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CELL-FREE TRANSLATION OF MESSENGER RNA FOR HUMAN BISPHOSPHOGLYCEROMUTASE

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mRNAs prepared from different human tissues were translated in a cell-free reticulocyte lysate system and, when present, the neosynthesized bisphosphoglyceromutase (BPGM) was specifically isolated by immunoprecipitation and analyzed by polyacrylamide gel electrophoresis. Analysis of the translation products showed that bisphosphoglyceromutase was synthesized in vitro with its mature molecular weight and messenger RNA specifying the synthesis of BPGM exhibited a sedimentation coefficient of 12 S in human reticulocytes. This synthesis seems to be highly tissue specific since we could not evidence any synthesis of this enzyme using mRNA obtained from non erythroid tissue. The BPGM synthesis represents 0.1% of the total neosynthesized non heme proteins in human reticulocyte and ten times less (0.01%) in human fetal liver.

Bisphosphoglyceromutase (BPGM) (E.C. 2.7.5.4.) is a glycolytic enzyme that controls the concentration of 2,3-DPG in erythrocytes. It consists of two identical subunits the molecular weight of which is 31 000 daltons (1). 2,3-DPG facilitates the supply of oxygen to the tissues by binding to hemoglobin, with a higher affinity for the deoxy form. The regulatory properties of the enzyme involved in 2,3-DPG metabolism have been the subject of intensive investigation. Several lines of evidence have shown that BPGM is also responsible for the total diphosphoglycerate phosphatase activity (E.C. 3.1.3.13) in the red cells (2)(3)(4). A third activity is slightly displayed by BPGM but provided, for the main part, by phosphoglyceromutase (E.C. 2.7.5.3) (5). This enzyme probably has a structure very close to that of BPGM but its role is completely distinct. Studies have also focused on the erythroid specificity of BPGM, its biosynthesis in differentiating erythroid cells, and its expression and role during animal development. Recent studies by Narita et al. (6) showed that in rabbit reticulocytes and bone marrow erythroid cells, the accumulation of 2,3-DPG is primarily attribuable to the in-

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crease in BPGM activity. This increased activity is obtained through an increase in the amount of enzyme. The physiological function of 2,3-DPG in animal development has been extensively investigated in several species but remains poorly understood(7).

Although the sequence of the human BPGM has been recently described (8), it is clear that in order to investigate further these fundamental questions, one needs a molecular probe which will allow studies at the RNA or DNA levels. Cell-free synthesis and detection of neosynthesized human enzymes are a possible first step for the characterization of the corresponding messenger RNA. We report here the first cell free synthesis of human BPGM,preliminary results of which have already been reported (9), and we show that this synthesis is only possible when the template RNA is extracted from erythroid tissues such as reticulocytes or fetal liver.

MATERIAL AND METHODS

Blood with hyper-reticulocytosis (15 to 20%) was withdrawn from patients with sickle cell anemia during exchange transfusion. Human adult liver was obtained after surgical partial hepatectomy and human fetal liver was obtained from therapeutic abortion at 20 weeks of pregnancy.

Oligo (dT) cellulose (type 3) was obtained from Collaborative Research. ^{35}S Methionine (1 200 Ci/mmol) was purchased from Amersham. Calf liver tRNA was obtained from Boehringer. Immunoprecipitin formalin fixed staph A cells were obtained from BRL. Acrylamide, bisacrylamide were supplied by Fluka. Low molecular weight protein markers were obtained from Pharmacia, En ^{3}hance autoradiography enhancer from NewEngland Nuclear and Royal X omatic AR films from Eastman Kodak.

- Specific antibodies preparation

Bisphosphoglyceromutase was purified according to the method previously described (10). Rabbit antibodies against bisphosphoglyceromutase were obtained as follows : two rabbits were injected intramuscularly with 1 ml of a mixture of 1 mg of purified enzyme in NaCl (0.9%) and 1 ml Freund's complete adjuvant. Two weeks later and after another two weeks, the rabbits were reimmunized with the same amount of enzyme and Freund's incomplete adjuvant. At this time, blood was withdrawn and clotted. The serum was tested and stored at - 80°C.

Double immunodiffusion on Ouchterlony and immunoneutralization showed that the antibodies were specific for bisphosphoglyceromutase (10). There was no cross reaction with phosphoglyceromutase.

- Isolation of mRNA

RNA was isolated from reticulocytes as previously described (11) and from adult and fetal livers using the lithium chloride method (12). Poly(A^+) RNA was purified by oligo(dT) cellulose chromatography according to Aviv and Leder (13).

