Role of Eukaryotic Initiation Factor 5 in the Formation of 80 S Initiation Complexes*

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The function of eukaryotic initiation factor 5 (eIF-5) from rabbit reticulocyte lysate has been studied by sucrose gradient preparation of 40 S and 80 S initiation complexes. eIF-5 is required for transfer of initiator tRNA from 40 S preinitiation complexes to puromycin-reactive 80 S complexes. The transfer is dependent upon GTP hydrolysis and is associated with release of eIF-2 and eIF-3 from the 40 S subunit. The GTP-dependent loss of eIF-2 and eIF-3 is catalyzed by eIF-5 in the absence of 60 S subunits or when subunit joining is prevented by edeine, but not when GTP is replaced by GuoPP(NH)p. Unstable 40 S subunit-Met-tRNA\textsubscript{f} complexes generated by eIF-5 can form puromycin-reactive 80 S complexes when 60 S subunits are added in the absence of added GTP. In addition, kinetic evidence is presented that indicates GTP hydrolysis occurs prior to 80 S complex formation.

With the availability of reticulocyte initiation factors of high purity, it has become possible to assign specific functions or steps during the initiation process to most of the individual protein factors. eIF-5, formerly called IF-M2A (1), has been prepared in high purity from rabbit reticulocyte ribosomal wash (2). It consists of a single peptide of approximately 125,000 daltons and is required for amino acid polymerization reactions including methionyl-puromycin synthesis, hemoglobin synthesis, and polyphenylalanine synthesis. In addition, eIF-5, in the absence of other protein factors, has GTPase activity which requires 40 S and 60 S ribosomal subunits for its expression. The exact function of eIF-5 is not known, but work from several laboratories has suggested this protein has "joining" activity; that is, it is required for the joining of 60 S subunits to 40 S subunits containing template and initiator tRNA (3–5).

We have recently defined conditions which permit isolation of 40 S and 80 S ribosomal preinitiation complexes in unfixed sucrose gradients with yields of 10 to 60% of starting material (6). This has permitted us to study in greater detail the sequence of reactions leading to formation of an 80 S initiation complex. In this report we present evidence that eIF-5, in an energy-dependent step, releases eIF-2 and eIF-3 from the 40 S subunit independent of, and perhaps prior to, joining of the 60 S subunit. These findings suggest that eIF-5 should not be considered as a factor which actively joins 40 S subunit complexes with 60 S subunits, but rather in a step separate from, but closely linked to, joining of the 60 S subunit to form the complete 80 S initiation complex.

MATERIALS AND METHODS

Preparation of Materials—Initiation factors from rabbit reticulocyte lysate were prepared and purified as previously described; eIF-2 (7), eIF-3 (8), eIF-4C and eIF-4D (9), eIF-5 (2), eIF-2A (10). The nomenclature used is that recommended by the International Symposium on Protein Synthesis, October 1976 (1). Initiation factors were radiolabeled by reductive methylation using [\textsuperscript{3}H]CH\textsubscript{3}O and sodium borohydride (11).

Ribosomal subunits were prepared from reticulocyte polyribosomes by puromycin dissociation (12, 13). [\textsuperscript{3}H]Met-tRNA\textsubscript{f} (specific activity 15 Ci/mmol) was prepared from fractionated rat liver tRNA as previously described (13). [\textsuperscript{3}H]Met-tRNA\textsubscript{f} (specific activity 200 Ci/mmol) was prepared from unfractionated rat reticulocyte tRNA using a crude aminoacyl-tRNA synthetase preparation preparation from Escherichia coli (13).

