# PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES XII:* REQUIREMENT OF mRNA (AUG CODON) FOR Met-tRNA$_f^{Met}$ BINDING TO 40S RIBOSOMES

N. K. Gupta, B. Chatterjee and A. Majumdar

Department of Chemistry
University of Nebraska-Lincoln
Lincoln, Nebraska 68508

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Summary

A Millipore filtration assay method has been developed for studies of codon directed Met-tRNA$_f^{Met}$ binding to 40S ribosomes. Under the assay conditions, the stimulation of Met-tRNA$_f^{Met}$ binding to Millipore filters by added 40S ribosomes is strongly dependent on the presence of AUG codon, and partially purified peptide chain initiation factors. Crude 0.5 M KCl ribosomal wash is inactive in catalyzing this binding reaction due to the presence of an inhibitor in this preparation.

Studies of the detailed mechanism of protein synthesis initiation in mammalian cells have been greatly impeded due to lack of convenient assay methods for studies of specific interactions between initiator tRNA and initiation factor(s) with ribosomes and messenger RNA. In mammalian protein synthesis initiation, one of the peptide chain initiation factors IF1, forms a stable ternary complex with Met-tRNA$_f^{Met}$ and GTP and this complex is quantitatively retained on Millipore filters (2-8). This complex formation does not require $Mg^{++}$, ribosomes, or messenger RNA and the complex formation is inhibited by addition of ribosomes and $Mg^{++}$. Consequently, it has not been possible to use a standard Millipore filtration assay method for studies of initiation complex formation between ribosomes, initiator tRNA, and messenger RNA. Several laboratories have used density gradient centrifugation methods for these studies and have reported (6,11-13) that the binding of Met-tRNA$_f^{Met}$ to 40S ribosomal subunit is the first step in eukaryotic protein synthesis initiation and that this binding is mRNA independent.

Recently, we reported (1,9) that Met-tRNA$_f^{Met}$:IF1:GTP complex formed with partially purified initiation factors dissociates extensively upon addition of 5 mM $Mg^{++}$. Using this information, we have now developed a convenient Millipore filtration assay method for studies of AUG codon directed Met-

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$tRNA_f^{Met}$ binding to 40S ribosomes. The assay is carried out in two stages. In stage I, Met-$tRNA_f^{Met}$ is bound to 40S ribosomes in the presence of 2 mM $Mg^{++}$, peptide chain initiation factors, and AUG codon. In stage II, more $Mg^{++}$ (5 mM final concentration) is added to dissociate excess Met-$tRNA_f^{Met}$: IF1:GTP complex. Ribosome bound Met-$tRNA_f^{Met}$ is then assayed by Millipore filtration. Under the assay conditions, stimulation of Met-$tRNA_f^{Met}$ binding to Millipore filters by added 40S ribosomes is entirely dependent on the presence of AUG codon and peptide chain initiation factors. These results differ from previous reports (6,11-13) that Met-$tRNA_f^{Met}$ binding to 40S ribosomes is mRNA independent. They also indicate a similarity in one basic step in peptide chain initiation in both pro- and eukaryotic cells.

## Materials and Methods

The preparations of preincubated reticulocyte ribosomes and ribosomal 0.5 M KCl wash (I fraction) were the same as previously described (1-3). The crude I fraction was further purified by a three step purification procedure as has been described previously (1).

Reticulocyte 40S ribosomal subunits were prepared following the procedure of Schreier and Staehelin (10). The pelleted 40S subunits were suspended in 0.25 M sucrose and stored in liquid nitrogen. The final suspension contained 40-50 $A_{260}$ units per ml.

Poly r(U-G) directed methionine transfer reaction and Millipore filtration assay for [$^{35}$S]Met-$tRNA_f^{Met}$ binding to IF1 have been described (1-3). Other materials and methods were the same as described previously (1-3).

