TRANSLATION OF EXOGENEOUS mRNA IN A WHEAT EMBRYO CELL-FREE SYSTEM; EFFECT OF COMPOSITION OF CELL-FREE SYSTEM

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The optimal conditions of globin mRNA translation, the effect of spermin and hemin, and the efficiency of rabbit globin synthesis in a cell-free system from wheat embryos were studied.

The cell-free system prepared from wheat embryos was introduced by Marcus and coworkers[1,2] as a tool for testing the translation of plant viral RNA's. This system translates also bacterial mRNA's (refs[3,4]). Two groups of authors[5,6] used this system to translate rabbit globin mRNA. The wheat embryo cell-free system has received widespread application for testing the biological activity of eukaryotic mRNA's. Quiescent wheat embryos contain complete endogeneous mRNA's in ribonucleoprotein form which are not translated for some reason and polyribosomes are not formed[7].

In our studies on erythropoiesis we have used to advantage a simple cell-free system from rat liver[8] enriched by ribosomal subunits[9] for testing the biological activity of globin mRNA. Recently we have employed a cell-free system from wheat embryos which can easily be prepared and is characterized by a high efficiency and very low endogeneous proteosynthesis. The course of proteosynthesis controlled by mRNA added to the wheat embryo cell-free system is linear for a longer period than in the system from rat liver. In this study we investigated the optimal conditions of translation of exogeneous rabbit globin mRNA and determined the efficiency of the system.

EXPERIMENTAL

Chemicals. L-Amino acids were from Nutritional Biochemicals Corp. USA; GTP*, creatine phosphate, creatine phosphokinase, and hemin chloride were from Calbiochem, Switzerland ATP and CTP from Boehringer, FRG, 2-mercaptoethanol and dithiothreitol from Koch-Light,

* Abbreviations used: mRNA, messenger ribonucleic acid; GTP, guanosine-5′-triphosphate; CTP, cytidine-5′-triphosphate; ATP, adenosine-5′-triphosphate.

England, and spermine from Serva, FRG. L-Leucine-(4,5-^{3}H) (s.a. 53 Ci/mmol) was a product of the Radiochemical Centre Amersham, England. L-Leucine-(U-^{14}C) (s.a. 172 mCi/mmol) was purchased from the Institute for Production, Research, and Use of Radioisotopes, Prague. Untoasted wheat embryos were a commercial product of "Bar-Rav" Mill, Tel Aviv, Israel.

The postmitochondrial supernatant of wheat embryos (S-23) was prepared[1,6] as follows: 0·20 g of wheat embryos was allowed to swell for 1 min in 1·80 ml of a homogenization medium (25 mM Tris-acetate buffer, pH 7·6; 100 mM-KCl, 1 mM magnesium acetate, 2 mM-$CaCl_2$, and 6 mM 2-mercaptoethanol) and subsequently vigorously ground with sea sand for 30 s in a pre-cooled mortar. The homogenate was centrifuged for 12 min at 23000*g* and 2°C. The postmitochondrial supernatant was dialyzed for 2 h against 2 × 250 ml of dialyzation medium (10 mM Tris-acetate buffer, pH 7·6, 75 mM-KCl, 3·5 mM magnesium acetate and 6 mM 2-mercaptoethanol) at 2—4°C. The quantity of ribosomes in the postmitochondrial supernatant was determined after their separation in a 10—40% sucrose gradient (12 ml) prepared in 75 mM-KCl, 25 mM Tris-HCl buffer, pH 7·4, and 5 mM magnesium acetate. After the application of the sample the gradients were centrifuged for 3 h at 180000*g* and 4°C. The gradients were fractionated and the absorbance of the individual fractions was measured at 260 and 280 nm. The quantity of ribosomal RNA was determined from a nomogram[10] and the quantity of ribosomes from the formula[11] for the concentration of ribosomes ($11{\cdot}2A_{260}$ = 1 mg/ml). Postmitochondrial supernatant S-23 contained 2·5 mg of ribosomes in 1 ml.

