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Natural mRNA Is Required for Directing Met-tRNA$_f$ Binding to 40S Ribosomal Subunits in Animal Cells: Involvement of Co-eIF-2A in Natural mRNA-Directed Initiation Complex Formation†

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ABSTRACT: Two protein factors, eIF-2 as well as a high molecular weight protein complex from reticulocyte ribosomal high-salt wash which we term Co-eIF-2, promote Met-tRNA$_f$ binding to 40S ribosomes. This binding is dependent on the presence of an AUG codon or natural mRNAs [Roy et al. (1984) *Biochem. Biophys. Res. Commun. 122*, 1418–1425]. Co-eIF-2 contains two component activities, Co-eIF-2A and Co-eIF-2C. Previously, we have purified an 80-kDa polypeptide containing Co-eIF-2A activity and showed that this polypeptide is a component of Co-eIF-2 and is responsible for Co-eIF-2A activity in Co-eIF-2 [Chakravarty et al. (1985) *J. Biol. Chem. 260*, 6945–6949]. We now report purification of a protein complex (subunits of M_r 180K, 110K, 65K, 63K, 53K, 50K, 43K, and 40K) containing Co-eIF-2C activity and devoid of Co-eIF-2A activity. In SDS–PAGE, the purified Co-eIF-2C preparation and an eIF-3 preparation (purified in Dr. A. Wahba's laboratory) separated into seven similar major polypeptides (M_r 110K, 65K, 63K, 53K, 50K, 43K, and 40K). The 50-kDa polypeptide in Co-eIF-2C was immunoreactive with a monoclonal antibody against eIF-4A (50 kDa). We have studied the roles of purified Co-eIF-2A and Co-eIF-2C activities in ternary and Met-tRNA$_f$·40S ribosome complex formation. The results are as follows: (1) At low and presumably physiological factor concentration (30 nM), eIF-2 did not form detectable levels of ternary complex. Moreover, such complex formation was totally dependent on the presence of Co-eIF-2A and/or Co-eIF-2C. (2) Only the ternary complex formed with Co-eIF-2A was stable in the presence of physiological concentrations of natural mRNAs. (3) Co-eIF-2C but not Co-eIF-2A contained guanine nucleotide exchange factor (GEF) activity. (4) In the presence of Mg^{2+} and natural mRNAs, the ternary complex formation by eIF-2 was completely dependent on the presence of both Co-eIF-2A and Co-eIF-2C. Co-eIF-2C is presumably required to promote GDP displacement from eIF-2·GDP while Co-eIF-2A is required to stabilize the ternary complex toward natural mRNAs. (5) The combined presence of eIF-2 together with Co-eIF-2C efficiently promoted AUG-dependent Met-tRNA$_f$·40S ribosome complex formation. However, Co-eIF-2A was required besides eIF-2 + Co-eIF-2C for Met-tRNA$_f$·40S ribosome complex formation dependent on a natural mRNA. Several mRNAs, including globin mRNA, polio RNA, brome mosaic viral RNA, and cowpea mosaic viral RNA, actively stimulated (3–6-fold) Met-tRNA$_f$ binding to 40S ribosomes under the assay conditions. A mechanism for the early steps in peptide chain initiation leading to Met-tRNA$_f$·40S·mRNA complex formation has been proposed.

The first step in peptide chain initiation in animal cells is the formation of a ternary complex between Met-tRNA$_f$, the eukaryotic peptide chain initiation factor 2 (eIF-2)[1] and GTP: Met-tRNA$_f$·eIF-2·GTP. The next step is the transfer of Met-tRNA$_f$ to 40S ribosomes in the presence of mRNA and formation of the Met-tRNA$_f$·40S·mRNA complex. Two outstanding questions in this study and addressed in this paper are related to (1) characteristics and requirements of the peptide chain initiation factors in Met-tRNA$_f$·40S·mRNA complex formation. Several laboratories have reported requirements of multiple protein factors for such complex formation. However, there are significant controversies regarding the characteristics of the protein factors and their roles in peptide chain initiation [for recent reviews, see Ochoa (1983), Moldave (1985), Proud (1986), Gupta (1987), and Gupta et al. (1987)]. (2) The requirement of mRNA for Met-tRNA$_f$ binding to 40S ribosomes is the second question. Earlier studies in several laboratories using crude peptide chain initiation factor preparations have indicated that Met-tRNA$_f$

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[1] Abbreviations: eIF-2, eukaryotic peptide chain initiation factor 2, which forms the ternary complex Met-tRNA$_f$·eIF-2·GTP; Co-eIF-2, a high molecular weight protein complex which contains Co-eIF-2A and Co-eIF-2C activities; Co-eIF-2A, stimulates ternary complex formation and also stabilizes the complex toward physiological mRNA; Co-eIF-2C, promotes GDP displacement from eIF-2·GDP in the presence of Mg^{2+} and also stimulates ternary complex formation; GEF, guanine nucleotide exchange factor which promotes GDP displacement from eIF-2·GDP in the presence of Mg^{2+}; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BMV, brome mosaic viral RNA; CPMV, cowpea mosaic viral RNA.

