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Brief Communication

Homocystinuria: Biogenesis of Cystathionine β-Synthase Subunits in Cultured Fibroblasts and in an in Vitro Translation System Programmed with Fibroblast Messenger RNA

FLEMMING SKOVBY,[1] JAN P. KRAUS, AND LEON E. ROSENBERG

SUMMARY

Rabbit antiserum raised against pure human hepatic cystathionine β-synthase was used to precipitate synthase from extracts of radiolabeled cultured fibroblasts derived from 17 homocystinuric patients and two controls. Size analysis of the immunoprecipitates by SDS/polyacrylamide gel electrophoresis revealed that 15 of the 17 synthase-deficient lines synthesized synthase subunits indistinguishable in size from the control (M_r = 63,000). One mutant fibroblast line, previously shown to lack catalytic activity and antigenically cross-reacting material, contained no immunoprecipitable product. Analyses of immunoprecipitated polypeptides synthesized in vitro by cell-free translation of mRNAs prepared from selected mutants confirmed and extended the results from cell extracts. This experimental approach also allowed us to determine the biochemical and genetic defect in a patient with barely detectable synthase subunits in cell extracts. His cultured fibroblasts and those of his father contained two mRNA species, separable by size, coding for equal amounts of two immunoprecipitable polypeptides: one of normal size (M_r = 63,000); the other ~7,000 daltons smaller (M_r = 56,000). His mother's fibroblasts made only the M_r = 63,000 species. We conclude that this patient is a compound heterozygote, and that one of his mutant alleles

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[1] All authors: Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510. Address correspondence to J. P. K.

results in the synthesis of a synthase polypeptide missing about 60 amino acid residues.

INTRODUCTION

Deficiency of cystathionine β-synthase (L-serine hydrolyase [adding homocysteine], E.C.4.2.1.22), an autosomal recessively inherited inborn error of the transsulfuration pathway, is the leading cause of homocystinuria in man. The catalytic properties of mutant synthase in liver tissue and in cultured fibroblasts derived from affected individuals have been described in detail by a number of investigators, including ourselves [1]. Studies of the molecular structure of mutant synthase, on the other hand, have been limited by the difficulties encountered in purification of the normal enzyme and by the lack of specific antisynthase antibodies. We have shown that synthase purified from human liver is composed of identical subunits [2]. Using an antiserum raised in rabbits against the pure liver synthase, we then studied the biosynthesis of normal synthase in cultured human fibroblasts and in a cell-free translation system programmed by mRNA from normal fibroblasts. The only translational product recovered was a polypeptide with an apparent M_r of 63,000 on SDS/polyacrylamide gel electrophoresis [3]. Accordingly, we concluded that cystathionine β-synthase in cultured human fibroblasts is composed of identical subunits with an M_r of 63,000. We report here the initial results of an analysis of synthase subunit synthesis in our large collection of cultured fibroblasts from individuals with cystathionine β-synthase deficiency and document the first example of compound heterozygosity in a patient with homocystinuria.

MATERIALS AND METHODS

Cell Culture

Cultured skin fibroblasts from two controls, two obligate heterozygotes, and 17 synthase-deficient patients were used. The synthase-deficient fibroblast lines employed, denoted by laboratory accession numbers as used by Lipson et al. [4] and Skovby et al. [5], were subdivided as previously [5] into those with (CRM^+) and those without (CRM^-) detectable "steady-state" cross-reacting material.* The CRM^+ lines were numbers 42, 338, 339, 340, 341, 343, 366, 375, 380, 382, 458, 676, 729, and 860; the CRM^- ones were numbers 342, 344, and 599. Conditions for culturing and harvesting fibroblasts have been described [5, 6].

Labeling and Extraction of Cells

Fibroblasts were grown to confluence in 100-mm Petri dishes. Prior to labeling, the cells were incubated for 1 hr with leucine-free Eagle's minimal essential medium (Gibco, Grand Island, N.Y.), supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 μg/ml kanamycin. The cells were then labeled for 6 hrs in 4 ml of the same medium

* "Steady-state" CRM refers to the usually measured amount of cross-reacting antigen present in cells or body fluids when the rates of antigen synthesis and breakdown are in equilibrium. Such CRM is to be distinguished from the amount of antigen measured here, which reflects almost exclusively the rate of antigen synthesis.

containing 200 μCi L-[4,5-^{3}H]leucine (Amersham, Arlington Heights, Ill.). Harvesting of the cells was carried out as described [5, 6], except that 1% fetal calf serum was added to the phosphate-buffered saline. The cell pellets were stored at −70°C until used. Cell extracts were prepared by sonication of the cell pellets in 30 mM sodium phosphate, pH 6, and centrifugation for 1 hr at 20,000 rpm in a Sorvall SS 34 rotor.

