Polypeptide chain initiation in eukaryotes: Initiation factor MP in Artemia salina embryos*

WITOLD FILIPOWICZ†, JOSE M. SIERRA, AND SEVERO OCHOA
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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ABSTRACT The activity of IF-MP, a polypeptide chain initiation factor that forms a ternary complex with eukaryotic initiator Met-tRNA and GTP and promotes binding of the initiator to 40S ribosomes, is very low in undeveloped Artemia salina embryos but increases over 20-fold following re-sumption of development upon hydration of the cysts. The factor is present in both the ribosomal salt wash and high-speed supernatant. Its specific activity is 50 times higher in the wash but its total activity is only about twice as high in the wash as in the supernatant. As is true of IF-MP from other eukaryotic sources, the A. salina factor is specific for eukaryotic Met-tRNA and sensitive to SH-reagents, and its activity is GTP dependent.

The prokaryotic protein synthesis initiation factor IF-2 has two eukaryotic counterparts: IF-M1 and IF-MP in the Anderson nomenclature (1, 2). IF-M1, discovered in 1970 in salt washes of rabbit reticulocyte ribosomes (3, 4), is widely distributed (5–10). It catalyzes the AUG-dependent binding of pro- or eukaryotic fMet-tRNA, and less effectively Met-tRNA, (11), as well as the poly(U)-dependent binding of prokaryotic acPhe-tRNA and less effectively Phe-tRNA (12), to eukaryotic cytoplasmic 40S ribosomes in the absence of GTP or other nucleotides. Upon addition of 60S ribosomes and puromycin to the rather unstable 40S complex, aminoaeryl-puromycin is formed in good yield.

IF-MP, discovered in 1971 in ribosomal salt washes of mouse fibroblasts (13), is present in rabbit reticulocytes (14–16) and other cells (17, 18). Its distribution is roughly the same as that of IF-M1. IF-MP promotes the binding of Met-tRNA, to 40S ribosomes via formation of a Met-tRNAf-GTP ternary complex in the presence or absence of template (13-20). IF-MP, unlike IF-M1, (a) requires GTP for activity, (b) is specific for eukaryotic Met-tRNA, and less reactive with the formylated species, (c) forms a stable 40S complex from 40S or 80S ribosomes, and (d) requires template and additional factors for formation of the 80S initiation complex and Met-puromycin synthesis.

It appears that only IF-MP may be functional in the eukaryotic cytoplasm because mRNA translation in Artemia systems that contain IF-M1 but are free of other initiation factors (21) requires IF-MP and is not inhibited by antibody against A. salina IF-M1 (paper in preparation). This is consistent with the reversal by IF-MP of the inhibition of globin synthesis observed in reticulocyte lysates in the absence of hemin (22, 23). Since the occurrence of IF-MP in A. salina had not previously been reported, we looked for this factor in ribosomal washes and supernatants of undeveloped and developing embryos. IF-MP activity is very low in undeveloped embryos but increases markedly with development.

MATERIALS AND METHODS

Preparation of IF-MP. IF-MP was partially purified from a 0.5 M KCl ribosomal wash of developing A. salina embryos. Development and preparation of the unwashed ribosomal fraction were as described (21) except that the ribosomes were pelleted by centrifugation for 4 hr at 125,000 × g in a Spinco 60 Ti rotor. The ribosomal pellet from 300 g of cysts is gently suspended in 100 ml of low salt buffer A (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.6, 70 mM KC1, 9 mM Mg(AcO), 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol), stirred for 2.5 hr at 0°, and centrifuged for 20 min at 55,000 × g to remove glycerogen. The clear portion of the supernatant is collected and centrifuged at the same speed for 10 hr. The low salt ribosomal pellet is then suspended in 60 ml of buffer B [20 mM Hepes, pH 7.6, 0.1 mM EDTA, 0.5 M KC1, 5 mM Mg(AcO), 2 mM dithiothreitol, 5% glycerol], stirred for 4 hr at 0°, and finally centrifuged for 5 hr at 125,000 × g. The high salt wash is dialyzed overnight against buffer C (20 mM Hepes, pH 7.6, 80 mM KC1, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol) and applied to a DEAE-cellulose column (Whatman DE-52, 1.5 × 7 cm) equilibrated with the same buffer. The column is washed with buffer C containing 80 mM KC1, and the IF-MP activity is eluted with buffer C containing 180 mM KC1. This preparation also contained other initiation factors. For assay of IF-MP levels in cytosol and ribosomal washes of undeveloped and developed embryos (Table 1), the high speed supernatant (S-125) and the 0.5 M KC1 ribosomal wash fractions were applied to a DEAE-cellulose column in buffer C containing 100 mM KC1, and IF-MP was eluted with buffer C containing 350 mM KC1. Protein was determined by the method of Lowry et al. (24) using bovine serum albumin as standard.