Cell-free system. The standard incubation mixture contained (unless stated otherwise in the legend to the individual figures) 25 mM Tris-acetate buffer, pH 7·6, 50 mM-KCl, 3 mM magnesium acetate, 2 mM dithiothreitol, 20 L-amino acids (30 μM each, corrected for the concentration of labeled leucine), 1 mM ATP, 0·25 mM GTP, 8 mM creatine phosphate, creatine phosphokinase (60 μg/ml of cell-free system), dialyzed S-23 (1 ml in 3 ml of cell-free system), 5·5 μCi of L-leucine-(U-^{14}C) or 100 μCi of L-leucine(4·5-^{3}H)/1 ml of cell-free extract, and mRNA as shown in the legends to the individual figures. The preincubation of the cell-free system with the amino acids and the energy source is not necessary and was omitted. The incubation was allowed to proceed 90 min at 25°C with gentle shaking in a water bath. At the end of the incubation 0·1 ml samples were applied to Whatman 3 MM paper discs which had been wetted with 0·1 ml of 2% bovine serum albumin for quantitative precipitation of proteins. The paper discs with the sample applied were dried for 15 s in a stream of cold air and then treated according to Mans and Novelli[12]. Radioactivity was measured in a liquid scintillation mixture SLD-31 (Spolana, Neratovice) in Mark II Nuclear Chigaco liquid scintillation counter. The efficiency of the measurement for ^{3}H was 31% and for ^{14}C 82%.

Globin messenger RNA from rabbit reticulocytes was prepared from total RNA isolated from rabbit reticulocyte polyribosomes[13] by affinity chromatography on a poly(U)-Sepharose column[14].

Identification of products of proteosynthesis. A 3 ml sample of the cell-free system after 120 min incubation was taken for the identification of rabbit globin. Rabbit hemoglobin (50 mg) was added as a carrier and the globin was precipitated with acetone and hydrochloric acid[15] in the presence of 50 mM 2-mercaptoethanol. *1)* The α- and β-chains of the globin were subsequently resolved by chromatography on CM-cellulose according to Dintzis[16]. The radioactivity of L-leucine-(4,5-^{3}H) incorporated was determined in 0·4 ml samples of effluent after the evaporation of the volatile buffer. *2)* Polyacrylamide gel electrophoresis. A sample of the cell-free system was treated according to Aviv and Leder[17]. The 10% polyacrylamide gel (0·6 × 10 cm) contained 0·1% sodium dodecyl sulfate and 0·1 M phosphate buffer, pH 7·0 (ref.[18]). The electrophoresis was allowed to proceed 6 h at 8 mA/gel. The gel was subsequently cut into 1 mm strips which

were dried (4 at a time) in scintillation vials, immersed in SLD-31 scintillation liquid, and subjected to radioactivity measurement.

RESULTS AND DISCUSSION

Protein synthesis in the cell-free system from wheat embryos to which mRNA has been added is a linear function of time for at least 1 h (Fig. 1*a*, curve *1*). Endogeneous proteosynthesis is negligible (Fig. 1*a*, curve 2).

The cell-free system can be saturated with as little as 5–6 μg of mRNA in 1 ml of the cell-free system (Fig. 1*b*). The addition of 18 S RNA or 28 S RNA brings about only a small increase of proteosynthesis (Fig. 1*c*, curve *1* and 2).

The optimal concentration of K^+-ions is 50 mM (Fig. 2*a*) and of Mg^{2+}-ions 2 mM (Fig. 2*b*). Spermine, a polyamine whose addition enhances the synthesis of certain proteins[19,20] in the wheat embryo cell-free system, strongly inhibits globin synthesis in this system (Fig. 2*c*),

The optimal concentration of GTP is higher than 0·25 mM which is conventionally used (Fig. 3*a*). The optimal concentration of CTP is 0·8 mM and of creatine phosphate 15–18 mM (Fig. 3*b*, 3*c*).

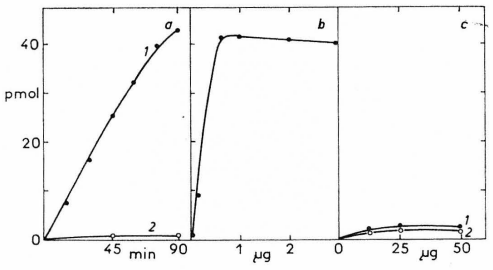

FIG. 1

Time Course of L-Leucine-(U-^{14}C) Incorporation into Protein and Saturation of Cell-Free System by Different RNA's

The cell-free system was incubated *a* with rabbit globin mRNA (10 μg/ml) *1* and in the absence of mRNA 2. The time of incubation is given in min; *b* with different amounts of rabbit globin mRNA 90 min; *c* with different amount of 18 S *1* and 28 S RNA 2 90 min. The radioactivity incorporated into the protein[12] was determined in aliquots. The quantity of exogeneous RNA is given in μg/100 μl of cell-free system and the degree of incorporation in pmol of leucine incorporated per 100 μl of cell-free system.

