Two Eukaryotic Initiation Factors (IF-I and IF-II) of Protein Synthesis that Are Required to Form an Initiation Complex with Rabbit Reticulocyte Ribosomes

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ABSTRACT The formation of an initiation complex with rabbit reticulocyte ribosomes and certain templates is dependent upon the action of two initiation factors (IF-I and IF-II) of protein synthesis isolated from 0.5 M KCl extracts of rabbit reticulocyte polyribosomes. IF-I mediates the GTP-dependent, but template-independent, binding of the cytoplasmic initiator transfer RNA (rabbit liver) to a 40S ribosomal subunit. It is present in the absence of a 60S ribosomal subunit and of AUG, AUG(U)_m, or rabbit-globin messenger ribonucleoproteins (but not of rabbit-globin messenger RNAs). IF-II causes the transfer of the initiator transfer RNA to an initiation complex containing a 80S ribosome. Although 5'-guanylylmethylenediphosphonate can replace GTP as the cofactor used in binding the initiator transfer RNA to IF-I or to a 40S ribosomal subunit, the subsequent transfer of the initiator transfer RNA to an initiation complex requires GTP and cannot take place in the presence of 5'-guanylylmethylenediphosphonate. After the formation of this initiation complex, the initiator transfer RNA is bound in the P site of the 80S ribosome since exposure of the complex to puromycin releases the methionine residue from the initiator transfer RNA as methionyl-puromycin.

IF-I and IF-II are dissociated from polysomes by 0.5 M KCl as a complex having a molecular weight of about 370,000, and they remain in this form during and after gel filtration. However, the two factors can be separated by appropriate step elutions from DEAE-cellulose. After separation of the two activities, the molecular weights of IF-I and IF-II are about 150,000 and 220,000, respectively.

The major components of an initiation complex, involved in eukaryotic cytoplasmic protein biosynthesis, have been identified and include the 40S and 60S ribosomal subunits, the initiator tRNA (Met-tRNA\(^{\text{Met}}\)), and an mRNA containing an appropriate initiator AUG codon (1). However, the identities, physical properties, and functions of individual initiation factors that control the formation of this initiation complex have not been well characterized. Previous reports (2-5) from our laboratory have described one initiation factor (IF-I), isolated from 0.5 M KCl extracts of rabbit reticulocyte polyribosomes, which is required for the template-independent binding of Met-tRNA\(^{\text{Met}}\) (rabbit liver) to a 40S ribosomal subunit (rabbit reticulocytes). In the absence of ribosomal subunits, IF-I forms a stable ternary complex (2, 4-8) with GTP [or 5'-guanylylmethylenediphosphonate (GDPCP) (2)] and with the cytoplasmic initiator tRNA from all eukaryotic sources tested (rabbit reticulocytes, rabbit liver, chicken liver, and rat liver) (4); these ternary complexes are retained by Millipore filters (2, 4-8). Ternary complex formation is blocked by ammonium aurintricarboxylate (3) or by prior reaction of IF-I with any one of the sulphydryl reagents tested [N-ethylmaleimide, cystamine, and 5,5'-dithiobis(2-nitrobenzoate)] (4). IF-I has been purified from a variety of eukaryotic tissues and cells (rabbit reticulocytes, rabbit liver, chicken reticulocytes, chicken liver, and chick embryonic leg muscle), and no differences were found between these IF-I preparations with respect to either their biological activities or their physical properties (4). The molecular weights of all IF-I activities in 0.5 M KCl polysomal extracts were determined to be approximately 370,000 (4).

We now report the detection, purification, and function of a second eukaryotic initiation factor (IF-II) of protein synthesis isolated from rabbit reticulocyte IF-I of 370,000 molecular weight. The IF-I and IF-II activities in the complex of 370,000 molecular weight were separated by sequential step elutions from DEAE-cellulose. IF-II is required for the transfer of Met-tRNA\(^{\text{Met}}\) (rabbit liver) from a 40S ribosomal subunit to an initiation complex in the presence of 60S ribosomal subunits. This transfer is stimulated greatly by the initiator AUG triplet, by the defined oligoribonucleotide AUG(U)_m, and by rabbit-globin messenger ribonucleoproteins (mRNPs), but not by rabbit-globin mRNAs. After the IF-II-mediated transfer of the initiator tRNA, the tRNA is bound to the P site of the 80S ribosome where the methionine residue is available for reaction with puromycin. All functions of IF-II require GTP in an enzymatically hydrolyzable form.

