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Genetics

Molecular cloning of L-methylmalonyl-CoA mutase: Gene transfer and analysis of *mut* cell lines

(methylmalonic acidemia/inborn errors of metabolism/cobalamin/cDNA/gene expression)

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ABSTRACT **L-Methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) is a mitochondrial adenosylcobalamin-requiring enzyme that catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA. This enzyme is deficient in methylmalonic acidemia, an often fatal disorder of organic acid metabolism. Antibody against human placental MCM was used to screen human placenta and liver cDNA expression libraries for MCM cDNA clones. One clone expressed epitopes that could affinity-purify antibodies against MCM. A cDNA corresponding in length to the mRNA was obtained and introduced into COS cells by DNA-mediated gene transfer. Cells transformed with this clone expressed increased levels of MCM enzymatic activity. RNA blot analysis of cells genetically deficient in MCM indicates that several deficient cell lines have a specific decrease in the amount of hybridizable mRNA. These data confirm the authenticity of the MCM cDNA clone, establish the feasibility of constituting MCM activity by gene transfer for biochemical analysis and gene therapy, and provide a preliminary picture of the genotypic spectrum underlying MCM deficiency.**

L-Methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) catalyzes the isomerization of L-methylmalonyl-CoA to succinyl-CoA, which is an intermediate step in the catabolism of valine, isoleucine, threonine, and methionine, odd-chain fatty acids, and cholesterol (1). Genetic deficiency of MCM leads to methylmalonic acidemia (MMA), in which there is accumulation of precursors and abnormal metabolites of L-methylmalonyl-CoA. Severe forms of MMA can be associated with fulminant metabolic acidosis, widespread secondary aberrations in metabolic homeostasis, mental retardation, or neonatal death (1, 2). Benign forms of MCM deficiency can be associated with methylmalonic aciduria without detrimental effects (3).

MCM is a mitochondrial enzyme that requires adenosylcobalamin (vitamin B_{12}) as a cofactor (1). There are several distinct phenotypes of MCM apoenzyme deficiency (designated *mut*). Some individuals exhibit residual apoenzyme activity with abnormal kinetics, stability, or posttranslational processing (designated *mut*$^-$), whereas others have no detectable apoenzyme activity (designated *mut*^{0}) (1, 2). Dietary deficiency of vitamin B_{12}, or defects in transport or derivatization of vitamin B_{12} (designated *cbl*), lead to secondary defects in MCM activity.

MCM has been purified from human placenta (4) and liver (5). The mature protein is a homodimer of 72,000- to 77,000-Da subunits (4, 5). We report cloning of a cDNA for human MCM from a human liver cDNA library, use of this clone to constitute human MCM enzymatic activity in cultured cells by DNA-mediated gene transfer, and preliminary analysis of MCM mRNA expression in various forms of MMA.

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MATERIALS AND METHODS

Purified Human MCM and Anti-MCM Antibody. MCM was purified from human placenta by affinity chromatography on 5′ deoxyadenosylcobalamin-Sepharose and DEAE-cellulose anion-exchange chromatography as described (4). The purified material comprises a single band of 72,000 Da on Coomassie blue-stained $NaDodSO_4$/polyacrylamide gels. Chicken antisera against human MCM have been described (4, 6). These antisera exhibited crossreactivity with human albumin that was removed by affinity chromatography against human albumin (Sigma) attached to cyanogen bromide-activated Sepharose 4B (Pharmacia). After five passes over the affinity column, the antisera exhibited no crossreactivity with human albumin on electrophoretic transfer blots.

Screening Human cDNA Libraries. Construction of a human liver cDNA library in λgt11 has been described (7). A human placenta cDNA library in λgt11 was provided by Brian Knoll (University of Texas Health Science Center, Houston) (8). Both libraries were screened with chicken anti-human MCM antibody and ^{125}I-labeled goat anti-chicken IgG by using a modification of the method of Young and Davis (9–12). The human liver cDNA library was rescreened with ^{32}P-labeled DNA fragments as described (7). Screening methods are described (13).

