. The sedimentation profile of each incubation stage was determined in 5-ml sucrose gradients (5–20%) in the same buffer–salt mixtures used in the corresponding incubation. Centrifugation was at 130,000 × *g* for 55 min at 14°. The gradients were monitored at A_{260} in an ISCO fractionator, and fractions were collected dropwise. Each fraction of each gradient was diluted in the corresponding buffer–salt mixture, collected on nitrocellulose membranes, and counted as described in Table 1. [^{35}S]Met-tRNA$_f$ (●– – –●); A_{260} (——).

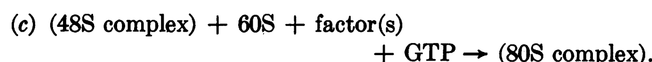
tRNA$_f$ at elevated $MgCl_2$ concentrations (8–16 mM) that was observed (18) with untreated subunits that retained their initiation factors. Although active in protein synthesis, such subunits imposed obvious restrictions upon detailed studies of the initiation sequence *in vitro*. From the current studies, it is apparent that stripped subunits, which depend on exogenous factors and are effective at low $MgCl_2$ concentrations, permit a stepwise analysis of the initiation process.

DISCUSSION

In the studies presented here, a partially purified ribosome factor, designated *C*, is implicated in eukaryotic protein initiation by its ability to specifically bind Met-tRNA$_f$ in a ternary complex with GTP. The possibility that factor *C* represents a ribosomal binding site for initiator Met-tRNA is supported by stepwise formation of 48S and 80S complexes that are dependent upon prior assembly of the ternary complex [*C*-Met-tRNA$_f$-GTP] as follows:

(*a*) *C* + Met-tRNA$_f$ + GTP → [*C*-Met-tRNA$_f$-GTP].


(*b*) [*C*-Met-tRNA$_f$-GTP] + 40S + factor(s) + GTP → (48S complex).


(*c*) (48S complex) + 60S + factor(s) + GTP → (80S complex).

The 80S complex formed by this sequence is functional to the extent that in the presence of puromycin, the methionyl moiety is quantitatively released to form methionyl-puromycin. The specific nature of the factor and GTP requirements in the proposed scheme is still under investigation. Preliminary data indicate that each step in the sequence is mediated by separate factors; in addition, the GTP requirement in reactions (*b*) and (*c*), which has been demonstrated to some extent in other eukaryotic systems (10–12, 25, 26), has also been confirmed (unpublished data).

An interesting anomaly of the above scheme is the apparent absence of an mRNA requirement in the sequential formation of the 80S complex. Since mRNA is an essential component of the true 80S initiation complex, the possibility cannot be excluded that the factor or subunit preparations contain trace amounts of mRNA fragments. However, preliminary data from studies in progress indicate that a factor-dependent addition of exogenous viral mRNA to the sequence considerably augments binding of Met-tRNA$_f$ in an [80S–Met-tRNA$_f$–mRNA] initiation complex. An analysis of the sequence to determine in which step mRNA was inserted revealed two essential features that support the contention that the ternary complex is involved in protein initiation: (*i*) in the stepwise sequence leading to the formation of the 80S initiation complex, no assembly of ribosomal complexes occurred without the prior formation of the ternary complex; and, (*ii*) the binding of the ternary complex to the 40S subunit preceded the incorporation of mRNA (unpublished data).

It is not clear if the entire ternary complex binds to the 40S subunit in reaction (*b*), or if the complex acts primarily as a carrier for initiator Met-tRNA$_f$. However, the similarity of the eukaryotic complex to the prokaryotic ternary complex [IF-2–fMET-tRNA$_f$–GTP] of Rudland *et al.* (15) and of Lockwood *et al.* (16) is manifest. If a mechanism analogous to the prokaryotic complex is assumed to occur in eukaryotes, it is probable that the eukaryotic ternary complex binds intact to the 40S subunit.

Addendum. After submission of this manuscript for publication, evidence for a similar eukaryotic ternary complex [factor–Met-tRNA$_f$–GTP] with a factor derived from reticulocyte ribosomes was published by Chen, Y. C., Woodley, C. L., Bose, K. K., & Gupta, N. K. (1972) *Biochem. Biophys. Res. Commun.* **48,** 1–9.

We thank Miss Gilda Castillo for excellent technical assistance. These studies were supported by the National Institutes of Health (CA-08751), the American Cancer Society (NP-36 I), and the Muscular Dystrophy Associations of America.

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