MATERIALS AND METHODS

The preparations of high-specific-activity L-[\(^{35}\)S]methionine (9), [\(^{35}\)S]Met-tRNA\(^{\text{Met}}\) (rabbit liver) (10, 11), and template- and factor-free rabbit reticulocyte ribosomes (12) have been described. Rabbit-globin mRNPs were isolated from polysomes by treatment with EDTA followed by zone velocity sedimentation through preformed sucrose density gradients (13), and then were recovered by high-speed centrifugation (5). Globin mRNAs were prepared by phenol deproteinization of the globin mRNPs. Assays for IF-I activity by Millipore filter retention of the IF-I-[\(^{35}\)S]Met-tRNA\(^{\text{Met}}\)-GTP ternary complex (2, 5) and by IF-I-dependent [\(^{35}\)S]Met-tRNA\(^{\text{Met}}\) binding

Abbreviations: Met-tRNA\(^{\text{Met}}\), methionyl-tRNA that can be enzymatically formylated (except for Met-tRNA\(^{\text{Met}}\) from plants) by Escherichia coli enzymes and that functions as the initiator tRNA in the cytoplasm of eukaryotes; GDPCP, 5'-guanylylmethylenediphosphonate; mRNPs, globin mRNAs with associated proteins that are released from rabbit reticulocyte polysomes by treatment with EDTA; P site, peptidyl-tRNA binding site on the ribosome; A site, aminoacyl-tRNA binding site on the ribosome; IF, initiation factor.
Fig. 1. Steps leading to the formation of an initiation complex. Binding reactions were prepared as described in Methods. As specified below, 825 pmol of AUG or 680 pmol of AUG(U)₅₉ were added before the second incubation. Reactions were layered on 10–25% (w/v) exponential sucrose gradients made in buffer B. The gradients were centrifuged at 2° for 6 hr at 32,000 rpm in a Spinco type SW 40 Ti rotor. Additions are as follows: (A) IF-I; (B) IF-I + AUG; (C) IF-I + AUG(U)₅₉; (D) IF-I + IF-II; (E) IF-I + IF-II + AUG; (F) IF-I + IF-II + AUG(U)₅₉.

Fig. 2. Formation of an initiation complex with rabbit globin mRNPs and mRNAs. Binding reactions and gradients are described in the legend to Fig. 1. Additions before the second incubation are as follows: (A) 0.16 A₅₅₀ nm unit of rabbit-globin mRNPs; (B) 0.25 A₅₅₀ nm unit of rabbit-globin mRNAs (pH 7.4 at 20°)–5 mM MgCl₂–1 mM 2-mercaptoethanol.

In addition to the above solutions, which contain the IF-I ternary complex reaction mixture, to the second incubation also are added 1.5 A₅₅₀ nm units of rabbit reticulocyte ribosomes (12) and, as specified in the legends, 10 µg of IF-II, and AUG, AUG(U)₅₉, globin mRNPs, or globin mRNAs. Initiation complex assembly is analyzed by zone velocity sedimentation through preformed sucrose density gradients (Figs. 1–3) or by formation of [³⁵S]methionyl-puromycin (Table 1 and following section).

Reaction of [³⁵S]Met-tRNA⁰⁺ in an Initiation Complex with Puromycin (8). An initiation complex is generated by two successive incubations, as described in the preceding section. After the second incubation, 44 µmol of puromycin are added in the cold. The solution is warmed and is incubated at 25° for an additional 30 min. One milliliter of cold 0.1 M sodium phosphate (pH 8.0) is added, followed by 1.5 ml of ethyl acetate. The [³⁵S]methionyl-puromycin is extracted into the ethyl acetate phase by vigorous mixing on a Vortex mixer.