Epitope Selection. Epitope selection was used to affinity-purify antibodies reacting with the λgt11 fusion proteins (14). Two thousand plaque-forming units of phage were plated on 100-mm plates. Phage were grown 5 hr at 42°C, overlaid with nitrocellulose filters presoaked in 10 mM isopropyl thiogalactopyranoside, incubated 1 hr at 37°C, and then incubated at 4°C overnight. Filters were incubated sequentially in Blotto for 20 min (10), in Blotto with 50 μl of chicken anti-MCM overnight, and three times in 50 mM Tris·HCl/150 mM NaCl/0.5% Triton X-100. Antibody was eluted in three 1-min washes in 3 ml of 5 mM glycine HCl, pH 2.3/150 mM NaCl/0.5% Triton X-100/100 μg of bovine serum albumin per ml neutralized with 1 ml of 200 mM Tris·HCl (pH 7.4). Protein standards (Bio-Rad low molecular weight) included lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine albumin, and phosphorylase B.

DNA-Mediated Gene Transfer. The vector 91023B, which contains the adenovirus major late promoter, tripartite leader sequence, and Va genes, was provided by Randy Kaufman (Genetics Institute) (15, 16). Partial *Eco*RI fragments of MCM cDNA or human phenylalanine hydroxylase (PAH) cDNA (PAH247; ref. 17) were subcloned into the *Eco*RI site. Plasmids were transfected into the COS cell line (18) by calcium/phosphate coprecipitation (19, 20).

Abbreviations: MCM, L-methylmalonyl-CoA mutase; MMA, methylmalonic acidemia; PAH, phenylalanine hydroxylase; CRM, crossreactive material.

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Analytical Procedures. Cell extracts were prepared 48 hr after transfection by scraping, resuspension in 28 mM $NaPO_4$, pH 7.0/5 mM EDTA, sonication 3 × 30 sec, and centrifugation at 13,000 rpm in an Eppendorf microcentrifuge to remove particulate matter. MCM activity was assayed in the presence of adenosylcobalamin by using the perchloric acid/potassium permanganate method as described (21). Immunoreactive protein was identified after $NaDodSO_4$/polyacrylamide gel electrophoresis and electrophoretic transfer as described (22). Total RNA was prepared from cultured cells by using hot phenol (23). RNA transfer blotting was performed after formaldehyde/agarose gel electrophoresis as described (24, 25) by using MCM cDNA probes or a β-actin probe provided by Robert Schwartz (Baylor College of Medicine). Protein concentrations were determined by using the Bio-Rad protein assay.

Cell Lines. Cell lines GM2452, GM930, GM1673, and GM50 were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository. Cell lines 87-1645, 79-865, 81-1085, 87-1640, and 82-1211 were provided by Mark Batshaw and David Valle (The Johns Hopkins University School of Medicine).

RESULTS

Screening for MCM cDNA Clones. Chicken anti-MCM preabsorbed against human albumin identified a single band of crossreactive material (CRM) in extracts of human liver (Fig. 1, lane 1) that comigrated with purified MCM (Fig. 1, lane 2). This antibody was used to screen the human liver and placenta λgt11 cDNA libraries. Six independent clones were identified. No cross-hybridization was observed among the six clones on Southern blotting (data not shown), indicating that each clone represented a distinct cDNA species.

Identification of a MCM Clone by Epitope Selection. To identify authentic MCM clones, the chicken anti-MCM antibody was affinity-purified with each of the six clones by epitope selection, and the affinity-purified antibodies were used as probes against purified MCM protein or equal concentrations of protein standards. Antibodies selected by using clone MCM105 reacted with MCM protein (Fig. 1, lane 3) but not with protein standards (Fig. 1, lane 4). Antibodies selected by using the other clones did not react with MCM, though several showed weak reaction with albumin (one example is shown: Fig. 1, lanes 5 and 6). As a control, goat anti-PAH antiserum (26) was affinity-purified with clone MCM105 or a rabbit tryptophan hydroxylase clone pTRH479 (11). Anti-PAH, selected with MCM105, did not crossreact

