STUDIES ON THE FORMATION OF THE INITIATION COMPLEX IN EUKARYOTES

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SUMMARY: The initial interaction between a natural messenger RNA and ribosomes in a eukaryotic system was studied. Utilizing a population of 1.0 M KCl-washed ribosomes and isolated myosin mRNA, only the 40S subunits bound mRNA to form initiation complex I. This binding required the presence of initiation factor EF_3 and either GTP or its analogue, GDPCP. When unfractionated initiation factors, tRNA and GTP were added to the reaction mixture, the 60S ribosomal subunit joined with complex I to produce complex II. Complex II could not be demonstrated in the absence of GTP or when GTP was replaced by its analogue, GDPCP.

The isolation of an RNA species which we have tentatively identified as the messenger RNA directing the synthesis of the 200,000 molecular weight subunit of myosin (1,2) has made it possible to study the mechanism of initiation of protein synthesis in a eukaryotic system utilizing a natural mRNA. These studies have suggested that the mechanism for the initiation of protein systhesis is similar in both prokaryotes and eukaryotes (3,4). The first step in the formation of the initiation complex is the initiation factor dependent binding of mRNA to the 40S ribosomal subunit (complex I)(3). We show here that a protein factor (EF_3) is capable of binding ^{32}P-mRNA to the 40S ribosomal subunit in confirmation of our earlier studies (5). The formation of complex I is either stimulated or stabilized by the addition of GTP. The addition of both GTP and tRNA is required for the 40S subunit-RNA to associate with the 60S ribosomal subunit to form the completed initiation complex (complex II), which sediments at 75S (3). Although GTP is required for optimal formation of complex I and for formation of complex II, it is not clear whether cleavage of GTP is necessary to form these complexes. We report here studies which suggest that 5'-guanylyl-methylene-diphosphate (GDPCP), an analogue of GTP, is an effective substitute for GTP in complex I formation, but is ineffective in the formation of complex II.

MATERIALS AND METHODS

For each experiment 1.0 M KCL-washed ribosomes and initiation factors were freshly prepared from 14 day embryonic chick muscle as previously described (2,6). The designation of these eukaryotic initiation factors as EF_1, EF_2, and EF_3 is based on the elution from DEAE cellulose and is analogous to the prokaryotic factors F_1, F_2, and F_3. The DEAE cellulose chromatography of the initiation factors was performed as previously reported (5). ^{32}P-labeled 25-27S myosin mRNA was prepared as reported previously (1), except the large polysomes were collected directly by centrifugation of the cytoplasmic supernatant for 30 minutes at 240,000 x g through 5 ml of 40 per cent sucrose in 0.01 M tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 0.25 M KCl. Unstripped tRNA was prepared from adult chicken muscle by the method of Von Ehrenstein (6).

All incubation mixtures for binding ^{32}P-mRNA to ribosomes contained 0.5 mg of 1.0 M KCl-washed ribosomes in 0.5 ml of MIB buffer [0.02 M tris-HCL (pH 7.6), 0.005 M $MgCl_2$, 0.15 M KCl, and 0.006 M mercaptoethanol]. Initiation factors, GTP or its analogue GDPCP, and tRNA were added to each incubation mixture in the combinations and amounts indicated in the individual figure legends. The reaction mixtures were incubated for 5 minutes at 5°C (no significant differences were noted in reactions incubated at 25°C) and subsequently analysed by sucrose density gradient centrifugation as previously described (3). Fractions were collected from the gradients onto nitrocellulose membrane filters and washed with 2 ml MIB buffer. The radioactivity was determined on a low background counter (less than 2 counts per minute background).

