Distinct Functions of Eukaryotic Translation Initiation Factors eIF1A and eIF3 in the Formation of the 40 S Ribosomal Preinitiation Complex*

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We have used an in vitro translation initiation assay to investigate the requirements for the efficient transfer of Met-tRNAf (as Met-tRNAf:eIF2-GTP ternary complex) to 40 S ribosomal subunits in the absence of mRNA (or an AUG codon) to form the 40 S preinitiation complex. We observed that the 17-kDa initiation factor eIF1A is necessary and sufficient to mediate nearly quantitative transfer of Met-tRNAf to isolated 40 S ribosomal subunits. However, the addition of 60 S ribosomal subunits to the 40 S preinitiation complex formed under these conditions disrupted the 40 S complex resulting in dissociation of Met-tRNAf from the 40 S subunit. When the eIF1A-dependent preinitiation reaction was carried out with 40 S ribosomal subunits that had been preincubated with eIF3, the 40 S preinitiation complex formed included bound eIF3 (40 S:eIF3-Met-tRNAf:eIF2-GTP). In contrast to the complex lacking eIF3, this complex was not disrupted by the addition of 60 S ribosomal subunits. These results suggest that in vivo, both eIF1A and eIF3 are required to form a stable 40 S preinitiation complex, eIF1A catalyzing the transfer of Met-tRNAf:eIF2-GTP to 40 S subunits, and eIF3 stabilizing the resulting complex and preventing its disruption by 60 S ribosomal subunits.

Initiation of translation in eukaryotic cells occurs by a sequence of partial reactions requiring the participation of a large number of specific proteins called eukaryotic translation initiation factors (eIFs). In vitro studies using purified initiation factors have shown that the overall initiation reaction commences with the binding of the initiator Met-tRNAf to the Met-tRNAf:eIF2-GTP ternary complex to 40 S subunits to form the 40 S preinitiation complex (40 S:eIF3-Met-tRNAf:eIF2-GTP). In the next step, the 40 S preinitiation complex binds to the capped 5'-end of mRNA in a reaction requiring initiation factors eIF4F, eIF4A, and eIF4B and then scans the mRNA to locate the correct initiation AUG codon where the preinitiation complex is positioned on the mRNA to form the 40 S initiation complex (40 S:eIF3-mRNA-Met-tRNAf:eIF2-GTP). Subsequently, the 60 S ribosomal subunit joins the 40 S complex in an eIF5-dependent reaction to form a functional 80 S initiation complex (80 S:mRNA-Met-tRNAf,eIF3). We have investigated each of these partial reactions to define their requirements. Recently, we described (4) an efficient in vitro translation initiation assay in which the 17-kDa initiation factor eIF1A is necessary and sufficient for the transfer of the initiator Met-tRNAf (as Met-tRNAf:eIF2-GTP ternary complex) to isolated 40 S ribosomal subunits in the absence of mRNA (or an AUG codon) to form the 40 S preinitiation complex (40 S-Met-tRNAf:eIF2-GTP). Several laboratories have, however, reported that the multimeric initiation factor eIF3 that binds to the 40 S ribosomal subunit in vitro in the absence of other initiation components (5) also stimulated the transfer of the Met-tRNAf:eIF2-GTP ternary complex to 40 S subunits to form the 40 S preinitiation complex (40 S:eIF3-Met-tRNAf:eIF2-GTP) (6–9). There is also evidence that in vivo, the majority of native 40 S ribosomal subunits contain bound eIF3 (10, 11). Thus, the possibility exists that in vivo, in the presence of eIF1A, the Met-tRNAf:eIF2-GTP ternary complex binds to a 40 S:eIF3 complex rather than to free 40 S ribosomal subunits to form the 40 S preinitiation complex. In this paper, we investigate the role of both eIF1A and eIF3 in the formation of the 40 S preinitiation complex in vitro. We show that these two proteins perform distinct functions in 40 S preinitiation complex formation. Whereas eIF1A alone is necessary and sufficient for the quantitative transfer of Met-tRNAf eIF2-GTP to isolated 40 S ribosomal subunits, in reaction mixtures containing both 40 S and 60 S ribosomal subunits, eIF3, in addition to eIF1A, is required to form a stable 40 S preinitiation complex.