Immunoprecipitation of Radiolabeled Cystathionine β-Synthase from Fibroblast Extract

The extract of cells from one dish, 0.15 ml, was mixed with 0.24 ml of a detergent solution containing 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, 1% leucine, and 0.1% SDS, pH 7. Prior to immunoprecipitation, the amounts of extracts were adjusted to contain the same amounts of acid-precipitable radioactivity. Antisynthase antiserum, 3 μl, was added, and the mixture was incubated at 4°C for 16–20 hrs. The immunoprecipitates were sedimented at 7,000 *g* for 10 min at 4°C and washed five times with a detergent solution containing 150 mM NaCl, 10 mM Tris/HCl, 1% Triton X-100, 1% deoxycholate, and 0.1% SDS, pH 7.2 (method A). Alternatively (method B), after incubation of the extract with antisynthase antiserum, the immunoprecipitates were sedimented with 30 μl of 10% (w/v) fixed *Staphylococcus aureus* cells (Bethesda Research, Gaithersburg, Md.) and washed as described in method A. While the use of *Staph. aureus* cells increased the recovery of antigen from mutant fibroblast extracts, it also resulted in nonspecific precipitation of other radiolabeled proteins. These were solubilized by washing the immunoprecipitate in 0.025 N NH_4OH, pH ~11, for 30 min at room temperature. The immunoprecipitates were dissolved for SDS/ polyacrylamide gel electrophoresis by boiling for 2 min in buffer containing 1% SDS and 5% β-mercaptoethanol [5].

Preparation of mRNA and in Vitro Synthesis of Cystathionine β-Synthase

Roller bottles (850 cm^2, Corning) containing confluent monolayers of fibroblasts from two controls, four CRM$^+$ mutants (339, 366, 375, 676), three CRM$^-$ mutants (342, 344, 599), and two obligate heterozygotes (the parents of 366) were harvested under sterile conditions (10–16 bottles per cell line). The cell pellets, weighing 1.5–5.2 g, were stored at −70°C until used. Total RNA was prepared as described by Chirgwin et al. [7], except that the frozen cells were broken by sonication. Polyadenylated RNA was isolated by oligo(dT)cellulose chromatography according to the supplier's instructions (type 2, Collaborative Research, Lexington, Mass.). The poly(A$^+$) RNA (~100 μg) was then fractionated on a linear sucrose gradient as described by Kraus and Rosenberg [8]. Each fraction was precipitated with ethanol after addition of 12 μg of carrier calf liver tRNA (Boehringer-Mannheim, Indianapolis, Ind.) and NaCl to a final concentration of 200 mM. The precipitates were washed with 70% ethanol and, after lyophilization, dissolved in 50 μl of sterile H_2O. In vitro translations of 3 μl of mRNA from each fraction were performed using a rabbit reticulocyte lysate translation kit according to the supplier's instructions (Bethesda Research). Each translation (40 μl) contained 75 μCi of L-[^{35}S]methionine (Amersham). All immunoprecipitations of in vitro synthesized polypeptides were performed as described [8].

Polyacrylamide Gel Electrophoresis and Fluorography

Electrophoresis in SDS/9% polyacrylamide gels was performed according to Laemmli [9]. Fluorography was done on dried gels following impregnation with Autofluor (National Diagnostics, Somerville, N.J.).

RESULTS

Immunoprecipitation of Mutant Cystathionine β-Synthase from Fibroblast Extracts

Figure 1 shows an SDS/polyacrylamide gel electrophoresis of immunoprecipitated synthase from control and seven CRM$^+$ mutant fibroblast extracts. Pre-

