Function of Eukaryotic Translation Initiation Factor 1A (eIF1A) (Formerly Called eIF-4C) in Initiation of Protein Synthesis*

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We have used an efficient in vitro translation initiation system to show that the mammalian 17-kDa eukaryotic initiation factor, eIF1A (formerly designated eIF-4C), is essential for transfer of the initiator Met-tRNAf (as Met-tRNAf,eIF2-GTP ternary complex) to 40 S ribosomal subunits in the absence of mRNA to form the 40 S preinitiation complex (40 S Met-tRNAf,eIF2-GTP). Furthermore, eIF1A acted catalytically in this reaction to mediate highly efficient transfer of the Met-tRNAf,eIF2-GTP ternary complex to 40 S ribosomal subunits. The 40 S complex formed was free of eIF1A indicating that its role in 40 S preinitiation complex formation is not to stabilize the binding of Met-tRNAf to 40 S ribosomes. Additionally, the eIF1A-mediated 40 S initiation complex formed in the presence of AUG codon efficiently joined 60 S ribosomal subunits in an eIF5-dependent reaction to form a functional 80 S initiation complex. In contrast to other reports, we found that eIF1A plays no role either in the subunit joining reaction or in the generation of ribosomal subunits from 80 S ribosomes. Our results indicate that the major function of eIF1A is to mediate the transfer of Met-tRNAf to 40 S ribosomal subunits to form the 40 S preinitiation complex.

The initiation of translation in eukaryotic cells occurs by a sequence of well defined partial reactions that require a large number of specific proteins called eukaryotic translation initiation factors (eIFs)1 (for reviews, see Refs. 1–5). According to the current accepted view of initiation (4), the first step involves the binding of GTP and the initiator Met-tRNAf, to initiation factor eIF2 to form the Met-tRNAf,eIF2-GTP ternary complex, which is then transferred to the 40 S ribosomal subunit in a reaction that is stimulated approximately 2-fold by the multi-subunit initiation factor eIF3 to form the 40 S preinitiation complex (eIF3-40 S Met-tRNAf,eIF2-GTP). The 40 S preinitiation complex then binds to the capped 5’ end of mRNA in a reaction requiring eIF4F, eIF4A, and eIF4B and begins scanning the mRNA until it selects the appropriate initiation AUG codon where the 40 S complex is positioned on the mRNA through codon-anticodon base pairing to form the 40 S initiation complex (40 S mRNA Met-tRNAf,eIF2-GTP). Initiation factor eIF5 then interacts with the 40 S initiation complex resulting in the hydrolysis of 40 S subunit-bound GTP. The hydrolysis leads to the release of the initiation factors bound to the complex and the concomitant joining of the 60 S ribosomal subunit to the 40 S complex to form the functional 80 S initiation complex (1–5). It has been reported that a 17-kDa protein, eIF1A (formerly called eIF-4C), stimulates formation of both the 40 S and 80 S initiation complexes about 2- and 3-fold, respectively (6–9). The Saccharomyces cerevisiae homologues of eIF1A and another low molecular weight initiation factor, eIF1, have been shown to be essential genes (10–12) that play important roles in translation initiation. However, the function of these proteins in the initiation pathway is not clearly understood.

In our laboratory, we have been studying each partial reaction of the initiation pathway separately to define the requirements of each reaction. We have previously shown (13) that when the Met-tRNAf,eIF2-GTP ternary complex was incubated with 40 S ribosomal subunits and AUG at an elevated Mg2+ concentration of 5 mM (the physiological Mg2+ concentration is about 1–2 μM), the ternary complex was efficiently transferred to 40 S ribosomal subunits to form the stable 40 S initiation complex (40 S AUG Met-tRNAf,eIF2-GTP) without the requirement for any additional initiation factors. This 40 S initiation complex served as an efficient substrate for the eIF5-mediated joining of 60 S ribosomal subunits to form a functional 80 S initiation complex (13–16). These reactions have been utilized in our laboratory to purify and characterize eIF5 from both mammalian cells (14–18) and S. cerevisiae (19, 20). However, we have now observed that when Met-tRNAf,eIF2-GTP ternary complex was incubated with 40 S ribosomal subunits and AUG at a more physiological Mg2+ concentration of 1–2 mM, little or no transfer of Met-tRNAf to 40 S subunits occurred. During the course of purification of initiation factors from 0.5 M KCl ribosomal wash proteins from rabbit reticulocyte lysates, we observed that the addition of a protein fraction eluting from the phosphocellulose column with 1 M KCl to the 40 S initiation reaction mixture at 1 or 2 mM Mg2+ supported efficient transfer of the ternary complex to 40 S ribosomal subunits. Purification and characterization of the stimulatory factor present in the 1 M phosphocellulose eluate showed that the activity responsible for this transfer is the 17-kDa-eIF1A. This result was surprising in view of the previous reports (6–8) that eIF1A stimulated 40 S initiation complex formation only 1.5–2-fold.