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binding to 40S ribosomes in animal cells, as in prokaryotes, is dependent on polyribonucleotide messengers containing initiation codons (Dettman & Stanley, 1972; Ilan & Ilan, 1973; Gupta et al., 1975; Smith et al., 1976; Chatterjee et al., 1976). However, several other laboratories using purified factor preparations have claimed that in mammalian protein synthesis, Met-tRNA$_f$ binds to 40S ribosomes in the absence of mRNA and subsequent addition of mRNA has no significant effect on such a binding reaction (Staehelin et al., 1975; Jagus et al., 1981; Hershey, 1982).

We have recently reported (Roy et al., 1984) that two protein factors, eIF-2 and Co-eIF-2, promoted a natural mRNA-dependent Met-tRNA$_f$ binding to 40S ribosomes. Co-eIF-2 is a high molecular weight protein complex and contains two component activities. (1) *Co-eIF-2A* stimulates ternary complex formation by eIF-2 as well as stabilizes the complex in the presence of natural mRNAs (Das Gupta et al., 1978; Roy et al., 1981). We have isolated an 80-kDa polypeptide containing Co-eIF-2A activity and have provided evidence that this Co-eIF-2A polypeptide is a component of Co-eIF-2 and is responsible for Co-eIF-2A activity in Co-eIF-2 (Chakravarty et al., 1985). (2) *Co-eIF-2C* promotes GDP displacement from eIF-2·GDP in the presence of Mg^{2+} (GEF activity) and thus stimulates ternary complex formation (Bagchi et al., 1985a,b). This activity of GEF resembles a similar activity present in reticulocyte cell supernatant and also in ascites ribosomal salt wash and termed RF (Siekierka et al., 1981; Matts et al., 1983; Salimans et al., 1984), GEF (Siekierka et al., 1984; Clemens et al., 1982; Pannier & Henshaw, 1983; Goss et al., 1984), and eIF-2B (Konieczny & Safer, 1983). However, the polypeptide composition of the purified ribosomal salt wash factor (Co-eIF-2C), as reported here, appears somewhat different from those reported for other GEF preparations (Siekierka et al., 1982b; Konieczny & Safer, 1983). Moreover, this ribosomal salt wash factor contains other activities besides GEF (Bagchi et al., 1985a,b). For these reasons, we have tentatively retained the term Co-eIF-2C to describe this similar but not identical factor prepared by other laboratories.

In this paper, we report purification of a protein complex containing Co-eIF-2C activity and devoid of Co-eIF-2A activity. Both Co-eIF-2A and Co-eIF-2C activities were essential for ternary complex formation in the presence of physiological concentrations of eIF-2, Mg^{2+}, and natural mRNAs. The subsequent transfer of Met-tRNA$_f$ to 40S ribosomes, under the assay conditions, was almost completely dependent on the presence of either AUG codon or natural mRNA. Only two factors, eIF-2 + Co-eIF-2C, were required to promote AUG-directed Met-tRNA$_f$ binding to 40S ribosomes whereas the additional factor, Co-eIF-2A as well as eIF-2 + Co-eIF-2C, was required to promote similar Met-tRNA$_f$ binding to 40S ribosomes in the presence of a natural mRNA.

Materials and Methods

Most of the materials used in these studies were the same as described previously (Chakravarty et al., 1985; Bagchi et al., 1985a,b). eIF-2 activity was purified by using DEAE-cellulose (fraction II), phosphocellulose (fraction III), and CM-Sephadex chromatography (fraction IV) (Das et al., 1982; Datta et al., 1988). Fraction IV eIF-2 preparation contains a 67-kDa polypeptide besides the three eIF-2 subunits (Datta et al., 1988). Co-eIF-2A activity was purified as described previously (Chakravarty et al., 1985).

eIF-3 and eIF-4A preparations were purified in Dr. Wahba's laboratory (Mississippi University, Jackson, MS) following the published procedures (Grifo et al., 1983) and were generously donated to us. Dr. Hans Trachsel (Bern, Switzerland) kindly supplied us with monoclonal antibodies against eIF-4A. Polio viral RNA is a gift from Dr. Asim Dasgupta (UCLA). Dr. Leslie Lane (The University of Nebraska, Lincoln, NE) provided us with brome mosaic virus and cowpea mosaic virus. Viral RNA was prepared following the procedure of Mandels and Bruening (1968).

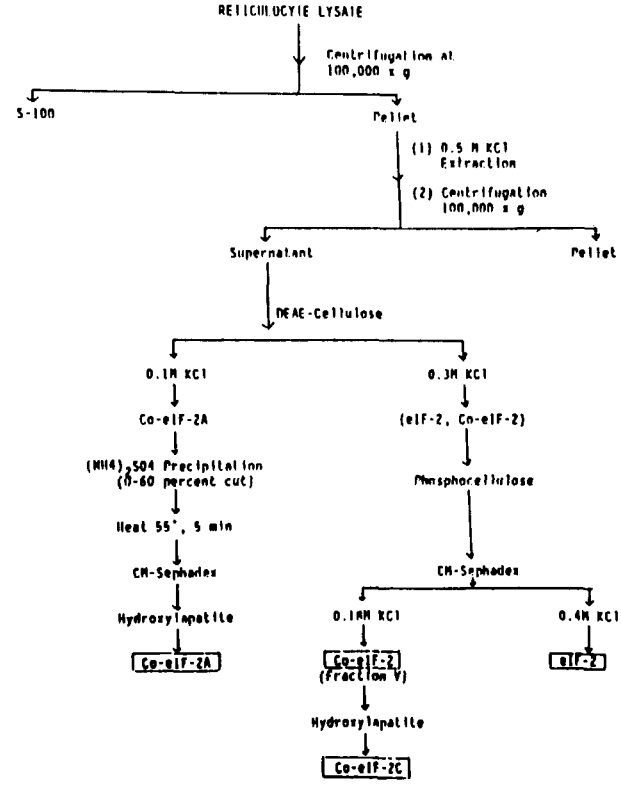

FIGURE 1: Purification scheme for peptide chain initiation factors.