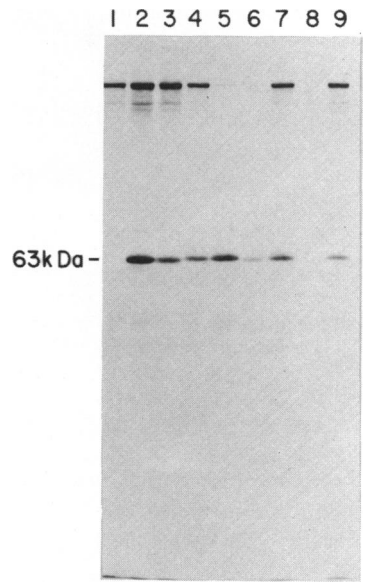

FIG. 1.—Immunoprecipitation of cystathionine β-synthase subunits from extracts of radiolabeled control and steady-state CRM^+ mutant fibroblasts. Fluorogram of SDS/9% polyacrylamide gel was exposed for 64 hrs. Synthase was immunoprecipitated with extracts containing equal amounts of acid-precipitable radioactivity. *Lane 1*, control extract precipitated with normal rabbit serum; *lane 2*, control extract precipitated with antisynthase antiserum. Extracts of the following mutant fibroblast lines were precipitated with antisynthase serum: *lane 3*, 676; *lane 4*, 375; *lane 5*, 339; *lane 6*, 42; *lane 7*, 338; lane 8, 366; *lane 9*, 382.

cipitation of radiolabeled control extract with normal rabbit serum often yielded an artifactual protein band of high molecular weight (lane 1). In all mutants (lanes 3–9) except mutant 366 (lane 8), the radiolabeled polypeptides specifically precipitated by antisynthase antiserum had apparent M_r of 63,000, identical to that observed in the control (lane 2). Similar findings were obtained from study of the other seven CRM^+ mutants (results not shown). Under these conditions, mutant 366 (lane 8) yielded no detectable band. After prolonged fluorography (6 weeks), however, an M_r = 63,000 band was observed in addition to another, smaller band with an M_r of ~56,000. Visual comparison of the radiolabeled synthase subunits from extracts containing equal amounts of acid-precipitable radioactivity revealed that all mutant extracts contained substantially less radiolabeled synthase than did the control. No further attempts were made to quantitate the newly synthesized radiolabeled synthase subunits in the pulsed mutant extracts, or to correlate the data shown in figure 1 with the previously determined CRM values that reflected the steady-state levels of immunologically identifiable synthase present after several days of growth in cell culture [5].

The three mutant cell lines previously found to have no detectable steady-state cross-reacting material [5] were studied by two methods of immunoprecipitation: sedimentation of immunocomplexes by centrifugation (method A) and sedimentation by centrifugation after addition of *Staph. aureus* cells (method B). Figure 2 compares the results obtained using these methods on identical extracts from

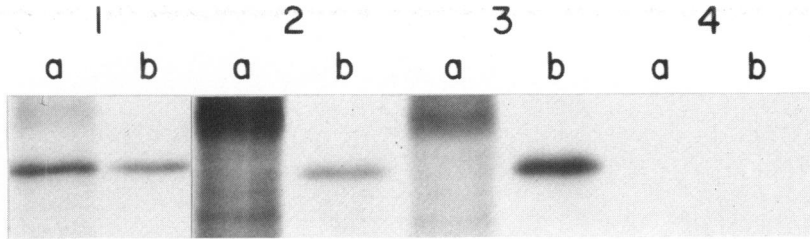

FIG. 2.—Immunoprecipitation of cystathionine β-synthase subunits from extracts of radiolabeled control and steady-state CRM⁻ mutant fibroblasts. Fluorogram of SDS/9% polyacrylamide gel. See MATERIALS AND METHODS for details of immunoprecipitation methods A (*lanes a*) and B (*lanes b*). *Lanes 1a* and *b*, control extracts (fluorogram exposed 24 hrs); *lanes 2a* and *b*, extracts of mutant fibroblast line 342; *lanes 3a* and *b*, extracts of mutant fibroblasts 344; *lanes 4a* and *b*, extracts of mutant fibroblasts 599. Fluorogram of *lanes 2–4* was exposed for 72 hrs.

control and mutant fibroblasts. Whereas the recovery of synthase subunit from control extract was equal with the two methods (lanes 1a and b), only method B resulted in the precipitation of an M_r = 63,000 polypeptide in mutants 342 (lane 2b) and 344 (lane 3b). No immunoprecipitable product was found in extracts from mutant line 599 with either method (lanes 4a and b).

Immunoprecipitation of Mutant Cystathionine β-Synthase from Polypeptides Synthesized in Vitro

The cell-free synthesis of cystathionine β-synthase was achieved in a reticulocyte lysate translation system programmed with mRNA from cultured fibroblasts from selected mutants and obligate heterozygotes. Figure 3*A* shows the radiolabeled synthase subunits immunoprecipitated from in vitro translations of mRNA from control fibroblasts (lane 1) and from three CRM⁺ mutant cell lines (339, 375, 676) (lanes 2–4); figure 3*B* presents analogous findings from control (lane 1) and three CRM⁻ mutant lines (343, 344, 599) (lanes 2–4). In agreement with results presented above using pulsed intact cells, a single polypeptide with an apparent M_r of 63,000 was observed in all the mutant lines except line 599, where no radiolabeled product was recovered (fig. 3*B*, lane 4).

