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Polypeptide chain initiation in eukaryotes: Mechanism of formation of initiation complex*

(*Artemia salina*/reticulocyte initiation factors/protein synthesis)

CESAR NOMBELA†§, NOHELLY A. NOMBELA†§, SEVERO OCHOA†, BRIAN SAFER‡, W. FRENCH ANDERSON‡, AND WILLIAM C. MERRICK‡

† Roche Institute of Molecular Biology, Nutley, New Jersey 07110; and ‡ Molecular Hematology Branch, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT ***Artemia salina* ribosomal subunits and highly purified reticulocyte initiation factors (IF) are used to study the mechanism of formation of the puromycin-sensitive initiation complex Met-tRNA$_i$·80S ribosome·AUG. A complex with equimolar amounts of 40S subunit, GTP, and Met-tRNA$_i$ is formed at low Mg^{2+} concentration with a requirement for IF-MP (homogeneous) but not AUG or other factors. An 80S complex is formed only upon the further addition of AUG, IF-M2A, and IF-M2B, but not of either factor alone. This complex contains no GTP or GDP. A 40S complex, which cannot be converted to an 80S one, is formed when the nonhydrolyzable analog GMPPCP is substituted for GTP. IF-M2A has no effect on the formation of this complex, but IF-M2B enhances its formation.**

In prokaryotes initiation factor IF-2 selects the initiator aminoacyl-tRNA for binding, together with GTP, to a complex of mRNA and a small ribosomal subunit. When the large subunit joins this complex, GTP is hydrolyzed and the bound IF-2 is released for further rounds of initiation (2). In eukaryotes IF-MP functions similarly to form the small subunit initiation complex, but joining of the large subunit, to form an 80S complex, requires additional factors (3–9). Using reticulocyte ribosomes and factors, and methionyl-puromycin (Met-puro) synthesis as a measure of 80S complex formation, the additional factors were found to be IF-M2A and IF-M2B (7). Eukaryotes have another IF-2-like factor, IF-M1, which in the absence of GTP or other nucleotides catalyzes the formation of an AUG-dependent 40S complex. This complex, upon addition of 60S subunits, can be directly converted to a puromycin–reactive 80S complex (7, 10, 11). However, IF-M1 does not appear to participate in mRNA translation in the *Artemia salina* system (1).

We have continued the studies on the formation of the initiation complex promoted by IF-MP using reticulocyte initiation factors and *A. salina* ribosomal subunits since *A. salina* ribosomes maintain good functional integrity (11) and can use reticulocyte initiation factors for translation (1). We find that IF-MP forms from 80S ribosomes a 40S initiation complex that contains equimolar amounts of Met-tRNA$_i$ and GTP. This complex is converted to a puromycin-reactive, non-GTP (or GDP) containing 80S complex upon addition of AUG and both IF-M2A and IF-M2B. The latter factor can also enhance 40S complex formation.

Abbreviations: IF, initiation factor; Met-tRNA$_i$, the methionyl-tRNA species that initiates protein synthesis; Met-puro, methionyl-puromycin.

* This is paper no. VII in the series, "Polypeptide Chain Initiation in Eukaryotes." Preceding paper of this series is ref 1.

§ Present address: Department of Microbiology, University of Salamanca, Spain.

MATERIALS AND METHODS

The preparation of rabbit liver [^{35}S]Met-tRNA$_i$ has been described (12). *A. salina* [^{35}S]Met-tRNA$_i$ was prepared as described (8). [8-^{3}H]GTP (8.1 Ci/mmol) was from New England Nuclear and [8-^{3}H]GMPPCP (5.5 Ci/mmol) from ICN. The sources of other materials were as before (1).

Ribosomal Subunits and Factors. 40S and 60S ribosomal subunits from undeveloped *A. salina* embryos were used throughout unless otherwise stated. They were prepared as described (13). Reticulocyte 40S subunits were prepared as previously (11). The rabbit reticulocyte initiation factors were the same as in the preceding paper (1).

Factor-Dependent Binding of [^{35}S]Met-tRNA$_i$ to Ribosomes. The incubations leading to formation of 40S and 80S initiation complexes were carried out in stepwise (6) or one-step fashion.

