# DISTRIBUTION OF INITIATION FACTORS IN CELL FRACTIONS FROM MAMMALIAN TISSUES.

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Received October 25, 1971

## SUMMARY

Cytosol from ascites cells catalyzed the binding of Phe-tRNA to 40S ribosomal subunits, the synthesis of polyphenylalanine at low concentrations of magnesium, and the translation of encephalomyocarditis virus (EMC) RNA by ribosomes reconstituted from subunits. Thus, ascites cytosol contained the mammalian initiation factors M1, M2, and M3; M2 could be removed by precipitation at pH 5.2. Cytosol from rat liver or from muscle on the other hand contained only M1; the fraction extracted from liver or muscle microsomes with 1 M KCl (salt-wash-fraction) contained M2 as well as M1. There was no translation of EMC RNA even when cytosol from rat muscle or from liver was supplemented with the homologous salt-wash-fraction; thus neither fraction has active M3 for the translation of EMC RNA.

## INTRODUCTION

Three factors are required for the initiation of protein synthesis in eukaryotes (1). Two of the factors, M1 and M2, catalyze the binding of met-$tRNA_f$ to ribosomes (2); M3 is needed to translate natural mRNA (1). Although initiation factors are generally prepared from ribosomes (1, 3, 4), cytosol from ascites cells will support translation of encephalomyocarditis virus (EMC) RNA

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even without supplementation with factors extracted from ribosomes (5). It would be valuable if EMC RNA could be used to study initiation and synthesis of viral specific proteins in cell-free systems from mammalian tissues where the endogenous mRNA's are heterogeneous and uncharacterized. However, we found that cytosol from rat skeletal muscle (or liver) was unable to support the translation of EMC RNA by either muscle or ascites ribosomes, although the same ribosomes could translate EMC RNA if ascites cytosol was used. The inability of cytosol from mouse brain or rabbit reticulocytes to support translation of EMC RNA had been reported before (5).

One interpretation of these results is that there is a specific M3 for translation of EMC RNA that is lacking from most tissues. Before considering that possibility it should be determined if cytosol from the 'unresponsive' tissues contain the other initiation factors (i.e. M1 and M2). We decided then to determine the distribution of initiation factors in cell fractions from several mammalian tissues.

## MATERIALS AND METHODS

The following have been described before: the growth of ascites cells--obtained from Dr. A. T. Burness of The Sloan-Kettering Research Laboratory--in HA/ICR Swiss mice (males 20-25 g), and of the K2 strain of EMC virus (6); the isolation of viral RNA (6); the preparation of rat liver and skeletal muscle ribosomes (7), of ascites cell ribosomes (6), of ribosomal subunits (8), and of tissue cytosol--100,000 g supernatant (7); the assay of TI and TII (9), and of M1 (40S binding factor) (10). Liver ribosomes were used in all the experiments in which polyphenylalanine (polyphe) synthesis was assayed; the ribosomes were treated with deoxycholate and 1 M KCl (11) to remove translation and initiation factors. The [$^3$H] phenylalanine (5 Ci/mmole) was purchased from New England Nuclear Corporation; the rats (males, 100-120 g) from Sprague-Dawley.

Preparation of salt-wash-fractions. Microsomes

from 130 g of skeletal muscle or from 60 g of rat liver were suspended in 5 or 10 ml respectively of a buffer containing Tris-HCl pH 7.5 (10 mM), $MgCl_2$ (5 mM), β-mercaptoethanol (10 mM) and KCl (1 M). The suspension was maintained at $0^{\circ}$ for 1 hr with occasional stirring and then the ribosomes sedimented at 65,000 rpm in the Spinco SW65 rotor for 2 hr at $0^{\circ}$. The upper portion of the supernatant was carefully removed and dialyzed against buffer--Tris-HCl, pH 7.5 (10 mM), β-mercaptoethanol (1 mM) and KCl (100 mM). The material that was extracted is referred to as the "salt-wash-fraction"; it was stored in liquid nitrogen.