Other Preparations. A. salina 40S and 60S ribosomal sub-
units were prepared essentially as described (7). *A. salina* (undeveloped embryos) and rat liver aminoacyl-tRNA synthetases were prepared by DEAE-cellulose chromatography (7) of postribosomal supernatant fractions. *E. coli* aminoacyl-tRNA synthetases (25) were kindly supplied by Dr. J. Ofengand of this Institute. Crude *A. salina* tRNA was isolated from postribosomal supernatant of undeveloped embryos. It was separated from contaminating high-molecular-weight RNA by Sephadex G-100 chromatography. Partially purified tRNA\textsubscript{Met} was prepared by BD-cellulose chromatography of crude tRNA (26). *A. salina* [\textsuperscript{35}S]Met-tRNA\textsubscript{i} (12,000 to 30,000 cpm/pmol) was prepared by acylation of crude tRNA with *A. salina* aminoacyl-tRNA synthetases. *A. salina* [\textsuperscript{14}C]Met-tRNA\textsubscript{i} (450 cpm/pmol) was prepared by acylation of partially purified tRNA\textsubscript{Met} with *E. coli* aminoacyl-tRNA synthetases.

Rabbit reticulocyte [\textsuperscript{35}S]Met-tRNA\textsubscript{i} (12,000 cpm/pmol), [\textsuperscript{35}S]Met-tRNA\textsubscript{i} (12,000 cpm/pmol), and [\textsuperscript{3}H]Met-tRNA\textsubscript{i} (1060 cpm/pmol) were prepared by acylation of crude rabbit reticulocyte tRNA (kindly supplied by Dr. N. K. Gupta, University of Nebraska) with *E. coli* aminoacyl-tRNA synthetases. Leucovorin was used as formyl donor in acylation mixtures when [\textsuperscript{35}S]Met-tRNA\textsubscript{i} was prepared. Rabbit liver [\textsuperscript{14}C]Met-tRNA\textsubscript{Met} (450 cpm/pmol) was prepared by acylation of purified Met-tRNA\textsubscript{Met} (kindly supplied by Drs. W. C. Merrick and W. F. Anderson, NIH, Bethesda, Md.) with rat liver aminoacyl-tRNA synthetases.

*E. coli* [\textsuperscript{14}C]Met-tRNA\textsubscript{i} (450 cpm/pmol) and [\textsuperscript{14}C]PhetRNA (850 cpm/pmol) were prepared by acylation of crude *E. coli* tRNA (Schwarz/Mann) with *E. coli* aminoacyl-tRNA synthetases. Highly purified *E. coli* f[\textsuperscript{35}S]Met-tRNA\textsubscript{i} (31,900 cpm/pmol) was similarly prepared from *E. coli* tRNA\textsubscript{Met} (about 95% pure) kindly supplied by Dr. G. D. Novelli, Oak Ridge National Laboratory.

Homogeneous rabbit reticulocyte IF-MP (2) was the kind gift of Drs. W. C. Merrick and W. F. Anderson.