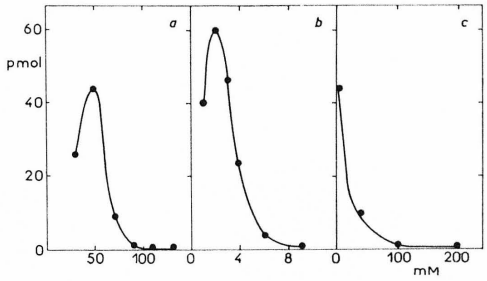

FIG. 2

Incorporation of L-Leucine-(4,5-^{3}H) as Function of Concentration of Different Cations

Concentrations of *a* K^+ in 3·0 mM Mg^{2+}; *b* Mg^{2+} in 50 mM K^+; *c* spermine in 3·0 mM Mg^{2+} and 50 mM K^+. All samples contained rabbit globin mRNA (25 μg/1 ml of cell-free system) and the radioactivity of the protein precipitated by trichloroacetic acid was determined after 90 min incubation[12]. The concentrations of K^+, Mg^{2+}, and spermine are mM and the degree of incorporation is expressed in pmol of leucine incorporated per 100 μl of cell-free system.

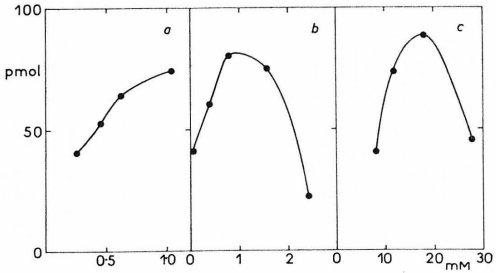

FIG. 3

Effect of Concentration of GTP, CTP, and Creatine Phosphate on Protein Synthesis Controlled by Rabbit Globin mRNA

The samples were incubated with L-leucine-(4,5-^{3}H) and mRNA (25 μg/1 ml of cell-free system) under standard conditions. The radioactivity of the protein precipitated by trichloroacetic acid was determined after 120 min incubation[12]. The concentrations of *a* GTP, *b* CTP, and *c* creatine phosphate are mM and the degree of incorporation is expressed in pmol of leucine incorporated per 100 μl of cell-free system.

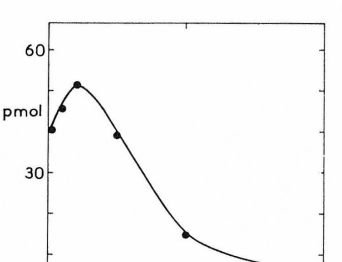

FIG. 4

Effect of Hemin on Protein Synthesis Directed by Rabbit Globin mRNA

The incubation of the samples with L-Leucine-(4,5-^{3}H) and mRNA (25 μg/1 ml of cell-free system) was carried out under standard conditions described under Experimental. The radioactivity of the protein precipitated by trichloroacetic acid[12] was determined after 90 min incubation. The concentration of hemin is μM and the degree of incorporation is expressed in pmol of leucine incorporated per 100 μl of cell-free system.

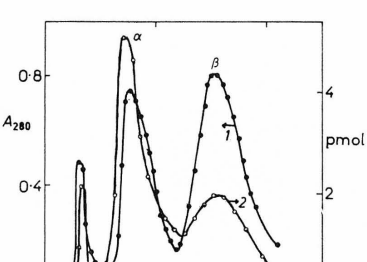

FIG. 5

Chromatography on CM-cellulose of Rabbit Globin Synthetized in Wheat Embryo Cell-free System

The composition of the cell-free system is described under Experimental. After 120 min incubation with L-leucine-(4,5-^{3}H) and mRNA (10 μg/1 ml of cell-free system) the sample was treated as described under Experimental. The effluent volume is given in ml, the quantity of protein is given as absorbance at 280 nm *1*, and the degree of incorporation in pmol of leucine incorporated per 1 ml of effluent *2*.