In this paper, we have used homogeneous eIF1A isolated from rabbit reticulocyte lysates as well as bacterially expressed recombinant eIF1A to investigate the role of this protein in the formation of 40 S and 80 S ribosomal initiation complexes. The function of this protein as a ribosomal subunit anti-association factor has also been studied. The results we have obtained demonstrate that eIF1A is essential for the transfer of Met-
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tRNA (as Met-tRNAf-eIF2-GTP ternary complex) to 40 S ribosomal subunits prior to the binding of mRNA to form the 40 S preinitiation complex. In contrast to reports from other laboratories (6–9), we find that eIF1A has no demonstrable role either in the joining of 60 S ribosomal subunits to the 40 S initiation complex or in the dissociation of 80 S ribosomes.

**EXPERIMENTAL PROCEDURES**

**tRNA, Ribosomes, and Purified Proteins—**The preparation of [3S- or [H]-labeled rabbit liver Met-tRNAf (10,000–30,000 cpm/pmol) and ribosomal subunits from Artemia salina eggs as described previously (21, 22) for the isolation of recombinant eIF5 was isolated from *Escherichia coli* as described (23). Initiation factors eIF2 and eIF3 were purified from 300 ml of rabbit reticulocyte lysates (Green Hectares Co.) as follows. The preparation of crude ribosomal 0.5 M KCl wash proteins and their subsequent fractionation by successive stepwise elution from DEAE-cellulose and phosphocellulose columns followed by FPLC-Mono Q (HR5/5) chromatography were as described previously for the isolation of rabbit reticulocyte eIF5 from this laboratory (16). During the Mono Q gradient step, eIF2 eluted at ~270 mM KCl while eIF3 eluted at ~400 mM KCl. Fractions containing eIF2 activity were pooled and further purified by FPLC-Mono S chromatography by an adaptation of the procedure of Dholakia and Wahba (24). Purified eIF2 exhibited three distinct polypeptide bands, corresponding to the α, β, and γ units of eIF2 (25). For further purification of eIF2, the pooled Mono Q-eIF3 fraction was concentrated to about 1 ml by Centricon-30 filtration and then purified by centrifugation at 40,000 rpm in two 15–40% (w/v) glycerol gradients containing 20 mM Tris-Cl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, and 650 mM KCl for 20 h in an SW41 rotor at 2 °C. Fractions containing eIF3 activity were pooled, dialyzed against buffer A (20 mM Tris-Cl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol) containing 100 mM KCl to reduce the KCl concentration to 100 mM, and then fractionated on a FPLC-Mono S column (1 ml bed volume). eIF3 activity eluted at about 400 mM KCl. Active fractions were pooled, dialyzed for about 5 h against buffer A containing 55% glycerol and 100 mM KCl, and then stored in small aliquots at −70 °C.

**Purification of the Stimulatory Protein (eIF1A) from Rabbit Reticulocyte Ribosomal Salt-wash Proteins—**Crude 0.5 M KCl ribosomal wash proteins (120 mg) prepared from 300 ml of rabbit reticulocyte lysates (16) were loaded onto a 40-ml bed volume of a DEAE-cellulose column that had been equilibrated in buffer A + 100 mM KCl. After washing the column with buffer A containing buffer A + 300 mM KCl and dialyzed against buffer A + 75 mM KCl to reduce the ionic strength of the fraction to that of buffer A, 100 mM KCl, the dialyzed DEAE-cellulose eluate was then applied to a 12-ml bed volume of a phosphocellulose column, equilibrated in buffer A + 100 mM KCl. The column was washed with 5 ml of this buffer, and bound proteins were then eluted with 40 ml of buffer A containing increasing concentrations of KCl as follows: (a) 300 mM KCl, (b) 650 mM KCl, and (c) 1 M KCl. The phosphocellulose, 1 M KCl eluate (0.5 mg of protein) was dialyzed against buffer A + 100 mM KCl to reduce the ionic strength to that of buffer A + 100 mM KCl and then applied to a 1-bed volume FPLC-Mono Q column. The column was washed with 5 ml of this buffer, and bound proteins were then eluted with two consecutive linear gradients in buffer A (1 ml/min) as follows: 100 mM → 250 mM KCl (total volume 5 ml), followed by 250 mM → 600 mM KCl (total volume 25 ml). Fractions containing eIF1A activity (eluting at about 340 mM KCl) were pooled and stored in small aliquots at −70 °C.