EXPERIMENTAL PROCEDURES
tRNA, Ribosomal Subunits, Purified Proteins, and Antibodies—The preparation of 35S-labeled rabbit liver Met-tRNAf (20,000–100,000 cpm/pmol) and ribosomal subunits from Artemia salina eggs was described previously (12, 13). Initiation factor eIF2 was purified from rabbit reticulocyte lysates by established procedures (4); homogeneous bacterially expressed recombinant human eIF1A was isolated as described (4). Purified eIF3 was isolated from rabbit reticulocyte lysates as we described recently (9). It consists of six major polypeptides of 110, 67, 42, 40, 36, and 35 kDa but lacked the 170-kDa polypeptide reported by others (14–17) to be a constituent of the eIF3 complex. These eIF3 preparations are highly active in forming AUG-dependent 40 S initiation complex that is fully competent in joining 60 S ribosomal subunits to form a functional 80 S initiation complex (9). Rabbit IgG antibodies specific for mammalian eIF1A were obtained as described (4), and total IgY antibodies specific for mammalian eIF3 subunits were isolated from egg yolks of laying hens immunized with purified rabbit reticulocyte eIF3 (9). Immunoblot analysis was as described previously (4, 9).

Assay for Formation of the 40 S Preinitiation Complex—Formation of
the 40 S preinitiation complex was measured by eIF1A-dependent transfer of Met-tRNA\(_{f}\) (as the Met-tRNA\(_{f}\)-eIF2-GTP ternary complex) to 40 S ribosomal subunits at 1 mM Mg\(^{2+}\) in the absence of mRNA or AUG as follows. Reactions were carried out in two stages. In stage 1, reaction mixtures (50 \(\mu\)l each) containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM 2-mercaptoethanol, 1 mM MgCl\(_2\), 0.6 \(A_{260}\) unit of 40 S ribosomal subunits, and 30–100 ng of purified recombinant eIF1A were incubated for 4 min at 37 °C for 4 min and then chilled in an ice-water bath. Each mixture was then supplemented with 45 \(\mu\)l of a preformed \(^{[35]}\)Met-tRNA\(_{f}\)-eIF2-GTP ternary complex reaction mixture containing 2 pmol of bound \(^{[35]}\)Met-tRNA\(_{f}\) (30,000 cpm/pmol) and 1 \(\mu\)g of nuclease-free bovine serum albumin, 40 pmol of \(^{[32]}\)P-GTP (20,000–50,000 cpm/pmol), and 0.8–1.2 \(\mu\)g of purified rabbit reticulocyte eIF2 were incubated at 37 °C for 4 min to promote formation of the \(^{[35]}\)Met-tRNA\(_{f}\)-eIF2-GTP ternary complex. Reaction mixtures were then chilled in an ice-water bath, and the MgCl\(_2\) concentration was raised to 5 mM and then resolved on sucrose gradient centrifugation as described under “Experimental Procedures.” Panel A, eIF1A added but no 60 S ribosomal subunits; E, eIF1A + 60 S ribosomal subunits added. Panel B, four reaction mixtures were prepared and incubated as described under panel A except that all reaction mixtures contained 2.5 \(\mu\)g of eIF3 and, where indicated, 50 ng of eIF1A during the first incubation. After the addition of 45 \(\mu\)l of a preformed \(^{[35]}\)Met-tRNA\(_{f}\)-eIF2-GTP ternary complex reaction mixture containing 2 pmol of bound \(^{[35]}\)Met-tRNA\(_{f}\), one of the reaction mixtures containing eIF3 alone (○) and the other containing both eIF3 and eIF1A (●) were treated with 0.6 \(A_{260}\) unit of 60 S ribosomal subunits. The Mg\(^{2+}\) concentration of all reaction mixtures was then raised to 5 mM. After incubation at 37 °C for 5 min, the mixtures were chilled in an ice-water bath, and the formation of the 40 S preinitiation complex was analyzed by sucrose gradient centrifugation as described under “Experimental Procedures.” ●, eIF1A alone; ○, eIF1A + 60 S ribosomal subunits added. #, eIF1A + 60 S ribosomal subunits. In both panels, the ascending arrows indicate \(^{35}\)S radioactivity recovered at the top of each gradient tube representing unrecovered free \(^{[35]}\)Met-tRNA\(_{f}\) used during the initial ternary complex formation as well as any unrecovered \(^{[35]}\)Met-tRNA\(_{f}\)-eIF2-GTP ternary complex. The position of sedimentation of the 40 S particle was determined in a parallel reaction and is indicated.