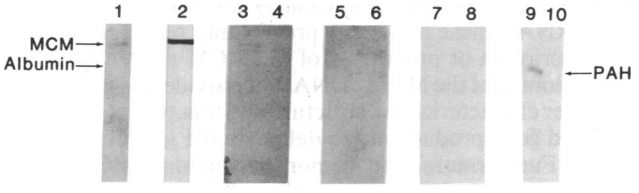

FIG. 1. Identification of MCM cDNA clones by epitope selection. Lane 1, MCM in human liver identified with chicken anti-MCM antibody. Lane 2, purified MCM stained with Coomassie blue. Lanes 3 and 4, antibody selected against clone MCM105 showing reactivity with purified MCM (lane 3) but not protein standards (lane 4). Lanes 5 and 6, antibody selected by using a putative clone from the placenta cDNA library showing no reactivity to purified MCM (lane 5) but crossreactivity to albumin (lane 6). Lanes 7 and 8, control experiment: anti-PAH epitope selected against clone MCM105 showing the absence of reactivity to either purified MCM (lane 7) or protein standards (lane 8). Lanes 9 and 10, control experiment: anti-PAH selected against rabbit tryptophan hydroxylase showing reactivity to human liver protein (100 μg, lane 9) but not protein standards (lane 10).

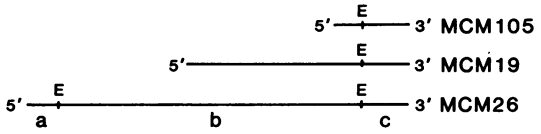

FIG. 2. Schematic of MCM clones. Clone MCM105, isolated by antibody screening, contains two internal *Eco*RI fragments (b and c). Clones MCM19 and MCM26, isolated by screening with MCM105, contain *Eco*RI fragments hybridizing to fragments b and c. Clone MCM26 contains an additional fragment (a) representing the 5′ end of the cDNA.

with MCM or the protein standards (Fig. 1, lanes 7 and 8), whereas anti-PAH selected against TRH479 reacted strongly with PAH in rat liver extracts (Fig. 1, lane 9). These results indicated that clone MCM105 expressed specific epitopes common to purified MCM.

Identification of MCM mRNA in Human Liver. *Eco*RI fragments from each of the six clones were hybridized to total human liver RNA. Two *Eco*RI fragments from clone MCM-105 (designated b and c, Fig. 2) each hybridized to mRNA large enough to encode a protein of 72,000–77,000 Da (Fig. 3, lanes 2 and 3). The other clones hybridized to smaller mRNA species (one example is shown: Fig. 3, lane 4).

Gene Transfer and Expression of MCM cDNA. The 600-base-pair *Eco*RI fragment from clone MCM105 was used to screen one million plaques from the human liver cDNA library. Twenty positive phage were identified. The two phage with the longest inserts (MCM19 and MCM26) contained *Eco*RI fragments (a and b) that corresponded to the two *Eco*RI fragments of clone MCM105 (Fig. 2). Clone MCM26 contained an additional *Eco*RI fragment (a) (Fig. 2) that did not hybridize with MCM105 or the other clones. Fragment a hybridized to mRNA that comigrated with that identified by the b or c fragment (Fig. 3).

Partial *Eco*RI digestion was performed on phage MCM26 and fragments were cloned into the expression vector 91023B. These subclones were transfected into COS cells, and cellular extracts were assayed for MCM activity. The results of two independent experiments are given in Table 1. Low levels of MCM are present in COS cells (a derivative of the monkey kidney cell line CV-1). The level of activity was increased 2- to 5-fold by transfection with subclone MCM60 containing fragments a and b (Table 1). This result indicated that the a and b fragments contained a functional open reading frame and were present in MCM60 in a sense orientation. No increase in activity occurred following transfection with subclone MCM75 or MCM62 containing fragments b and c in different orientations or with human PAH cDNA as a control (Table 1).