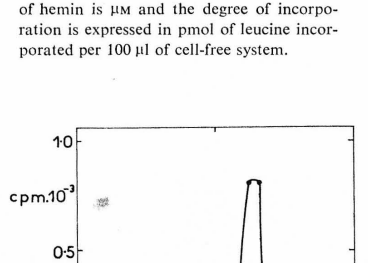

FIG. 6

Polyacrylamide Gel Electrophoresis of Products of Cell-Free System in Sodium Dodecyl Sulfate

The protein sample (50 μl of 2% solution) was placed on top of the gel. The gel length is in cm and the radioactivity of L-leucine-(4,5-^{3}H) incorporated in counts/min.

Hemin, which is essential for globin synthesis in a lysate of rabbit reticulocytes[21], stimulates at 20 μM concentration globin synthesis in the wheat embryo cell-free system but inhibits the synthesis at concentrations higher than 50 μM (Fig. 4).

The wheat embryo cell-free system synthetizes the α- and β-chains of rabbit globin after the addition of rabbit globin mRNA (Fig. 5). The product of proteosynthesis has been also identified by polyacrylamide gel electrophoresis where maximal radioactivity coincides with the globin zone (Fig. 6).

After the addition of globin mRNA the wheat embryo cell-free system incorporated 0·6–3·2 nmol of leucine/1 mg of ribosomal RNA in 60 min at 25°C. Since this cell-free system is saturated at a concentration of 6 μg of mRNA/1 ml and since 1 ml of the cell-free system contains 0·81 mg of ribosomes, then 0·4–2·2 chains of rabbit globin per 1 mol of mRNA are synthetized in 60 min at 25°C.

The wheat embryo cell-free system shows a high efficiency for translation of rabbit globin mRNA. The optimal conditions of translation which we have examined, slightly differ from those described by other authors[5,6].

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REFERENCES

1. Marcus A., Luginbill B., Feeley J.: Proc. Nat. Acad. Sci. U.S.A. *59*, 1243 (1968).
2. Marcus A., Efron. D., Weeks D. P. in the book: *Methods in Enzymology* XXX, Part F (L. Grossman, K. Moldave, Eds), p. 749. Academic Press, New York 1974.
3. Wang S., Marcu K. B., Inouye M.: Biochem. Biophys. Res. Commun. *68*, 1194 (1976).
4. Gallis B. M., McDonnell J. P., Hopper J. E., Young E. T.: Biochemistry *14*, 1038 (1975).
5. Efron D., Marcus A.: FEBS (Fed. Europ. Biochem. Soc.) Lett. *33*, 23 (1973).
6. Roberts B. E., Paterson B. M.: Proc. Nat. Acad. Sci. U.S.A. *70*, 2330 (1973).
7. Weeks D. P., Marcus A.: Biochim. Biophys. Acta *232*, 684 (1971).
8. Burke G. T., Goldstein J., Redman C. M.: FEBS (Fed. Europ. Biochem. Soc.) Lett. *37*, 221 (1973).
9. Borová J., Fuchs O., Poňka P., Neuwirt J.: This Journal *41*, 2448 (1976).
10. Warburg O., Christian W.: Biochem. Z. *310*, 384 (1942).
11. Rich A. in the book: *Methods on Enzymology* XIIA. Part A (L. Grossman, K. Moldave, Eds), p. 487. Academic Press, New York–London 1967.
12. Mans R. J., Novelli G. D.: Arch. Biochem. Biophys. *94*, 48 (1961).
13. Perry R. P., LaTorre J., Kelley D. E., Greenberg J. R.: Biochim. Biophys. Acta *262*, 220 (1972).
14. Lindberg V., Persson T.: Eur. J. Biochem. *31*, 246 (1972).
15. Rossi-Fanelli A., Antonini E., Caputo A. Biochim. Biophys. Acta *30*, 608 (1958).
16. Dintzis H. M.: Proc. Nat. Acad. Sci. U.S.A. *47*, 247 (1961).
17. Aviv H., Leder P.: Proc. Nat. Acad. Sci. U.S.A. *69*, 1408 (1972).

18. Weber K., Osborn M.: J. Biol. Chem. *244*, 4406 (1969).
19. Kemper B., Hebener J. F., Mulligan R. C., Potts J. T., Rich A.: Proc. Nat. Acad. Sci. U.S.A. *71*, 3731 (1974).
20. Thang M. N., Thang D. C., DeMaeyer E., Montagnier L.: Proc. Nat. Acad. Sci. U.S.A. *72*, 3975 (1975).
21. Howard G. A., Adamson S. D., Herbert E.: Biochim. Biophys. Acta *213*, 237 (1970).

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