**RESULTS**

**Formation of the 40 S Preinitiation Complex: Requirements for the Binding of the Initiator Met-tRNA\(_{f}\) to 40 S Ribosomal Subunits in the Absence of mRNA**—We have shown previously (4) that the 17 kDa-initiation factor eIF1A was both necessary and sufficient for the transfer of Met-tRNA\(_{f}\) to 40 S ribosomal subunits (4). Whereas the multimeric initiation factor eIF3, which binds to 40 S ribosomal subunits in the absence of other factors (5), also can promote this transfer reaction, the efficiency of Met-tRNA\(_{f}\) transferred by eIF1A was far greater than with an excess of eIF3 alone (4). Furthermore, the catalytic reutilization of eIF1A in the transfer reaction was independent of eIF3. It was not immediately apparent that the binding of eIF3 to 40 S ribosomal subunits played any role in the formation of the 40 S preinitiation complex.

The reactions described above were performed with isolated 40 S ribosomal subunits. To investigate complex formation under more physiological conditions, namely when both 40 S and 60 S ribosomal subunits were present in the same reaction, a preformed \(^{[35]}\)Met-tRNA\(_{f}\)-eIF2-GTP ternary complex was incubated with 40 S ribosomal subunits and eIF1A in the presence of 60 S ribosomal subunits, and the reaction products were analyzed by sucrose gradient centrifugation (Fig. 1). Although eIF1A, as expected, mediated the efficient transfer of \(^{[35]}\)Met-tRNA\(_{f}\) to 40 S ribosomal subunits to form the 40 S preinitiation complex, the presence of 60 S ribosomal subunits severely inhibited the formation of the 40 S preinitiation complex.
C

The addition of 60 S subunits reduced the amount of Met-tRNA$_f$ bound to 40 S subunits from 1.2 pmol to about 0.02 pmol (Fig. 1A). If however, the 40 S ribosomal subunits were preincubated with both eIF1A and eIF3, the 40 S preinitiation complex was formed efficiently even in the presence of 60 S ribosomal subunits (Fig. 1B). About 1.8 pmol of Met-tRNA$_f$ was bound to 40 S ribosomes in the absence of 60 S subunits; the addition of 60 S subunits to the preinitiation reaction resulted in the binding of nearly 1.4 pmol of Met-tRNA$_f$ to 40 S ribosomal subunits. The efficient formation of the 40 S preinitiation complex under these conditions (i.e. in the presence of eIF3 and 60 S ribosomal subunits) was still dependent on the presence of eIF1A in the reaction mixture. Omission of eIF1A from a preinitiation reaction containing only eIF3 resulted in a marked decrease in the amount of Met-tRNA$_f$ bound to 40 S ribosomes (Fig. 1B). However, even under these conditions, the small amount of the 40 S preinitiation complex formed in the presence of eIF3 alone was unaffected by the addition of 60 S ribosomal subunits (Fig. 1B). These results demonstrate that in the presence of 60 S ribosomal subunits, efficient formation of the 40 S preinitiation complex requires the participation of both eIF3 and eIF1A in the reaction. It should be noted here that in these 40 S preinitiation reactions containing 60 S ribosomal subunits, no 80 S initiation complexes were formed because initiation factor eIF5 was not added to these reactions.

