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Cell-free Translation of Human Uroporphyrinogen Decarboxylase mRNAs

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Uroporphyrinogen decarboxylase was synthesized in a reticulocyte lysate cell-free system under the direction of messenger RNAs isolated from human fetal liver and from human reticulocytes. The enzyme was specifically isolated by immuno affinity chromatography. Analysis of the translation products showed that uroporphyrinogen decarboxylase was synthesized in vitro with its mature molecular weight. This enzyme represented 0.04 % of the total neosynthesized proteins under the direction of fetal liver mRNA and about ten times less (0.005 %) with reticulocyte mRNA.

Uroporphyrinogen decarboxylase (URO decarboxylase) (E.C. 4.1.1.37) is a cytosolic enzyme which catalyzes the sequential removal of the four carboxyl groups of the carboxymethyl side chains in uroporphyrinogen to yield coproporphyrinogen (1). This reaction is the fifth step of heme synthesis.

URO decarboxylase has recently been purified to homogeneity from human erythrocytes (2). Specific antibodies have been used to investigate the enzyme abnormality in patients with familial porphyria cutanea tarda (PCT) and hepato-erythropoietic porphyria (HEP), diseases characterized by a defect of uroporphyrinogen decarboxylase activity. In most of the patients the immunoreactive protein is decreased to the same extent as the catalytic activity (3, 4). Therefore further investigation of the molecular lesion requires studies at the mRNA or/and at the gene level. Moreover, enzymes of the heme biosynthetic pathway are induced in a coordinate manner during early stages of erythroïd differentiation (5). This leads to an increased heme syn-

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thesis which may in turn affect globin genes expression (6). However, molecular mechanisms underlying this coordinate expression are not known. Further understanding of this process clearly requires measurement of specific messenger RNAs and characterization of the corresponding genes.

Cell-free translation followed by specific isolation of the polypeptides under investigation allows to detect developmental or pathological changes in corresponding mRNAs and is also a preliminary step for mRNA purification and cDNA cloning. Here we report the first study of cell-free synthesis and immuno affinity purification of URO decarboxylase directed by messenger RNA isolated from human fetal liver and human reticulocytes.

MATERIAL AND METHODS

Human fetal liver was obtained from therapeutic abortion at 20 weeks of pregnancy. Blood with hypereticulocytosis (> 15 %) was withdrawn from patients with sickle cell anemia during exchange transfusion. [^{35}S]-methionine and [^{14}C]-formaldehyde were purchased from Amersham. Glutaraldehyde-activated ultrogel and oligo (dT) trisacryl were supplied by Industrie Biologie Française ; acrylamide, bisacrylamide, Royal X-omatic R_1 films were obtained from Eastman Kodak ; soluene, aquasol, En^{3}hance (autoradiography enhancer) were from New England Nuclear ; tRNA from calf liver was obtained from Boehringer. Low molecular weight markers were obtained from Pharmacia.

URO decarboxylase was purified to homogeneity according to de Verneuil et al (2). Low molecular weight protein markers and purified URO decarboxylase were [^{14}C] labelled according to Dottavio-Martin and Ravel (7).

Rabbit antibodies against URO decarboxylase were raised in rabbits as previously described (2) in the laboratory of Drs A. Kappas and S. Sassa (Rockefeller University, N.Y.) ; immunoglobulins were coupled to glutaraldehyde activated ultrogel at a concentration of 3-5 mg immunoglobulin/mg of gel (8).

Isolation of mRNAs

mRNA was isolated from reticulocytes according to Goossens and Kan (9) and from fetal liver using the lithium chloride method (10). Poly (A^+) RNA was purified by oligo (dT) trisacryl chromatography according to the manufacturer.

Cell-free synthesis and analysis of the translation products

Messenger RNA was denaturated at 65°C for 5 minutes then rapidly cooled on ice and translated in a micrococcal nuclease-treated rabbit reticulocyte lysate system (11) ; after incubation for 90 minutes at 30°C, a 5 µl sample was withdrawn for estimation of [^{35}S]-methionine incorporated in proteins and the remainder of the mixture was diluted with two volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 0.02 % sodium azide, 0.3 mM phenylmethylsulfonylfluoride, 10 mM

EDTA, 10 mM methionine, 1 % (v,v) Triton X-100 and 1 % (w,v) sodium deoxycholate. Neosynthesized URO decarboxylase was isolated by affinity chromatography using microcolumns which contained 10 µl of antibody-ultrogel according to the method of Kahn and coworkers (8). Elution was obtained by boiling the gel in 50 µl of 0.0625 M Tris/HCl buffer (pH 6.8), 5 % (v,v) ß mercaptoethanol, 10 % glycerol and 0.001 % (w,v) bromophenol blue. Translation products were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (12). The gel was treated with En^{3}hance, dried and autoradiographed. Radioactivity was measured by scintillation counting with 10 ml of aquasol using the preset [^{14}C] channel of a liquid scintillation counter.