Purification of Co-eIF-2C. The 0.18 M KCl eluant from CM-Sephadex chromatography (Figure 1) containing both Co-eIF-2(A + C) activities was used as the starting material. The eluate solution was dialyzed against 20 mM potassium phosphate buffer (pH 7.6) containing 10 mM β-mercaptoethanol, 50 μM EDTA, and 10% (v/v) glycerol. The dialyzed protein was loaded onto a hydroxylapatite column preequilibrated with 20 mM potassium phosphate buffer, washed with 200 mM potassium phosphate buffer, and eluted with a linear gradient of 200–500 mM potassium phosphate buffer. The activity of Co-eIF-2C was present in the fraction at approximately 450 mM potassium phosphate concentration. The peak fractions were pooled, concentrated by using an Amicon microconcentrator (Centricon-10), dialyzed against buffer B containing 0.1 M KCl, 20 mM Tris-HCl, 50 μM EDTA, 5 mM β-mercaptoethanol, and 10% (v/v) glycerol, and stored in liquid nitrogen.

Other methods and experimental procedures have been described in the legends of tables and figures.

Results

Figure 1 is a diagrammatic presentation of the steps taken for purification of eIF-2, Co-eIF-2, Co-eIF-2A, and Co-eIF-2C activities. The starting material was 0.5 M KCl wash of reticulocyte ribosomes. As reported previously (Chakrabarty et al., 1985), Co-eIF-2 protein complex contains both Co-eIF-2A and Co-eIF-2C activities, and Co-eIF-2A activity in this protein complex is due to the presence of an 80-kDa polypeptide. Upon further fractionation using hydroxyapatite chromatography, Co-eIF-2C preparation devoid of 80-kDa

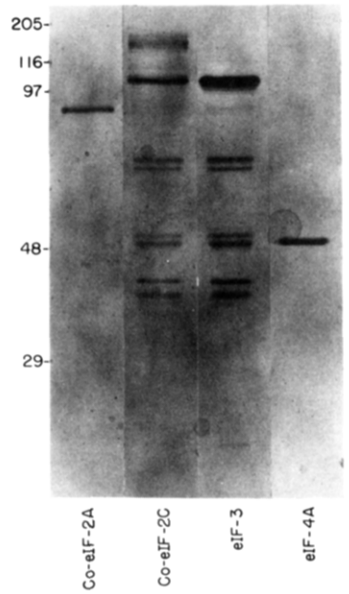

FIGURE 2: SDS–polyacrylamide gel electrophoresis of different factor preparations. The purified proteins were used for polyacrylamide (15%) gel electrophoresis in the presence of sodium dodecyl sulfate at pH 8.3. The electrophoresis was performed at 150 V for 6 h, followed by staining in 0.1% Coomassie brilliant blue R-250. Concentrations of proteins used were as follows: Co-eIF-2A, 5 μg; Co-eIF-2C, 12 μg; eIF-3, 16 μg; eIF-4A, 8 μg.

polypeptide and devoid of Co-eIF-2A activity was obtained. Figure 2 shows SDS–PAGE of Co-eIF-2A, Co-eIF-2C, eIF-3, and eIF-4A.

Most of our results reported here have been obtained by using eIF-2 preparations containing an extra polypeptide (67 kDa) (Datta et al., 1988). Upon further fractionation using a glycerol density gradient procedure, eIF-2 containing the usual three subunits (α, β, and γ) and devoid of the 67-kDa polypeptide can be obtained (Datta et al., 1988). In several experiments, we have used this eIF-2 containing three subunits to form initiation complexes, and the results obtained were similar. Purified Co-eIF-2A showed a single polypeptide band in SDS–PAGE (Figure 2). Careful analysis using different marker proteins revealed that the molecular weight of this Co-eIF-2A polypeptide was slightly higher than previously reported (80K) and was close to 90K. Both the Co-eIF-2C preparation purified in our laboratory and a sample of the eIF-3 preparation from Dr. Wahba's laboratory gave seven apparently identical polypeptide bands (M_r 110K, 65K, 63K, 53K, 50K, 43K, and 40K). In addition, the Co-eIF-2C preparation gave a diffuse higher molecular weight polypeptide band (180K). Purified eIF-4A (from Dr. Wahba's laboratory) gave a single polypeptide band at 50 kDa. The results of an immunoblot experiment using monoclonal antibodies against eIF-4A showed that the 50-kDa polypeptide component in Co-eIF-2C was immunoreactive with these antibodies (Figure 3).