**Role of eIF1A and eIF3 in the Formation of the 40 S Preinitiation Complex**—The simplest interpretation of the results obtained above is that the addition of 60 S subunits to 40 S preinitiation reactions at 5 mM MgCl$_2$ resulted in the nonenzymatic association of the ribosomal subunits to form 80 S ribosomes prior to 40 S preinitiation complex formation. It is well established that formation of the 40 S preinitiation complex requires free 40 S subunits; 80 S ribosomes cannot participate in this reaction. Initiation factor eIF3 has been reported to bind to free 40 S ribosomal subunits in the absence of initiator Met-tRNA$_f$ and other initiation factors and prevent the Mg$^{2+}$-dependent association between 40 S and 60 S ribosomal subunits to form 80 S ribosomes (5, 10, 18, 19; also see Ref. 3). Similar antiassociation activity has also been reported for eIF1A (20). In light of this reported antiassociation activity of these two initiation factors, the possibility exists that the presence of both eIF1A and eIF3 in the preinitiation reaction prevented association of the ribosomal subunits, thereby making free 40 S subunits available for 40 S preinitiation complex formation. We therefore investigated whether eIF3 and eIF1A indeed function as ribosomal subunit antiassociation factors in the absence of Met-tRNA$_f$-eIF2-GTP ternary complex. Isolated 40 S ribosomal subunits were incubated with eIF1A and eIF3 at 1 mM Mg$^{2+}$; the Mg$^{2+}$ concentration of the reaction mixture was then raised to 5 mM, 60 S ribosomal subunits were added, and the level of 80 S ribosome formation was determined by sucrose gradient centrifugation (Fig. 2). In control incubations in which no protein factors were added, ribosomal subunits, as expected, remained dissociated at 1 mM Mg$^{2+}$; at 5 mM Mg$^{2+}$, the ribosomal subunits associated to form 80 S ribosomes (Fig. 2, left panel, A and B). However, prior incubation of 40 S ribosomal subunits with eIF3 and eIF1A followed by the addition of 60 S ribosomal subunits failed to prevent association of the subunits to form 80 S ribosomes (Fig. 2, left panel, C). Under similar assay conditions, eIF6, which binds to 60 S ribosomal subunits, prevents the association of 40 S and 60 S ribosomal subunits (data not shown here; see Refs. 21–23). These results demonstrate that in contrast to other reports (18–20), eIF1A and eIF3 by themselves do not have ribosomal subunit antiassociation activity in the absence of Met-tRNA$_f$ and other initiation components.

It should be noted that the lack of antiassociation factor activity of eIF3 was not caused by the absence of binding of eIF3 to 40 S ribosomal subunits. This was shown by incubating eIF3 with 40 S ribosomal subunits followed by the addition of 60 S particles either at 1 mM Mg$^{2+}$ (to keep the subunits

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**Fig. 2. Effect of eIF3 and eIF1A on the association of 40 S and 60 S ribosomal subunits.** Left panel, Three reaction mixtures (A, B, and C), each of 125-μl volume and containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol (reaction buffer), 1 mM MgCl$_2$, and 0.6 F$_{260}$ unit of 40 S ribosomal subunits were prepared. Reaction mixture C contained in addition 5 μg of eIF3 and 0.3 μg of eIF1A. After incubation for 4 min at 37 °C, 1.2 F$_{260}$ units of 60 S ribosomal subunits was added to each reaction mixture, and the Mg$^{2+}$ concentration of reactions B and C was raised to 5 mM, but that for reaction A was maintained at 1 mM. All reaction mixtures were incubated for an additional 4 min at 37 °C, chilled, and then layered onto 7.5–30% sucrose gradients in reaction buffers containing either 1 mM MgCl$_2$ (for reaction A) or 5 mM MgCl$_2$ (reactions B and C) and centrifuged at 48,000 rpm for 105 min in an SW 30.1 rotor. Each gradient was then fractionated in an ISCO gradient fractionator, and the absorbance profile at 254 nm was monitored. The sedimentation positions of 40 S, 60 S, and 80 S ribosomes are indicated. Right panel, binding of eIF3 to 40 S ribosomal subunits. Two reaction mixtures, A and B (25 μl each), containing the reaction buffer, 1 mM MgCl$_2$, and 0.6 F$_{260}$ unit of 40 S ribosomal subunits, were incubated with 10 μg of purified eIF3 for 4 min at 37 °C. Subsequently, 1.2 F$_{260}$ units of 60 S ribosomal subunits was added to each reaction, and the Mg$^{2+}$ concentration of reaction B was adjusted to 5 mM, but that for reaction A was maintained at 1 mM. After an additional incubation for 3 min at 37 °C, the chilled reaction mixtures were centrifuged in sucrose gradients as described under the left panel, except that the MgCl$_2$ concentrations in the gradient buffers in reactions A and B were 1 and 5 mM, respectively. Aliquots of each gradient fraction were then assayed for eIF3 by immunoblot analysis using chicken anti-eIF3 antibodies as probes. A, 40 S + 60 S subunits + eIF3 at 1 mM MgCl$_2$; B, 40 S + 60 S + eIF3 at 5 mM MgCl$_2$. The positions of sedimentation of 80 S ribosomes, 40 S ribosomal subunits, and free eIF3 are indicated.
concomitant formation of 80 S ribosomes (compare Fig. 2, right immunoblot, panel A). However, at 5 mM Mg$_{2+}$, the presence of 60 S ribosomal subunits resulted in the release of eIF3 from 40 S ribosomal subunits (Fig. 2, right immunoblot, panel B) with the concomitant formation of 80 S ribosomes (compare Fig. 2, A and C, of left panel). The release of eIF3 from the 40 S subunits is not caused by the elevated Mg$_{2+}$ concentration alone, since in the absence of 60 S ribosomal subunits, eIF3 remained bound to 40 S particles even at 5 mM Mg$_{2+}$ (data not shown). These results suggest that although eIF3 binds stably to 40 S ribosomal subunits in the absence of Met-tRNA$_f$-eIF2-GTP ternary complex, eIF3 alone or in the presence of eIF1A is devoid of ribosomal subunit antiassociation factor activity. Thus, the formation of the 40 S preinitiation complex by eIF3 and eIF1A in the presence of 60 S ribosomal subunits is not the result of any ribosomal subunit antiassociation activity of these two initiation factors.