Stepwise reaction samples contain *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.4, 50 mM; KCl, 100 mM; and dithiothreitol, 2 mM. Other additions are as follows: *Step 1.* Final volume 50 μl; GTP, 0.15 mM; rabbit liver [^{35}S]Met-tRNA$_i$, 6 pmol; ApUpG (AUG), 0.05 A_{260} unit; without or with IF-MP as specified in the legends. Incubation is for 6 min at 30°. When IF-MP is present, the reaction leads to formation of a ternary complex, [^{35}S]Met-tRNA$_i$·IF-MP·GTP, which is retained on Millipore filters. *Step 2.* Final volume, 60 μl. $Mg(OAc)_2$ is added, to a concentration of 2 mM, along with either 40S subunits or a mixture of 40S + 60S subunits in the amounts specified in the legends. Incubation is for 15 min at 30°. In this step [^{35}S]Met-tRNA$_i$ is transferred to ribosomes. *Step 3.* Final volume, 64 μl. The $Mg(OAc)_2$ concentration is raised to 5 mM and the samples are incubated for 10 min at 0° to allow for destruction of excess ternary complex (6). *Step 4.* After addition of 60S ribosomal subunits, if not already present, and standing for a few more minutes at 0° the samples are analyzed by sucrose density gradient centrifugation. IF-M2A and/or IF-M2B are added, when desired, at the step and in the amounts specified in the legends.

One-step reaction samples contain, in a final volume of 60 μl, Hepes buffer, pH 7.3, 50 mM; KCl, 100 mM; $Mg(OAc)_2$, 2 mM; dithiothreitol, 2 mM; AUG, 0.05 A_{260} unit; *A. salina* [^{35}S]Met-tRNA$_i$, 9.8 pmol; either 40S ribosomal subunits, 0.41 A_{260} unit, or a mixture of 40S, 0.44 A_{260} unit, and 60S 1.13 A_{260} unit, subunits; [^{3}H]GMPPCP, 0.015 mM; and factors as specified in the legends. Time and temperature of incubation are also given in the legends.

For analysis of complex formation the samples are layered over 5 ml of a 15–25% linear sucrose gradient in 20 mM Hepes, pH 7.3, 100 mM KCl, 5 mM $Mg(OAc)_2$, and centri-

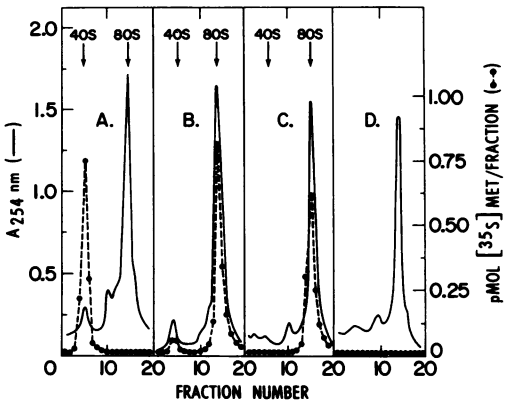

FIG. 1. Factor requirements for formation of 40S and 80S initiation complexes. Stepwise reaction as described in *Materials and Methods*. (A) IF-MP, 3.3 μg, at step 1; 40S subunits, 0.36 A_{260} unit, at step 2; 60S subunits, 0.96 A_{260} unit, at step 4. (B) IF-MP at step 1; 40S subunits at step 2; 60S subunits, IF-M2A, 1.3 μg, IF-M2B, 30 μg, at step 4. (C) IF-MP, IF-M2A, IF-M2B at step 1; rabbit reticulocyte 40S subunits, 0.315 A_{260} unit, and *A. salina* 60S subunits, 0.96 A_{260} unit, added at step 2. (D) As in C but without IF-MP. Specific radioactivity of [^{35}S]Met-tRNA$_i$, 8030 cpm/pmol.

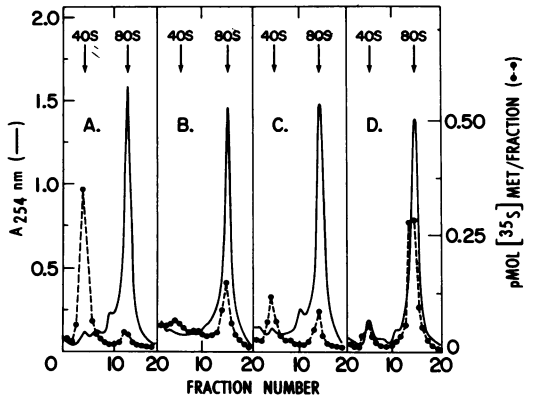

FIG. 2. Factor requirements for formation of 40S and 80S initiation complexes. Conditions of Fig. 1 except that a mixture of 40S subunits, 0.27 A_{260} unit, and 60S subunits, 0.96 A_{260} unit, was added throughout at step 2. Reticulocyte factors in the same amounts as in Fig. 1 were added at step 1. (A) IF-MP; (B) IF-MP and IF-M2A; (C) IF-MP and IF-M2B; (D) IF-MP, IF-M2A, IF-M2B. Specific radioactivity of [^{35}S]Met-tRNA$_i$, 7200 cpm/pmol.