Effects of Co-eIF-2A and Co-eIF-2C on Ternary Complex Formation by eIF-2. The results presented in Figure 4 show the extent of ternary complex formation by eIF-2 at different eIF-2 concentrations and the influence of the addition of Co-eIF-2A and Co-eIF-2C on ternary complex formation. In these experiments, ternary complex formation was studied in the absence of Mg^{2+}. As shown here, at a very low and presumably physiological concentration of eIF-2 (0.25 μg per 75-μL reaction mixture; approximately 30 nM), eIF-2 did not form detectable levels of ternary complex. Importantly, addition of either Co-eIF-2A or Co-eIF-2C stimulated ternary complex formation by eIF-2 in excess of 20-fold. At higher

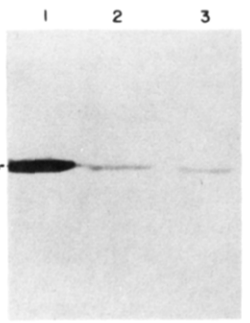

FIGURE 3: Electrotransfer and immunoblots of eIF-4A and Co-eIF-2C. The proteins eIF-4A and Co-eIF-2C were subjected to SDS–PAGE and electrotransferred to nitrocellulose sheets following the procedure provided by Bio-Rad (Trans-Blot Cell Operating Instructions, 1985). The nitrocellulose sheets were then treated as described in the Bio-Rad Immunoblot Kit (The Bio-Rad Immuno Blot Assay Kit, 1987). Monoclonal antibodies against eIF-4A were used. Lane 1, eIF-4A (4 μg); lane 2, Co-eIF-2C (30 μg); lane 3, Co-eIF-2C (15 μg).

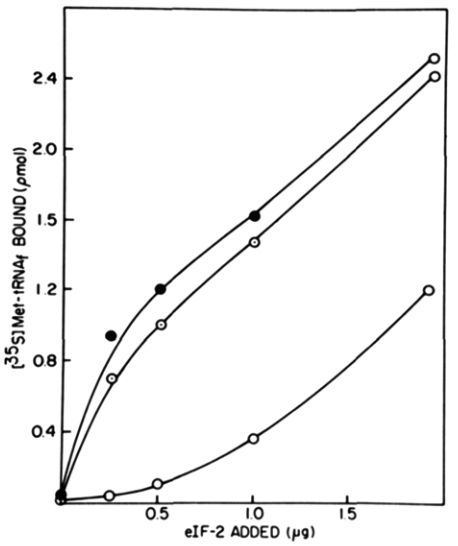

FIGURE 4: Ternary complex formation by eIF-2 in the presence of Co-eIF-2C and Co-eIF-2A. Standard reaction mixtures (in a total volume of (75 μL) contained 20 mM Tris-HCl, pH 7.8, 2 mM dithiothreitol, 70 mM KCl, 10 μg of bovine serum albumin, varying concentrations of eIF-2, and, where indicated, 12 μg of Co-eIF-2C or 10 μg of Co-eIF-2A. The reaction mixtures were incubated at 37 °C for 5 min. At the end of the incubation period, the reactions were stopped by addition of 3 mL of ice-cold wash buffer and were assayed by the standard Millipore filtration assay procedure. Factors added: none (○); Co-eIF-2A (⊙); Co-eIF-2C (●).

eIF-2 concentrations, the ternary complex formation by eIF-2 increased, and although both Co-eIF-2A and Co-eIF-2C significantly stimulated ternary complex formation by eIF-2, the extent of the stimulation with increasing eIF-2 concentration became progressively lower.

These observations suggest that at low and presumably physiological concentrations of eIF-2 [reticulocyte lysates contain 30–40 nM eIF-2 (Safer et al., 1979)], the ternary complex formation by eIF-2 is totally dependent on Co-eIF-2 activities (Co-eIF-2A and/or Co-eIF-2C). We assume that the stimulation observed in both cases is due to a physical binding of the cofactor to the ternary complex and subsequent shifting of the equilibrium in favor of the quaternary complex formation.

We have previously reported that the ternary complex formed with eIF-2 alone is unstable in the presence of natural mRNAs and addition of Co-eIF-2A stimulated ternary complex formation by eIF-2 and also stabilized the complex in the presence of natural mRNAs (Roy et al., 1981). The results

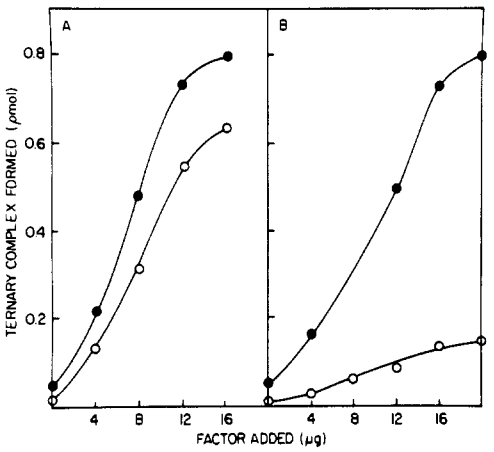

FIGURE 5: Effects of addition of globin mRNA on ternary complex formation in the presence of Co-eIF-2A or Co-eIF-2C. Reaction conditions were the same as described in Figure 3. 0.25 μg of eIF-2 and varying concentrations of either Co-eIF-2A (panel A) or Co-eIF-2C (panel B) were used in the presence (○) or absence of 4 μg of globin mRNA (●). Reaction mixtures were analyzed by using the standard Millipore filtration procedure.