Isolation of Initiation Complexes by Sucrose Gradient Centrifugation—Initiation complex formation was performed in 100-μl reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 90 mM KCl, 2 mM MgCl\textsubscript{2}, 0.1 mM dithiothreitol, 0.3 μg A\textsubscript{260} unit of AUG-[\textsuperscript{3}H]Met-tRNA\textsubscript{f} as indicated in figure legends, 30 to 50 pmol of 40 S subunits, 0.8 mM GTP or GuoPP(NH)p, 4 mM phosphoenolpyruvate, and 5 IU of pyruvate kinase. Other components (60 S subunits, eIF-2, eIF-3, eIF-4C, eIF-4D, and eIF-5) were added as indicated in figure legends. Reaction mixtures were incubated at 30°C for 15 min and layered on prechilled linear 10 to 34% sucrose gradients containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl\textsubscript{2}, 100 μM GuoPP(NH)p. Centrifugation was performed using a Beckman 50.5 rotor at 55,000 rpm for 150 min (for 40 S subunit complexes) or 135 min (when 60 S subunits were included). Gradients were displaced by heavy sucrose through an ISCO flow cell to record absorbance at 254 nm. Radioactivity of gradient fractions was determined using 10 ml of Hydromix made 8% in H\textsubscript{2}O and a Packard model 2475 liquid scintillation spectrometer.

Isolation of 40 S Subunit Complexes by Gel Filtration—The preparation of complexes containing 40 S subunits, eIF-2, GTP, and Met-tRNA\textsubscript{f} was achieved by a two-step process. The first step was an incubation of a 1-ml reaction mixture at 35°C for 15 min. The reaction mixture contained: 20 mM Hepes/KOH, pH 7.5, 2 mM MgCl\textsubscript{2}, 100 mM KCl, 1 mM dithiothreitol, 3.0 A\textsubscript{260} units of AUG, 40 μg of eIF-2, 400 pmol of 40 S subunits, 400 pmol of [\textsuperscript{3}H]Met-tRNA\textsubscript{f} (specific activity 200 mCi/mmol), and 80 μM [\textsuperscript{32}P]GTP (specific activity 400 Ci/mmol).

The second step was the isolation of preinitiation complexes by gel filtration. Following incubation, the 1-μl reaction mixture was chilled and applied to a column (0.9 x 20 cm) of Sepharose 6B equilibrated with 20 mM Hepes/KOH, pH 7.5, 100 mM KCl, 5 mM MgCl\textsubscript{2}, and 1 mM dithiothreitol. The column was maintained at 4°C. Complexes of 40 S subunits, eIF-2, GTP, and Met-tRNA\textsubscript{f} were eluted with equilibrating buffer at a flow rate of 20 ml/h. Free 40 S subunits and complexes of 40 S subunits with eIF-2, GTP, and Met-tRNA\textsubscript{f} eluted in the void volume whereas eIF-2, GTP, and Met-tRNA\textsubscript{f} eluted in...
the included volume. For subsequent analyses, the two-peak, 1-ml fractions of 40 S complexes were pooled and either 50- or 100-μl aliquots were combined with an equal volume of reaction mixture to examine 80 S complex formation. GTP hydrolysis, or stability of the 40 S complex as described in the appropriate figure or table legend.

Assays—Assays for methionyl-tRNA synthetase, GTP hydrolysis, and retention of preinitiation complexes on Millipore filters were as previously described (13). When these assays were performed using either 50 or 100 μl of preformed 40 S complexes, an equal volume of reaction mixture was added to achieve the following final concentrations: 100 mM KCl; 20 mM HEPES/KOH, pH 7.5; 2 mM MgCl₂; 1 mM dithiothreitol; and 0.3 A₅₅₀ unit of AUG/100 μl. No GTP, phosphonopyruvate, or pyruvate kinase was added.

In addition, eIF-4C, -4D, -5, and 60 S subunits were added to form 80 S complexes as indicated in the appropriate legends. These components were added at levels optimal for the initial 1 ml reaction mixture used to generate 40 S complexes and therefore represent a 2- to 3-fold excess above the amount present in the more dilute column fractions.