**Expression of eIF1A in E. coli and Purification of the Bacterially Overproducing Recombinant Protein—**The open reading frame of eIF1A cDNA (10) was synthesized by reverse transcriptase-polymerase chain reaction of HeLa poly(A) RNA using Life Technologies, Inc. kit. The primer sequences used were as follows: N terminus, 5′-dCGGATCCATATGGCCGAAAGATATAA-3′; and C terminus, 5′-dCGTCTAGAGAATCTCTTATGCTCATAATATCATTC-3′. The 460-nucleotide long polymerase chain reaction product was sequenced to ensure error-free DNA sequence and cloned into the Ndel/EcoRI sites of pET-5a plasmid (26) (Novagen). This PET-5a-eIF1A expression vector in which the eIF1A coding sequence is under the transcriptional control of a T7 RNA polymerase promoter was used to transform E. coli BL21 (DE3) cells (Novagen). Cells containing T7 RNA polymerase were induced with 0.1 mM isopropyl-β-D-thiogalactoside and grown at 37 °C. The yield was about 3 mg of homogeneous protein. eIF1A was monitored at different purification steps by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining as well as by immunoblot analysis using rabbit polyclonal anti-eIF1A antibodies as the probe (data not shown).

**Purification of [35S]-Labeled eIF1A—** E. coli BL21 (DE3) harboring the pET-5a-eIF1A expression plasmid was grown at 37 °C in 500 ml of M9 medium (27) containing 1 mM MgSO4 and 50 μg/ml ampicillin to an A600 of about 1.0. The cells were then pelleted by centrifugation and resuspended in 500 ml of prewarmed M9 medium containing 0.4 mM MgSO4 and 50 μg/ml ampicillin. Cells were grown for 1 h at 37 °C with vigorous aeration, induced with 1 mM isopropyl-β-D-thiogalactoside, and then grown for an additional 150 min. The culture was then supplemented with 5 mM [35S]methionine and [35S]cysteine (Tran35S-label, DuPont NEN), and the cells allowed to grow with vigorous aeration for an additional 30 min. Cells were then harvested by centrifugation, washed with ice-cold 50 mM Tris-Cl, pH 8.0, followed by an additional washing with 0.9% NaCl, and then quick-frozen. Homogeneous [35S]-eIF1A (~5,000 cpm/ml protein) was isolated from the frozen cells using the purification protocol described above for the isolation of unlabeled recombinant eIF1A.

**Immunological Methods—**Approximately 60 μg of purified rabbit reticulocyte eIF1A were subjected to SDS-polyacrylamide (10%) gel electrophoresis. The 17-kDa-eIF1A polypeptide was excised with a scalpel and used as an antigen for the preparation of antisera against eIF1A in New Zealand White rabbits, following a protocol similar to that previously used for anti-eIF5 antibodies (15). The antibodies were affinity purified against purified rabbit reticulocyte stimulatory protein (eIF1A) by an adaptation of the procedure of Olmedo (28) as described for affinity purification of anti-eIF5 antibodies by Ghosh et al. (15). Immunoblot analysis of eIF1A was performed using rabbit polyclonal anti-eIF1A antibodies as probes following a procedure previously used in this laboratory for immunoblot analysis of eIF5 (15).

**Assay of eIF1A Activity—** eIF1A activity was routinely assayed for its ability to mediate the transfer of [35S]- or [H]-labeled Met-tRNA from Met-tRNAf-eIF2-GTP ternary complex to 40 S ribosomal subunits in the presence of 1 mM Mg2+. Reactions were carried out in two stages as follows. In stage 1, 0.8–1.2 μg of purified eIF2, 8 pmol of [35S]Met-tRNAf, and 6 μM GTP were incubated in reaction mixtures (50 μl) containing 20 mM Tris-Cl, pH 7.5, 100 mM KCl, 5 mM 2-mercaptoethanol, and 4 μg of nuclelease-free bovine serum albumin for 4 min at 37 °C to promote formation of the [35S]Met-tRNAf-eIF2-GTP ternary complex. In stage 2, the above reaction mixtures were supplemented with eIF1A (10–200 ng of protein), 0.05 mM γ32P-ATP, 1.5 μM GTP, and 0.6 μCi 3H-labeled rabbit liver Met-tRNAf, and MgCl2 (1 mM final concentration). Following incubation at 37 °C for 4 min, reaction mixtures (125 μl each) were chilled in ice water and layered onto 7.5–30% (w/v) sucrose gradients (5 ml each) containing 20 mM Tris-Cl, pH 7.5, 1.5 mM MgCl2, 100 mM KCl, 5 mM 2-mercaptoethanol, and centrifuged at 48,000 rpm for 105 min in a SW 60.1 rotor. Fractions (200–350 μl) were collected from the bottom of each tube, and the radioactivity was measured by counting in Aquasol in a liquid scintillation spectrometer. Under the conditions of this assay, detection of the eIF1A-dependent guanine nucleotide exchange factor is proportional to the amount of eIF1A added. The efficiency of 40 S initiation complex formation was calculated relative to [35S]Met-tRNAf-eIF2-GTP ternary complex formed in stage 1 incubation.