fuged for 90 min at 4° and 47,000 rpm in the SW 50.1 rotor in a Spinco model L-2 65B preparative ultracentrifuge. The gradients are analyzed in an ISCO model 640 density gradient fractionator, equipped with a model UA-4 monitor and a model 614 recorder. Fractions are collected for determination of the ribosome-bound radioactivity. Each fraction is filtered through a Millipore filter; the filter is dried under an infrared lamp, and radioactivity is determined in Omnifluor (New England Nuclear) in a scintillation counter. Radioactivity of single-labeled samples was determined in a Beckman model LS-100C counter with an efficiency of 87% for ^{35}S. Radioactivity of double-labeled samples was determined in a Beckman model LS-250 counter with an efficiency of 20% for ^{3}H and 60% for ^{35}S.

RESULTS

Formation of 40S and 80S initiation complexes

The initiation factor requirements for formation of 40S and 80S initiation complexes (stepwise incubation) are shown in Figs. 1 and 2. In Fig. 1 (panels A and B) the 40S complex was formed prior to addition of 60S subunits. Note that with IF-MP alone (panel A) only a 40S complex is formed and that, in the additional presence of IF-M2A and IF-M2B, most of the bound [^{35}S]Met-tRNA$_i$ radioactivity moves out of the 40S into the 80S region of the gradient, forming an 80S complex. Note further that, whether an 80S complex is formed or not, the bulk of the 40S and 60S subunits associate to form 80S ribosomes. Thus, under conditions in which the uncomplexed subunits join to form 80S couples, the complex formed by [^{35}S]Met-tRNA$_i$ with the 40S subunit fails to join the 60S subunit unless IF-M2A and IF-M2B are present. In panels C and D (Fig. 1) a previously made hybrid mixture of 40S reticulocyte and 60S *A. salina* subunits was added at step 2, in the presence of either IF-MP, IF-M2A, and IF-M2B, or only IF-M2A and IF-M2B. This experiment shows that IF-MP is indispensable for initiation complex formation.

The experiments of Fig. 2, in which premixed *A. salina* 40S and 60S subunits (i.e., 80S ribosomes) were added throughout at step 2, show that a 40S initiation complex is formed from 80S ribosomes with homogeneous IF-MP as the only factor (panel A). They also show that both IF-M2A and IF-M2B must be present for conversion of the 40S to an 80S initiation complex (panels B, C, and D). Note, however, that the 40S complex is labilized by either factor, for in the presence of IF-M2A (panel B) or IF-M2B (panel C) much of the radioactivity disappears from the 40 S, with only a small amount of radioactivity appearing in the 80S region of the gradient.

Whereas formation of a 30S initiation complex in prokaryotes is template-dependent (2), the formation of the IF-MP-mediated 40S initiation complex in eukaryotes occurs in the absence or presence of template. However, template is required for 80S complex formation (3–5, 7, 9). This is confirmed by the data of Table 1. Template binding stabilizes the ribosomal binding of aminoacyl-tRNA and conversely (9, 14). Which initiation step requires template depends on

Table 1. Template requirements for formation of 40S and 80S initiation complexes

Additions			[^{35}S]Met-tRNA$_i$ bound (pmol)	
Factors (μg)	Ribosomes	ApUpG	to 40S	to 80S
IF-MP (2.2)	40S	+	0.70	
IF-MP (2.2)	40S	—	0.75	
IF-MP (2.2), IF-M2A (1.3), IF-M2B (21)	40S + 60S	+	0.09	0.70
IF-MP (2.2), IF-M2A (1.3), IF-M2B (21)	40S + 60S	—	0.02	0.02

Stepwise reaction. *A. salina* ribosomal subunits 40S, 0.36 A_{260} unit, 60S, 0.96 A_{260} unit. The specific radioactivity of [^{35}S]Met-tRNA$_i$ was 7628 cpm/pmol. Values were calculated from the radioactivity in the 40S and 80S regions of the gradient.