RESULTS

Previous studies in our laboratory had determined the requirements for the formation of 40 S preinitiation complexes, as analyzed by sucrose gradients, and 80 S initiation complexes, as analyzed by methionyl-tRNA synthetase (3, 6). In order to monitor the fate of radiolabeled initiation factors during the process of 80 S initiation complex formation, conditions for the formation of such complexes were studied. The stringent requirement for eIF-5 and GTP in the formation of 80 S complexes as assayed by methionyl-tRNA synthetase is also observed by sucrose gradient analysis (Fig. 1). For this analysis, 40 S and 60 S reticulocyte ribosomal subunits were incubated with Met-tRNAf, the initiator codon AUG, initiation factors, and either the energy source GTP or the nonhydrolyzable analog of GTP, GuoPP(NH)P. The samples were placed on sucrose gradients and after centrifugation the absorbance at 254 nm was used to identify the location of the 40 S, 60 S, and 80 S subunit complexes within the sucrose gradient. In Panel A is shown an incubation containing eIF-2, eIF-3, GTP, and eIF-4C and -4D. There is an absorbance peak at the top of the gradient due to unbound tRNA and GTP. Approximately 12 pmol of Met-tRNAf are bound to the 40 S subunit and in the 80 S region of the gradient and in the absence of eIF-5, no Met-tRNAf is found in the 80 S peak. There is, however, a small 80 S ribosome peak by absorbance. Radiolabeled eIF-2 is also found in the 40 S subunit region of the gradient in an approximate 1:1 stoichiometry with Met-tRNAf consistent with the idea that the ternary complex of Met-tRNAf, eIF-2, and GTP is bound to the 40 S subunit. In contrast, eIF-3 is found in two places in the gradient, at approximately 18 S where the large eIF-3 complex migrates into the gradient on its own, and also in the 40 S subunit region where approximately 25 pmol are bound to 50 pmol of 40 S ribosomal subunits. With the addition of 4 pmol of eIF-5, there is a large increase in the 80 S ribosomal peak by absorbance and, in addition, approximately 6 pmol of Met-tRNAf is found in the 80 S region (Panel B). Note however, that eIF-2 and eIF-3 are not found in the 80 S region, but remain, in lower amounts, in the 40 S region. The incomplete conversion of 40 S preinitiation complexes to 80 S initiation complexes presumably reflects a lack of excess joining components (i.e. eIF-4C, -4D, -5, and 60 S subunits). The substitution of GuoPP(NH)P for GTP prevents the formation of an 80 S initiation complex as judged by the absence of [3H]Met-tRNAf and an absorbance peak in the 80 S region. Met-tRNAf and eIF-2 remain bound to the 40 S subunits in an amount equal to or greater than the binding seen in the control in Panel A. Thus, eIF-5 is required for transfer of Met-tRNAf from 40 S preinitiation complexes to 80 S initiation complexes and there is an accompanying release of eIF-2 and eIF-3 during the conversion. The formation of 80 S initiation complex from a 40 S complex apparently requires hydrolysis of GTP since it can be prevented by use of a nonhydrolyzable GTP analog. Thus, eIF-5 could function as a subunit joining factor with GTP hydrolysis being required for 40 S and 60 S subunit joining. Alternatively, eIF-5 might have its effect at the 40 S subunit level alone, independent of the 60 S subunit. In order to examine this question further, we have studied the effect of eIF-5 on 40 S preinitiation complexes in the absence of 60 S subunits.

The effect of eIF-5 on 40 S preinitiation complexes is shown in Fig. 2. The 40 S subunits were incubated with [3H]Met-tRNAf, eIF-2, eIF-3, GTP, and AUG. About 10 pmol of Met-tRNAf were bound to 40 S subunits, presumably as ternary complex (open circles). The addition of 4 pmol of eIF-5 results in a loss of about 90% of the Met-tRNAf from the 40 S subunit region (open triangles). The use of GuoPP(NH)P (or GuoPP(CH₃)P, data not shown) instead of GTP in the incubation prevents any loss of Met-tRNAf from 40 S subunit preinitiation complex (closed circles). Thus, eIF-5 apparently