These results suggested that the inhibition of eIF1A-mediated 40 S preinitiation complex formation by 60 S ribosomal subunits in the absence of eIF3 could be caused by displacement of the 40 S subunits from the 40 S preinitiation complex formed in an eIF1A-dependent reaction. To investigate this possibility, a preformed [$^{35}$S]Met-tRNA$_f$-eIF2-GTP ternary complex was incubated with 40 S ribosomal subunits in presence of eIF1A to form the 40 S preinitiation complex that was detected by sucrose gradient centrifugation (Fig. 3A). When such a preformed 40 S preinitiation complex was incubated with 60 S ribosomal subunits and then subjected to sucrose gradient centrifugation, the amount of Met-tRNA$_f$ bound to 40 S ribosomes was reduced markedly (Fig. 3A). If, however, 40 S ribosomal subunits were first incubated with eIF3 and then used to form an eIF1A-dependent 40 S preinitiation complex, the subsequent addition of 60 S ribosomal subunits did not destabilize the preinitiation complex (Fig. 3A). Western blot analysis (using anti-eIF3 and anti-eIF1A antibodies as probes) of the gradient fractions derived from the reaction in which the 40 S preinitiation complex was formed in the presence of eIF1A and eIF3 was carried also out (Fig. 3B). As shown, eIF3 was detected in the region of the gradient where the 40 S preinitiation complex also sedimented (Fig. 3B, top panel). In contrast, anti-eIF1A antibodies did not detect eIF1A in this region; eIF1A was detected near the top of the gradient at a position expected for a 17 kDa-protein (Fig. 3B, lower panel). These results show that although eIF1A is released from the 40 S subunit after formation of the 40 S preinitiation complex, eIF3 remains bound to the complex. Furthermore, in contrast to the complex lacking eIF3, the eIF3-bound complex was not disrupted by 60 S ribosomal subunits. It should be noted that in data not presented here, we have shown that 60 S-mediated disruption of the 40 S preinitiation complex in the absence of eIF3 leads to the concomitant formation of 80 S particles devoid of bound Met-tRNA$_f$.

Further Characterization of the Requirements for the Stabilization of the 40 S Preinitiation Complex—The results described above suggest that the 40 S preinitiation complex formed in an eIF1A-dependent reaction was stable in the presence of 60 S ribosomal subunits only when the initiation reaction also contained eIF3. Because eIF1A and eIF3 were present in these initiation reactions, it was unclear whether eIF1A, which is essential for the formation of the 40 S preinitiation complex, also plays a cooperative role with eIF3 in the stabilization of the 40 S preinitiation complex. To investigate this possibility, a preformed [$^{35}$S]Met-tRNA$_f$-eIF2-GTP ternary complex was incubated with 40 S ribosomal subunits and eIF1A in the absence of AUG. The preinitiation complex formed was then isolated free of unreacted reaction components by sucrose gradient centrifugation (see “Experimental Procedures”). Western blotting using anti-eIF1A antibodies confirmed that the purified isolated 40 S preinitiation complex was devoid of eIF1A (data not shown here; see Ref. 4). The stability of such an isolated 40 S preinitiation complex was then investigated (Fig. 4). When the isolated 40 S preinitiation complex was again subjected to sucrose gradient centrifugation, most of the bound Met-tRNA$_f$ dissociated from the 40 S ribosomal subunit (Fig. 4). Released [$^{35}$S]Met-tRNA$_f$ sedimented near the top of the gradient (data not shown in the figure).2 These results indicate that an isolated 40 S preinitiation complex,

In a separate experiment, we observed that nearly 70–80% of the released [$^{35}$S]radioactivity was retained on nitrocellulose membrane filters, indicating that Met-tRNA$_f$ was released from the 40 S subunit as [$^{35}$S]Met-tRNA$_f$-eIF2-GTP ternary complex.