Fig. 3. Demonstration of the requirement for GTP during the formation of an initiation complex. Binding reactions and gradients are described in the legend to Fig. 1, except that 70 nmol of GDPCP were substituted for GTP during the first incubation. In (C) 90 nmol of GTP were added before the second incubation after ternary complex formation had taken place with GDPCP. Additions are as follows: (A) IF-I + GDPCP; (B) IF-I + GDPCP + IF-II + AUG; (C) IF-I + GDPCP + IF-II + AUG + GTP.
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Fig. 4. Molecular-weight determinations of IF-I and of IF-II. (A) Logarithmic plot of molecular weight against elution volume. 1, Urease; 2, complex of IF-I and IF-II; 3, catalase; 4, IF-II separated from IF-I; 5, aldolase; 6, IF-I separated from IF-II. (B) Relative activities of initiation factors in the column eluate. IF-I, complex with IF-II; I, relative value of 100 = 37 pmol of [35S]Met-tRNA\textsubscript{Met} bound per ml. IF-II, separated from IF-I; 100 = 7.1 pmol of [35S]methionyl-puromycin formed per ml. IF-I, separated from IF-II; 100 = 1.9 pmol of [35S]Met-tRNA\textsubscript{Met} bound per ml. All assays were described in Methods.

The series of events that culminate in the formation of an initiation complex with rabbit reticulocyte ribosomes is illustrated in Fig. 1. The binding of [35S]Met-tRNA\textsubscript{Met} to ribosomes and ribosomal subunits is analyzed by zone velocity centrifugation of reaction mixtures through preformed sucrose density gradients since the IF-I-initiator tRNA-GTP ternary complex is retained on Millipore filters (2). In the presence of IF-I and GTP, [35S]Met-tRNA\textsubscript{Met} is bound to a 40S ribosomal subunit (Fig. 1A). The addition of the initiator AUG triplet, or of the defined oligoribonucleotide AUG(U)\textsubscript{3}, does not stimulate this binding (Fig. 1B and C). After the binding of [35S]Met-tRNA\textsubscript{Met} to a 40S ribosomal subunit is directed by IF-I, the sedimentation coefficient of the smaller ribosomal subunit is increased by 4S (Figs. 1A, B, C, and 3). Upon the binding of IF-II to the initiation intermediate depicted in Fig. 1B and C, [35S]Met-tRNA\textsubscript{Met} is transferred to an initiation complex containing an 80S ribosome (Fig. 1E and F). This transfer is promoted by the oligoribonucleotides AUG and AUG(U)\textsubscript{3} (compare Fig. 1D with Fig. 1E and F). An initiation complex also can be formed in the presence of rabbit-globin mRNPs (Fig. 2A). The mRNA-associated proteins are required for the formation of this complex; globin mRNAs derived from the mRNPs by phenol deproteinization do not contribute to a level of initiation complex formation greater than that observed in the absence of any exogenous template (compare Fig. 1D with 2B). If the IF-I-[35S]Met-tRNA\textsubscript{Met}·GTP ternary complex is formed with the GTP analogue, GDP·PCP (2), [35S]Met-tRNA\textsubscript{Met} subsequently can be bound to a 40S ribosomal subunit (Fig. 3A). However, in this case, the IF-II-mediated transfer of the [35S]Met-tRNA\textsubscript{Met} to an initiation complex containing an 80S ribosome is prevented (Fig. 3B). If free GTP (0.9 mM) is added to the second incubation, after the IF-I ternary complex has been formed in the presence of GDP·PCP during the first incubation, very little of the IF-II-mediated transfer of the initiator tRNA is observed (Fig. 3C).

Table 1. Reaction of puromycin with [35S]Met-tRNA\textsubscript{Met} bound in an initiation complex

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative amount of [35S]methionyl-puromycin formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF-I + AUG</td>
<td>14</td>
</tr>
<tr>
<td>IF-II + AUG</td>
<td>0</td>
</tr>
<tr>
<td>IF-I + IF-II</td>
<td>35</td>
</tr>
<tr>
<td>IF-I + IF-II + AUG</td>
<td>100</td>
</tr>
<tr>
<td>IF-I + IF-II + AUG(U)\textsubscript{3}</td>
<td>86</td>
</tr>
<tr>
<td>IF-I + IF-II + rabbit-globin mRNAPs</td>
<td>81</td>
</tr>
<tr>
<td>IF-I + IF-II + rabbit-globin mRNAs</td>
<td>41</td>
</tr>
</tbody>
</table>

