TRANSLATION OF MESSENGER RNA FRACTIONS EXTRACTED FROM FREE AND MEMBRANE BOUND RAT FOREBRAIN RIBOSOMES IN A RABBIT RETICULOCYTE CELL-FREE SYSTEM*

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SUMMARY

Messenger RNA fractions were obtained from free and membrane bound rat forebrain ribosomes by alkaline phenol extractions. These RNA fractions stimulated protein synthesis in a cell-free rabbit reticulocyte system partially dependent on the addition of exogenous mRNA. The polypeptide products of protein synthesis with RNA fractions derived from free and membrane bound brain ribosomes and reticulocyte ribosomes were compared by polyacrylamide gel electrophoresis and found to have different distributions.

INTRODUCTION

The brain as well as other tissues contains free and membrane bound ribosomes (1-3). Studies in liver have suggested that ribosomes attached to membranes are responsible for synthesis of proteins for export (e.g. serum proteins) and free ribosomes synthesize proteins for intracellular use (e.g. ferritin)(4-6). Thus, there could be differences in mRNA populations associated with free and membrane bound ribosomes in the liver. In order to study this possibility in the brain, RNA was extracted from free and membrane bound ribosomes and added to a rabbit reticulocyte cell-free protein synthesis system dependent on exogenous mRNA as described by Crystal et al. (7). The products of protein synthesis were comapred by polyacrylamide gel electrophoresis.

Shafritz et al. (8) have recently reported the use of the mRNA dependent reticulocyte [cell-free] system for translation of rabbit liver mRNA fractions, and have identified ferritin as a specific protein product. These investiga-

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tors have also studied the translation of mRNA fractions derived from free and membrane bound liver polysomes (9). Zomzely-Neurath and coworkers (10) have reported the de novo synthesis of S-100 protein in a reconstituted cerebral cell-free system containing polysome-derived mRNA and 40S and 60S ribosomal subunits. The preparation of cerebral polysomes used in this study (10) was obtained by detergent treatment of a mixture of free and membrane bound polysomes. In the present study free and membrane bound brain ribosomes were first separated by discontinuous sucrose gradient centrifugation (3) prior to the use of detergents so that the characteristics of the respective RNA extracts in protein synthesis could be studied separately.

METHODS

Preparation of rabbit reticulocyte protein synthesis system: The reticulocyte cell-free protein synthesis system was prepared as described by Crystal et al. (7). In order to remove endogeneous mRNA, reticulocyte ribosomes were exposed to 0.05 µg per ml of pancreatic ribonuclease. Polymerization assays (100 µl, 30°C, 30 min.) included: 20 mM Tris-HCl, pH 7.5, 4.0 mM $MgCl_2$, 86 mM KCl, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 3 mM phosphoenolpyruvate, 0.1 i.u. of pyruvate kinase, 0.02 mM (^{14}C)-L-leucine, 0.04 mM (^{12}C)-amino acids minus leucine, 0.15 A_{260} unit of rabbit liver tRNA, 150 µg concentrated ribosomal wash fraction (12), 100 µg of supernatant fraction (7), 0.049 A_{260} unit of ribosomes and various amounts of mRNA fractions. The tRNA was obtained from General Biochemicals and further purified by Sephadex G-200 filtration as previously described (11). After incubation, protein was precipitated by the addition of 10% trichloroacetic acid and collected on nitrocellulose filters as previously described (11). The filters were dissolved in Bray's solution (13) and counted at an efficiency of 75% for ^{14}C in a liquid scintillation counter.

Preparation of ribosomes for RNA extraction: Reticulocyte lysate ribosomes were prepared as described by Crystal et al. (7). Free and membrane bound ribosome fractions from forebrains of 17 day old, male, Sprague Dawley rats were prepared as described by Murthy (3). The forebrain was considered to be

all tissue rostral to the colliculi. All buffers contained 1 mM dithiothreitol and 0.1 mM EDTA in addition to buffer components described previously by Murthy (3), except that heparin (1.5 mg per 100 ml) was used as a ribonuclease inhibitor instead of rat liver inhibitor. Prior to extraction of RNA, ribosome preparations were suspended in 1% (w/v) Triton X-100 and 1% (w/v) sodium deoxycholate (14). Ribosomes were then collected by centrifugation at 100,000 x g for 2 hrs. The ribosome pellets were suspended in 0.1 M NaCl, 0.2 M Tris-HCl, pH 9.1, and 0.01 M EDTA ("pellet buffer").

