SEPARATION OF TWO FACTORS, M_1 AND M_2, REQUIRED FOR POLY U DEPENDENT POLYPEPTIDE SYNTHESIS BY RABBIT RETICULOCYTE RIBOSOMES AT LOW MAGNESIUM ION CONCENTRATION.

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SUMMARY

Two factors have been isolated from a 0.5 M KCl ribosomal wash fraction of rabbit reticulocytes which are required for poly U directed polypeptide synthesis at 5mM Mg^{++}. These factors, M_1 and M_2, are required in addition to saturating levels of the reticulocyte soluble enzyme transfer factors present in crude ribosomal 105,000 x g supernatant. A rapid assay for this low Mg^{++} polypeptide synthesis, using ^{14}C-phe-tRNA, has been developed.

Recently, Miller and Schweet (1) reported a shift in the Mg^{++} optimum for poly U directed polyphenylalanine synthesis to a lower concentration ("Mg^{++} shift") in the rabbit reticulocyte cell-free system when a 0.5M KCl ribosomal wash fraction was used in conjunction with KCl-washed reticulocyte ribosomes. This fraction, not required for polyphenylalanine synthesis at higher Mg^{++} concentration, was also necessary for the synthesis of complete hemoglobin molecules using endogenous hemoglobin-mRNA, since in its absence only nascent globin polypeptide chains were completed. The active component in the ribosomal high salt wash fraction was shown to be protein in nature by heat inactivation studies, pronase and N-ethyl maleimide sensitivity, and T_1 ribonuclease resistance, but attempts at further characterization were unsuccessful.

The present communication reports the isolation of two separate factors, M_1 and M_2 (named for their mammalian origin), from the 0.5M KCl wash fraction of rabbit reticulocyte ribosomes, and describes a rapid assay for identifying these factors.

METHODS

Preparation of components: Immature New Zealand white rabbits 1 1/2 to 2 kg were utilized for all studies. Induction of reticulocytosis by phenyl-

hydrazine injection and the preparation of lysate, 0.5M KCl washed ribosomes, a crude 105,000 x g ribosomal supernatant fraction, and a 0.5M KCl ribosomal wash fraction were performed according to procedures reported by Miller and Schweet (1).

Polymerization Assays: Polymerization of ^{14}C-phe was performed by the procedure described by Miller and Schweet (1), as modified from the original method of Allen and Schweet (2) for the reticulocyte cell-free system. Reactions were stopped by the addition of ice cold 10% trichloroacetic acid (TCA), and radioactivity of hot TCA insoluble material was determined as previously described (3). Polymerization using ^{14}C-phe-tRNA, as originally reported in the rabbit reticulocyte system by Arlinghaus, Favelukes, and Schweet (4), was determined as described in the legend of Table I.

RESULTS

"Mg^{++} shift" Assay: The poly U dependent incorporation of ^{14}C-phenylalanine into hot TCA insoluble polypeptide at 37°C in the presence of 0.5M KCl washed rabbit reticulocyte ribosomes, deacylated tRNA, crude reticulocyte supernatant, and an ATP generating system was found to be maximal at 15 mM Mg^{++}. When a 0.5M KCl ribosomal wash fraction was added, there was a shift in the Mg^{++} concentration for optimal polypeptide synthesis to 6mM and an increase in the rate of polymerization. In order to bypass the possible influence of Mg^{++} concentration on phenylalanine-tRNA synthetase, an assay was developed using previously acylated ^{14}C-phe-tRNA as substrate. As can be seen in Figure 1, the optimal Mg^{++} concentration in the absence of ribosomal wash was 10mM, while in the presence of ribosomal wash the optimum was 5-6mM and the activity of the system was increased. Under the conditions used, supernatant and ribosomal wash fractions were present in saturating amounts, but not in excess where inhibition may occur; additional ^{14}C-phe-tRNA did not significantly increase activity. The reactions were linear with time to 3 minutes and linear with increasing ribosome concentration to 0.3 A^{260} units per incubation, so that all values given represent rate points. The requirements for the polymerization assay, using ^{14}C-phe-

TABLE I

Requirements for polymerization of ^{14}C-phe-tRNA by 0.5M KCl washed rabbit reticulocyte ribosomes in the presence and absence of a 0.5M KCl ribosomal wash fraction.

Deletions from reaction mixture	A. Plus ribosomal wash (5mM Mg^{++})[a]	B. Minus ribosomal wash (10mM Mg^{++})[a]
Complete system	3.20	2.15
Minus ribosomes	0.15	0
Minus Mg^{++}	0.02	0
Minus poly U	0.02	0.02
Minus supernatant	1.35	0.01
Minus ribosomal wash	0.52	----
Plus ribosomal wash	----	1.42

