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Nucleotide sequence of medium-chain acyl-CoA dehydrogenase mRNA and its expression in enzyme-deficient human tissue

(carnitine deficiency/mitochondria/cDNA clone/β-oxidation/genetic defect)

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ABSTRACT **Medium-chain acyl-CoA dehydrogenase (MCAD; acyl-CoA:(acceptor) 2,3-oxidoreductase, EC 1.3.99.3) is one of three similar enzymes that catalyze the initial step of fatty acid β-oxidation. Definition of the primary structure of MCAD and the tissue distribution of its mRNA is of biochemical and clinical importance because of the recent recognition of inherited MCAD deficiency in humans. The MCAD mRNA nucleotide sequence was determined from two overlapping cDNA clones isolated from human liver and placental cDNA libraries, respectively. The MCAD mRNA includes a 1263-base-pair coding region and a 738-base-pair 3′-nontranslated region. A partial amino acid sequence (137 residues) determined on peptides derived from MCAD purified from porcine liver confirmed the identity of the cDNA clone. Comparison of the amino acid sequence predicted from the human MCAD cDNA with the partial protein sequence of the porcine MCAD revealed a high degree (88%) of interspecies sequence identity. RNA blot analysis shows that MCAD mRNA is expressed in a variety of rat (2.2 kilobases) and human (2.4 kilobases) tissues. Blot hybridization of RNA prepared from cultured skin fibroblasts from a patient with MCAD deficiency disclosed that mRNA was present and of similar size to MCAD mRNA derived from control fibroblasts. The isolation and characterization of MCAD cDNA is an important step in the definition of the defect underlying MCAD deficiency and in understanding its metabolic consequences.**

The acyl-CoA dehydrogenases are mitochondrial flavoenzymes that catalyze the initial step in fatty acid β-oxidation. This reaction involves the 1,2-dehydrogenation of acyl-CoA thioesters with formation of the *trans*-2-enoyl-CoA product (1). Medium-chain acyl-CoA dehydrogenase (MCAD; acyl-CoA:(acceptor) 2,3-oxidoreductase EC 1.3.99.3) is one of three straight-chain acyl-CoA dehydrogenases present in mammalian tissues (1). MCAD is active with acyl chain lengths of C_4–C_{16} and exhibits overlap in substrate range with the other two enzymes, short- and long-chain acyl-CoA dehydrogenase (SCAD, LCAD), depending on the species studied (2–6). Isolation and purification of these three enzymes have been accomplished in a variety of species (2–6). The enzymes have remarkably similar molecular weights, isoelectric points, and amino acid compositions (3, 6). Despite these similar properties, each enzyme has a distinct pattern of substrate specificity. Moreover, cross-reactivity among antisera raised against the individual rat proteins is not observed (6). The tissue distribution and primary structure of these enzymes have not been determined in any species.

Clinical syndromes secondary to genetic defects in each of the acyl-CoA dehydrogenases have been described recently and are now recognized as secondary causes of the systemic carnitine deficiency syndrome (7–13). Assays performed on peripheral blood leukocytes and skin fibroblasts of these patients reveal marked reductions in the activity of the individual acyl-CoA dehydrogenases (12, 13). Clinical features of MCAD deficiency include fasting hypoglycemia, hepatic dysfunction, and encephalopathy, often resulting in death in infancy (14, 15).

The steady-state levels of MCAD protein and its mRNA have not been evaluated in MCAD-deficient tissues nor has the defect been defined at the molecular level in any patient. Evaluation of MCAD synthesis in cultured skin fibroblasts from 13 enzyme-deficient patients demonstrated MCAD protein indistinguishable in subunit molecular weight compared to controls with normal MCAD activity (16). We have isolated cDNA clones encoding human MCAD as a preliminary step in determining the molecular defects in MCAD deficiency and to define the primary structure and tissue-specific expression of MCAD. We report the complete cDNA-derived amino acid sequence of MCAD and the expression of the mRNA encoding MCAD in tissues with normal MCAD activity and in the cultured skin fibroblasts from a MCAD-deficient patient.