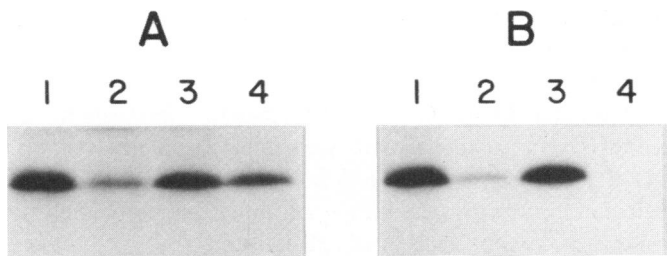

FIG. 3.—Immunoprecipitation of cystathionine β-synthase subunits synthesized in vitro using mRNAs from control and mutant cells. Fluorogram of SDS/9% polyacrylamide gel. *A*: *lane 1*, control mRNA; *lanes 2–4*, mRNA from steady-state CRM⁺ mutant fibroblast lines; *lane 2*, 676; *lane 3*, 375, *lane 4*, 339. *B*: *lane 1*, control mRNA; *lanes 2–4*, mRNA from steady-state CRM⁻ mutant fibroblast lines; *lane 2*, 342; *lane 3*, 344; *lane 4*, 599.

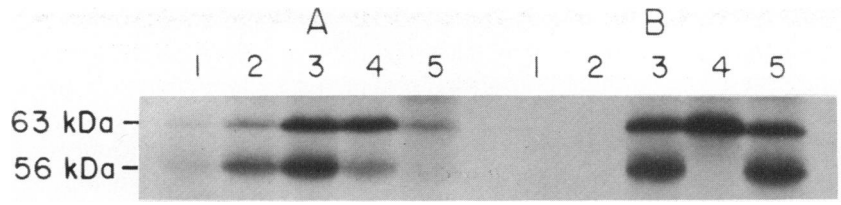

FIG. 4.—Immunoprecipitation of cystathionine β-synthase subunits synthesized in vitro using mRNA from mutant cell line 366 and from his parents' lines. Fluorogram of SDS/9% polyacrylamide gel was exposed for 2 days. *A*: *lanes 1–5*, fractions 16–20 of mRNA from mutant 366 separated by sucrose gradient centrifugation. *B*: *lanes 1–3*, mRNA from mutant 366 (same gradient fraction as used in *A*, *lane 3*); *lane 1*, precipitated with normal rabbit serum; *lane 2*, precipitated with antisynthase antiserum in the presence of excess purified human liver synthase; *lane 3*, precipitated with antisynthase antiserum; *lane 4*, immunoprecipitation of polypeptides made using the peak gradient fraction of synthase mRNA from the mother's cultured fibroblasts; *lane 5*, immunoprecipitation of polypeptides made using the peak gradient fraction of synthase mRNA from the father's cultured fibroblasts.

Identification of Compound Heterozygote

When mRNA from mutant 366 (fig. 1, lane 8) was fractionated on a sucrose gradient and an aliquot of each fraction was translated in vitro, two polypeptides in about equal amounts and with M_r's of 63,000 and 56,000, respectively, were precipitated by antisynthase antiserum (fig. 4*A*). The slightly earlier elution from the sucrose gradient of the mRNA coding for the M_r = 56,000 subunit (fig. 4*A*) indicates that the two polypeptides were encoded by two different mRNAs. Neither polypeptide was recovered when cell-free translations were incubated with normal rabbit serum (fig. 4*B*, lane 1) or with antisynthase antiserum in the presence of excess human liver synthase (fig. 4*B*, lane 2). The simplest explanation for these findings is that cultured fibroblasts from mutant 366 contain two mRNAs, separable by size, coding for two synthase subunits differing in molecular weight by 7,000.

We then proceeded to investigate the mRNAs prepared from cultured fibroblasts obtained from the parents of patient 366. When mRNA from the father's cells was fractionated, translated in vitro and immunoprecipitated for synthase, two polypeptides with the same mobilities as those in the synthase-deficient patient were found (fig. 4*B*, lane 5), whereas only synthase subunits with a normal M_r of 63,000 were immunoprecipitated using mRNA prepared from the mother's cultured fibroblasts (fig. 4*B*, lane 4). These findings demonstrate that patient 366 inherited the mutant allele coding for the abnormally small synthase subunit from his father.