Table I: Factor Requirements for Ternary Complex Formation in the Presence of Mg^{2+} and a Natural mRNA[a]

	[^{35}S]Met-tRNA$_f$ bound to Millipore filters (pmol)			
	$-Mg^{2+}$		$+Mg^{2+}$	
factors added	-mRNA	+mRNA	-mRNA	+mRNA
eIF-2	0.05	0.02	0.01	0.01
eIF-2 + Co-eIF-2C	0.72	0.11	0.45	0.05
eIF-2 + Co-eIF-2A	0.71	0.58	0.18	0.05
eIF-2 + Co-eIF-2C + Co-eIF-2A	0.82	0.70	0.60	0.53

[a] Standard Millipore filtration assay conditions were used. Where indicated, 0.2 μg of eIF-2, 10 μg of Co-eIF-2A, 12 μg of Co-eIF-2C, and 4 μg of globin mRNA were added.

presented in Figure 5 show the effects of addition of globin mRNA on ternary complex formed by eIF-2 in the presence of Co-eIF-2A (Figure 5A) and Co-eIF-2C (Figure 5B). The complex formed with Co-eIF-2A was significantly stable in the presence of globin mRNA whereas the complex formed with Co-eIF-2C dissociated extensively in the presence of mRNA.

GEF Activity. It is now generally agreed that the bulk of reticulocyte eIF-2 is isolated as eIF-2·GDP and that GEF activity is necessary to displace GDP from eIF-2·GDP in order to form ternary complex in the presence of Mg^{2+} (Siekierka et al., 1982b; Bagchi et al., 1985). We tested the purified factor preparations for GEF activity (Figure 6). Apparently, only Co-eIF-2 and Co-eIF-2C are able to promote GDP displacement from eIF-2·GDP. Conversely, eIF-2 and Co-eIF-2A showed no significant activity.

Promotion of Ternary Complex Formation by eIF-2 in the Presence of Mg^{2+} and Natural mRNA Requires the Participation of Co-eIF-2A as Well as Co-eIF-2C. The results presented in Table I show that both Co-eIF-2A and Co-eIF-2C are required for ternary complex formation by eIF-2 in the presence of Mg^{2+} and a natural mRNA. In the presence of Mg^{2+}, only Co-eIF-2C promoted a significant extent of ternary complex formation by eIF-2, presumably because Co-eIF-2C promoted GDP displacement from eIF-2·GDP. However, the Co-eIF-2C-stimulated ternary complex formed with eIF-2, in the presence or in the absence of Mg^{2+}, was unstable when globin mRNA was also present. As shown in Table I, addition of Co-eIF-2A alone gave small but significant stimulation of

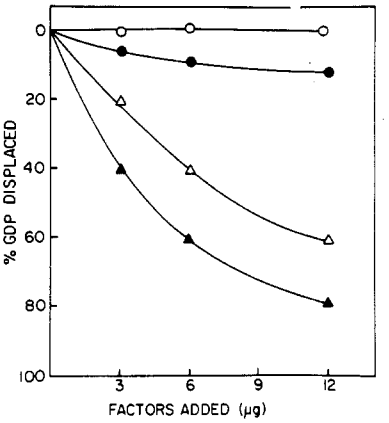

FIGURE 6: Assay for GEF activity in different factor preparations. A two-stage assay procedure was followed. In stage I, eIF-2·[^{3}H]GDP was preformed at 37 °C for 5 min. Reaction mixtures (in 30 μL) contained 1 μg of eIF-2, 0.5 μL of [^{3}H]GDP (5400 cpm/pmol), 20 mM Tris-HCl, pH 7.8, 2 mM dithiothreitol, 70 mM KCl, and 4 μg of bovine serum albumin. The reactions were incubated for 5 min at 37 °C. The reaction mixtures were then mixed with magnesium acetate (final concentration 1 mM) and 100 μM GDP. In stage II, 50 μL of the reaction mixture was further incubated at 37 °C for 2 min in the presence of varying concentrations of different factors, and the [^{3}H]GDP released was measured by the standard Millipore filtration assay method. Factors added: eIF-2 (○); Co-eIF-2A (●); Co-eIF-2C (△); Co-eIF-2 (▲).

ternary complex formation. The nature of this stimulation is not clear as the complex formed was unstable in the presence of globin mRNA. However, when Co-eIF-2A was added in the presence of Co-eIF-2C, the stimulated ternary complex was significantly stable when globin mRNA was included in the reaction mixture.

Requirements for Met-tRNA$_f$·40S Ribosome Complex Formation in the Presence of AUG Codon and Natural mRNAs. We have previously reported that eIF-2 together with Co-eIF-2 promoted Met-tRNA$_f$·40S ribosome complex formation dependent on globin mRNA provided GTP hydrolysis during the complex formation was prevented by replacement of GTP by a nonhydrolyzable GTP analogue, GMP-PNP, or by inclusion of 5′-nucleoside-diphosphate kinase (NDK) and ATP together with GTP in the reaction mixture. The results presented in Figure 7 show which factor is required for the formation of Met-tRNA$_f$·40S ribosome complex in the presence of AUG codon (Figure 7A) or globin mRNA (Figure 7B). As before, [^{35}S]Met-tRNA$_f$ binding to 40S ribosome was assayed by density gradient centrifugation followed by Millipore filtration. Apparently, Met-tRNA$_f$ binding to 40S ribosomes under the assay condition was completely dependent on the presence of either AUG codon (Figure 7A) or globin mRNA (Figure 7B); control experiments without AUG codon or globin mRNA showed negligible binding in each case. Only two factors, eIF-2 and Co-eIF-2C, were required for efficient Met-tRNA$_f$ binding to 40S ribosomes in the presence of AUG codon. Moreover, the addition of Co-eIF-2A had no apparent significant effect on such binding activity (Figure 7A). However, as shown in Figure 7B, the same factor combination, namely, eIF-2 together with Co-eIF-2C, was almost completely inactive in promoting globin mRNA dependent Met-tRNA$_f$·40S complex formation. Such complex formation required Co-eIF-2A in addition to eIF-2 + Co-eIF-2C.