- Fractionation of mRNA sucrose gradient centrifugation

Poly(A^+) containing RNA (200 µg in 300 µl of water) was heated at 70°C for 3 min., cooled on ice and layered into 12 ml of 5 to 20% sucrose gradients in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. After centrifugation at 33 000 r.p.m. for 16 h. at 4°C in a Beckman SW-41 rotor, the gradients were fractionated using an ISCO density gradient fractionator. Absorbance was monitored at 260 nm, and 0.4 ml fractions were collected. After ethanol precipitation, RNA pellets were resuspended in 20 µl of water. For in vitro translation, 1 µl of the suspension was added to 10 µl of lysate.

- Cell free synthesis, immunoprecipitation and analysis of translation products

Messenger RNA was heated at 65°C for 5 min. then rapidly cooled in ice and translated in a micrococcal nuclease-treated rabbit reticulocyte lysate system according to Pelham and Jackson (14) in the presence of calf liver tRNA during 90 min. at 30°C. Then a 5 µl sample was TCA precipitated for estimation of ^{35}S methionine incorporation in neosynthesized proteins. Radioactivity was measured by scintillation counting with 10 ml of aquasol in a liquid scintillation counter and the radioactive neosynthesized bisphosphoglyceromutase was immunoprecipitated with specific antibodies according to Opperman (15).

Translation products were analyzed by SDS-PAGE as described by Laemmli (16). The gel then was treated with En ^{3}hance, dried and autoradiographed.

RESULTS

In order to investigate on the presence of mRNA sequences encoding BPGM in different tissues, mRNAs extracted from these tissues were assayed in a rabbit reticulocyte lysate cell-free system and the translation products were immunoprecipitated with anti-BPGM antibodies. mRNA sequences isolated from reticulocyte direct the synthesis of a single BPGM radioactive band. This band appears just above the 30 000 daltons marker (Fig.1,lane 2),as does the purified BPGM (31 000 daltons) on coomassie stained gel (not shown). An additional confirmation was obtained by immunological competition with the pure protein : as expected, an excess of cold enzyme prevented binding of the radioactive neosynthesized BPGM (Fig. 1, lane 3).

In order to roughly estimate the size of the specific mRNA, total poly(A^+) mRNA were fractionated by sucrose gradient centrifugation. BPGM mRNA is found in the 12 S fraction as shown by immunoprecipitation of translation products from the different fractions (Fig. 2, lane 3).

When fetal liver mRNA is assayed in the same manner in the cell-free system, a second round of immunoprecipitation was ne-

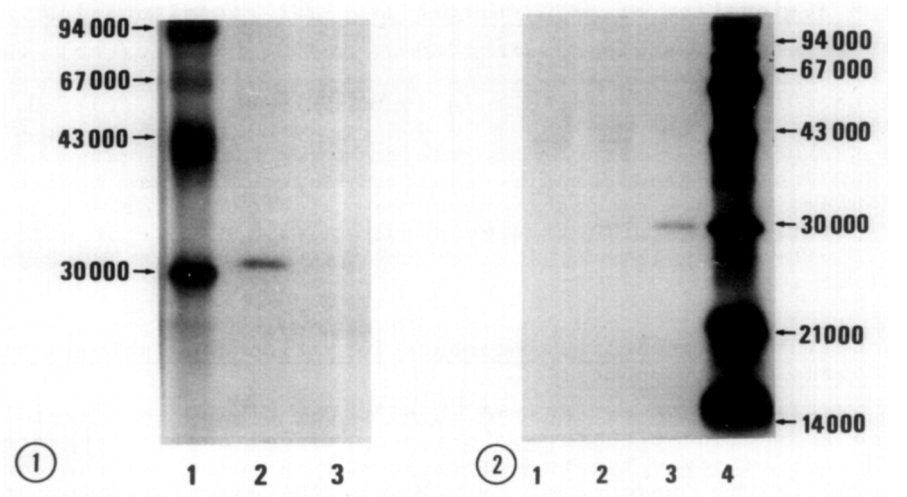

Fig. 1. SDS polyacrylamide gel electrophoresis of cell-free translation products from mRNA isolated from human reticulocytes, after immunoprecipitation with BPGM specific antibodies.

Lane 1 : ^{14}C labelled protein markers : muscle phosphorylase B (Mr : 93 000), bovine serum albumine (Mr : 68 000), ovalbumin (Mr : 45 000), carbonic anhydrase (Mr : 30 000)

Lane 2 : ^{35}S neosynthesized BPGM purified by immunoprecipitation.

Lane 3 : Same than in lane 2 but the immunoprecipitation of ^{35}S neosynthesized BPGM has been done in presence of an excess of cold pure BPGM.