RESULTS AND DISCUSSION

The proteins removed from muscle ribosomes by a 1 M KCl wash have been separated into several fractions by DEAE cellulose chromatography. A fraction, EF_3, was found to be responsible for binding mRNA to ribosomes attached to nitrocellulose membrane filters (5). When the binding assay is performed using sucrose density gradient centrifugation, this same fraction (EF_3) is

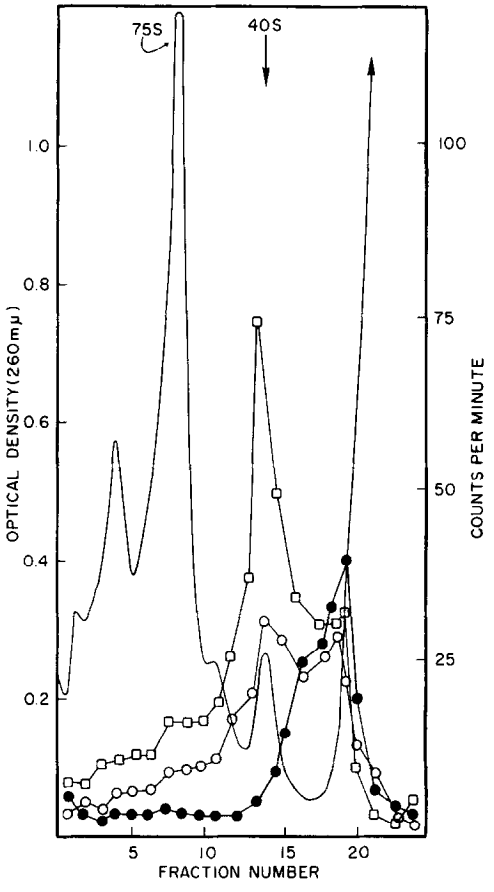

Fig. 1. Initiation Factor Dependence of Complex I Formation. ^{32}P-26S-mRNA (500 cpm/assay) was incubated with 0.5 mg of washed ribosomes, 0.5 mM GTP, and 30 μg of the three separated factor preparations: EF_1 (●–●–●–●), EF_2 (○–○–○–○), or EF_3 (□–□–□–□). The mixtures were layered onto 10%-30% sucrose density gradients and centrifuged 5 hours at 25,000 rpm. The solid line in the figure is the optical density at 260 mμ as recorded continuously while fractions were collected directly onto nitrocellulose filters for counting.

required for the binding of ^{32}P-mRNA to the 40S ribosomal subunit (Fig. 1). EF_1 has no binding activity, while EF_2 binds ^{32}P-mRNA only to a limited extent. The binding activity of EF_2 is likely a result of EF_3 contamination of our EF_2 preparation. These results confirm our previous observations concerning the role of EF_3 in the formation of the initiation complex. The fact that EF_3 reacts directly with the 40S ribosomal subunit gives further evidence of the direct role of native subunits in the process of initiation of protein synthesis.

We have consistently observed the presence of ribosomal subunits in both 1 M KCl-washed and unwashed ribosomal preparations when analysed in sucrose density gradients containing 0.02 M tris-HCl (pH 7.4), 0.15 M KCl, and 0.005 M $MgCl_2$. Both washed and unwashed preparations show optical density profiles similar to that illustrated in Fig. 1 (also see reference 3). These results, and the inability to demonstrate dissociation activity in the initiation factor preparations (unpublished observations), suggest that ribosomal subunits involved in the initiation of protein synthesis differ from subunits obtained in high monovalent salt solutions, which readily form monomeric ribosomes when returned to low salt buffers (7,8). This implied heterogeneity of ribo-