Fig. 3. Role of eIF3 in stabilizing a preformed 40 S preinitiation complex against the destabilizing effect of 60 S ribosomal subunits. Panel A. The reactions were carried out in two separate stages. In stage 1, four separate reaction mixtures, each of 50-µl total volume and containing buffer A (see legend to Fig. 1), 0.3 A$_{260}$ unit of 40 S ribosomal subunits, and 100 ng of purified eIF1A, were prepared. Two of the reaction mixtures (A, △) also contained 2.5 µg of eIF3; the other two (C, ○) lacked eIF3. After the addition of 45 µl of a preformed ternary complex reaction mixture containing 2 pmol of [$^{35}$S]Met-tRNA$_f$-eIF2-GTP ternary complex to each reaction, all mixtures were incubated at 37 °C for 5 min to form the 40 S preinitiation complex. In stage 2, two of the reaction mixtures, one containing only eIF1A (○) and the other containing both eIF1A and eIF3 (△) were treated with 0.6 A$_{260}$ unit of 60 S ribosomal subunits. After raising the Mg$^{2+}$ concentration of all reaction mixtures to 5 mM, they were incubated at 37 °C for 5 min, chilled in an ice-water bath, and the amount of the 40 S preinitiation complex remaining was determined by sucrose gradient centrifugation as described under “Experimental Procedures.” Panel B, aliquots (25 µl) of sucrose gradient fractions of the reaction mixture containing both eIF1A and eIF3 but no 60 S subunits (A) were subjected to separate Western blot analysis using either anti-eIF3 or anti-eIF1A antibodies as probes. The positions of migration of purified eIF3 and eIF1A, probed with specific antibodies, are shown in the leftmost lane of each gel.
purified free of unreacted reaction components, cannot sustain a second round of sedimentation through sucrose gradients even in the absence of 60 S ribosomal subunits, indicating an intrinsic instability of the 40 S preinitiation complex. If, however, the isolated 40 S preinitiation complex was incubated with eIF3 and then subjected to sucrose gradient centrifugation, most (approximately 88%) of the Met-tRNAf remained near the top of the gradient (indicated by arrowf). These values are not shown in the figure.

DISCUSSION

An obligatory intermediate step in translation initiation in eukaryotic cells is the initial binding of Met-tRNAf to 40 S ribosomal subunits (in the absence of mRNA) to form the 40 S preinitiation complex (1–3). This is then followed by the scanning of the mRNA by the 40 S preinitiation complex to locate and then recognize the initiation AUG codon to form the 40 S initiation complex. In this paper, we have investigated the role of eIF1A and eIF3 in the formation of the 40 S preinitiation complex. Results presented here show that although eIF3 is essential for the formation of the 40 S preinitiation complex, the presence of eIF3 bound to the 40 S ribosomes is required for the stability of the 40 S preinitiation complex. eIF1A plays no role in this stabilization effect.

The 60 S ribosomal subunit-mediated dissociation of Met-tRNAf from the 40 S preinitiation complex, formed in the absence of eIF3, occurred prior to its recognition of the AUG codon. If the 40 S preinitiation complex was allowed to interact with AUG to form an initiation complex (40 S-AUG-Met-tRNAf-eIF2-GTP), subsequent incubation with 60 S ribosomal subunits did not cause dissociation of Met-tRNAf even in the absence of eIF3 (Fig. 5). This experiment also indicates that the 40 S preinitiation complex formed in the absence of eIF3 is an unstable complex with a conformation distinct from that of the 40 S initiation complex.
subunits preincubated with eIF3, the resulting 40 S preinitiation complex (40 S·eIF3-Met-tRNA\textsubscript{f}·eIF2-GTP) was resistant to the disruptive action of 60 S ribosomal subunits. These results along with the previous observations made by Thompson et al. (10) and Smith and Henshaw (11), that the majority of native 40 S ribosomal subunits contain bound eIF3, suggest that in the presence of eIF1A, the Met-tRNA\textsubscript{f}·eIF2-GTP ternary complex binds to a 40 S·eIF3 complex rather than to free 40 S ribosomal subunits. Thus, in vivo, when 40 S and 60 S ribosomal subunits are present in the same milieu, both eIF1A and eIF3 are required to form a stable 40 S preinitiation complex, eIF1A catalyzing the transfer of Met-tRNA\textsubscript{f}·eIF2-GTP to 40 S ribosomal subunits, and eIF3 stabilizing the complex against 60 S-mediated disruption.