[a] Values expressed as μμmoles ^{14}C-phe-tRNA polymerized. Incubations, in a total volume of 50 μl, were performed at 37°C for 2 minutes and contained 15mM Tris-Cl, pH 7.5, 100mM KCl, 1mM GTP (neutralized to pH 7.0 with KOH), 5mM PEP (neutralized to pH 7.0 with KOH), 0.3 international units of pyruvate kinase, 30mM β-mercaptoethanol, 0.5 A^{260} units poly U, 0.2 A^{260} units 0.5M KCl washed reticulocyte ribosomes, 6 μμmoles E. coli ^{14}C-phe-tRNA (750 cpm/μμmole), 0.57 mg ribosomal supernatant protein, 40 μg ribosomal wash protein, and $MgCl_2$ as indicated. Reactions were linear for 3 minutes with increasing ribosome concentration up to 0.3 A^{260} units. Supernatant and ribosomal wash fractions were used at saturating levels. Radioactivity in hot 10% TCA insoluble material was determined as noted in Methods.

tRNA as substrate, are given in Table I. At 5mM Mg^{++}, there was a six fold stimulation in the rate of polypeptide synthesis upon the addition of the ribosomal wash fraction, whereas at 10mM Mg^{++}, there was a decrease in rate.

Separation of 0.5M ribosomal wash factors: The 0.5M KCl ribosomal wash was separated into two factors (M_1 and M_2) by DEAE cellulose column chromatography as shown in Figure 2 (see legend for details of separation). Figure 2A gives the activity of each fraction assayed in the presence of fraction V (M_2), using the activity of fraction V alone (1.40 μμmoles) as the blank. Figure 2B gives the activity of each fraction assayed in the presence of fraction I (M_1), using the activity of fraction I alone (0.53 μμmoles) as the blank. In sepa-

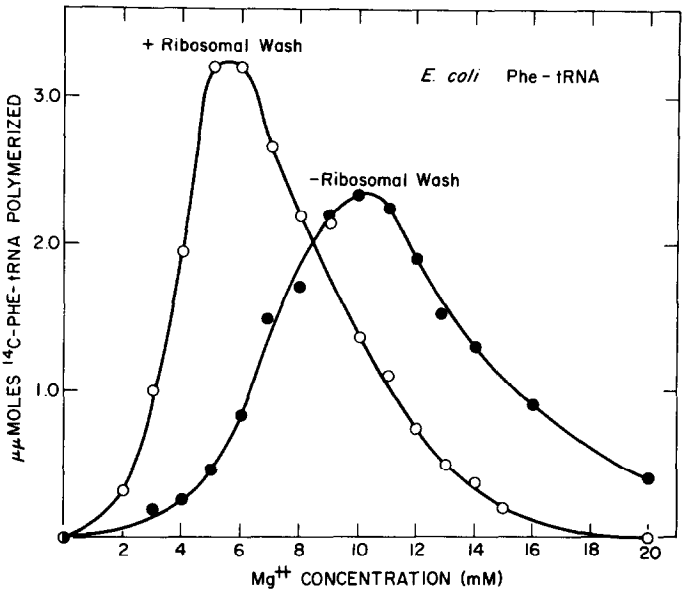

Figure 1.
The effect of the 0.5M KCl ribosomal wash fraction on the Mg^{++} concentration optimum for poly U directed polypeptide synthesis. Assay conditions are listed in the legend to Table 1. Incubations were at 37°C for 2 minutes and contained 6 μμmoles E. coli ^{14}C-phe-tRNA, 0.2 A^{260} units of 0.5M KCl washed ribosomes, and 0.57 mg supernatant protein. 40 μg ribosomal wash protein were added where indicated. A similar "Mg^{++} shift" was obtained using rabbit reticulocyte ^{14}C-Phe-tRNA instead of E. coli ^{14}C-Phe-tRNA.

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rate experiments listed in Table II it can be seen that the activity obtained by addition of ribosomal wash to supernatant can be reproduced by replacement of the ribosomal wash with the separate factors M_1 and M_2. That these two factors are distinct from supernatant factors is shown by the fact that full activity at 5mM Mg^{++} cannot be obtained unless all components are present: supernatant plus ribosomal wash (Tables I and II) or supernatant plus M_1 and M_2 (Table II).

DISCUSSION

A specific requirement for low Mg^{++} concentration in the synthesis of polypeptides with fidelity in bacterial cell-free systems is well known (5-9). This "Mg^{++} shift" can be produced in the E. coli system using the synthetic messenger poly U, when N-acetyl-phe-tRNA in addition to phe-tRNA is used in