MCM cDNA in Normal and Deficient Fibroblasts. The b fragment of clone MCM26 was used as a probe for hybridizable mRNA in a series of *mut* or *cbl* cell lines (Fig. 4*A*). Hybridizable mRNA was present in cell lines from individ-

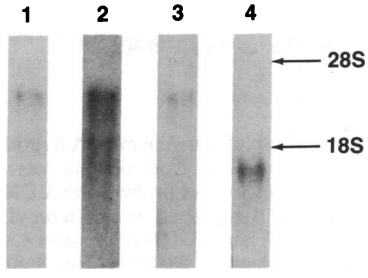

FIG. 3. RNA transfer blot of human liver RNA (20 μg) probed with *Eco*RI fragments from MCM clones. Lane 1, fragment a; lane 2, fragment b; lane 3, fragment c; lane 4, RNA probed with a putative clone from placental library.

Table 1. MCM activity in COS cells following DNA-mediated gene transfer of partial fragments from MCM cDNA

Sample	MCM activity, nmol of succinate formed per mg of protein	
	Experiment 1	Experiment 2
Human liver	37	80
COS cells	—	5.6
+ PAH	7.1	—
+ MCM60	16.9	37
+ MCM62	3.0	—
+ MCM75	7.3	10.3

COS cells were transfected with plasmids containing the 91023B expression vector and partial *Eco*RI fragments from clone MCM26 or human PAH cDNA. MCM enzyme activity was assayed in sonicated cellular extracts. Clone MCM60 contains fragments a and b; clones MCM75 and MCM62 contain fragments b and c in opposite orientations. The results of two independent transfection experiments are shown.

uals deficient in cobalamin metabolism (GM2452, lane 1; 81-1085, lane 6) that contain normal amounts of MCM apoenzyme activity. Hybridizable MCM mRNA was present in several *mut* cell lines (GM930, lane 2; 79-865, lane 5; 82-1211, lane 7; 81-1085, lane 8; 87-1640, lane 9) though in two other cell lines (GM1673, lane 3; GM50, lane 4) hybridizable mRNA was absent or present at extremely low levels. To demonstrate that the difference in hybridizable MCM mRNA in these cell lines was specific, the same filter was reprobed with a cDNA to β-actin (Fig. 4*B*). β-Actin mRNA was present in all lanes at levels equivalent to, or higher than, levels in the control *cbl* fibroblast cell lines.

DISCUSSION

This work describes the cloning of a cDNA for human MCM from a human liver cDNA library by using an antiserum against placental MCM as a probe. This antiserum was not monospecific, as evidenced by the fact that it recognized albumin and six unrelated cDNA species in the human and placenta cDNA libraries. Several independent lines of evidence indicate that the MCM clone identified in this report represents an authentic MCM cDNA. (*i*) This clone could be used to epitope-select antibodies crossreactive with partially purified MCM. (*ii*) This clone hybridized to mRNA species sufficiently large to contain the open reading frame for a 72,000- to 77,000-Da protein. (*iii*) A cDNA of corresponding length was able to increase MCM enzymatic activity in cultured cells following DNA-mediated gene transfer. (*iv*) Several cell lines that are deficient in MCM enzymatic activity have a specific decrease in the amount of hybridizable mRNA with the MCM26 b fragment probe. We were unable to obtain amino acid sequence data from purified MCM and thus are unable to confirm the authenticity of this clone by direct comparison of protein and nucleic acid sequences.