**Ternary Complex Assay.** Unless otherwise noted, samples contain, in a final volume of 0.05 ml, Hepes buffer pH 7.6, 20 mM; KC1, 100 mM; Mg(AcO)\textsubscript{2}, 3 mM; dithiothreitol, 1 mM; partially purified IF-MP, about 35 µg of protein; *A. salina* [\textsuperscript{35}S]Met-tRNA\textsubscript{i}, 3.3 pmol; and GTP, 0.14 mM when present. The reaction components are added at 0° in the order listed above. After incubation for 5 min at 30° the reaction is stopped by dilution with 4 ml of cold wash buffer [20 mM Tris-HCl pH 7.5, 100 mM KC1, 3 mM Mg(AcO)\textsubscript{2}], and the samples are filtered through nitrocellulose membranes (Millipore Hapwo 2500, 0.45 µm pore size). The filters are washed twice each with 4 ml of the same buffer and dried, and the retained radioactivity is measured in Omnifluor in a Beckman LS-100 Scintillation counter. One unit is taken as the amount of factor causing a GTP-dependent binding of 1 pmol of Met-tRNA\textsubscript{i} under the conditions of the assay.

**Sucrose Density Gradient Centrifugation Analysis.** For 40S initiation complex assay, samples containing, in a final volume of 0.1 ml, Hepes buffer pH 7.3, 20 mM; KC1, 100 mM; Mg(AcO)\textsubscript{2}, 3 mM; dithiothreitol, 1 mM; IF-MP, 84 µg of protein; *A. salina* [\textsuperscript{35}S]Met-tRNA\textsubscript{i}, 10 pmol; and GTP or 5'-guanylyl-methylenediphosphonate (GMPPCP), 0.14 mM when present, are first incubated for 5 min at 30°. Then,

### Table 1. IF-MP levels in cytosol and ribosomal wash fractions from undeveloped and developing embryos

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Factor fraction derived from</th>
<th>[\textsuperscript{35}S]Met-tRNA\textsubscript{i} retained on Millipore filters (pmol)</th>
<th>Total units*</th>
<th>IF-MP specific activity†</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>-GTP</td>
<td>+GTP</td>
<td>Difference</td>
</tr>
<tr>
<td>Undeveloped</td>
<td>Cytosol (0.18 mg)</td>
<td>0.043</td>
<td>0.046</td>
<td>0.003</td>
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<tr>
<td>Developing</td>
<td>Cytosol (0.17 mg)</td>
<td>0.043</td>
<td>0.103</td>
<td>0.060</td>
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<tr>
<td>Undeveloped</td>
<td>Wash (0.028 mg)</td>
<td>0.06</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>Developing</td>
<td>Wash (0.026 mg)</td>
<td>0.10</td>
<td>0.60</td>
<td>0.50</td>
</tr>
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</table>

Standard assay was with *A. salina* Met-tRNA\textsubscript{i} (4.15 pmol) and factor fractions as indicated.
* Per 100 g of dry cysts.
† Units/mg of protein.

![Fig. 1](image-url)  
**Fig. 1.** Ternary complex formation as a function of the concentration of (A) Mg\textsuperscript{2+}, (B) IF-MP, and (C) Met-tRNA\textsubscript{i}, and (D) as a function of time. Curves 1 (●), with GTP; curves 2 (○), without GTP. Panel A, curve 3, (△), with GTP but without IF-MP. The wash buffer for each of the panel A assays had the same Mg\textsuperscript{2+} concentration as the corresponding assay sample. Conditions were of the standard assay except for the variable component and for the fact that panel C samples contained only 21 µg of IF-MP. The batch of IF-MP used for panels A and D was different from that used for B and C.
Fig. 2. Formation of ternary and 40S complexes as assayed by sucrose density gradient centrifugation. Assay conditions were as described in Materials and Methods. Sedimentation was from left to right. Upper panels, with GTP; lower panels, with GMPPCP. A and C, no subunits; B and D, complete system. A$_{255}$ nm (---); $^{35}$S radioactivity (o-o-); $^{38}$S radioactivity in assays (panel B) without GTP or IF-MP (o-o-o).

After addition of A. salina 40S subunits (0.85 A$_{260}$ unit), they are further incubated for 7 min at 30°. After incubation the samples are chilled in ice, layered on 4.5 ml of a linear 15–25% sucrose gradient in Hepes buffer pH 7.3, 20 mM; KCl, 80 mM; Mg(AC)$_2$, 5 mM; dithiothreitol, 0.5 mM, and centrifuged for 2 hr at 55,000 rpm and 2° in a Spinco SW 56 rotor. The gradients are monitored at 254 nm in an ISCO fractionator, and 0.2-mI fractions collected. Each fraction is diluted and filtered, and its radioactivity is measured as described for ternary complex assay.