![Fig. 1. The function of eIF-5 in 80 S initiation complex formation. Fifty picomoles of 40 S subunits, 100 pmol of eIF-3, 50 pmol of eIF-2, 0.3 A₅₅₀ unit of AUG, and 30 pmol of [3H]Met-tRNAf were incubated in 100 μl containing 20 mM Tris-HCl, pH 7.5, 85 mM KCl, 3 mM MgCl₂, 0.4 mM GTP or GuoPP(NH)P, 2 mM phosphonopyruvate, 5 IU/ml of pyruvate kinase, and 0.1 mM dithiothreitol. After 15 min incubation at 30°C, 40 pmol of 60 S subunits and 2 μg of mixed eIF-4C and -4D were added and the incubation was continued for 10 additional min. The samples were layered on 10 to 38% sucrose gradients containing 100 μM GuoPP(NH)P. Panel A, GTP was in the incubation; Panel B, same as A, but 4 pmol of eIF-5 were added with 60 S subunits; Panel C, same as B but GuoPP(NH)P instead of GTP was in the incubation. Since [3H]eIF-2 and [14C]eIF-3 could not be distinguished in the same tube, duplicate samples were run, one with [14C]eIF-2 and the other with [3H]eIF-3. Both are plotted on the same figure.](http://www.jbc.org/)}
breaks down the ternary complex on the 40 S subunit in an energy-dependent manner, and it functions catalytically in that 4 pmol of eIF-5 results in loss of approximately 8 to 9 pmol of Met-tRNAf from 40 S subunits. In addition, 60 S subunits are not required for the action of eIF-5 on 40 S subunit preinitiation complexes. If eIF-5 functions prior to 60 S subunit joining or independent of 60 S joining, then the existence of an intermediate 40 S subunit-Met-tRNAf complex might be suspected. Such a complex might be expected to be unstable since eIF-2 and GTP would not be present. Instability of this complex would explain the paradoxic loss of Met-tRNAf as well as eIF-2 from 40 S subunits when eIF-5 acts in the absence of 60 S subunits. We have obtained evidence for the existence of this complex by forming 40 S preinitiation complexes by a preliminary incubation at 30°C for 15 min and then adding eIF-5 and incubating for varying periods of time at 30°C. The results are presented in Table I. In the absence of eIF-5, about 13 pmol of eIF-2 and Met-tRNAf are bound to 30 pmol of 40 S subunits. If eIF-5 is added and the sample is immediately placed on ice (prior to centrifugation) about half of the eIF-2 is lost and 15% of Met-tRNAf is lost. With progressively longer incubations with eIF-5, both eIF-2 and Met-tRNAf are lost from the 40 S subunit complex but Met-tRNAf is selectively retained. This result suggests that the function of eIF-5 is to break down the 40 S subunit preinitiation complex to a 40 S subunit-Met-tRNAf complex which is itself highly unstable and survives the isolation procedure poorly. This eIF-5-directed phenomenon, which can be referred to as “an eIF-5-dependent release,” occurs in the presence or absence of eIF-3 and AUG, and in all instances requires the presence of GTP rather than a nonhydrolyzable analog. An indication of the instability of the 40 S-Met-tRNAf bond is the “trailing” of the Met-tRNAf radioactivity from the 40 S region to the top of the gradient in the samples treated with eIF-5 but omitting 60 S subunits (e.g., Fig. 4).

The data shown above suggest that the requirement for eIF-5 in 80 S complex formation directed by AUG may be distinct from the direct joining of the 40 S and 60 S subunits and perhaps involves a prior independent but associated step leading to formation of a 40 S subunit-Met-tRNAf complex not containing initiation factors. It can be argued that the omission of 60 S subunits is a highly artificial situation of no direct relevance in vivo. Secondly, it can be argued that traces of 60 S subunits which may be present in a 40 S preparation may be playing an important catalytic role in our observations. Thus, we chose a second method to distinguish the eIF-5-dependent release of initiation factors from subunit joining, the use of the antibiotic edeine. Edeine has the property of preventing the joining of 40 S and 60 S ribosomal subunits (14). In Fig. 3, Met-tRNAf binding to 40 S subunits in the absence of eIF-5 and edeine is shown by open triangles. Approximately 10 pmol of Met-tRNAf were bound. The addition of eIF-5 resulted in the loss of all 40 S subunit-associated Met-tRNAf and the appearance of about 4 pmol of Met-tRNAf in the 80 S ribosomal region (open circles). When edeine is present in the absence of eIF-5, 40 S subunit binding is reduced to about 3 pmol (closed triangles). Addition of eIF-5 in the presence of edeine yields little or no 40 S subunit binding of Met-tRNAf and none of the Met-tRNAf appears in the 80 S ribosome region (closed circles). Therefore, edeine prevents formation of 80 S complexes containing Met-tRNAf, primarily by blocking subunit joining, but it does not prevent the labelization of Met-tRNAf that accompanies the eIF-5-dependent release of eIF-2. It is also clear that edeine interferes with the binding of the ternary complex to 40 S subunits, but to a lesser extent than the blockage of subunit joining.