## RESULTS AND DISCUSSION

Cytosol from rat skeletal muscle was unable to support translation of EMC RNA by either muscle or ascites

Table I. Translation of EMC RNA.

| Ribosomes | Cytosol | Phe Incorporated (pmole) |
|---|---|---|
| Ascites | Muscle | 0.07 |
| Muscle | Muscle | 0.03 |
| Ascites | Ascites | 10.6 |
| Muscle | Ascites | 6.4 |
| Ascites† | Ascites | 9.2 |
| Muscle† | Ascites | 5.6 |

Ribosomes (25 μg of ribosomal RNA) were incubated for 45 min at $37^{\circ}$ in 50 μl containing Tris-HCl pH 7.5 (1 μmole), KCl (6.25 μmole), magnesium acetate (0.25 μmole), β-mercaptoethanol (0.3 μmole), ATP (0.05 μmole), GTP (0.005 μmole), creatine phosphate (0.25 μmole), creatine kinase (10 μg), 19 unlabelled amino acids (2.5 mμmole of each), [$^3$H] phenylalanine (0.25 mμmole), EMC-viral RNA (5 μg) and cytosol (250 μg). The ribosomes were preincubated (6) to decrease endogenous synthesis of protein. The reaction mixture was treated as described by Mathews and Korner (6) and radioactivity was determined by liquid scintillation spectrometry with 16% efficiency. Thus 1000 cpm was equivalent to 0.57 pmole of Phe incorporated.

† The ribosomes (25 μg of ribosomal RNA) were reconstituted from subunits (8).

ribosomes, yet ascites cytosol prepared in the same way was quite active (Table I). Translation by muscle ribosomes (even by muscle ribosomes reconstituted from subunits, hence unlikely to have any initiation factors--reference 9) did occur in the presence of ascites cytosol, albeit translation was less efficient than with ascites ribosomes (Table I).

We wanted to know the reason for the failure of muscle cytosol to support translation of EMC RNA, so we tested first whether it had the initiation factors M1 and M2. Shafritz and Anderson (12) showed that in the presence of M1 and M2 polyphe synthesis is maximum at low concentrations of magnesium (the transfer factors TI and TII must be present as well), whereas in their absence synthesis is only significant at high magnesium concentrations. We, therefore, examined the magnesium dependency of polyphe synthesis by liver ribosomes that had been treated with deoxycholate and 1 M KCl (11) and, therefore, were likely to be free of initiation factors. In the presence of ascites cytosol,

Table II. Binding of Phe-tRNA to 40S Ribosomal Subunits.

| Addition | Amount (μg) | Phe Bound (pmole) |
|---|---|---|
| None | — | 0.13 |
| Liver Cytosol | 250 | 0.85 |
| Liver Cytosol (pH 5.2-treated) | 250 | 1.14 |
| Ascites Cytosol | 250 | 0.64 |
| Ascites Cytosol (pH 5.2-treated) | 250 | 1.54 |
| Muscle Cytosol | 150 | 1.11 |
| Liver Salt-Wash-Fraction | 600 | 1.07 |
| Redissolved pH 5.2 Precipitate | 200 | 0.20 |

Liver 40S ribosomal subunits (2.14 μg of ribosomal RNA) were incubated for 30 min at 37° with various preparations of factors and the binding of [$^3$H] Phe-tRNA (40 μg - 32,000 cpm) was determined (10). The reaction mixture (0.1 ml) also contained (in μmole): Tris-HCl (pH 7.5), 2.5; KCl, 8; GTP, 0.05; β-mercaptoethanol, 1; $MgCl_2$, 0.5; and 10 μg poly U. The counting efficiency was 16%. Thus 1000 cpm were equivalent to 0.57 pmole of Phe-tRNA bound.

synthesis was maximum when the magnesium concentration was 6 mM, whereas with muscle cytosol maximum synthesis occurred when the concentration was about 12.5 mM (Fig. 1). (We can not account for the decreased effectiveness of muscle cytosol even at high magnesium concentrations.) The difference in magnesium optima for ascites and muscle cytosol was seen even though the amount of both was saturating. The magnesium optimum (12.5 mM) with liver cytosol was the same as with muscle cytosol (results not shown).

The results indicated that muscle and liver cytosol lacked M1 or M2 or both. We knew (10 and Table II), however, that liver and muscle cytosol contained a factor (almost certainly M1--unpublished results) which catalyzed the binding of Phe-tRNA to liver 40S ribosomal subunits, thus it must be M2 that was deficient.