Preparation of mRNA fractions: Messenger RNA subfractions from the ribosomal preparations were extracted by a modification of the method of Brawerman et al. (15). The ribosome suspension (in pellet buffer) was made 0.5% (w/v) in sodium dodecyl sulfate and allowed to sit at room temperature for 5 min.; an equal volume of redistilled phenol containing 8-hydroxyquinoline (0.1 gm per 100 ml of phenol) was added and the mixture shaken for 10 min. at 2°C. The phenol phase was reextracted with 2 vols. of pellet buffer. The aqueous phases were combined and the nucleic acid was precipitated by the addition of 2 vols. of ethanol and allowed to remain at -20°C for 6 hrs. The precipitate was collected by centrifugation, redissolved in pellet buffer and precipitated with ethanol as above. The final nucleic acid precipitate was dissolved in buffer containing 2 mM Tris-HCl, pH 7.5, and 1 mM KCl and dialyzed against this buffer for 24 hrs. at 2°C.

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis in the prescence of 0.5% (w/v) sodium dodecyl sulfate was carried out according to the procedure of Fairbanks et al. (16); alternatively the method of Davis (17) was utilized. Each sample was prepared for electrophoresis by dialysis against 1 liter of 10 mM sodium bicarbonate and 5 mM 2-mercaptoethanol for 18 hrs. at 2°C with 2 changes of buffer. After electrophoresis the gels were sliced with a vertical Canalco gel slicer into pieces about 1.5 mm thick. Each gel fraction was counted in Amersham-Searle NCS tissue solubilizer and ammonia as described by Ward et al. (18) and counted by scintillation spectrometry. Count-

TABLE I

Requirements for protein synthesis in the reticulocyte cell-free system

Contents of reaction mixture	^{14}C-leucine incorporated* pmoles
Complete system	34.4
Minus ribosomal wash fraction	0.1
Minus tRNA	16.4
Minus ribosomes	0.2
Minus reticulocyte mRNA fraction	0.2

*Incubations in a total volume of 100 µl were performed at 30°C for 35 min. 0.049 A_{260} unit of ribosomes were added to each reaction unless otherwise stated. Other incubation conditions are given in the METHODS section. A "0 time" blank of 0.7 pmole was subtracted from each value. Specific activity of the (^{14}C)-L-leucine was 331 mCi per mmole.

ing efficiency for double label experiments was 51% for ^{14}C and 23% for ^{3}H with a spillover of ^{14}C into the ^{3}H channel of 13.5% and of ^{3}H into the ^{14}C channel of 0.1%.

Preparation of polypeptide products for polyacrylamide gel analysis: The volume of protein synthesis reaction mixtures (see above) was increased 15 fold (1.5 ml total volume) and contained a saturating amount of an mRNA fraction, 2.2 A_{260} units of reticulocyte ribonuclease treated ribosomes, 2.3 mg of ribosomal wash fraction and other components required for protein synthesis as previously described. Incubations were for 35 min. at 30°C. The reaction mixture was then cooled to 2°C and centrifuged at 100,000 x g for 1.5 hrs. to remove ribosomes and nascent peptide chains.

RESULTS AND DISCUSSION

Some general characteristics of the mRNA dependent rabbit reticulocyte cell-free protein synthesis system used in this study are presented in Table I. Protein synthesis was dependent on the addition of a reticulocyte mRNA fraction, ribosomes and a concentrated ribosomal wash fraction which contains initiation

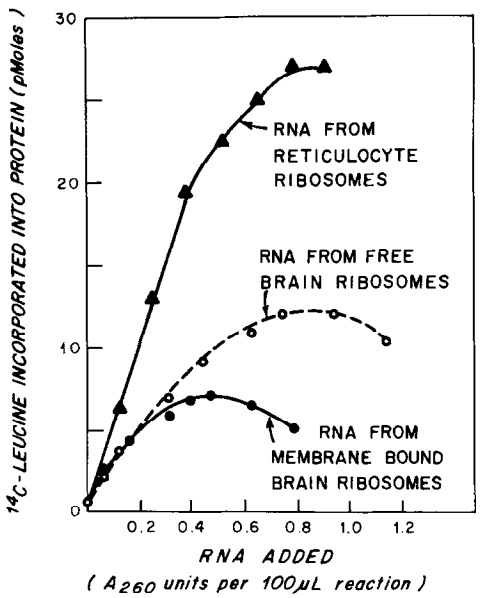

Fig. 1. Effect of RNA fractions on protein synthesis in the rabbit reticulocyte cell-free system. Incubations were for 30 min. at 30°C. Each reaction mixture contained components described in METHODS. The specific activity of the (^{14}C)-L-leucine was 331 mCi per mmole.