MATERIALS AND METHODS

Protein Purification, Determination of Amino Acid Sequence of Porcine MCAD, and Preparation of Antiserum. MCAD was purified to homogeneity ($A_{275/450}$ = 5.16) from mitochondria isolated from pig liver using a modification of the protocol described by Hall and Kamin (17). Assays were done using electron transfer flavoprotein as the intermediate electron acceptor as described by Beinert (18). Relative activities with substrate acyl chain lengths $C_4/C_8/C_{16}$ were 0.15/1/0.15, respectively.

Purified pig liver mitochondrial MCAD protein (6 nmol) was digested with trypsin or cyanogen bromide (19). The cyanogen bromide digests were preceded by reduction and carboxymethylation (20). After separation by reverse-phase HPLC (19), the peptides were subjected to automated Edman degradation in an Applied Biosystems (Foster City, CA) gas-phase sequenator (470A) using the "no-vac" program as described by the manufacturer. Initial yield was at least 60%, with repetitive yields of at least 94% per cycle. The phenylthiohydantoin-derivatized amino acids present in each cycle were identified by HPLC analysis (21).

Rat liver MCAD, SCAD, and LCAD were purified by the method of Ikeda *et al.* (6) for use in the Ouchterlony analysis.

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Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; LCAD, long-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase.

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Mitochondria were isolated from human liver by the method of de Duve *et al.* (22). The isolated mitochondria were ruptured by freeze–thawing in 5 mM potassium phosphate (pH 8.0).

Anti-porcine MCAD was prepared by primary immunization with 500 μg of purified protein in an equal volume of complete Freund's adjuvant injected intraperitoneally into a female New Zealand White rabbit. A booster injection of 250 μg of purified protein in incomplete Freund's adjuvant was administered 4 wk later.

Isolation, DNA Sequence Analysis, and Blot Analyses of the Human MCAD cDNA Clone. A cDNA library derived from mRNA isolated from human liver constructed in the vector λgt11 was kindly provided by Savio Woo (Baylor University). The library was screened with MCAD antibody using the protocol described by Young and Davis (23) with ^{125}I-labeled staphylococcal protein A (Amersham) used for antibody labeling. A second cDNA library derived from human placental mRNA, in which the cDNA was size-selected and constructed in λgt11, was kindly provided by Evan Sadler (Washington University). DNA sequencing was performed in either M13 bacteriophage vector or pUC13 and -19 plasmids using the dideoxy-chain-termination method (24). Either the M13 universal primer or synthetic oligonucleotides were used as primers. RNA was isolated from tissues and cultured skin fibroblasts using the proteinase K technique (25). Protein blot analysis was performed using anti-porcine MCAD and ^{125}I-labeled staphylococcal protein A according to the method of Burnette (26). RNA blot hybridization was performed using GeneScreen membrane (New England Nuclear) according to the manufacturer's recommended protocol. cDNA probes were prepared by labeling to high specific activity with ^{32}P as described by Feinberg and Vogelstein (27).

Fibroblast Culture and Enzyme Assays. Cultured skin fibroblasts from patient K.J. were donated by R. Hillman (Division of Genetics, Children's Hospital, Washington University Medical Center). The cells were grown in minimal essential medium with 10% fetal bovine serum at 37°C in 5% CO_2/95% air. Confluent monolayers were harvested for RNA isolation. Enzyme assays for acyl-CoA dehydrogenase activities were performed on cultured skin fibroblasts at Children's Hospital of Philadelphia by Daniel Hale and Paul Coates and are expressed as nmol of electron transfer flavoprotein reduced per min per mg of protein. Fibroblast LCAD activity from patient K.J. was normal at 2.16. MCAD activity was 0.21, <10% of normal control levels. SCAD activity was 0.74, ≈1/3rd of normal control levels.