Binding reactions for initiation complex formation and extraction of methionyl-puromycin are described in Methods. A relative value of 100 = 0.11 pmol of [35S]methionyl-puromycin formed under the conditions of the assay. Controls without ribosomes (0.04 pmol) have been subtracted from each value. The amounts of AUG and AUG(U)\textsubscript{3} are given in the legend to Fig. 1, and the amounts of mRNPs and mRNAs are given in the legend to Fig. 2.
puromycin (Table 1). The data presented in Fig. 2 already indicate that rabbit-globin mRNAs cannot participate in the formation of an initiation complex; this fact is confirmed by the inability of these mRNAs to function as the template required before the formation of \( ^{25}S \) methionyl-puromycin. The data in Table 1 clearly indicate that the actions of both IF-I and IF-II are required for the assembly of an initiation complex containing \( ^{25}S \)Met-tRNA\(^{\text{met}}\) in the puromycin-reactive ribosomal P site.

Molecular weights of IF-I and IF-II have been determined by gel filtration through a calibrated column of Sepharose 6B. IF-I from several eukaryotic tissues and cell types (including rabbit reticulocytes) (4) and IF-I plus IF-II from rabbit reticulocytes, while still in a 0.5 M KCl polysomal extract, elute as a single species with a molecular weight of 370,000 (Fig. 4). However, if gel filtrations are carried out independently after IF-I and IF-II are separated by DEAE-cellulose chromatography, the elution volumes of the two initiation factors yield molecular weights for IF-I and IF-II of about 150,000 and 220,000, respectively (Fig. 4).

**DISCUSSION**

We have demonstrated that at least two distinct eukaryotic initiation factors of protein synthesis, IF-I and IF-II, are required for the formation of an initiation complex with rabbit reticulocyte ribosomes. IF-I promotes GTP-dependent, but template-independent, binding of Met-tRNA\(^{\text{met}}\) to a 40S ribosomal subunit (4, 5). Several other reports (8, 21, 22) have suggested that the binding of Met-tRNA\(^{\text{met}}\) to a 40S ribosomal subunit is the first step in initiation of eukaryotic protein synthesis, and that this binding is mRNA-independent. The IF-I-mediated binding of Met-tRNA\(^{\text{met}}\) causes an increase in approximately 4 S in the sedimentation coefficient of the smaller ribosomal subunit. This increase probably is due, at least in part, to a condensation of the structure of the ribosomal subunit, since the gain in molecular weight of the subunit, due to the binding of the initiator tRNA (molecular weight = 25,000) and of IF-I (molecular weight = 150,000) cannot be entirely responsible for such an increase in the sedimentation coefficient.

In the presence of certain templates, IF-II mediates the transfer of Met-tRNA\(^{\text{met}}\) from a 40S ribosomal subunit to an initiation complex containing an 80S ribosome. Initiation complex formation in the presence of AUG-containing oligoribonucleotides requires only two initiation factors, IF-I and IF-II. If, however, natural mRNAs (which contain a relatively high degree of secondary structure) are used as templates during initiation complex formation, the mRNA-associated proteins clearly are required. This requirement suggests that the mRNA-associated proteins establish the proper reading frame on the natural mRNAs by making accessible to the ribosome the correct AUG codon to serve as the initiator codon. Blobel (23) has reported that there are two proteins attached to rabbit-globin mRNAs with molecular weights of 52,000 and 78,000, and there is evidence (24) that at least one of the mRNA-associated proteins is necessary for binding rabbit-globin mRNAs to deoxycholate-washed 40S ribosomal subunits obtained from rabbit reticulocytes. Hahn and Hahn (25) demonstrated that at least one protein from mRNPs is necessary for the translation of natural mRNAs in an insect cell-free system derived from *Tenebrio*. These requirements for mRNA-associated proteins observed in two very different eukaryotic systems indicate that these proteins play an integral role in the initiation of eukaryotic protein synthesis, and that they may regulate the translational efficiencies of the mRNAs.