## Results

The Millipore filtration assay method was used for studies of Met-$tRNA_f^{Met}$ binding to 40S ribosomes. In this assay, the DEAE-cellulose purified initiation factor preparations (Fraction IV) were found to be most effective. The characteristics of such binding reaction using a mixture of DEAE-cellulose fractions, IF(1+2+3) are shown in Table I. Under the assay conditions, no significant stimulation of Met-$tRNA_f^{Met}$ binding was observed upon addition of 40S ribosomes alone and the addition of both 40S ribosomes and AUG codon gave a clear stimulation (4-5 fold above background binding) of Met-$tRNA_f^{Met}$ binding. This AUG directed Met-$tRNA_f^{Met}$ binding reaction was dependent on the addition of initiation factors, GTP and $Mg^{++}$. This assay is carried out in two stages. In stage I, Met-$tRNA_f^{Met}$ is bound to 40S ribosomes in the presence of 2 mM $Mg^{++}$, AUG codon and peptide chain initi-

TABLE I

REQUIREMENTS FOR THE MILLIPORE FILTRATION ASSAY FOR AUG DIRECTED [$^{35}$S]Met-tRNA$_f^{Met}$ BINDING TO 40S RIBOSOMES

| Reaction Mixtures | Radioactivity Bound to Millipore Filters (cpm) | | |
|---|---|---|---|
| | -AUG -ribosomes | -AUG +40S ribosomes | +AUG +40S ribosomes |
| Complete | 4,300 | 4,680 | 20,360 |
| -Stage II | 19,240 | 14,360 | 20,420 |
| -IF | 100 | 120 | 210 |
| -GTP | 2,120 | 2,680 | 3,900 |
| -$Mg^{++}$ | 28,660 | 18,070 | 18,780 |

A two stage procedure was used. In stage I, the complete incubation mixture contained in a total volume of 0.07 ml: 21.4 mM Tris-HCl, pH 7.5; 80 mM KCl, 2.14 mM dithiothreitol; 0.28 mM GTP; 0.05 $A_{260}$ unit A U G; 2.14 mM $MgCl_2$; 10 µg bovine serum albumin; 0.015 ml Fraction IV initiation factor mixture IF(1+2+3) (1:1:1 mixture of Fraction 19, 26 and 31); 8 pmole [$^{35}$S]Met-tRNA$_f^{Met}$ (1 x $10^5$ cpm); and 0.1 $A_{260}$ unit 40S ribosomes. The reaction was started by simultaneous addition of [$^{35}$S]Met-tRNA$_f^{Met}$ and 40S ribosomes. The reaction was incubated at 37° for 5 minutes. At the end of the incubation, 5 µl $MgCl_2$ solution (45 mM) was added and the incubation was continued in an ice bath for 10 minutes. The reaction was then terminated by addition of 3 ml cold washing buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, and 5 mM $MgCl_2$). The solution was then used for Millipore filtration assay as described previously (1).

In the experiments, where stage II was omitted, the reactions were terminated after first stage reaction at 37° for 5 minutes using 3 ml cold washing buffer containing 2 mM $Mg^{++}$ and in the experiments where $Mg^{++}$ was omitted, the reactions were terminated after stage II using 3 ml cold washing buffer minus $Mg^{++}$.

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ation factors. In stage II, more $Mg^{++}$ is added to dissociate excess Met-tRNA$_f^{Met}$:IF1:GTP complex. Ribosome bound Met-tRNA$_f^{Met}$ is stable to $Mg^{++}$ and is assayed by Millipore filtration. As shown in Table I, when the reaction was terminated at the end of stage I, the background binding due to Met-tRNA$_f^{Met}$:IF1:GTP complex was very high and the assay method could not distinguish between ribosome bound Met-tRNA$_f^{Met}$ and free Met-tRNA$_f^{Met}$:IF1:GTP complex.

When the reaction was carried out in the absence of $Mg^{++}$ and the incubation was terminated with cold wash buffer minus $Mg^{++}$, increased Met-$tRNA_f^{Met}$:IF1:GTP complex was formed as expected, and this complex was retained on Millipore filters. This binding in the absence of $Mg^{++}$ was somewhat lowered upon addition of 40S ribosomes and addition of AUG codon did not have any significant effect.

The results presented in Table II describe the activities of different ribosomal salt wash fractions at different stages of purification for catalysis of Met-$tRNA_f^{Met}$ binding to 40S ribosomes. The crude 0.5 M KCl ribosomal wash was completely inactive in the binding reaction. Fraction III was active, although with increased concentration of this initiation factor preparation significant binding of Met-$tRNA_f^{Met}$ to 40S ribosomes was observed in the absence of AUG codon. As mentioned before, DEAE-cellulose purified IF(1+2+3) fraction combination was very efficient in the binding reaction.