**Isolation of the 40 S Initiation Complex—** An incubation reaction mixture (175 μl) was prepared and incubated as described above under “Assay of eIF1A Activity” with the following modifications. During the
Formation of the ternary complex (stage 1 incubation), 40 pmol of \[^{35}S\]Met-tRNA, (20,000 cpm/pmol), 12 µg of purified eIF2, and 18 µM GTP were added, and during the formation of the 40 S initiation complex (stage 2), 1.8 A260 units of 40 S ribosomal subunits, 0.12 A260 unit of AUG, and 0.6 µg of purified recombinant eIF1A were added. After incubation to form the 40 S initiation complex, the chilled reaction mixture was subjected to 7.5-30% sucrose gradient centrifugation to form the stable 40 S initiation complex. In contrast, the AUG-directed system directly measured the requirements for the initial transfer of Met-tRNA, to the 40 S ribosomal subunit by codon-anticodon base pairing to form the stable 40 S initiation complex (40 S-AUG-Met-tRNA,eIF2-GTP). In this system, the requirements for the mRNA-binding proteins eIF4A, eIF4B, and eIF4F, which are essential for the scanning of mRNA, are bypassed.

The requirements for the initial transfer of Met-tRNA,eIF2-GTP ternary complex to 40 S ribosomes prior to mRNA binding were examined. For this purpose, preformed \[^{35}S\]Met-tRNA,eIF2-GTP ternary complex was incubated with 40 S ribosomal subunits in the presence of AUG codon, and the reaction products were then analyzed by sucrose gradient centrifugation. When the initiation reaction was carried out at an elevated Mg\(^{2+}\) concentration of 5 mM, \[^{35}S\]Met-tRNA, (presumably as Met-tRNA,eIF2-GTP ternary complex) was nearly quantitatively transferred to 40 S ribosomes without the requirement of any additional protein factors (Fig. 1 and Refs. 13-16). In contrast, when the initiation reaction was carried out at the more physiological Mg\(^{2+}\) concentration of 1 or 2 mM (only the results with 1 mM Mg\(^{2+}\) are shown), little or no 40 S initiation complex was formed (Fig. 1). To identify one or more initiation factor(s) that may be required for the formation of the 40 S preinitiation complex at 1–2 mM Mg\(^{2+}\), we fractionated the 0.5 M KCl wash proteins derived from rabbit reticulocyte polysomes through successive DEAE and phosphocellulose chromatographic steps as described under “Experimental Procedures.” As shown in Fig. 1, supplementation of the components of the 40 S initiation reaction with the protein fraction eluting from phosphocellulose column with 1 M KCl resulted in highly efficient transfer of \[^{35}S\]Met-tRNA, to 40 S ribosomal subunits. While only 0.15 pmol of Met-tRNA, was bound to 40 S ribosomes in the absence of the phosphocellulose fraction, the addition of this fraction resulted in the binding of 2.4 pmol of Met-tRNA, to 40 S ribosomes (a stimulation of nearly 18-fold). Initiation factor eIF3, in agreement with reports from other laboratories (7–9), also stimulated this transfer reaction approximately 3-fold. However, the efficiency of transfer of Met-tRNA, to 40 S ribosomes mediated by the stimulatory protein was far greater than that obtained with an excess of eIF3 (Fig. 1). The stimulatory activity present in the phosphocellulose fraction was further purified by gradient elution from an
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**FPLC-Mono Q column (see “Experimental Procedures”).** Analysis of this activity by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining showed a single polypeptide band of about 17 kDa (Fig. 2, panel A).