The results presented in Table II shows that several mRNAs tested such as globin mRNA, BMV RNA, CPMV RNA, and polio RNA promoted Met-tRNA$_f$ binding to 40S ribosomes and in each case, Co-eIF-2A presence was essential for such stimulation.

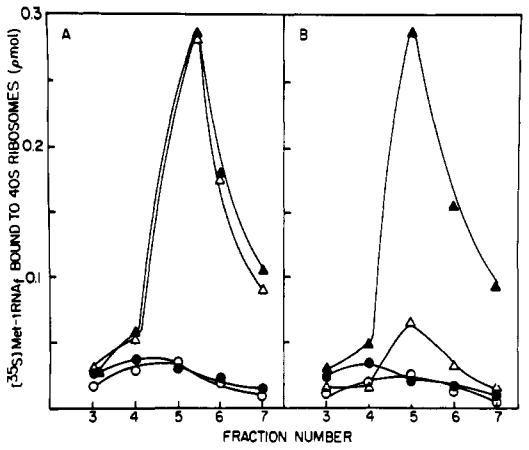

FIGURE 7: Requirement for Met-tRNA$_f$-40S initiation complex formation with AUG codon (panel A) and globin mRNA (panel B). A three-stage assay method was used. In stage I, the reaction mixtures contained (in a total volume of 75 μL) 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM magnesium acetate, 10 μg of bovine serum albumin, 2 mM dithiothreitol, 0.26 mM GMP-PNP, 0.2 μg of eIF-2, 15 μg of Co-eIF-2C, 10 pmol of [^{35}S]Met-tRNA$_f$ (20000 cpm/pmol), and 8 μg of Co-eIF-2A (where indicated). The reaction mixtures were incubated at 37 °C for 5 min. In stage II, 0.08 A_{260} unit of 40S ribosomes and, where indicated, 0.05 A_{260} unit of AUG codon (panel A) or 4 μg of globin mRNA (panel B) were added, and the reaction mixtures were further incubated at 37 °C for 5 min. In stage III, the reaction mixtures were mixed with magnesium acetate (final concentration 5 mM) and further incubated at 0 °C for an addition 15 min. Aliquots of the reaction mixtures (70 μL) were then layered on top of sucrose density gradients (15–27%) containing 20 mM potassium phosphate, pH 6.0, 100 mM KCl, 2 mM magnesium acetate, 1 mM dithiothreitol, and 50 μM EDTA. The gradients were centrifuged at 45000 rpm for 105 min in a 50.1 SW rotor. At the end of the run, the gradients were fractionated by using an ISCO-185 density gradient fractionator, and 0.35-mL fractions from the top of the gradient were collected into 3 mL of wash buffer containing 1 mM magnesium acetate. The solutions were then assayed by using the standard Millipore filtration procedure. AUG codon (panel A) or globin mRNA (panel B) was omitted from the control experiments: control, mRNA (or AUG) (○); +Co-eIF-2A (●); +Co-eIF-2C (△); Co-eIF-2A + Co-eIF-2C (▲).

Table II: Factor Requirements for Met-tRNA$_f$-40S Ribosome Complex Formation with AUG Codon and Different Natural mRNAs[a]

mRNA added	[^{35}S]Met-tRNA$_f$ bound to 40S ribosomes (pmol)	
	−Co-eIF-2A, +Co-eIF-2C	+Co-eIF-2A, +Co-eIF-2C
none	0.27	0.27
AUG	1.44	1.44
globin	0.29	1.16
CPMV RNA	0.25	0.84
BMV RNA	0.26	0.80
polio virus RNA	0.27	0.80

[a] Reaction conditions were the same as described in Figure 6. Where indicated, 4 μg of globin mRNA, 10 μg of CPMV RNA, 10 μg of BMV RNA, and 8 μg of polio viral RNA were added. Concentrations of protein factors used were as follows: eIF-2, 0.4 μg; Co-eIF-2A, 8 μg; and Co-eIF-2C, 12 μg.

Discussion

In this paper, we describe purification of polypeptides with Co-eIF-2A and Co-eIF-2C activities. The purified factors are freed of cross-contamination, and therefore the specific roles of each factor in ternary and Met-tRNA$_f$-40S ribosome complex formation may be analyzed individually. Some pertinent observations reported in this paper which are also related to reports from other laboratories are discussed below:

(i) We provide evidence that at physiological eIF-2 concentration, eIF-2 alone does not form detectable levels of ternary complex in vitro. Moreover, such complex formation is completely dependent on the presence of eIF-2 ancillary protein factors, namely, Co-eIF-2A and/or Co-eIF-2C. This observation is apparently in agreement with the reports by Benne et al. (1979) and also by de Haro et al. (1978). Benne et al. (1979) have reported that at low concentration (40 nM), eIF-2 did not form detectable levels of ternary complex, and de Haro et al. (1978) have reported that ternary complex formation by eIF-2 at low concentration was almost completely dependent upon addition of an eIF-2 stimulatory protein, ESP. This ESP protein isolated from wheat germ is apparently similar in its activity to Co-eIF-2A-like peptide as reported by several other laboratories (Treadwell et al., 1979; Osterhout et al., 1983; Seal et al., 1983). Reports from our laboratory (Ahmad et al., 1985a,b) and others (Lax et al., 1982; Osterhout et al., 1983; Woodley et al., 1981; Wahba & Woodley, 1984) have indicated that wheat germ and other low eukaryotic cells do not appear to contain peptide with Co-eIF-2C or GEF-like activity.