TABLE II

ACTIVITIES OF DIFFERENT RIBOSOMAL SALT WASH FRACTIONS FOR CATALYSIS OF [$^{35}$S]Met-$tRNA_f^{Met}$ BINDING TO 40S RIBOSOMES

| Factors Added | Amounts Added (µl) | Radioactivity Bound to Millipore Filters (cpm) | | |
|---|---|---|---|---|
| | | -AUG -ribosomes | -AUG +40S ribosomes | +AUG +40S ribosomes |
| 0.5 M KCl Wash (Fraction I) | 2 | 1,540 | 1,640 | 1,650 |
| | 5 | 4,170 | 4,750 | 4,530 |
| Ammonium Sulfate Fraction (Fraction III) | 2 | 2,380 | 2,400 | 9,630 |
| | 5 | 4,530 | 11,070 | 31,200 |
| DEAE-cellulose Fractions (Fraction IV) | 15 | 5,400 | 5,450 | 17,580 |
| Fraction (I + IV) | 2+15 | 4,700 | 6,350 | 5,030 |

Standard Millipore filtration assay conditions as described in Table 1 were used. Protein concentrations in different ribosomal salt wash fractions were: Fraction I, 12 mg per ml; Fraction III, 4.5 mg per ml; Fraction IV IF(1+2+3) (1:1:1 mixture) 1.8 mg per ml.

The inability of the crude ribosomal salt wash (Fraction I) to catalyze the binding reaction is due to the presence of an inhibitor in this preparation which strongly inhibits Met-tRNA$_f^{Met}$ binding to 40S ribosomes. Addition of a small amount of Fraction I (2 µl) to Fraction IV preparation (15 µl) completely inhibited the stimulatory activity of the latter fraction for AUG directed Met-tRNA$_f^{Met}$ binding. The characteristics of this inhibitor are not known at present. Apparently, this inhibitor is removed during the first DEAE-cellulose purification step. We have noted that a few Fraction III preparations were inactive in the binding reaction presumably due to incomplete removal of this inhibitor during purification.

Fig. 1 describes fractionation of peptide chain initiation factors using a typical DEAE-cellulose chromatographic procedure (1-3) and the abilities of different column fractions to catalyze various peptide chain initiation reactions including Met-tRNA$_f^{Met}$ binding to 40S ribosomes as assayed using Millipore filtration. The upper graph (A) describes the abilities of these factors to catalyze poly r(U-G) directed methionine transfer activity and to bind Met-tRNA$_f^{Met}$ in the absence and presence of 3 mM $Mg^{++}$. As reported previously (1-3), three peaks of methionine transfer activities (IF1, IF2 and IF3) were observed when assayed with poly r(U-G) messenger. The IF1 fractions also bind Met-tRNA$_f^{Met}$ in the presence of GTP. IF1 exists in at least two forms, IF1A and IF1B; Met-tRNA$_f^{Met}$:IF1A:GTP complex is stable to $Mg^{++}$ and Met-tRNA$_f^{Met}$:IF1B:GTP complex is extremely unstable in the presence of 3 mM $Mg^{++}$. The IF1B activity spreads well into IF2 activity region and some IF1B activity can also be detected under the IF3 peak. The lower graph (B) describes the activities of these fractions to catalyze Met-tRNA$_f^{Met}$ binding to 40S ribosomes as assayed using the Millipore filtration technique. At least two peaks of activities were observed. The first peak eluted between IF1 and IF2 region and the second peak was almost coincidental with the IF2 activity peak. The IF2 peak fractions showed no increase in binding upon addition of only 40S ribosomes. The binding was strongly stimulated when both 40S ribosomes and AUG codon were added. This column profile pattern has been repeated at least ten times with similar results.

Table III describes the activities of different DEAE-cellulose peak fractions added singly and in combinations to catalyze Met-tRNA$_f^{Met}$ binding to 40S ribosomes. IF1 (Fraction 19) does not give any significant stimulation of Met-tRNA$_f^{Met}$ binding to 40S ribosomes even in the presence of AUG codon. In other experiments when completely $Mg^{++}$ resistant Met-tRNA$_f^{Met}$:IF1A:GTP complexes were used, no Met-tRNA$_f^{Met}$ binding to 40S ribosomes was observed. Apparently, another factor or factors besides IF1A are necessary for Met-tRNA$_f^{Met}$ binding to ribosomes.