Cell-free synthesis was performed as described in Material and Methods in a total reaction mixture of 100 µl containing 4 µg of reticulocyte mRNA and 100 µCi ^{35}S methionine. Under these conditions radioactivity incorporation into TCA precipitable material was about 5.5×10^6 cpm.
Analysis of the radioactivity incorporated in neosynthesized BPGM was performed by scintillation counting of the corresponding slice of the gel solubilized in 0.5 ml of soluene.

Fig. 2. SDS polyacrylamide gel electrophoresis of cell-free translation products of mRNA from fetal liver after immunoprecipitation with BPGM specific antibodies.

Lanes 1-2 : Neosynthesized ^{35}S proteins after double immunoprecipitation with BPGM specific antibodies in presence (lane 1) and absence (lane 2) of cold pure protein, of translation products of fetal liver mRNA.

Lane 3 : Neosynthesized ^{35}S BPGM immunoprecipitated from translation products of 12 S sucrose gradient fraction of reticulocyte mRNA.

Lane 4 : ^{14}C labelled protein markers as in Fig. 1

cessary for the radioactive band to be visible above the background. This indicates that the rate of BPGM synthesis is very low in this tissue. Nevertheless a polypeptide of the expected

size was immunoprecipitated by anti-BPGM antibody, its identity being confirmed by immunological competition (Fig. 2,lanes 1 and 2) with the purified protein. In contrast, with adult liver mRNA, we could not detect any radioactive band after gel electrophoresis of the immunoprecipitated products, even after long exposures (not shown).

We have attempted to estimate the proportion of the mRNAs coding for BPGM in human reticulocyte and fetal liver by determination of the d.p.m. incorporated in gel slices by liquid scintillation counting after solubilization of the slices in 0.5 ml of soluene. The total incorporation of ^{35}S methionine during synthesis in the cell-free translation mixture was estimated by trichloracetic precipitation and scintillation counting. It appears that BPGM mRNA accounts for 0.01% of the poly(A^+) RNA in reticulocyte and only 0.005% in fetal liver cells.

DISCUSSION

We have demonstrated the presence of mRNA sequences coding for BPGM in human reticulocytes and fetal liver and roughly estimated their concentration. This has been done by isolation of total mRNA from these tissues, translation in a cell free rabbit reticulocyte lysate system and immunoprecipitation of the translation products with a specific anti-BPGM antibody.

When present, the neosynthesized BPGM was detected as a single radioactive polypeptide having a molecular weight of about 31 000 daltons. It migrates exactly as do the purified BPGM subunits in SDS- PAGE gels.Immunoprecipitation of this 31 000 daltons polypeptide is efficiently prevented by competition with the purified protein, and this confirms that this peptide is actually the subunit of BPGM. It also appears that adult and fetal proteins synthesized in vitro did not differ with respect to their size.

Sucrose gradient analysis of reticulocyte mRNA showed that BPGM mRNA has a sedimentation coefficient of 12 S. This is different from the sedimentation coefficient of carbonic anhydrase mRNA which has been reported to be 14 S (17), although the polypeptide has a similar molecular weight (30 000 daltons). This discrepancy is probably related to the large 3' non coding sequence present in carbonic anhydrase mRNA (17).

We have also estimated the proportion of the mRNAs coding for BPGM contained in the two erythroid tisues that we have

studied. Our results indicate that BPGM mRNA represents 0.01 % of total protein mRNA in a very specialized cell such as the reticulocyte, in which globin represents 90 % of the total proteins. It appears that BPGM mRNA is a rare species even in reticulocytes and our results agree with those of NARITA et al. (6) who found 0.04 % BPGM synthesis in rabbit recitulocytes.
BPGM is responsible for the production of 2,3-DPG, an effector of hemoglobin. These two molecules are present at almost equimolar amount in erythroid cells. In fetal liver cells BPGM mRNA represents 0.01 % of globin mRNA in which there is a high proportion of γ globin mRNA (90 % of non α globin mRNA). It is known that fetal hemoglobin ($\alpha_2\gamma_2$) does not bind to 2,3-DPG. If the synthesis of this compound is in some way controlled by its binding capacities to the hemoglobin molecule, the 2,3-DPG level and then the BPGM mRNA level, are expected to be low in fetal erythroid cells. Unfortunately we could not obtain sufficient material in convenient conditions to measure the 2,3-DPG level in fetal liver cells.

We have demonstrated the presence of small quantities of mRNA molecules coding for BPGM in human reticulocytes and fetal liver cells. The lack of this mRNA in adult liver, in our experimental conditions, demonstrates further the tissue specificity of this protein. Our results strongly confirm that this enzyme is a very useful marker of erythroid cell differentiation and it should be of interest to investigate how globin and BPGM synthesis are coordinated in differentiating erythroid cells.

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