DISCUSSION

Following the description of homocystinuria and the elucidation of its biochemical basis 2 decades ago [10–12], characterization of cystathionine β-synthase deficiency focused on the catalytic abnormalities of mutant enzymes [1]. An antiserum raised against pure synthase from human liver enabled us recently to quantitate the steady-state amounts of synthase antigen in cultured mutant fibroblasts. Out of 20 such cell lines examined, only three were devoid of detectable cross-reacting antigen [5]. We then documented that synthase in cultured skin fibroblasts from normal individuals is composed of identical subunits with an M_r of 63,000

[3]. The results described in the present study represent the subsequent step in our investigation of synthase deficiency and the first analysis at the subunit level of a large series of cultured fibroblasts from patients with homocystinuria due to deficiency of cystathionine β-synthase.

All but two of the 17 mutant cell lines currently studied contained only synthase subunits with the same apparent molecular weight as the normal subunit: 63,000. Although SDS/polyacrylamide gel electrophoresis does not detect abnormalities in size due to very small (i.e., few amino acids) deletions, our results nevertheless support our previously held notion that most mutant synthase molecules have substitutions of a single amino acid as a result of missense mutations in the structural gene.

Our previous analysis of synthase mutants by competitive immunotitration indicated that among six cell lines without measurable catalytic activity, three contained no detectable synthase antigen (cell lines 342, 344, 599) [5]. These results were initially confirmed by experiments when centrifugation alone was used to sediment mutant synthase-antisynthase antibody complexes. When, in addition, *Staph. aureus* cells were employed to sediment immunocomplexes, synthase subunits with a normal molecular weight were clearly observed in cell lines 342 and 344. Why, then, do these cell lines contain no "steady-state" CRM? Perhaps the interaction between these two mutant antigens and antisynthase antiserum is so weak that no immunocomplexes form in the presence of the competing normal synthase molecules used in the "steady-state" CRM assay [5], whereas the immunocomplexes formed in the absence of normal synthase can be recovered when a vehicle such as *Staph. aureus* cells is used. Alternatively, the synthase subunits formed by these two mutant lines may be so rapidly degraded that they are absent under steady-state conditions. These results underscore the limited interpretive value of undetectable steady-state CRM values with regard to the primary molecular lesion in a mutant line. No synthase subunit and no functional synthase mRNA were detected in mutant line 599. Many mechanisms, including a partial or complete gene deletion, abnormal mRNA processing or transport, could account for the synthase deficiency in this mutant.

Previous immunochemical studies of mutant cell line 366, derived from a mildly affected, pyridoxine-responsive, male homocystinuric patient with no detectable catalytic activity in extracts of cultured fibroblasts, demonstrated a small amount (~13%) of cross-reacting material [5]. Immunoprecipitation of extracts from his radiolabeled fibroblasts showed barely detectable synthase subunits. When mRNA was prepared from cultured fibroblasts and translated in vitro, however, the molecular pathology of this patient's synthase deficiency was more clearly revealed. Antisynthase antiserum precipitated two polypeptides differing in molecular weight by 7,000. In addition to the synthase subunit of normal size (M_r = 63,000), an abnormally small M_r = 56,000 polypeptide was also found in cell-free translations of size-fractionated mRNAs prepared from the patient's cultured fibroblasts and from those of his father, whereas only synthase subunits with a normal molecular weight were observed when mRNA prepared from the mother's cultured fibroblasts was used. These results demonstrate unequivocally that mutant 366 is a compound heterozygote. The M_r = 56,000

synthase subunit, whose gene was transmitted to the patient from his father, could result from either a partial gene deletion or a single base change leading to a smaller mRNA possibly by a generation of a new splice site. Furthermore, the easily detectable amounts of immunoprecipitable mutant polypeptides found after in vitro translation of this patient's mRNA (fig. 4) contrast with the barely detectable amounts of mutant synthase recovered from extracts of whole cells. This suggests that his molecular defect does not involve the rate of synthesis of the mutant enzyme subunits; rather, the scant amount of immunoprecipitable material found under steady-state conditions is more likely caused by instability of the mutant enzyme or its subunits in intact cells and/or in cell extracts.

Future studies, using methods more sensitive to change in charge and molecular weight than SDS/polyacrylamide gel electrophoresis, are likely to show that the majority of synthase mutants are compound heterozygotes. Once a suitable probe is available, however, recombinant DNA techniques might more easily identify compound heterozygosity at the gene level [13]. The availability of pure rat liver synthase mRNA in our laboratory [8] should make this approach feasible.

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