An independent assay for the completion of initiation complex assembly is the puromycin-induced release of the methionine moiety as methionyl-puromycin. The fact that this methionine residue rapidly and quantitatively reacts with puromycin indicates that the initiator tRNA is bound directly to the P site of the 80S ribosome in an initiation complex. No elongation factor (EF-2) is required to shift or "translocate" the initiator tRNA from the 80S ribosomal A site to the P site. Thus, with certain templates, the combined action of IF-I and IF-II results in the methionine moiety being in position to form the first peptide bond with the next amino-acid residue. This residue is esterified to the tRNA species to be bound to the A site, according to the base sequence of the mRNA, as a preformed complex with EF-1.

Initiation complex formation has a definite nucleotide requirement; this requirement can be met entirely by GTP. The GTP analogue, GDPCP, can substitute with equal efficiency for GTP in the IF-I-dependent binding of Met-tRNA\(^{\text{met}}\) to a 40S ribosomal subunit, but cannot substitute for GTP during the IF-II-mediated transfer reaction. Since free GTP cannot stimulate initiation complex formation once the IF-I ternary complex has been formed with GDPCP, the following conclusions may be drawn: (1) the isolated ternary complex of IF-I, Met-tRNA\(^{\text{met}}\), and GTP is a stable intermediate in binding Met-tRNA\(^{\text{met}}\) to a 40S ribosomal subunit; and (2) the GTP in this ternary complex subsequently is hydrolyzed during the transfer of the Met-tRNA\(^{\text{met}}\) from a 40S ribosomal subunit to an initiation complex, a reaction correlated with the completion of the function of IF-I and/or with the action of IF-II.

The fact that IF-I and IF-II are found as a high-molecular-weight complex in 0.5 M KCl polysomal extracts is intriguing. After separation of the factor activities on DEAE-cellulose, IF-I and IF-II have molecular weights of 150,000 and 220,000, respectively. From the results of assays for the initiation factor activities, it appears that both IF-I and IF-II are able to function properly under conditions where they exist as the lower molecular weight proteins and also under conditions where they exist together as a higher molecular weight complex. Since IF-I activities in 0.5 M KCl extracts of polysomes from all sources tested (rabbit reticulocytes, rabbit liver, chicken reticulocytes, chicken liver, and chick embryonic leg muscle) were found to possess molecular weights of 370,000 (4), it is highly probable that complexes of IF-I and IF-II are common to at least all of these cells and tissues.

One of the initiation factor activities (IF-I) described in this paper is similar to the activity exhibited by a combination of two factors (IFE\(_2\) plus IFE\(_3\)) found by Schreier and Staehelin (8) in 0.5 M KCl extracts of rabbit reticulocyte polysomes. One of their factors (IFE\(_2\)) resembles IF-I in the ability to form a GTP-dependent ternary complex with Met-tRNA\(^{\text{met}}\), and in two physical properties: (1) elution characteristics from DEAE-cellulose, and (2) sedimentation (5 S to 6 S) in glycerol density gradients (L.M.C. and W.M.S., Jr., unpublished data). However, unlike IF-I, IFE\(_2\) alone is unable to promote template-independent Met-tRNA\(^{\text{met}}\) binding to a 40S ribosomal subunit (8). In order to carry out this reaction, IFE\(_2\) requires the presence of IFE\(_3\), an extremely
large substance that sediments at 15 S or 17 S in glycerol density gradients (8, 26). We have never observed any requirement for such a factor at any stage during the assembly of an active initiation complex. Perhaps our second factor, IF-II, which, with certain templates, is capable of completing the formation of an 80S initiation complex, corresponds with one or more of three other briefly described initiation factors (IFE4, IFE5, or IFE6) (8, 26). However, no information concerning the purifications, physical properties, or functions of these factors has been published, beyond a mention that IFE4 is required to form an 80S initiation complex (8), and a brief note that IFE5 (influenced by IFE1 and IFE2) is necessary for this same reaction, which then was stated to be ATP-dependent (26).

In summary, we have defined the requirements of the steps that culminate in the assembly of a functional eukaryotic initiation complex, and have described in detail two of the specific initiation factors (IF-I and IF-II) that control the formation of this initiation complex.

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