If eIF-5 leads to formation of a 40 S subunit-Met-tRNAf complex free of eIF-2, eIF-3, and GTP, and this complex is an intermediate leading to 80 S ribosomal complex formation, it should be possible to isolate such a complex and show that it can participate in a subsequent reaction to yield a functional 80 S initiation complex. We examined the 40 S subunit-Met-tRNAf complex by harvesting it from the 40 S subunit region of a sucrose gradient and testing whether methionyl-tRNAf synthesis could occur by adding 60 S subunits, eIF-4C, eIF-4D, and puromycin in the presence of GuoPP(NH)p. Normally, methionyl-tRNAf synthesis is dependent upon eIF-2 (or eIF-2A), eIF-5, and GTP (instead of GuoPP(NH)p). The results are presented in Fig. 4. Panel A is a control sample in which 40 S and 60 S subunits, eIF-2, -3, -4C, -4D, and -5, and GTP were incubated. The sample was centrifuged through a sucrose gradient containing GTP. The centrifugation was for 135 min in Panel A and 150 min in Panels B and C. The 80 S peak (Panel A) was harvested and puromycin was added to the sample. Salts and [Mg2+] were adjusted to conditions optimal for methionyl-tRNAf synthesis and the volume was increased to overcome the inhibitory effect of sucrose. The picomoles of methionyl-tRNAf synthesized are shown by the vertical bar. Forty-seven per cent of the radioactivity in the 80 S peak was converted to methionyl-tRNAf; most of the remaining radioactivity was present as free methionine as a result of nonenzymatic deacylation. In a similar experiment, the same initiation factors were incubated with just 40 S subunits and subjected to centrifugation in a gradient containing GuoPP(NH)p (to block any subsequent effect of eIF-5). When 60 S subunits, puromycin and eIF-4C and -4D were added to the three peak fractions,
methionyl-puromycin synthesis occurred to the extent of 16 to 38% of the Met-tRNA$_f$ contained in the gradient fractions. Since GuoPP(NH)p and not GTP was in the gradient, it appears that methionyl-puromycin synthesis and presumably 80 S initiation complex formation do not require GTP hydrolysis for subunit joining per se, although the prerequisite "eIF-5-dependent release" of eIF-2 and eIF-3 was dependent on GTP hydrolysis (Fig. 1). To evaluate the possibility that eIF-5 was carried into the gradient fractions with the 40 S complex, [14C]eIF-5 was applied to a separate tube containing 40 S preinitiation complexes. The amount of eIF-5 in gradient fractions from that tube are plotted in Panel B. Clearly, less than 0.1 pmol of eIF-5 could be expected to be in each gradient fraction. The experiment in Panel C is the same as that presented in Panel B except that GuoPP(NH)p replaced GTP in the incubation media. The eIF-5-mediated release of eIF-2 was blocked and 40 S subunit complexes containing Met-tRNA$_f$ and [14C]eIF-5 were obtained. When 60 S subunits eIF-4C, 4D, and puromycin were added to isolated gradient fractions, very little methionyl-puromycin was synthesized, amounting to 4 to 5% of the Met-tRNA$_f$ contained in the two highest gradient fractions. Thus, when eIF-5 function was blocked by GuoPP(NH)p, the 40 S preinitiation complexes formed were unable to combine with 60 S subunits to synthesize methionyl-puromycin. These data suggest that a 40 S subunit-Met-tRNA$_f$ complex, free of eIF-2, eIF-3, and eIF-5 is nearly as efficient as 80 S ribosomal complexes in methionyl-puromycin synthesis when 60 S subunits and eIF-4C and -4D are added. Furthermore, GTP hydrolysis does not appear to be required after eIF-5 has mediated the release of eIF-2 and eIF-3.