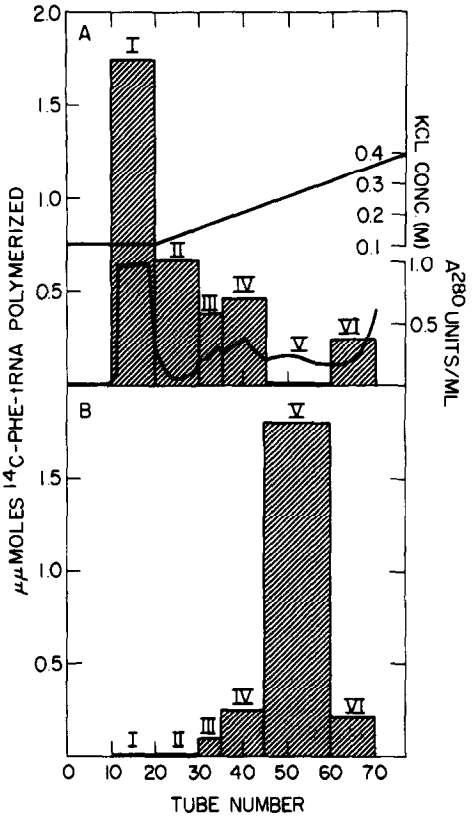

Figure 2

The separation of ribosomal wash factors by DEAE cellulose chromatography. Twenty ml of ribosomal wash (4.0 mg/ml) were diluted to 0.1M KCl with buffer solution (10mM Tris-Cl, pH 7.5, 1mM dithiothreitol, and 0.1mM EDTA) and placed over a 1.6 x 25 cm column of recycled, defined, and equilibrated microgranular DEAE cellulose. After the hemoglobin had passed through the column (hemoglobin is not retained under these conditions), a linear gradient was started from 0.1M to 0.4M KCl. Five ml fractions were collected. The tubes were pooled into six fractions (the first fraction containing the entire hemoglobin band) and the fractions concentrated by ultrafiltration. Absorbance at 280 mμ was monitored continuously with an Isco flow cell system, and represented predominantly protein; at 0.35M KCl nucleic acid material began to elute. Initially, all fractions were tested separately and in various combinations for their ability to stimulate polypeptide synthesis at 5mM Mg^{++}. This basic chromatographic procedure has been performed three times with similar results. A. The various fractions were tested in combination with fraction V (M_2), the latter always present in saturating amounts. Incubations and assay procedures were similar to those given in Table I, in this instance using 12.5 μg M_2 protein. A blank of 1.40 μμmoles, representing activity for supernatant plus M_2 alone, was subtracted from each sample. B. Each fraction was tested for polymerization at 5mM Mg^{++} in the presence of saturating levels of fractions I (M_1). Incubations were as noted in A, except that 62.5 μg of M_1 protein were used in place of M_2. A blank of 0.53 μμmoles, representing activity for supernatant plus M_1 alone, was subtracted from each sample.

TABLE II

Dependence on factors M_1 and M_2 for polymerization of ^{14}C-phe-tRNA by 0.5M KCl washed rabbit reticulocyte ribosomes

Additions to washed ribosomes	μμmoles ^{14}C-phe-tRNA polymerized
Supernatant and ribosomal wash	2.96
Supernatant	0.74
Ribosomal wash	1.40
Supernatant + M_1	0.42
Supernatant + M_2	1.45
Supernatant + M_1 + M_2	3.21
M_1 + M_2	0.01

Incubations, in a total volume of 50 μl, were performed at 37°C for 2 minutes and contained 0.2 A^{260} units of washed ribosomes, 6 μμmoles *E. coli* ^{14}C-phe-tRNA, 5mM Mg^{++} and other components as noted in the legend to Table I. Supernatant, 0.57mg protein; ribosomal wash, 40 μg protein; M_1, 62.5 μg protein; and M_2, 12.5 μg protein were added as indicated. Values obtained in the absence of ribosomes, approximately 0.12 μμmoles in each instance, have been subtracted.

conjunction with NH_4Cl washed ribosomes and protein factors F_1 and F_2 (10). Hardesty and his co-workers (11-13) have reported a "Mg^{++} shift" in poly U-directed protein synthesis in the rabbit reticulocyte system dependent upon the presence of deacylated $tRNA^{Phe}$. Miller and Schweet (1) reported a requirement for a 0.5M KCl ribosomal wash fraction when using rabbit reticulocyte salt-washed ribosomes in de novo hemoglobin polypeptide synthesis (which occurs only in the presence of low Mg^{++} concentration) and this same fraction was also required for a "Mg^{++} shift" in poly U-directed polyphenylalanine synthesis. We have confirmed the former observation of Miller and Schweet in a previous study (14), and the latter in the present study using their method of assay. It has further been demonstrated, using the rapid assay described in Methods, that the "Mg^{++} shift" is not due to a Mg^{++} concentration effect on phenylalanyl-tRNA synthetase. Furthermore, since the ribosomal wash dependent activity decreases when

the Mg^{++} concentration is raised from 6 to 10 mM (in the presence of saturating levels of supernatant) while the activity due to supernatant alone increases, it would appear that the effect of the ribosomal wash could not be attributed to the transfer factors already present in the supernatant. The isolation of two distinct factors from the ribosomal wash, M_1 and M_2, further supports this conclusion, although the possibility that these factors are subunits of the transfer factors cannot be excluded. In studies to be reported elsewhere, neither M_1 nor M_2 alone (in conjunction with supernatant) can cause the "Mg^{++} shift" for phe-tRNA polymerization; M_1 and M_2 have been shown to be distinct from partially purified transferase I (T_1) and transferase II (T_2); and N-Acetyl-phe-tRNA produces an additional small downward shift in the Mg^{++} optimum in the presence of supernatant, M_1, and M_2. The mechanism for the "Mg^{++} shift", the influence of deacylated $tRNA^{Phe}$, and the possible role of factors M_1 and M_2 in natural hemoglobin-mRNA translation in the rabbit reticulocyte system are currently under investigation.

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