The apparent molecular weight of the stimulatory protein and its strong affinity for phosphocellulose are properties similar to those of the initiation factor eIF1A (formerly eIF-4C) (4). Mass spectrometric analysis (not shown) showed that the purified fraction containing the stimulatory activity contained one major protein species of Mr 16,332. This value is identical to the molecular weight of eIF1A lacking the N-terminal methionine as deduced from the human eIF1A-cDNA clone (10). Further confirmation that the stimulatory activity purified here was indeed eIF1A was derived from the following observations. First, recombinant eIF1A was purified to apparent electrophoretic homogeneity from overproducing E. coli cells as described under “Experimental Procedures.” The homogenous recombinant eIF1A protein (Fig. 2, panel A, lane b) was as active as the stimulatory protein purified from rabbit reticulocyte lysates in mediating the transfer of Met-tRNAf to 40 S ribosomal subunits (Fig. 3, panels A and B). eIF3 was far less active than eIF1A in this transfer reaction. Even in the presence of excess eIF3, the amount of Met-tRNAf transferred never reached the value obtained with limiting concentrations of eIF1A (Fig. 3, panel B). Second, specific antisera were raised in rabbits against purified denatured rabbit reticulocyte stimulatory protein (Mono Q fraction) and affinity purified against the homologous protein from wheat germ (30), was found to be heat stable. Incubation of recombinant eIF1A at 90 °C for 4 min had no effect on its ability to mediate the transfer of Met-tRNAf to 40 S ribosomes (data not shown). Furthermore, in agreement with the requirements of eIF2 and GTP for the eIF1A-mediated transfer of Met-tRNAf to 40 S ribosomes, we observed that when the 40 S incubation mixture contained [3H]Met-tRNAf and [γ-32P]GTP, eIF1A mediated the transfer of nearly identical molar amounts of both 3H and 32P to 40 S particles (Fig. 4A, upper panel). Immunoblot analysis of gradient fractions for eIF2 using rabbit anti-eIF2 antibodies showed that eIF2 also co-sedimented with the 40 S particles (Fig. 4A, lower panel). In the absence of eIF1A, insignificant amounts of [3H]Met-tRNAf and [γ-32P]GTP were transferred to 40 S ribosomes, and no immunoreactive eIF2 polypeptides sedimented with the 40 S ribosomes (Fig. 4B, upper and lower panels). These results indicate that eIF1A mediated the transfer of the entire Met-tRNAf-eIF2-GTP ternary complex to 40 S ribosomes. In other experiments (data not presented) when a preformed [3H]Met-tRNAf-eIF2-GTP ternary complex was first isolated by Seph腺ex G-75 gel filtration and then incubated at 1 mM MgCl2 with 40 S ribosomes, AUG codon, and eIF1A, the entire ternary complex was transferred to 40 S ribosomal subunits to form the 40 S initiation complex.

### eIF1A-mediated Transfer of Met-tRNAf to 40 S Ribosomes in the Absence of AUG Codon—Experiments presented thus far measured the ability of eIF1A to mediate the transfer of the Met-tRNAf-eIF2-GTP ternary complex to 40 S ribosomes in the presence of AUG. To demonstrate that eIF1A is indeed required for the initial transfer reaction and AUG plays a passive role in this reaction presumably by stabilizing the binding of the transferred Met-tRNAf to 40 S ribosome, the preformed [35S]Met-tRNAf-eIF2-GTP ternary complex was incubated with 40 S ribosomes and eIF1A in the absence of the AUG codon in reactions containing 1 mM MgCl2. The products formed were then analyzed by sedimentation through sucrose gradients in
buffers containing 1 or 5 mM MgCl₂. Elevated Mg²⁺ concentration is known to stabilize ribosomal binding of aminoacyl-tRNAs and thus has been used to analyze 40 S and 80 S initiation complexes by sucrose gradient centrifugation (7, 8). As shown in Fig. 5, low levels of Met-tRNA₉ bound to 40 S ribosomes were detected in gradients containing 1 mM Mg²⁺, in the presence or absence of eIF1A. In contrast, when analysis was carried out in sucrose gradients containing 5 mM Mg²⁺, [³⁵S]Met-tRNA₉ bound to 40 S ribosomes was readily observed (Fig. 5). The binding of Met-tRNA₉ to 40 S ribosomes under these conditions was still dependent on the presence of both eIF1A and eIF2 (Fig. 5) as well as on GTP in the reaction (data not shown). These results demonstrate that eIF1A mediates the transfer of Met-tRNA₉ (as Met-tRNAₑIF2-GTP ternary complex) to 40 S ribosomes prior to the binding of mRNA to form the 40 S preinitiation complex.

**Fig. 5. Analysis of eIF1A-mediated transfer of [³⁵S]Met-tRNAₑIF1A to 40 S ribosomal subunits in the absence of AUG codon.** Reaction mixtures for the transfer of [³⁵S]Met-tRNAₑIF1A to 40 S ribosomal subunits were prepared and incubated as described under "Assay of eIF1A Activity" (see "Experimental Procedures") except that AUG was omitted from the reaction mixtures. One set of reaction mixtures (○) contained 200 ng of recombinant eIF1A, whereas the other set (●) did not contain eIF1A. Following incubation at 37 °C for 5 min to form the 40 S preinitiation complex, reaction mixtures were analyzed by 7.5–30% sucrose gradients in buffers containing either 1 mM (——) or 5 mM (●●●●●●●●) MgCl₂ as described under "Experimental Procedures." A reaction mixture (▲) containing eIF1A but not eIF2 was also analyzed.