While the above experiments gave insight into the function of eIF-5 in protein synthesis initiation, two problems were encountered. First, due to the loss of radiolabeled GTP during complex isolation on sucrose density gradients, the hydrolysis of GTP could only be inferred by comparative studies with GTP and GuoPP(NH)p. Second, kinetic studies on the conversion of 40 S complexes to 80 S complexes were not possible as the isolation of 40 S complexes was slow and contained high concentrations of sucrose. To circumvent these problems, 40 S preinitiation complexes were isolated by gel filtration (Fig. 5). It can be seen that such 40 S preinitiation complexes contain approximately equimolar amounts of [gamma-32P]GTP and [14C]Met-tRNA$_f$. While these complexes contain free 40 S subunits, the other components (eIF-2, GTP, and Met-tRNA$_f$) were eluted in subsequent column fractions.

In order to follow the conversion of the 40 S preinitiation complexes to 80 S initiation complexes, eIF-4C, -4D, -5, and 60 S subunits were incubated with the isolated 40 S preinitiation complexes and the formation of 80 S complexes was determined as sensitivity of the [14C]Met-tRNA$_f$ to puromycin. At the same time, the hydrolysis of GTP was determined (as organic extractable phosphate). The results of two such experiments are presented in Table II. From an initial input of 40 S complexes with a slight molar excess of Met-tRNA$_f$ over GTP, approximately one-half of these complexes were converted to 80 S complexes (as judged by methionyl-puromycin synthesis) and there was slightly less than an equimolar hydrolysis of GTP (as judged by phosphate released from GTP).

A kinetic analysis of the formation of 80 S initiation complexes from isolated 40 S preinitiation complexes is presented in Fig. 6. As is apparent GTP hydrolysis is rapid with an appreciable degree of GTP hydrolysis occurring at 0°C during the time required to add the "joining components" (eIF-4C, -4D, -5, and 60 S subunits), mix the solutions, and transfer to 37°C. On the other hand, methionyl-puromycin synthesis is preceded by a period of GTP hydrolysis, which is rapid and nearly complete at 37°C.
Figure 6. Isolation of 40 S preinitiation complexes by gel filtration. The conditions for the formation of 40 S preinitiation complexes and the subsequent separation of these complexes from reactants by chromatography using a column (0.9 x 20 cm) of Sepharose 6B are given under “Materials and Methods.” Absorbance at 280 nm was monitored continuously with a Zeiss PM 2 UV spectrophotometer. Radioactivity was determined by liquid scintillation spectrometry of 50-μl aliquots mixed in 10 ml of Hydromix (Yorktown). The elution positions of 40 S subunits, eIF-2, tRNA or Met-tRNA, and GTP were determined in separate experiments as absorbance at 280 nm and in some instances by radioactivity ([14C]Met-tRNA, specific activity 290 mCi/mmol; [γ-32P]GTP, specific activity 406 mCi/mmol). Fractions of 1 ml were collected every 3 min.

Table II
Conversion of 40 S preinitiation complexes to 80 S initiation complexes

The preparation and assay of 40 S preinitiation complexes by Sepharose 6B chromatography was as described under “Materials and Methods.” One hundred-microliter aliquots of the pooled 40 S preinitiation complexes were mixed with an equal volume of buffer which also contained puromycin, eIF-4C, -4D, -5, and 60 S subunits. The 200-μl reaction mixture was incubated for 30 min at 37°C.

| Experiment | Met-tRNA<sub>6</sub> | GTP | Methionylpuromycin | PO<sub>4</sub> released |
|------------|------------------|-----|--------------------|----------------------|
|            | pmol             | pmol| pmol               | pmol                 |
| Experiment 1 | 1.84             | 1.30| 0.92               | 0.88                 |
| Experiment 2 | 1.73             | 1.44| 0.94               | 0.65                 |

Figure 6. Kinetics of PO<sub>4</sub> release and methionylpuromycin synthesis using isolated 40 S preinitiation complexes. The preparation of 40 S preinitiation complexes and subsequent assay of 50-μl aliquots was as described under “Materials and Methods” and in the legend to Table II. The initial input of [14C]methionine and [32P]PO<sub>4</sub>, were 0.83 and 0.52 pmol, respectively. Control analyses indicated that greater than 80% of the input radioactivity was in the parent compounds, [14C]Met-tRNA<sub>6</sub> and [γ-32P]GTP.