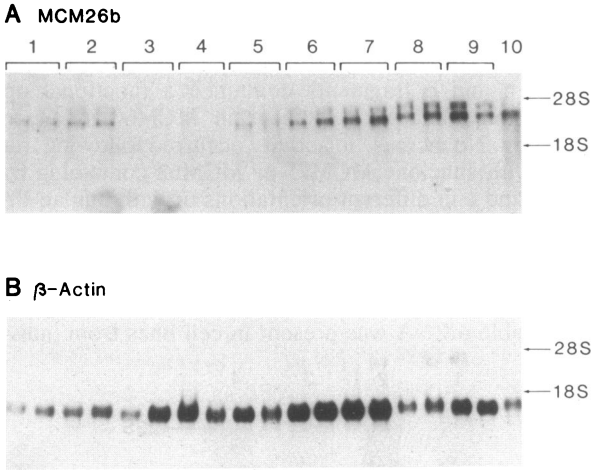

FIG. 4. RNA transfer blot showing mRNA hybridizable with the MCM26 b fragment in fibroblasts from nine patients with MMA and human liver. (*A*) RNA transfer blot probed with MCM26 b fragment. (*B*) The same filter as in *A* reprobed with β-actin cDNA. Lanes 1, GM2452; lanes 2, GM930; lanes 3, GM1673; lanes 4, GM50; lanes 5, 79-865; lanes 6, 87-1645; lanes 7, 82-1211; lanes 8, 81-1085; lanes 9, 87-1640; lane 10, human liver. RNA was prepared from primary fibroblast cell lines from patients with *mut* or *cbl* defects and from human liver. Duplicate samples representing independent isolations of RNA are shown. Lanes 1–9 contain 20 μg of total cellular RNA and lane 10 contains 10 μg of total liver RNA.

The MCM cDNA clone MCM26 contains three *Eco*RI fragments (Fig. 3). All three fragments hybridize to identical mRNA species, and the length of clone MCM26, ≈2400 bases, corresponds to the length of this mRNA. Subclone MCM60, which directs the expression of MCM enzymatic activity, contains only two of the three *Eco*RI fragments, a and b. Thus, these two fragments, comprising ≈2000 bases, apparently contain the entire open reading frame and represent the 5′ end of the cDNA. The third fragment presumably contains 3′ untranslated sequences. This orientation has been confirmed by preliminary sequencing that identified a poly(A) tail on the c fragment. A doublet of hybridizing mRNA is observed in fibroblast mRNA probed with MCM26 (Fig. 4, lanes 1–9) that is less prominent in liver mRNA (Fig. 3, lanes 1–3; Fig. 4, lane 10). All hybridizing bands are coincidentally decreased in several *mut* fibroblast cell lines, suggesting that this pleomorphism represents alternative processing of mRNA from a single locus.

The observation that some MCM-deficient cell lines contain absent or decreased hybridizing mRNA extends the recognized heterogeneity within *mut* MCM deficiency. Previous studies have identified *mut* cell lines that express no detectable CRM (6), CRM without enzyme activity (6), or enzyme activity with abnormal kinetics, abnormal stability (27), or abnormal posttranslational modification (28). Cell lines 81-1085 (a *cbl-B* mutant) and GM2453 (a *cblD* mutant) that have normal apoenzyme activity have normal hybridizable mRNA (6). Cell lines 79-865 and 82-1211, from individuals with clinically mild disease and mut^- deficiency and cell lines 87-1640 and 87-1645, from individuals with clinically mild vitamin B_{12} unresponsive MMA, have hybridizable mRNA as does cell line GM930. In contrast, cell line GM1673, a mut^0 line that is known to be CRM^- (6), and cell line GM50 have absent or extremely low levels of hybridizable mRNA. These mutations presumably represent defects in transcription or processing of the MCM mRNA.

The cloning of the MCM cDNA will provide an opportunity to further characterize the structure and function of the MCM gene and gene product and to delineate the genotype of *mut* MMA. Furthermore, the demonstration that MCM enzymatic activity can be constituted by gene transfer introduces the possibility of somatic gene therapy (29, 30) for this disorder, which continues to have high morbidity and mortality despite conventional therapy (2).

Note Added in Proof. The authenticity of the human MCM cDNA clones has been confirmed by identity between portions of the amino acid sequence predicted from the cDNA sequence and the amino acid sequence of several tryptic fragments of the human liver methylmalonyl CoA mutase protein determined by Leon Rosenberg and his colleagues (W. A. Fenton, F. Kalousek, and L. E. Rosenberg, personal communication).

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