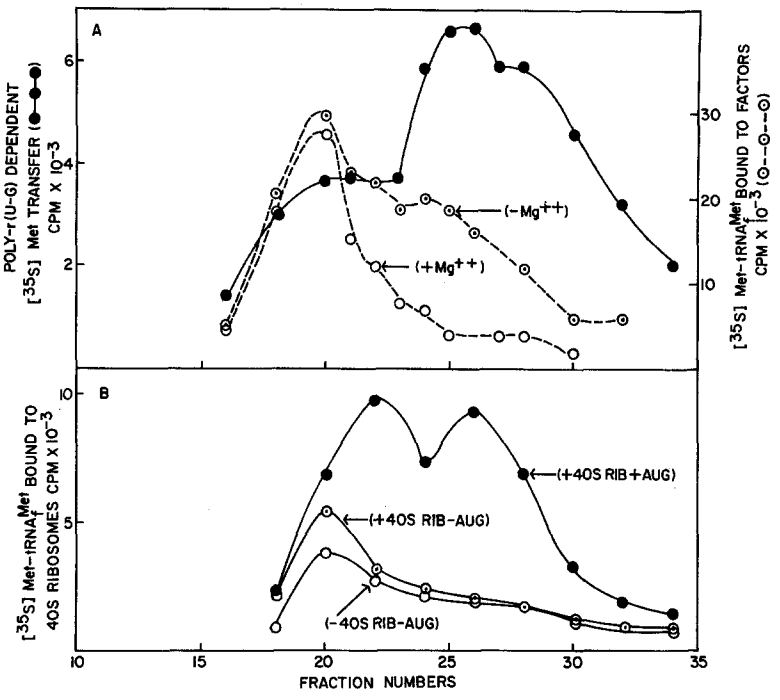


Fig. 1. DEAE-cellulose chromatography of initiation factor(s) (Fraction III). The preparation of Fraction III and DEAE-cellulose chromatography have been described previously (1). Fig. A describes the activities of the fractions for catalysis of poly r(U-G) directed methionine transfer reaction and for binding of Met-$tRNA_f^{Met}$ in the absence and presence of 3 mM $Mg^{++}$. The assay methods were the same as described previously. Fig. B describes the activities of the fractions in catalyzing Met-$tRNA_f^{Met}$ binding to 40S ribosomes in the presence of AUG codon. Standard Millipore filtration assay conditions as described in Table I were used. A 0.005 ml aliquot of the fractions was used in the assay. Where indicated 40S ribosomes and AUG codon were omitted.

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The IF2 Fractions by themselves gave significant stimulation of Met-$tRNA_f^{Met}$ binding and this binding was strongly AUG dependent. It should be noticed (see Fig. 1) that these fractions contain significant amounts of IF1B activity and are also presumably contaminated with IF3 activity.

Addition of IF1 and IF3 in the presence of suboptimal concentrations of IF2 gave significant stimulation of Met-$tRNA_f^{Met}$ binding upon addition of AUG codon thus indicating that these factors also participate in the binding reaction.

## Discussion

The Millipore filtration method described in this paper offers a rapid

TABLE III

EFFECTS OF ADDITIONS OF DIFFERENT RIBOSOMAL SALT WASH FRACTIONS ON [$^{35}$S]Met-tRNA$_f^{Met}$ BINDING TO 40S RIBOSOMES

| Factors Added | Amounts Added (µl) | Radioactivity Bound to Millipore Filters (cpm) | | |
|---|---|---|---|---|
| | | -AUG -ribosomes | -AUG +40S ribosomes | +AUG +40S ribosomes |
| IF1 | 5 | 3,270 | 2,870 | 2,990 |
| (Fraction 19) | 10 | 5,600 | 5,370 | 6,610 |
| IF2 | 5 | 2,000 | 1,920 | 5,940 |
| (Fraction 26) | 10 | 3,910 | 5,210 | 16,060 |
| IF3 | 5 | 760 | 720 | 1,730 |
| (Fraction 31) | 10 | 1,710 | 1,810 | 4,160 |
| IF(1 + 2) | 5+5 | 8,050 | 4,700 | 13,660 |
| IF(1 + 3) | 5+5 | 5,680 | 2,950 | 8,410 |
| IF(2 + 3) | 5+5 | 3,110 | 3,800 | 9,260 |
| IF(1 + 2 + 3) | 5+5+5 | 6,600 | 5,750 | 17,670 |