RESULTS

IF-MP in undeveloped and developing embryos

Preparations from developing, but not from undeveloped, A. salina embryos can translate exogenous mRNA, e.g., brome mosaic virus, globin mRNA (21). Since undeveloped embryo preparations promote chain elongation, they must be deficient in a factor(s) required for initiating translation of mRNA. As seen in Table 1, IF-MP activity as measured by ternary complex formation with A. salina $[^{35}$S]$\text{Met-tRNA}$_i$ is very low in undeveloped embryos but increases markedly during development. The factor is present in both the high-speed supernatant and the ribosomal wash fraction to the extent of about 30% and 70% of the total, respectively, but its specific activity is about 50-fold higher in the ribosomal wash.

| Table 2. Effect of N-ethylmaleimide on ternary complex formation |
|--------------------------------|-----------------|
| System                          | $[^{35}$S]$\text{Met-tRNA}$_i$ retained on Millipore filters (pmol) | Inhibition (%) |
| Complete                         | 0.05            | 0.90                | 0.85             | 0               |
| Complete + NEM (1 mM)            | 0.05            | 0.58                | 0.53             | 38              |
| Complete + NEM (5 mM)            | 0.05            | 0.05                | 0                | 100             |
| Complete + NEM (20 mM)           | 0.04            | 0.04                | 0                | 100             |
| Complete + dithiothreitol (40 mM) | 0.04           | 0.83                | 0.78             | 8               |

Standard assay samples were preincubated either without or with N-ethylmaleimide (NEM), or dithiothreitol and N-ethylmaleimide, at the stated concentrations for 15 min at 25°, prior to adding A. salina $[^{35}$S]$\text{Met-tRNA}$_i$ (3.3 pmol) and GTP (0.14 mM). Unreacted N-ethylmaleimide was then inactivated with excess dithiothreitol (40 mM), and the assay was conducted as described in Materials and Methods. The last sample contained dithiothreitol throughout the assay.

<table>
<thead>
<tr>
<th>Table 3. Initiator specificity</th>
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<tbody>
<tr>
<td>aa-tRNA</td>
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<tr>
<td></td>
</tr>
<tr>
<td>$[^{35}$S]$\text{Met-tRNA}$_i$</td>
</tr>
<tr>
<td>$[^{1}$C]$\text{Met-tRNA}$_i$</td>
</tr>
<tr>
<td>$[^{3}$H]$\text{Met-tRNA}$_i$</td>
</tr>
<tr>
<td>$[^{35}$S]$\text{Met-tRNA}$_i$</td>
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<tr>
<td>$[^{1}$C]$\text{Met-tRNA}$_i$</td>
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<tr>
<td>$[^{1}$C]$\text{Met-tRNA}$_i$</td>
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<tr>
<td>$[^{3}$S]$\text{Met-tRNA}$_i$</td>
</tr>
<tr>
<td>$[^{1}$C]$\text{Met-tRNA}$_i$</td>
</tr>
<tr>
<td>$[^{35}$S]$\text{Met-tRNA}$_i$</td>
</tr>
<tr>
<td>$[^{1}$C]$\text{Phe-tRNA}$_i$</td>
</tr>
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</table>

Assay conditions as described in Materials and Methods with 6–10 pmol of aminoacyl-tRNA/sample. All assays except the last were with partially purified A. salina IF-MP (about 50 μg of protein per sample). Last assay was with highly purified rabbit reticulocyte IF-MP (2.5 μg of protein per sample).
Properties of factor

As found for IF-MP from other sources (13–20, 27), the retention of eukaryotic Met-tRNA\textsubscript{f} by nitrocellulose filters in the presence of the A. \textit{salina} factor is markedly GTP-dependent (Fig. 1). GDP (not shown) was inactive. The effect of GTP depends on the Mg\textsuperscript{2+} concentration. Under our conditions maximal stimulation by GTP occurred at 3 mM Mg\textsuperscript{2+} (Fig. 1A), a concentration that is optimal for natural mRNA translation in cell-free A. \textit{salina} systems (21). Fig. 1B (curve 1) shows that formation of the ternary complex is proportional to the concentration of IF-MP within a wide concentration range. Under our conditions the concentration of A. \textit{salina} Met-tRNA\textsubscript{f} giving maximal binding was about 0.1 \mu M (Fig. 1C), and formation of the ternary complex (Fig. 1D, curve 1) was essentially complete in 10 min.