**Activity**

activity eluted from the column at the position expected for free eIF1A protein of Mᵋ = 17,000 (Fig. 6B). These results indicate that although eIF1A is essential for the transfer of Met-tRNAₑIF1A (as Met-tRNAₑIF2-GTP ternary complex) to 40 S ribosomes, eIF1A is not included in the resultant 40 S preinitiation complex and thus does not contribute to the stability of the 40 S preinitiation complex once it is formed.

**Catalytic Reutilization of eIF1A in the Formation of the 40 S Preinitiation Complex**—Our observation that while eIF1A is
experimental procedures" except that [3H]Met-tRNAf (10,000 cpm/pmol) and 35S-eIF1A (3,000 cpm/pmol) replaced [35S]Met-tRNAf and unlabeled eIF1A as described under "Experimental Procedures." In a separate experiment, we have determined that the antibodies used detected 5 ng of eIF1A in the above Western blot analysis (data not shown). Panel B, 40 S preinitiation complex was formed in the presence of eIF1A as described under "Experimental Procedures," the chilled reaction mixture was applied to a 12-ml bed volume column of Sephadex G-75 that was previously equilibrated in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 5 mM MgCl2, and 5% glycerol. The column was then developed with the same buffer. Fractions (250 μl) were collected and assayed for 3H and 35S radioactivity by counting each fraction in Aquasol in a liquid scintillation spectrometer. The elution positions of the 40 S ribosomal initiation complex, 35S-eIF1A protein, and free [3H]Met-tRNAf were determined separately in the same column and are shown.

essential for the transfer of Met-tRNAf to 40 S ribosomes but is not associated with the resultant 40 S preinitiation complex suggested that the factor might be acting catalytically in the initiation reaction. To investigate this possibility, we measured eIF1A-mediated transfer of Met-tRNAf to 40 S ribosomes in the presence or absence of the AUG codon using a higher molar ratio of the substrate (Met-tRNAf, 35S-eIF1A, and eIF2-GTP ternary complex to 40 S and 60 S ribosomal subunits in the presence of 1 mM MgCl2 with eIF1A and then added the preformed [35S]Met-tRNAf-eIF2-GTP ternary complex and AUG to the reaction. Formation of the 40 S initiation complex was then analyzed by sucrose gradient sedimentation in the presence of 1 mM MgCl2.

To determine whether eIF1A had any effect on the eIF5-mediated subunit joining reaction, isolated 40 S initiation complex containing bound [35S]Met-tRNAf, was incubated with 60 S ribosomal subunits, limiting concentrations of eIF5 and increasing levels of eIF1A. As shown in Fig. 8, right panel, increasing levels of eIF1A had virtually no effect on the formation of the 80 S initiation complex. We conclude that eIF1A plays no role in the formation of the 80 S initiation complex.

Effect of eIF1A on the Association of Ribosomal Subunits—eIF1A has been reported to bind to 40 S ribosomal subunits and prevents the Mg2+-dependent association of 40 S and 60 S ribosomal subunits (6). To investigate whether eIF1A indeed functions as a ribosomal subunit anti-association factor, we carried out the following series of reactions. First, we incubated 40 S and 60 S ribosomal subunits in the presence of 1 mM MgCl2, with eIF1A and then added the preformed [35S]Met-tRNAf-eIF2-GTP ternary complex and AUG to the reaction. Formation of the 40 S initiation complex was then analyzed by sucrose density gradient centrifugation in the presence of 1 mM MgCl2.

As shown in Fig. 9, 40 S and 60 S ribosomal subunits, as expected, remained dissociated, and [35S]Met-tRNAf was efficiently transferred to the 40 S ribosomes (see panels A and E). At 1 mM MgCl2, ribosomal subunits are known to remain dissociated in the absence of any added protein (Ref. 31 and data
such conditions (5 mM Mg²⁺) 

reasons for the discrepancy between our results and those of Thomas et al. (6) and Goumans et al. (32) which showed that eIF1A acted as a ribosomal subunit anti-association factor are not completely clear. It should be noted, however, that these investigators following interaction of eIF1A with ribosomes added glutaraldehyde as a fixative agent to reaction mixtures prior to sucrose gradient analysis. The use of fixatives often introduces nonspecific binding interactions. Whether eIF1A interacts with other proteins, e.g. eIF3 to cause ribosome dissociation, remains to be investigated.