factors and other enzymes required for protein synthesis. The time course of protein synthesis was linear for 35 min. with all mRNA fractions. Carboxylmethyl cellulose chromatography of the products in a reaction mixture containing the reticulocyte mRNA fraction indicated that α and β hemoglobin chains were synthesized.

Forebrain mRNA fractions extracted from free and membrane bound ribosomes by alkaline phenol treatment (see METHODS) significantly stimulated protein synthesis in the reticulocyte system as shown in Fig. 1. The products of protein synthesis with reticulocyte RNA and the brain RNA fraction from free ribosomes were separated by polyacrylamide gel electrophoresis in 0.5% (w/v) sodium dodecyl sulfate (Fig. 2). Significant amounts of polypeptides of high molecular wt. were synthesized with the brain RNA fraction that were not synthesized with the reticulocyte RNA. In order to better differentiate products, a similar experiment was done with polyacrylamide gels without sodium dodecyl sulfate (Fig. 3). 83% of the product synthesized with the reticulocyte mRNA was

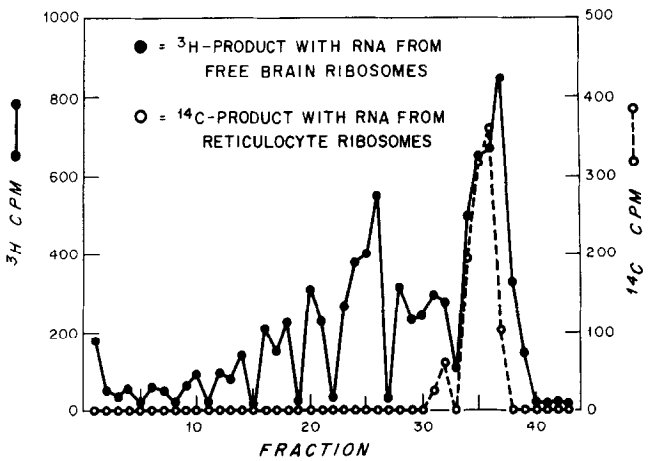

Fig. 2. Polyacrylamide gel electrophoresis in 0.5% (w/v) sodium dodecyl sulfate of peptides synthesized by the reticulocyte cell-free system containing either RNA from reticulocyte ribosomes (^{14}C product) or RNA from free brain ribosomes (^{3}H product). Reaction mixtures for product synthesis are described in METHODS and contained either (^{14}C)-L-leucine, specific activity 331 mCi per mmole or (^{3}H)-L-leucine, specific activity 6 Ci per mmole. 60 µl of the ^{3}H product (reaction mixture containing brain RNA) were combined with 15 µl of the ^{14}C product (reaction mixture containing the reticulocyte RNA) before analysis. The gel contained 8% acrylamide and 0.16% methylenebisacrylamide. The pyronin Y indicator was at Fraction 40.

present in fractions 18 to 21, whereas only 16% of the product synthesized with the mRNA fraction derived from free brain ribosomes was found in the same fractions. Similar results were obtained if the mRNA fraction was extracted from membrane bound forebrain ribosomes. Therefore, it is unlikely that more than 16% of the product synthesized in the reticulocyte system containing brain mRNA fractions was hemoglobin protein. This hemoglobin product from systems containing exogenous brain mRNA fractions was probably synthesized from endogenous hemoglobin mRNA not destroyed in the preparation of the reticulocyte ribosomes (see METHODS).