(ii) We report that the ternary complex formed by eIF-2 alone or in the presence of Co-eIF-2C is unstable in the presence of natural mRNAs and requires Co-eIF-2A for stabilization. Other laboratories have also observed that ternary complex is unstable in the presence of natural mRNAs (Kaempfer, 1981) and aurintricarboxylic acid (Lax et al., 1982; Osterhout et al., 1983; Woodley et al., 1981; Wahba & Woodley, 1984) and the complex can be stabilized by Co-eIF-2A from wheat germ (Lax et al., 1982; Osterhout et al., 1983) and *Artemia* (Woodley et al., 1981; Wahba & Woodley, 1984). We provide evidence that both Co-eIF-2A and Co-eIF-2C stimulated ternary complex formation by eIF-2 in the absence of Mg^{2+}. However, such ternary complex was stable in the presence of natural mRNAs only when formed with Co-eIF-2A and not with Co-eIF-2C. Co-eIF-2A is thus uniquely required for ternary complex formation in the presence of natural mRNAs.

(iii) For affecting maximum promotion of ternary and Met-tRNA$_f$-40S·mRNA complex, it was necessary to add a severalfold molar excess of Co-eIF-2A relative to eIF-2. It may be that excess Co-eIF-2A is required to drive the equilibrium toward the quaternary complex (Met-tRNA$_f$-eIF-2·GTP·Co-eIF-2A). However, a significant Co-eIF-2A effect on ternary complex formation could also be demonstrated at or near stoichiometric levels of eIF-2 and Co-eIF-2A [see Figure 5; also see Table IV of Das Gupta et al. (1978)]. It may be that at low and physiological eIF-2 concentrations, a significantly low Co-eIF-2A concentration is sufficient to promote ternary complex formation necessary for protein synthesis.

(iv) Only two protein factors, eIF-2 and a high molecular weight protein complex which we term Co-eIF-2C, are required for AUG-directed Met-tRNA$_f$-40S ribosome complex formation. The requirement of eIF-2 and a high molecular weight protein complex similar to Co-eIF-2C for AUG-stimulated Met-tRNA$_f$ binding to 40S ribosomes has been reported by several laboratories. Siekierka et al. (1982a) have reported the requirement of eIF-2 + a high molecular weight protein complex isolated from reticulocyte cell supernatant and termed GEF for AUG-stimulated Met-tRNA$_f$ binding to 40S ribosomes. Peterson et al. (1979) have reported a similar observation using eIF-2 and a high molecular weight protein complex, eIF-3.

In this paper, we report that the same factor combination which promoted AUG-directed Met-tRNA$_f$ binding to 40S ribosomes also promoted natural mRNA-driven Met-tRNA$_f$

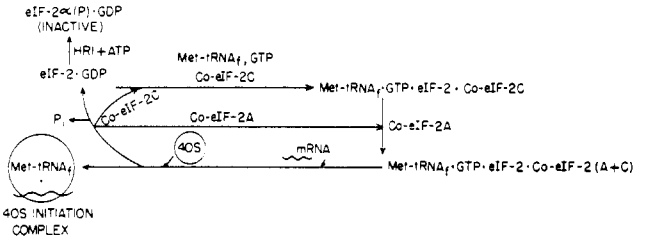

FIGURE 8: Tentative mechanism for the early steps in peptide chain initiation in animal cells leading to the Met-tRNA$_f$40S·mRNA complex formation.

binding to 40S ribosomes when an additional protein factor, purified almost to homogeneity, namely, Co-eIF-2A, was added to the reaction mixture. The simplest explanation for this observation is that Co-eIF-2A stabilized the ternary complex in the presence of natural mRNAs.

(v) Results employing SDS–PAGE showed that the purified Co-eIF-2C preparation reported here as well as the eIF-3 preparation from Dr. Wahba's laboratory possibly contain seven major common polypeptides (out of eight major polypeptides in Co-eIF-2C and seven in eIF-3). The 50-kDa polypeptide component in Co-eIF-2C (also a component of eIF-3) was immunoreactive with monoclonal antibodies against eIF-4A (Figure 3), indicating that eIF-4A may be a component of Co-eIF-2C. Consequently, it may be assumed that this Co-eIF-2C preparation contains eIF-3 activities and also one or more eIF-4 group activities. In addition, this Co-eIF-2C preparation contains significant GEF activity. The specific polypeptide component(s) necessary for GEF activity has (have) not yet been identified. It should be noted that we have purified Co-eIF-2C activity for its ability to stimulate ternary complex formation by eIF-2 and also by its GDP displacement (from eIF-2·GDP) activity. It may be that under our purification conditions, we have isolated in active form the polypeptide components responsible for GEF activity and present in an eIF-3-like protein complex such as Co-eIF-2C. Alternately, it is, however, possible that the GEF activity as well as one or more peptides responsible for mRNA binding activities in Co-eIF-2C preparation is present as a minor contaminant not detectable in SDS–PAGE.