DISCUSSION

eIF1A was originally isolated from mammalian cells (33–35) and wheat germ extracts (30, 36) based on its ability to stimulate protein synthesis in a partially reconstituted system derived from rabbit reticulocyte lysates or wheat germ extracts. Purified eIF1A stimulated the translation of globin mRNA in the reticulocyte system 3–5-fold (33–35) and in the translation of plant viral mRNAs in wheat germ extracts nearly 10-fold (30, 36). This stimulatory effect of eIF1A in purified in vitro

FIG. 7. Catalytic reutilization of eIF1A in the 40 S preinitiation complex formation. Three reaction mixtures (150 µl each) were prepared as described under “Assay of eIF1A Activity” (see “Experimental Procedures”) except that during formation of the ternary complex (stage 1 incubation), 40 pmol of ¹³⁵S Met-tRNA, 9,000 cpm/pmol, 12 µg of purified eIF2, and 18 µM GTP were added. Following incubation at 37 °C for 5 min to form the ternary complex (containing approximately 30 pmol of bound ¹³⁵S Met-tRNA), reaction mixtures were chilled in an ice-water bath, and MgCl₂ (1 mM final concentration), 1.8 A₂₆₀ units of 40 S ribosomal subunits, and 60 ng (3.5 pmol) of purified recombinant eIF1A were added to each reaction. This was followed by the addition of 0.16 A₂₆₀ unit of AUG codon to two reaction mixtures (● and ○), and no AUG codon was added to the third reaction (□). At this stage, the total volume of each reaction mixture was 200 µl. One of the reaction mixtures containing AUG codon (●) was incubated at 37 °C, and the other two reactions, one containing AUG (●) and the other without (○), were incubated at 16 °C. At the indicated time intervals, 40-µl aliquots of reaction mixtures were removed and chilled in ice water, and the amount of [¹³⁵S]Met-tRNA, bound to 40 S ribosomes was determined by 7.5–30% sucrose gradient centrifugation as described under “Experimental Procedures” except that the reaction mixture not containing AUG codon was analyzed in gradients containing 5 mM MgCl₂. The values shown in the figure represent the total amount of [¹³⁵S]Met-tRNA bound to 40 S ribosomes in the entire reaction mixture. It should be noted that three control reactions not containing eIF1A were prepared, incubated, and analyzed as above (data not shown). In these control reactions, the total amount of [¹³⁵S]Met-tRNA bound to 40 S ribosomes was between 0.4 and 0.8 pmol per reaction mixture at all time intervals examined. This value was subtracted from the results shown.

not shown). If, however, the [¹³⁵S]Met-tRNAeIF2-GTP ternary complex was added to ribosomal subunits that were preincubated at 5 mM MgCl₂ in the absence of eIF1A, no [¹³⁵S]Met-tRNA was transferred to the 40 S ribosomes (Fig. 9, panel B). This is presumably because at this elevated MgCl₂ concentration, the ribosomal subunits associated to form 80 S ribosomes (Fig. 9, panel F). If eIF1A acts as a ribosomal subunit dissociation or anti-association factor, the presence of eIF1A in reaction mixtures containing 40 S and 60 S ribosomal subunits should prevent 80 S ribosome formation, and addition of [¹³⁵S]Met-tRNAeIF2-GTP ternary complex would lead to 40 S initiation complex formation. However, as shown in Fig. 9, panels C and G, the presence of eIF1A did not prevent association of ribosomal subunits to form 80 S ribosomes, and in keeping with this no 40 S initiation complex was formed. Under such conditions (5 mM MgCl₂ and with AUG codon), in the absence of 60 S ribosomes, Met-tRNA, as expected (see Fig. 1 and Refs. 13 and 15), was efficiently transferred to 40 S ribosomal subunits in an eIF1A-independent manner (Fig. 9, panels D and H). These results demonstrate that eIF1A, by itself, does not act as a ribosomal subunit anti-association factor. The reasons for the discrepancy between our results and those of Thomas et al. (6) and Goumans et al. (32) which showed that eIF1A acted as a ribosomal subunit anti-association factor are
transcription systems coupled with the recent demonstration (10) that the S. cerevisiae gene encoding eIF1A is essential for cell growth and viability is at variance with the weak effect eIF1A has for in vitro formation of 40 S and 80 S initiation complexes (6–9). It has been reported that in vitro, eIF1A stimulates both AUG-dependent or globin mRNA-dependent 40 S initiation complex formation about 1.5–2-fold by stabilizing the binding of Met-tRNAf to 40 S ribosomal subunits which facilitates subsequent mRNA binding (6–8). It has also been reported that eIF1A has a pronounced effect on the eIF5-mediated joining of 60 S ribosomal subunits to the 40 S initiation complex to form the 80 S initiation complex (7, 9). The factor also has been implicated in the dissociation of 80 S ribosomes to 40 S and 60 S ribosomal subunits (6, 32). Based on these observations, eIF1A has been designated a pleiotropic factor in the initiation pathway (4).