RESULTS AND DISCUSSION

Figure 1 shows a fluorography of a SDS-PAGE of proteins synthesized in a cell-free system under the direction of mRNA isolated from human fetal liver. Although there is a large predominance of a radioactive

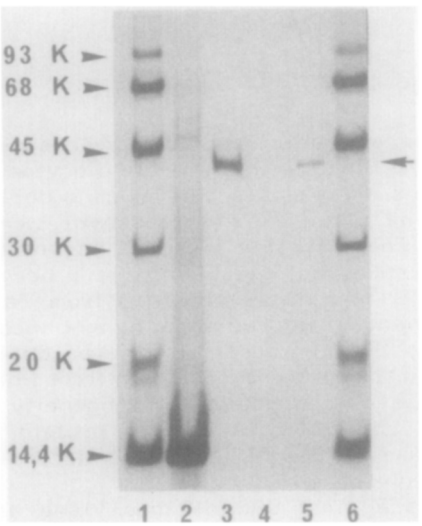

FIGURE 1

SDS-polyacrylamide gel electrophoresis of cell-free translation products using mRNA isolated from human fetal liver.

Channels 1 and 6 : [^{14}C]-labelled protein markers : muscle phosphorylase B (Mr = 93,000) ; bovine serum albumin (Mr = 68,000) ; ovalbumin (Mr = 45,000) ; carbonic anhydrase (Mr = 30,000) ; soybean trypsin inhibitor (Mr = 20,100) ; lactalbumin (Mr = 14,400).
Channel 2: total translation products.
Channel 3 : [^{14}C]-labelled URO decarboxylase purified from human erythrocytes.
Channel 4 : eluate of the immunoaffinity column previously saturated with excess of unlabelled URO decarboxylase.
Channel 5 : [^{35}S] neosynthesized URO decarboxylase purified by immunoaffinity column.

Cell-free synthesis was performed as described under "Material and Methods" in a total reaction mixture of 100 µl containing 4 µg of mRNA from fetal liver and 150 µCi [^{35}S]-methionine. Under these conditions radioactivity incorporation into trichloracetic precipitable material was about 3.5×10^6 cpm.

Fluorography of the gel was exposed for 2 days.

spot around 16,000 daltons, which corresponds to globin chains, numerous other polypeptides can be noticed. The presence of high molecular weight proteins warrants a good quality of the mRNA used in these experiments.

From the polypeptides synthesized, URO decarboxylase was isolated by immunoaffinity purification using microcolumns of immunoglobulins bound to ultrogel. The specificity of this procedure was demonstrated by immunological competition with the pure protein : as expected, an excess of cold enzyme prevented binding of the radioactive URO decarboxylase synthesized in the reticulocyte lysate (see Fig., channel 4).

A molecular weight of 43,000 daltons was found for neo-formed URO decarboxylase ; this value appears to be identical to that of the [^{14}C]-labelled purified protein. This finding indicates that URO decarboxylase is synthesized with its mature molecular weight as expected for a cytosolic enzyme.

Quantitative estimation of the radioactivity incorporated into URO decarboxylase was performed after SDS-PAGE of the cell-free translation products from human fetal-liver mRNA : the location of neosynthesized URO decarboxylase within the gel was determined by fluorography. Analysis of the radioactivity was performed by scintillation counting of the corresponding slice of the gel solubilized in 500 μl of soluene. URO decarboxylase represented 0.04 % of total TCA precipitable radioactivity.

Analysis of translation products directed by human reticulocyte mRNA using the same techniques indicated that i) adult and fetal URO decarboxylase synthesized in vitro did not differ with respect to their size ii) the amount of URO decarboxylase synthesized under the direction of mRNA from reticulocytes was around ten times lower than that obtained with fetal liver mRNA. Since fetal liver contains a large proportion of immature erythroid cells, this finding is in agreement with the observation that enzymes of the heme biosynthetic pathway are induced at early stages of erythropoiesis (5) and suggests that corres-

ponding mRNAs are destabilized before the reticulocyte stage. From these results it appears that fetal liver is a possible source of URO decarboxylase mRNA for molecular cloning of its cDNA.

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