We, therefore, cannot yet assume that our Co-eIF-2C preparation reported here is pure. However, using eIF-2 and this Co-eIF-2C preparation, we can now demonstrate an almost absolute requirement of a near-homogenous protein factor, namely, Co-eIF-2A, for natural mRNA-dependent Met-tRNA$_f$40S ribosome complex formation. The specific requirement of Co-eIF-2A for natural mRNA-dependent Met-tRNA$_f$40S ribosome initiation complex formation is thus established.

On the basis of the results presented here and previously, we propose a tentative model for peptide chain initiation in animal cells (Figure 8). According to this model, eIF-2 is isolated as eIF-2·GDP. A high molecular weight protein complex, Co-eIF-2C, promotes GDP displacement (GEF activity) from eIF-2·GDP and facilitates ternary complex formation. Co-eIF-2C also stimulates ternary complex formation by eIF-2 in the absence of Mg^{2+}, and this activity is not related to its GEF activity. We have suggested that Co-eIF-2C stimulates ternary complex formation as it becomes physically bound to the complex. The complex formed in the presence of Co-eIF-2C is unstable in the presence of natural mRNA. Co-eIF-2A stabilizes this complex presumably as this factor also becomes physically bound to the complex. The complex formed in the presence of Co-eIF-2C and Co-eIF-2A efficiently transfers Met-tRNA$_f$ to 40S ribosomes, and such a transfer reaction is almost completely dependent on the presence of AUG codon or natural mRNA. This transfer reaction is also accompanied by the hydrolysis of GTP in the complex and release of eIF-2 as eIF-2·GDP. We propose that Co-eIF-2C and Co-eIF-2A polypeptides are also released at this step for recycling. Protein synthesis inhibitors such as heme-regulated inhibitor (HRI) or double-stranded RNA-activated inhibitor (dsI) phosphorylate eIF-2 α-subunit and inhibit protein synthesis as Co-eIF-2C does not promote GDP displacement from eIF-2α(P)·GDP and ternary complex is not formed.

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Chromium(III) β,γ-Bidentate Guanine Nucleotide Complexes as Probes of the GTP-Activated cGMP Cascade of Retinal Rod Outer Segments†

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ABSTRACT: The exchange-inert Cr(III) β,γ-bidentate guanine nucleotide complexes Cr(III)GTP and Cr(III)Gpp(NH)p were used to probe the role of transducin in activating the retinal cGMP cascade. The Cr(III) nucleotide complexes were found to have lower binding affinity for transducin as compared to the Mg^{2+} complexes. However, the rate of hydrolysis of the transducin-bound Cr(III)GTP was similar to that of Mg(II)GTP. Cr(III)Gpp(NH)p activated the cGMP phosphodiesterase of photolyzed rod outer segment membranes up to 75% of the Mg(II)Gpp(NH)p level but lacked the ability to dissociate the transducin subunits from the rod outer segment membrane. This result implies that the activation of the phosphodiesterase by transducin–GTP complex is a membrane-associated event and the formation of a soluble complex of transducin–GTP with the inhibitory peptide of the phosphodiesterase may not be an obligatory step. Both the Δ and Λ screw sense stereoisomers of Cr(III)Gpp(NH)p were capable of activating the cGMP cascade with no apparent stereoselectivity. The nature of the interaction of the metal ion and GTP at the nucleotide-binding site of transducin is discussed together with the results from previous studies using the phosphorothioate GTP analogues [Yamanaka, G., Eckstein, F., & Stryer, L. (1985) *Biochemistry 24*, 8094–8101] and is compared to the site found in homologous GTP-binding proteins such as elongation factor Tu [Jurnak, F. (1985) *Science (Washington, D.C.) 230*, 32–36; la Cour, T. F. M., Nyborg, J., Thirup, S., & Clark, B. F. C. (1985) *EMBO J. 4*, 2385–2388]. The implications of the observed results on the molecular mechanism of visual signal transduction are discussed.

Visual excitation in vertebrate rod photoreceptor cells involves a light-activated cGMP cascade [for a review see Liebman et al. (1987), Hurley (1987), Stryer (1986), Applebury and Hargrave (1986), Chabre (1985), and Fung (1985)]. Photoexcitation of rhodopsin leads to the activation of a latent cGMP phosphodiesterase (PDE)[1] in the rod outer segments

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[1] Abbreviations: ROS, rod outer segment; T, transducin; T_α, the α-subunit of transducin; $T_{\beta\gamma}$, the β- and γ-subunit of transducin; PDE, cyclic GMP phosphodiesterase; $P_{\alpha\beta}$, the catalytic subunits of PDE; P_γ, the inhibitory peptide of PDE; Gpp(NH)p, guanosine 5′-(β,γ-imido)-triphosphate; Gpp(CH_2)p, guanosine 5′-(β,γ-methylene)triphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; Cr(III)GTP, $Cr^{III}(H_2O)_4$ β,γ-bidentate GTP complex; Cr(III)Gpp(NH)p, $Cr^{III}(H_2O)_4$, β,γ-bidentate Gpp(NH)p complex; SDS, sodium dodecyl sulfate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithiothreitol; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; PEI, poly(ethylene imine); CD, circular dichroism; EF-Tu, elongation factor Tu.

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