In the present work, we have used a model initiation system that directly measures the binding of Met-tRNAf (as Met-tRNA, eIF2-GTP ternary complex) to 40 S ribosomal subunit in the absence of mRNA to form the 40 S preinitiation complex. We have demonstrated that the transfer of Met-tRNAf (as Met-tRNA-eIF2-GTP ternary complex) to 40 S ribosomal subunit at physiological 1–2 mM MgCl2 requires the absolute participation of eIF1A. This effect did not require the presence of either mRNA or AUG in the reaction indicating that eIF1A is essential for the formation of 40 S preinitiation complex. In agreement with the results published from other laboratories (7–9), eIF3 also stimulated the transfer of Met-tRNAf-eIF2-GTP ternary complex to 40 S ribosomes. However, the efficiency of the transfer reaction was much greater with eIF1A than with eIF3 alone (15–20-fold stimulation of eIF1A vs eIF3 2–4-fold with eIF3). In the presence of eIF1A alone, the transfer of Met-tRNAf to 40 S ribosomes was nearly quantitative. Furthermore, under our conditions of analysis, we could not detect stable binding of eIF1A to the 40 S preinitiation complex thus convincing demonstrating that the protein is not required to stabilize the binding of Met-tRNAf to 40 S ribosomes, as suggested by others (4–9). Additionally, the absence of eIF1A in the 40 S initiation complex isolated by sucrose gradient centrifugation has allowed us to use the complex as a direct substrate for the 60 S subunit joining reaction. We demonstrate that eIF5 alone was sufficient to mediate subunit joining reaction to form the 80 S initiation complex, and eIF1A had no effect on this reaction. The factor also has no demonstrable role in generating 40 S and 60 S ribosomal subunits from 80 S ribosomes.

### Table I

| Additions | [35S]Met-tRNAf formed (pmol) |
|-----------|----------------------------|
| 1. Isolated 40 S initiation complex | 0.03 |
| 2. Same as 1 + 60 S | 0.10 |
| 3. Same as 1 + eIF5 | 0.03 |
| 4. Same as 1 + 60 S + eIF5 | 1.38 |

Our results raise substantial doubt in the current view (4) that eIF1A is a pleiotropic factor in initiation of translation with only a marginal effect (1.5–2-fold) on 40 S initiation complex formation but with a somewhat greater effect on the subsequent joining reaction. The reasons for the discrepancy are not immediately apparent. The previous experiments (6–9) that led to the current model (4) of the initiation pathway measured 40 S initiation complex formation in in vitro reactions that included all known initiation factors. The function of...
a specific protein, e.g. eIF1A, was then determined by its omission from the complete system and measuring ribosomal initiation complex formation in its absence. An inherent problem with such experiments is that a trace contamination of the protein being analyzed in other initiation factor preparations would underestimate the requirement of this factor in initiation complex formation. This will be especially true for a low molecular weight protein like eIF1A which acts catalytically in the formation of 40 S preinitiation complex. Furthermore, the discrepancy between our finding that eIF1A does not directly affect subunit joining and those that show it does (7, 9) can be explained. Unlike our experimental approach, presented in Fig. 8, previous investigators did not utilize an isolated 40 S initiation complex, free of unreacted reaction components, as the substrate for the subunits’ joining reaction. Rather, they formed the 40 S initiation complex in situ in the presence of either AUG codon (9) or globin mRNA (7) and all the required initiation factors except eIF1A and then added eIF1A, eIF5, and 60 S subunits to these reactions and measured the formation of 80 S initiation complex. Under these conditions, the observed increase of 80 S initiation complex formation by eIF1A can be accounted for by the stimulatory effect of this factor on the 40 S initiation complex formation.

Finally, it should be mentioned that although the present work demonstrates that eIF1A is essential to mediate nearly quantitative transfer of Met-tRNAf eIF2-GTP ternary complex to free 40 S ribosomal subunits, it is likely that in vivo the Met-tRNAf eIF2-GTP ternary complex binds, in the presence of eIF1A, to an eIF3-40 S complex rather than to free 40 S ribosomal subunits, since the majority of native 40 S subunits contains bound eIF3 (37, 38). We have, however, shown that the transfer of the ternary complex to 40 S subunits is inefficient in the presence of eIF3 alone, whereas eIF1A, by itself, is both necessary and sufficient to mediate highly efficient transfer of the ternary complex. These findings lead us to speculate that the major role of eIF3 is not in the initial transfer of the Met-tRNAf eIF2-GTP ternary complex to 40 S ribosomes but rather in the subsequent binding of mRNA to the 40 S preinitiation complex. Similar conclusions regarding eIF3 were also reached by Trachsel et al. (7).

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