Like IF-MP from other sources (17, 18), the A. \textit{salina} factor is sensitive to N-ethylmaleimide (Table 2). In further agreement with the properties of other preparations of this factor (2, 13-15, 18), A. \textit{salina} IF-MP forms a ternary complex with eukaryotic Met-tRNA\textsubscript{f}, but not Met-tRNA\textsubscript{m}, prokaryotic Met (or fMet)-tRNA\textsubscript{f}, or Phe-tRNA (Table 3). As shown by others (2, 14, 15), rabbit reticulocyte IF-MP is also unreactive towards eukaryotic aminocacyl-tRNAs other than Met-tRNA\textsubscript{f}. Since A. \textit{salina} IF-MP forms ternary complexes with Met-tRNA\textsubscript{f}, whether from A. \textit{salina} or rabbit reticulocytes (Table 3), it would appear that this factor is not species specific but only discriminates between prokaryotic and eukaryotic initiator tRNA. As further seen in Table 3, rabbit reticulocyte fMet-tRNA\textsubscript{f} is about one-half as active as the nonformylated species in ternary complex formation. Under our conditions (Table 3) binding of A. \textit{salina} Met-tRNA\textsubscript{f} by homogeneous rabbit reticulocyte IF-MP (2) is fully dependent on the presence of GTP.

Formation of 40S initiation complex

Ternary complex formation and subsequent transfer of the bound \textsuperscript{35}S)Met-tRNA\textsubscript{f} to 40S ribosomes, as analyzed by sucrose density gradient centrifugation, are illustrated in Fig. 2. In the absence of 40S subunits (Fig. 2A) there is only one peak of radioactivity near the top of the gradient. This peak undoubtedly corresponds to the \textsuperscript{35}S)Met-tRNA\textsubscript{f}-IF-MP-GTP ternary complex, for it decreases approximately to the extent that a new radioactive peak appears in the 40S region of the gradient upon addition of 40S subunits (Fig. 2B). No radioactive peaks are seen (Fig. 2B, open circles) in the absence of either IF-MP or GTP. Fig. 2 (C and D) further shows that the GTP analog, GMPPCP, can efficiently substitute for GTP in formation of both the ternary and the 40S initiation complex. However, the 40S complex formed with GMPPCP cannot be converted to an 80S complex (19, 20). Note that a 40S complex is formed in the absence of added template (16, 19, 20) despite the fact that ribosomal subunits prepared from undeveloped A. \textit{salina} embryos are devoid of messenger (21).

DISCUSSION

Our results with A. \textit{salina} IF-MP are in full accord with those reported with factor from other sources (13–20). Interestingly, the level of this factor in undeveloped A. \textit{salina} embryos is very low and undergoes a pronounced increase during development. In a subsequent communication (paper in preparation) we shall show that other initiation factors are also present in smaller amounts before development. IF-MP is found not only in the ribosomal wash, but also in the high-speed supernatant fraction. This would be expected if, like its prokaryotic counterpart IF-2 (28), IF-MP recycles between ribosomes and cytosol. Isolation of IF-MP from wheat germ cytosol has been reported (18).

We found that nucleoside triphosphates other than GTP promoted retention of \textsuperscript{35}S)Met-tRNA\textsubscript{f} by Millipore filters with the partially purified A. \textit{salina} but not with homologous rabbit reticulocyte IF-MP (see ref. 18). The effect of nucleoside triphosphates is due to the presence of nucleoside diphosphate kinase (EC 2.7.4.6). This enzyme catalyzes the transfer of the terminal phosphate of nucleotide triphosphates to diphosphates (e.g., NTP + GDP -> NDP + GTP) and is present along with GDP in the A. \textit{salina} but not the homogeneous reticulocyte preparation (paper in preparation). A similar observation with partially purified rabbit reticulocyte IF-MP was recently reported (29).

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