Polypeptide products from the cell-free reticulocyte system containing either RNA from free brain ribosomes (^{14}C-labeled) or RNA from membrane bound ribosomes (^{3}H-labeled) were compared by separation on polyacrylamide gels (Fig. 4). There were significant differences in the distribution of polypeptide products. The ratio of ^{14}C cpm to ^{3}H cpm in each gel fraction varied from 0.20 to 1.13. However, when both isotopes were used in the synthesis of

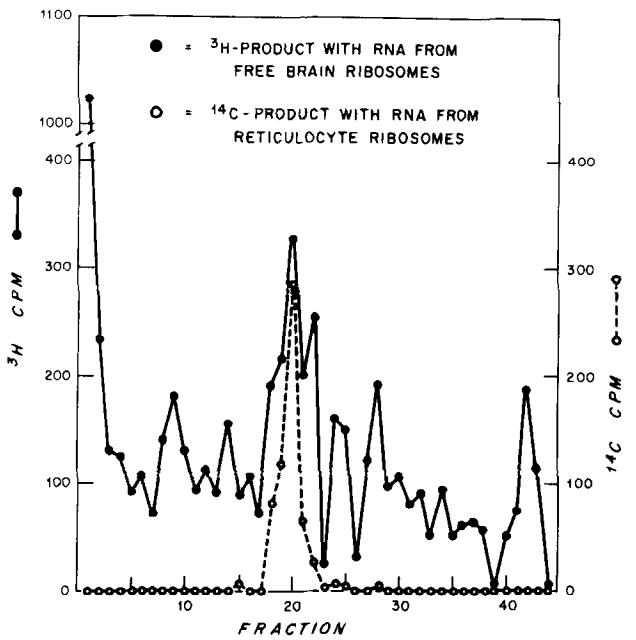

Fig. 3. Polyacrylamide gel electrophoresis of peptides synthesized by the reticulocyte cell-free system containing either RNA from reticulocyte ribosomes (^{14}C product) or RNA from free brain ribosomes (^{3}H product). Reaction mixtures for product synthesis are described in METHODS and contained the radioisotopes noted in Fig. 2. 50 µl of ^{3}H product were combined with 12 µl of ^{14}C product before analysis. The stacking gel contained 2.5% acrylamide and 0.62% methylenebisacrylamide. The separating gel contained 6% acrylamide and 0.20% methylenebisacrylamide. The front was Fraction 42 and a hemoglobin marker was at Fraction 20.

product with mRNA from free brain ribosomes, the distributions of polypeptide products were nearly identical and the ratio of ^{14}C cpm to ^{3}H cpm in each gel fraction varied only from 0.34 to 0.44. These results are consistant with the possibility that there are differences in the mRNA populations present in free and membrane bound forebrain ribosomes. There could be qualitative differences in mRNA species or the same species of mRNA may be present in both ribosome fractions in different relative amounts.

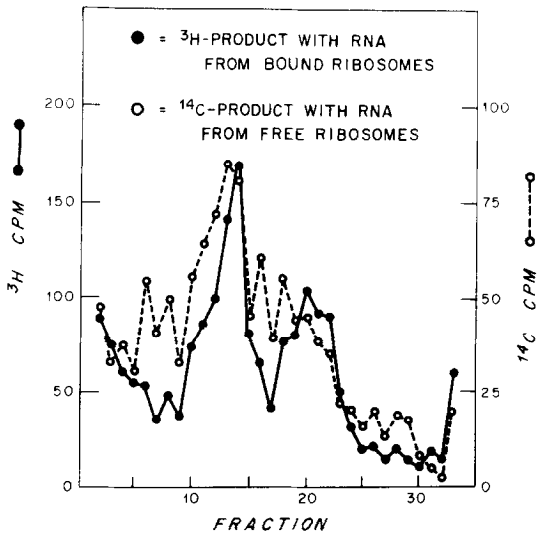

Fig. 4. Polyacrylamide gel electrophoresis of peptides synthesized by the reticulocyte cell-free system containing either RNA from free brain ribosomes (^{14}C product) or RNA from membrane bound brain ribosomes (^{3}H product). Reaction mixtures for product synthesis are described in METHODS and contained the radioisotopes noted in Fig. 2. 65 µl of ^{14}C product were added to 35 µl of ^{3}H product before analysis. The stacking gel contained 2.5% acrylamide and 0.62% methylenebisacrylamide. The separation gel contained 8% acrylamide and 0.16% methylenebisacrylamide. The front was at Fraction 34 (not shown) and contained 352 cpm of ^{3}H and 90 cpm of ^{14}C. Fraction 1 (also not shown) contained 566 cpm of ^{3}H and 314 cpm of ^{14}C.

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