Table 2. Met-puro synthesis with *A. salina* ribosomes and reticulocyte factors

Factor additions (μg)	Met-puro synthesis (pmol)
IF-MP (3.3)	0.00
IF-MP (3.3), IF-M2A (1.3)	0.09
IF-MP (3.3), IF-M2B (30)	0.06
IF-MP (3.3), IF-M2A (1.3), IF-M2B (30)	1.75

Conditions of Fig. 3, panel C, except for the use of unlabeled GTP, 10 μM. Puromycin, 75 μg, was added along with the ribosomes. After incubation for 15 min at 30° the samples were assayed for Met-puro synthesis as described (13). A blank (0.11 pmol) in the absence of puromycin was subtracted.

the intrinsic stability of the initiator tRNA–ribosome complex. Apparently IF-MP forms a stable Met-tRNA$_i$–40S complex without template, but the 80S complex is unstable. Table 2 shows that Met-puro synthesis requires IF-MP, IF-M2A, and IF-M2B, just as formation of the 80S complex does.

Interaction with GTP

As seen in Fig. 3, the 40S complex formed on incubation of IF-MP, 40S subunits, [^{35}S]Met-tRNA$_i$, and either [8-^{3}H]GTP (panel A) or [γ-^{32}P]GTP (panel C, inset), contains equimolar amounts of Met-tRNA$_i$ and GTP. With 40S + 60S subunits (panel B), the 40S complex is essentially the same but the bulk of the ribosomes are present as 80S couples. There is some trailing of ^{3}H radioactivity in the 60S region of the gradient due probably to nonspecific adsorption. When IF-M2A and IF-M2B are also present (panel C), much of the bound [^{35}S]Met-tRNA$_i$ radioactivity moves to the 80S region of the gradient unaccompanied by ^{3}H radioactivity. This suggests that GTP is released during, or just prior to, formation of the 80S complex.

As reported previously (3–5, 7, 9) substitution of the nonhydrolyzable analog GMPPCP for GTP allows 40S complex formation but precludes its conversion to an 80S complex (Fig. 4). When IF-MP (panel A) is supplemented with IF-M2A (panel B) there seems to be some loss of 40S-bound [^{35}S]Met-tRNA$_i$, but when supplemented with IF-M2B whether without (panel C) or with (panel D) IF-M2A, there is a pronounced increase of 40S-bound [^{35}S]Met-tRNA$_i$ and [^{3}H]GMPPCP; in addition, increased dissociation of 80S ribosomes is observed. IF-M2B also increased ribosomal dissociation in the absence of IF-MP (inset), i.e., under conditions where no 40S complex is formed. IF-M2B has been observed to stimulate (about 2-fold) the formation of the AUG-dependent 40S binding of Met-tRNA$_i$ promoted by the IF-MP-like factor IF-M1 (7)¶. In the presence of GMPPCP we find moderate stimulation of IF-MP-mediated binding of Met-tRNA$_i$ to 40S ribosomal subunits, particularly at low temperature (Table 3).

DISCUSSION

The following picture of eukaryotic initiation emerges from this and earlier work. 40S subunits arising from dissociation of runoff 80S ribosomes interact with IF-MP, GTP, and Met-tRNA$_i$, or their ternary complex, to form a 40S initiation complex. This complex, which is rather stable, contains equimolar amounts of Met-tRNA$_i$, GTP, and by analogy

¶ C. Nombela, N. A. Nombela, and S. Ochoa, unpublished observations.

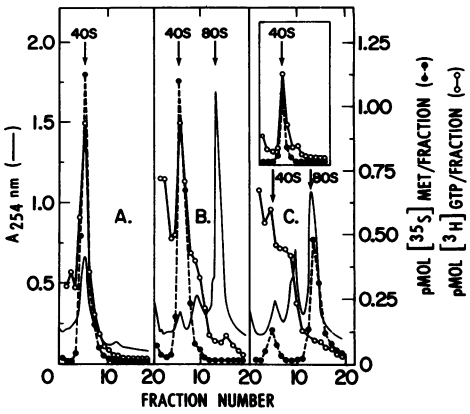

FIG. 3. Density gradient analysis of 40S and 80S complexes generated in the presence of labeled GTP. Reaction carried through steps 1 and 2 of stepwise procedure. Step 1 samples (final volume, 53 μl) contained [^{3}H]GTP (3,000 cpm/pmol), 10 μM, instead of nonlabeled GTP; *A. salina* (rather than rabbit liver) [^{35}S]Met-tRNA$_i$, 7350 cpm/pmol, 9.8 pmol; $Mg(OAc)_2$, 2 mM; and factors as indicated. Step 2 began with the addition of ribosomes. (A) IF-MP, 3.3 μg; 40S subunits, 0.51 A_{260} unit. (B) IF-MP; mixture of 40S subunits, 0.44 A_{260} unit, and 60S subunits, 1.13 A_{260} unit. (C) IF-MP; IF-M2A, 1.3 μg; IF-M2B, 30 μg; ribosomes as in B. Inset, same as A except with [γ-^{32}P]GTP (8086 cpm/pmol), 6.0 μM, instead of [8-^{3}H]GTP. After the step 2 incubation, samples were processed for sucrose gradient analysis.