Discussion

The factors required and the sequence of steps in the formation of an 80 S initiation complex are gradually being defined through the work of many laboratories. The preliminary step appears to be the formation of a soluble ternary complex which contains eIF-2, Met-tRNA<sub>6</sub>, and GTP (3-5, 15-32). Despite the general agreement on this point, there are reports that indicate two different observations on ternary complex formation (with highly purified eIF-2). The first group has observed that ternary complex formation is rapid, relatively insensitive to [Mg<sup>2+</sup>] and that the ternary complex can bind directly to 40 S subunits (3-5). The second group has found that ternary complex formation is slow, highly sensitive to [Mg<sup>2+</sup>] and may be stimulated by an additional protein(s) to enhance the yield of ternary complex (23, 33-38). Further considerations will be based on the observations of the first group (which are the basis for this study) as at present it is not possible to reconcile the differences between the two groups of reports.

The next general step in protein synthesis initiation is the binding of mRNA to the Met-tRNA<sub>6</sub> 40 S subunit complex. This step is catalyzed by eIF-1, eIF-4A, and eIF-4B (4, 5) and requires the utilization of ATP (4, 5, 37, 38). To date, there has been no report of any similar requirement when AUG codon or other synthetic mRNAs are used as template. Transfer of initiator tRNA from 40 S subunit complexes to 80 S initiation complexes requires 60 S subunits, GTP, and is catalyzed by eIF-4C and eIF-5 (3, 4, 6). During this last step, eIF-2 and eIF-3 are released from the 40 S subunit complexes (Refs. 3, 4, and 6, and Fig. 1). In this report, the role of eIF-5 in the initiation process is defined further. The initial observation with eIF-5 indicated that its presence could catalyze the release of eIF-2 and eIF-3 from 40 S preinitiation complexes in a reaction that required the hydrolysis of GTP (Ref. 6 and Fig. 2). The release of eIF-2 from 40 S preinitiation complexes appears to yield an unstable Met-tRNA<sub>6</sub> 40 S subunit complex which decomposes easily during the isolation procedure (Table I and Fig. 1). A similar observation had been made previously (39, 40), but had not been correlated with the loss of eIF-2 from 40 S preinitiation complexes. The unstable Met-tRNA<sub>6</sub> 40 S subunit complex can combine with 60 S subunits to form an 80 S initiation complex in the absence of added eIF-5 or GTP (Fig. 4).

Studies with 40 S preinitiation complexes isolated by gel filtration yielded additional information on the role of eIF-5. The data in Table II and Fig. 6 are consistent with the interpretation that eIF-5 induces a hydrolysis of eIF-2-bound GTP which is associated with (if not the cause of) the eIF-5-mediated release of eIF-2 from 40 S preinitiation complexes. Additional supporting evidence for this interpretation has been obtained from comparative kinetic studies on methionylpuromycin synthesis using either eIF-2 or eIF-2A to direct Met-tRNA<sub>6</sub> binding to 40 S subunits (41). This interpretation is also consistent with the observation that GTP hydrolysis precedes 80 S complex formation (as judged by methionylpuromycin synthesis, Fig. 6). A previous report from this
laboratory had indicated that eIF-5 mediated the hydrolysis of GTP only in the presence of both 40 S and 60 S subunits (2). Consistent with this finding is data obtained using gel-filtered 40 S preinitiation complexes which demonstrated that the eIF-5-mediated hydrolysis of GTP in 40 S preinitiation complexes occurs more rapidly in the presence of 60 S subunits (41). Thus it would seem that although eIF-5 may mediate hydrolysis of GTP in 40 S preinitiation complexes, the kinetic preference is for such hydrolysis in the presence of 60 S subunits. Exactly how this occurs and why GTP hydrolysis should so markedly precede methionylpuromycin synthesis are subjects for further research.

The data presented in this report are relatively consistent with similar studies which have used natural mRNA templates except for additional requirements for ATP and initiation factors (4, 5). It is quite possible, however, that the additional protein and nucleic acid may significantly alter the stability of intermediate preinitiation complexes and also affect the process of GTP hydrolysis (for example, compare results in Ref. 42 with Ref. 43). To examine this point further as well as to attempt to define the process of ATP hydrolysis associated with mRNA binding to 40 S subunits, similar experiments to those reported here will be attempted using globin mRNA and gel-filtered preinitiation complexes.

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