We tested then the initiation factor activity of fractions extracted from liver and muscle microsomes with 1 M KCl---salt-wash-fraction (Table III). We assayed for M2 alone by its ability to complement

Table III. Effect of Enzyme Fractions on Polyphenylalanine Synthesis.

| Addition | Phe Incorporated (pmole) | |
|---|---|---|
| | 5 mM $MgCl_2$ | 10 mM $MgCl_2$ |
| Liver Fractions | | |
| None | 0.03 | 0.03 |
| Salt-wash-fraction (300 μg) | 4.50 | 4.52 |
| Cytosol (pH 5.2-treated) (325 μg) | 2.36 | 6.12 |
| Cytosol + Salt-wash-fraction | 10.5 | 6.20 |
| Ascites Fractions | | |
| Redissolved pH 5.2 precipitate (80 μg) | 0.62 | 1.20 |
| Cytosol (pH 5.2-treated) (300 μg) | 1.11 | 11.40 |
| Cytosol + Redissolved pH 5.2 precipitate | 4.64 | 8.16 |

Liver ribosomes (3.75 μg of ribosomal RNA) were incubated with the enzyme fractions indicated in 5 mM or 10 mM $MgCl_2$ and the synthesis of polyphenylalanine assayed as in Fig. 1.

pH 5.2-treated ascites cytosol; M2 is precipitated from ascites cytosol at pH 5.2--see below. The salt-wash-fraction from liver microsomes caused a marked stimulation of polyphe synthesis when used to supplement pH 5.2-treated ascites cytosol; as was to be expected the stimulation was observed when the concentration of magnesium was 5 mM but not when it was 10 mM (Table III). Thus M2--the initiation factor absent from rat liver cytosol--can be extracted from microsomes. The salt-wash-fraction also had considerable M1, i.e. 40S binding activity (Table II), and appreciable aminoacyltransferase activity (TI and TII) for it supported polyphe synthesis by itself (Table III). Similar results were obtained with a salt-wash-fraction from muscle ribosomes (results not shown).

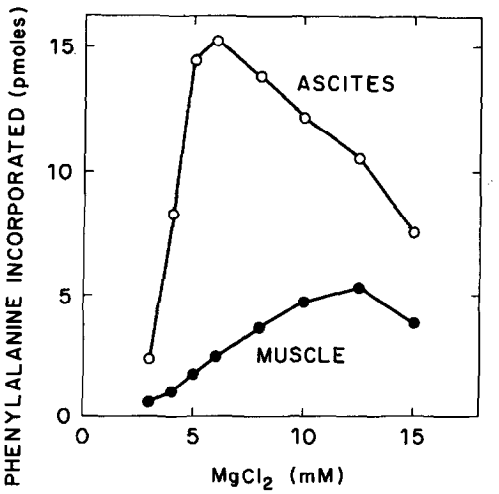


Figure 1. Effect of magnesium concentration on polyphenylalanine synthesis with ascites and muscle cytosol. Liver ribosomes (3.75 μg of ribosomal RNA) that had been treated with deoxycholate and 1 M KCl (11) were incubated for 30 min at 37° in 0.1 ml containing ATP (0.1 μmole), GTP (0.01 μmole), creatine phosphate (0.5 μmole), creatine phosphokinase (20 μg), [$^3$H] Phe-tRNA (80 μg of E. coli B tRNA aminoacylated with 19 non-radioactive amino acids and 20,000 cts/min [$^3$H] Phe), poly U (20 μg), Tris-HCl pH 7.5 (2 μmole), KCl (12.5 μmole), β-mercaptoethanol (0.6 μmole), ascites or muscle cytosol (375 μg of protein), and various amounts of $MgCl_2$. The polyphe was isolated (14) and the radioactivity determined by scintillation spectrometry with an efficiency of 5%. Thus 1000 cpm was equivalent to 1.8 pmole of Phe incorporated.

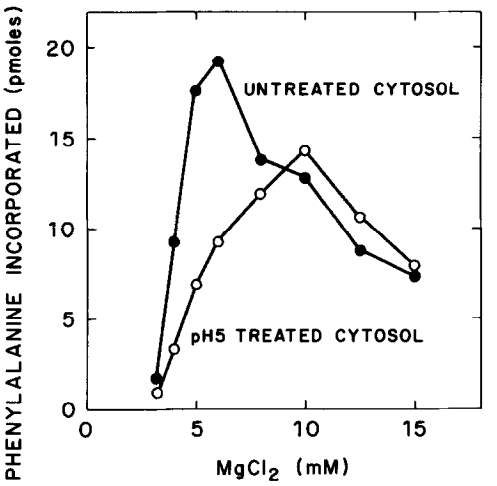


Figure 2. Effect of magnesium concentration on polyphenylalanine synthesis with pH 5.2-treated ascites cytosol. Liver ribosomes (3.75 μg of ribosomal RNA) were incubated with pH 5.2-treated or untreated ascites cytosol (375 μg of protein) and the synthesis of polyphe assayed as in Figure 1.