with the prokaryotic 30S complex, probably IF-MP. In prokaryotes, interaction of the 30S complex with a 50S subunit leads to GTP hydrolysis and recycling of IF-2. IF-2 is endowed with ribosome-dependent GTPase activity. In eukaryotes the equivalent interaction needs accessory factors, IF-M2A and IF-M2B. At this stage there is GTP hydrolysis which, to draw from the prokaryotic model, is probably accompanied by release of IF-MP. No IF-M3, ATP, or other factors (9) are required to form an 80S complex when AUG is the template (ref. 7, and this paper). GTP hydrolysis is probably catalyzed by IF-M2A, which has ribosome-dependent GTPase activity (15). At this time we have no further insight into the mode of action of the accessory factors but, as noted above, IF-M2A and IF-M2B, separately or together, labilize the 40S complex in the presence of GTP.

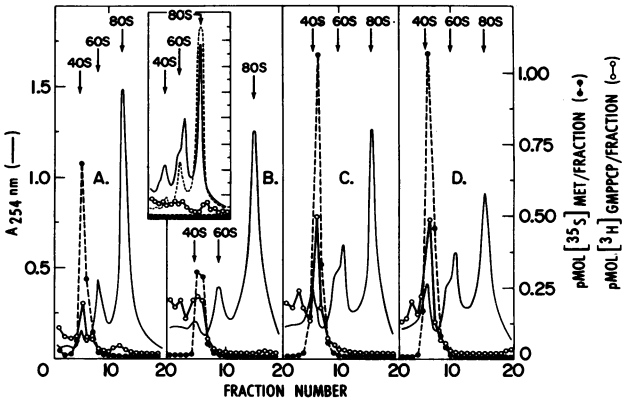

FIG. 4. Density gradient analysis of complexes formed in the presence of [^{3}H]GMPPCP. One-step reaction as described in *Materials and Methods*, with 40S + 60S ribosomal subunits throughout. (A) IF-MP, 3.3 μg; (B) IF-MP, IF-M2A, 1.3 μg; (C) IF-MP, IF-M2B, 30 μg; (D) IF-MP, IF-M2A, IF-M2B. Inset, as C, but without IF-MP; dashed line is the superimposed absorbance profile of panel A. The specific radioactivity of [^{3}H]GMPPCP was 1500 cpm/pmol.

Table 3. 40S ribosomal binding of [^{35}S]Met-RNA$_i$ in the presence of GMPPCP

Experiment no.	Factor additions	Incubation	[^{35}S]Met-tRNA$_i$ binding (pmol)
1	IF-MP	20 min at 30°	2.31
	IF-MP, IF-M2A	20 min at 30°	2.15
	IF-MP, IF-M2A, IF-M2B	20 min at 30°	2.88
2	IF-MP	30 min at 0°	1.21
	IF-MP, IF-M2B	30 min at 0°	2.50

Conditions of Fig. 4 with 40S subunits. Specific radioactivity of [^{35}S]Met-tRNA$_i$, 7200 cpm/pmol. Factor additions as in Table 2.

An unexpected result was the apparent ability of IF-M2B to cause dissociation of 80S ribosomes in the presence of the nonhydrolyzable analog of GTP, GMPPCP. This dissociation factor activity of IF-M2B may be of some significance because, unlike IF-3, IF-M3 does not have dissociation factor activity (16). Another fraction with dissociation factor activity has been described (16) but as yet is poorly characterized. Formation of initiation factor–40S complexes prevents their association to 60S subunits and promotes ribosome dissociation (17). The fact that a group of initiation factors is associated with "native" 40S subunits but absent from 80S ribosomes or polysomes (17, 18) speaks for recycling of eukaryotic initiation factors during protein synthesis, but direct evidence for recycling, as obtained with labeled prokaryotic factors (2), is not available.

An initiation complex formed by IF-M1 with reticulocyte or *A. salina* 40S subunits can be converted to a puromycin-sensitive 80S complex by the simple addition of *A. salina* but not reticulocyte 60S subunits (10, 11, 13). However, the latter subunits yield a puromycin-sensitive complex when IF-M2A, IF-M2B, and GTP are also added (7, 11). The reasons for this difference are not known.

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