The recruitment of the 40 S ribosomal subunits containing bound Met-tRNA\textsubscript{f}·eIF2-GTP ternary complex to 5′-cap structure of eukaryotic mRNAs occurs by interaction between eIF3 bound to the 40 S subunit with the eIF4G subunit of initiation factor eIF4F that is associated with the 5′-structure of mRNA (24, 25). In light of this finding, it is tempting to speculate on the physiological significance of the binding of eIF3 to 40 S subunits prior to the transfer of Met-tRNA\textsubscript{f} to form the 40 S preinitiation complex. First, as demonstrated in this work, the 40 S preinitiation complex formed in an eIF1A-dependent reaction in the absence of eIF3 is intrinsically unstable, and disruption of the complex by 60 S subunits is a reflection of this instability. This is consistent with our observation that the 40 S preinitiation complex not containing bound eIF3 and purified free of unreacted reaction components, by sucrose gradient centrifugation, is unstable to a second round of sucrose gradient centrifugation (Fig. 4). Second, the presence of eIF3 in the 40 S preinitiation complex is a stringent prerequisite for it to bind to mRNA. A 40 S preinitiation complex formed in the absence of eIF3 is a “dead-end” complex because it cannot bind mRNA, and the disruption by 60 S ribosomal subunits leads to the release of bound initiation factors that can then participate in effective initiation reactions. Thus, it can be argued that the 60 S subunit has a “proofreading” role ensuring that only a bona fide 40 S preinitiation complex, one with bound eIF3, and thus cued to bind mRNA in the subsequent step, is formed stably during translation initiation.

Our results also demonstrate that eIF3 and eIF1A do not prevent 40 S and 60 S ribosomal subunit association in the absence of other initiation components and may not be involved in the generation of free ribosomal subunits. It is worth noting that electronmicrographic studies of Srivastava et al. (26) showed that eIF3 binds to a site on the 40 S particle which is oriented away from the interaction site with the 60 S subunit. The ribosomal subunit antiassociation activity of eIF3 and eIF1A reported by other laboratories (10, 18–20) could be caused by the use of fixatives (e.g. glutaraldehyde) to stabilize the 40 S·eIF3 complex which may lead to nonphysiological interactions. The results presented here, however show that eIF3 does have antiassociation factor activity in the context of 40 S preinitiation reactions. In the absence of eIF3, 60 S subunits can displace the 40 S subunit present in the 40 S preinitiation complex and associate with 40 S subunits to form 80 S ribosomes. The presence of eIF3 bound to the 40 S preinitiation complex prevents 60 S subunits from displacing 40 S subunits from the preinitiation complex. Thus, under these conditions, 80 S ribosomes cannot be formed.

The eIF3 preparations used in the present study were purified based on their ability to stimulate the AUG-dependent binding of Met-tRNA\textsubscript{f} to 40 S ribosomal subunits to form the 40 S initiation complex (9). The subunit composition of these eIF3 preparations is somewhat different from that reported by others (see Ref. 3), notably the absence of p170 polypeptide in our purified eIF3 preparations (9). We demonstrated previously (9) that the p170 polypeptide is a “dissociable” subunit of mammalian eIF3. Thus although the presence of this polypeptide in eIF3 may be required for the 40 S preinitiation complex to bind mRNA, or for its ribosomal subunit antiassociation activity in the absence of other initiation components, this polypeptide is dispensable for reactions described in this paper.

Pestova et al. (27) have reported recently that initiation factors eIF1A and eIF1 act synergistically to mediate the assembly of the 40 S ribosomal initiation complex at the initiation codon. In light of our studies (Ref. 4 and this work) demonstrating that eIF1A is essential for the formation of the 40 S preinitiation complex, it is possible that the role of eIF1A on AUG selection observed by a Pestova et al. (27) may be the result of the effect of eIF1A on 40 S preinitiation complex formation prior to its binding to mRNA. Further experiments are clearly necessary to establish whether eIF1A, in addition to its function in the formation of the 40 S preinitiation complex, is also essential for the selection of the AUG codon on mRNA by the 40 S preinitiation complex.

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