Standard Millipore filtration assay conditions as described in Table I were used. Protein concentrations in different fractions were: IF1 (Fraction 19), 1.5 mg per ml; IF2 (Fraction 26), 2 mg per ml; IF3 (Fraction 31), 1.8 mg per ml.

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and convenient assay for codon directed Met-tRNA$_f^{Met}$ binding to 40S ribosomes. This assay method is clearly more advantageous than the density gradient centrifugation procedures presently being used for such studies. Density gradient centrifugation is time consuming and consequently the recovery of the initiation complexes is low. In the present Millipore filtration assay 1-3 pmole quantities of Met-tRNA$_f^{Met}$:40S ribosomes:AUG complexes can be conveniently analyzed in a short period of time.

Under the assay conditions, Met-tRNA$_f^{Met}$ binding to 40S ribosomes is entirely dependent on the presence of AUG codon. These results differ from recent reports of other laboratories (6,11-13) that the binding of Met-tRNA$_f^{Met}$ to a 40S ribosomal subunit is the first step in initiation of eukaryotic protein synthesis and this binding is m-RNA independent. However, it should be noted that those studies were done using density gradient centrifugation and in some cases the ribosomes and factor preparations were not pure. As noted

earlier, the recovery of different initiation complexes during density gradient centrifugation is low and may vary with different complexes. Also, less purified preparations such as 40S ribosomes in crude lysate or less purified initiation factor(s) may contain fragments of endogenous messenger RNAs with initiation sites, therefore, results from these studies cannot be conclusive. Our present experimental results using the Millipore filtration assay method clearly demonstrate that the initiator tRNA binding to 40S ribosomes is strongly dependent on the presence of AUG codon and in this respect the basic mechanism of initiation complex formation in both pro- and eukaryotic organisms is the same.

Hopefully, this Millipore filtration assay method will provide a convenient tool for analysis of the roles of different factors that are involved in peptide chain initiation in animal cells. Recent reports from our laboratory and elsewhere indicate that the mammalian peptide chain initiation factors can exist in different molecular forms and also in complex organization with different proteins. For example, the Met-$tRNA_f^{Met}$ binding factor, IF1, can exist in at least two forms (IF1A and IF1B) differing in molecular weights and sensitivity of their respective Met-$tRNA_f^{Met}$:IF1:GTP complexes towards $Mg^{++}$. Under the Millipore filtration assay conditions, IF1A fractions which formed the $Mg^{++}$ insensitive Met-$tRNA_f^{Met}$:IF1A:GTP complex did not stimulate Met-$tRNA_f^{Met}$ binding in the presence and absence of AUG codon. Apparently, another protein factor or factors are required for the binding of this complex to 40S ribosomes. Again, the IF2 fractions by themselves catalyzed very efficiently AUG directed Met-$tRNA_f^{Met}$ binding to 40S ribosomes. These fractions also catalyzed very efficiently poly r(U-G) and poly r(A-U-G) directed methionine transfer reactions (3). Apparently, the IF2 fractions contain all the components necessary for protein synthesis initiation including IF1B (see Fig. 1). In the presence of suboptimal concentrations of IF2, IF1 and IF3 stimulated Met-$tRNA_f^{Met}$ binding to 40S ribosomes, indicating that these two factors are necessary for the binding reaction. Precise roles of these factors in the binding reaction remain to be elucidated.

The crude ribosomal salt wash preparation was inactive in the binding reaction and also inhibited the binding reaction catalyzed by DEAE-cellulose purified factors. The nature of this inhibition is not clear at present. Presence of several inhibitors of reticulocyte protein synthesis have been reported. Some of these inhibitors specifically inhibit peptide chain initiation either by deacylating Met-$tRNA_f^{Met}$ (14-15) or by inhibiting the binding of Met-$tRNA_f^{Met}$ to 40S ribosomal subunit (16).

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