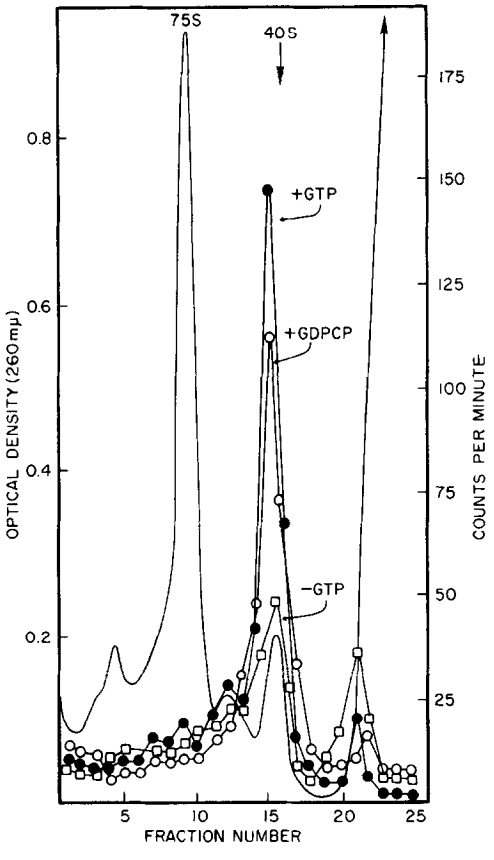

Fig. 2. Effect of GTP on Formation of Initiation Complex I in the Absence of tRNA. ^{32}P-26S-mRNA (600 cpm/assay) was incubated with 0.5 mg of washed ribosomes and 150 μg of unfractionated initiation factors in the absence of GTP (□-□-□-□), in the presence of 0.5 mM GTP (●-●-●-●), or in the presence of 0.5 GDPCP (0-0-0-0). The mixtures were analysed as described in Methods and Fig. 1. The solid line represents the optical density.

somes in eukaryotes is supported by the recent observations of Kabat (9).

In order to study the energy requirements for the formation of the initiation complex, GDPCP, an analogue of GTP, was tested for its ability to substitute for GTP in the binding of mRNA to the 40S ribosomal subunit (complex I) and the subsequent attachment of the 60S ribosomal subunit and tRNA (complex II). When GTP was omitted from the reaction mixture only a small amount of ^{32}P-mRNA was found to bind the 40S subunit. The addition of either GTP or GDPCP stimulated the binding of ^{32}P-mRNA (Fig. 2). These results confirm our previous observations concerning the requirement of GTP in complex I

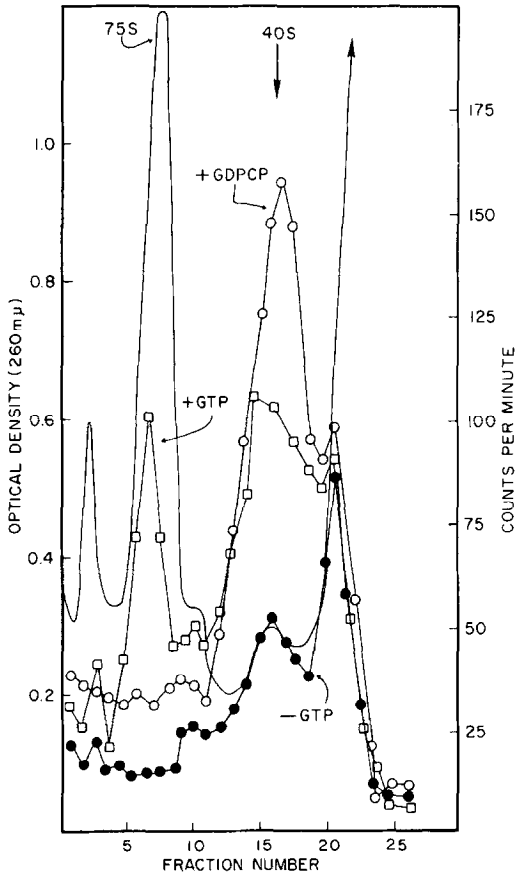

Fig. 3. Effect of GTP on Formation of Initiation Complex II in the Presence of tRNA. ^{32}P-26S-mRNA (1750 cpm/assay) was incubated with 0.5 mg of washed ribosomes, 100 μg tRNA, and 150 μg of unfractionated initiation factors in the absence of GTP (●–●–●–●), in the presence of 0.5 mM GTP (□–□–□–□), or in the presence of 0.5 mM GDPCP (0–0–0–0). The mixtures were analysed as described in Methods and Fig. 1. The solid line represents the optical density.

formation (3) and further suggest that GTP cleavage need not occur during this step of the initiation process. However, as shown in Fig. 3, the addition of unstripped tRNA results in the formation of complex II, sedimenting at 75S, only when GTP is present in the reaction mixture. GDPCP is ineffective in substituting for GTP in complex II formation. These results suggest that GTP cleavage may be necessary to form the complete initiation complex. Alternatively, complex II may be less stable with GDPCP present and thus dissociates in the course of centrifugation through a sucrose density gradient. Such a possibility has been suggested in similar analyses using bacterial ribosomes (4). Further studies will be required to clarify these alternatives. Nevertheless, the process of initiation of protein synthesis in eukaryotes appears to be similar to that found in prokaryotes, a possible exception being a greater heterogeneity of ribosomal populations found in eukaryotic cells.

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