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Liver ribosomes utilized ascites factors (Figs. 1 and 2). The corollary was also true: there was appreciable synthesis of polyphe by ascites ribosomes at low concentrations of magnesium in the presence of liver cytosol and liver salt-wash-fraction (results not shown). The results confirm (5) that the aminoacyltransferases and M1 and M2 are not species specific.

When the supernatant obtained after treatment of ascites cytosol at pH 5.2 was used, the synthesis of polyphe at low concentrations (4 to 6 mM) of magnesium was reduced, but synthesis at high concentrations was not affected (Fig. 2). Thus we confirm (15) that pH 5.2 precipitation removes (or inactivates) one or more of the initiation factors. Precipitation did not reduce the M1 in cytosol (Table II)—in fact it increased its specific activity--hence it must have been M2 that was lost.

The addition of redissolved pH 5.2 precipitate to the supernatant from ascites cytosol partially restored polyphe synthesis at low magnesium (Table II). Restoration must have been due to M2 as the redissolved precipitate had negligible 40S binding activity (M1)--Table II. The redissolved precipitate

did not support polyphe synthesis at high concentrations of magnesium (Table III), hence at least one (and probably both) transfer factors were lacking. pH 5.2 precipitation may be useful in isolating and purifying M2.

Having established that liver and muscle cytosol lacked M2, but that the factor could be extracted from microsomes, we were in a position to test whether a combination of cytosol and salt-wash-fractions would support translation of EMC RNA. What we found was that neither the combination from liver nor that from muscle were effective (Table IV). It is conceivable that a specific transfer RNA or some unrecognized accessory factor is required. However, it seems more likely that active M3 is lacking from liver (and muscle) cytosol and salt-wash-fractions or that the M3 necessary for translation of EMC RNA differs from that required for translation of endogenous template. Unfortunately it is not yet possible to decide between the alternatives.

Table IV. Translation of EMC RNA.

| Addition | | Phe Incorporated (pmole) |
|---|---|---|
| Cytosol | Salt-wash-fraction | |
| None | None | 0.38 |
| Ascites (250 μg) | None | 23.3 |
| Liver (120 μg) | None | 0.36 |
| Liver (120 μg) | Liver (60 μg) | 0.47 |
| Liver (120 μg) | Liver (120 μg) | 0.50 |
| Liver (120 μg) | Liver (240 μg) | 0.70 |
| Muscle (100 μg) | None | 0.54 |
| Muscle (100 μg) | Muscle (25 μg) | 0.49 |
| Muscle (100 μg) | Muscle (50 μg) | 0.52 |
| Muscle (100 μg) | Muscle (100 μg) | 0.42 |

Ascites cell ribosomes (25 μg of ribosomal RNA) were incubated as described in Table I.

M3, which is required for binding natural mRNA's to the ribosome, obviously is best assayed with an endogenous template. No rat liver or muscle mRNA has yet been isolated.

The results indicate that ascites cytosol contains the three initiation factors. The presence of M1 was demonstrated directly by the ability of ascites cytosol to catalyze binding of Phe-tRNA to 40S ribosomal subunits (Table II). M2 could be selectively precipitated from cytosol at pH 5.2 (Fig. 2 and Table III), hence must originally have been present. M3 is judged to be contained in ascites cytosol for it supports translation of EMC RNA by ribosomes reconstituted from subunits (Table I) which are not likely to contain initiation factors because of the method of their preparation. Moreover, translation of EMC RNA does not occur with rat muscle or liver fractions containing M1 and M2, and TI and TII (Table IV), hence M3 could not have been on the ribosome and perforce must have been contained in ascites cytosol.

This work increases the number of mammalian tissues in which individual initiation factors have been demonstrated, and describes how the distribution of those factors in cell fractions varies. (Of course, the exact location of the factors in situ is not known.) Information of the distribution of initiation and transfer factors in cell fractions is of practical importance in the study of the reaction mechanisms for protein synthesis.

ACKNOWLEDGEMENTS

We are grateful to Dr. M. Mathews for instruction in the preparation of EMC RNA and for assisting in carrying out some of the experiments. The expenses of the research were met by grants from the John A. Hartford Foundation and the National Institutes of Health (AM-04842). DPL is the recipient of a Fulbright-Hays Travel Grant. HKB is a Medical Scientist Trainee supported by an NIGMS grant (GM01939).

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