RESULTS

Characterization of Anti-Porcine MCAD Antiserum. Due to the difficulty in obtaining human tissue, porcine MCAD was purified by our established protocol (17) and used to generate antibody. To assess the anti-MCAD titer, specificity, and reactivity across species, Ouchterlony analysis was performed. A precipitin line with purified porcine MCAD and purified rat MCAD was observed. However, no reaction with purified rat LCAD and SCAD was present (data not shown). Therefore, the anti-porcine MCAD was monospecific for this straight-chain acyl-CoA dehydrogenase and cross-reactive with rat MCAD. To evaluate antibody cross-reactivity with human tissue, the anti-porcine MCAD was tested on a protein blot of mitochondrial proteins isolated from human liver. As shown in Fig. 1, a single signal is present in the lane containing the human liver proteins at a size (M_r, 42,000) similar to that of the purified porcine MCAD. The cross-reactivity of the anti-porcine MCAD with both rat and human MCAD suggested a high degree of protein sequence homology across species and justified the use of the antiserum in the screening of a human liver cDNA library.

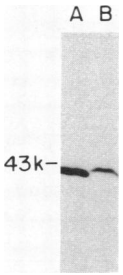

FIG. 1. MCAD protein blot analysis. Autoradiogram of a protein blot analysis after transfer of the proteins from a 12.5% NaDodSO$_4$/polyacrylamide gel. Prior to electrophoretic transfer, lane A contained 10 μg of purified porcine MCAD and lane B contained 50 μg of human liver mitochondrial proteins.

Determination of Partial Amino Acid Sequence of Porcine MCAD. Purified porcine MCAD was subjected to tryptic and cyanogen bromide digestion. After reverse-phase HPLC separation of the resultant peptides, sequence analyses were performed on 13 peptides and 137 amino acid residues were determined (see Fig. 3). Eight additional assignments were uncertain due to low yield or high background occurring during the particular cycle. NH_2-terminal sequencing was unsuccessful, presumably because the protein is blocked at this position.

Isolation of MCAD cDNA Clones and the Derived Amino Acid Sequence. Approximately 375,000 independent recombinants from a human liver cDNA library were screened with the porcine anti-MCAD antiserum. Twenty-one positive signals were present on primary screening. After secondary and tertiary screening, three putative clones were isolated. After partial restriction analysis and subcloning into M13 bacteriophage and pUC plasmid vectors, the DNA was subjected to sequence analysis. One of the three clones (M-3) was identified unequivocally as encoding MCAD by matching chemically determined amino acid sequence from porcine MCAD tryptic and cyanogen bromide peptides with that predicted by this human cDNA nucleotide sequence. The clone contains 1140 base pairs (bp), which encode MCAD and a 738-bp 3′-nontranslated region (Fig. 2). However, M-3 was only a partial clone because it did not contain the initiation codon. Therefore, a 571-bp cDNA fragment derived from the 5′ end of M-3 was used to screen ≈500,000 recombinants from the human liver library to isolate full-length clones. However, the two clones found were identical to M-3 based on restriction mapping and DNA sequence analysis. The same cDNA probe was then used to screen ≈500,000 recombinants from a human placenta library constructed from size-selected [>1 kilobase (kb)] cDNA. Of two

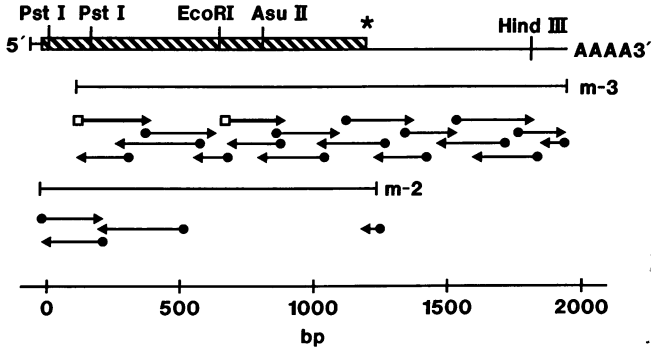

FIG. 2. Partial restriction map and sequencing strategy of clones M-3 and M-2. A region of ≈300 bp following the poly(A) tract in clone M-3, presumed to be a cloning artifact, is not shown. The map is drawn to scale. Thick arrows beginning with open boxes represent regions of DNA sequence determined with the M13 universal primer. Thin arrows beginning with closed circles denote sequence determined using synthetic oligonucleotide primers. Asterisk indicates the location of the stop codon. Hatched region denotes the coding region of the MCAD mRNA.

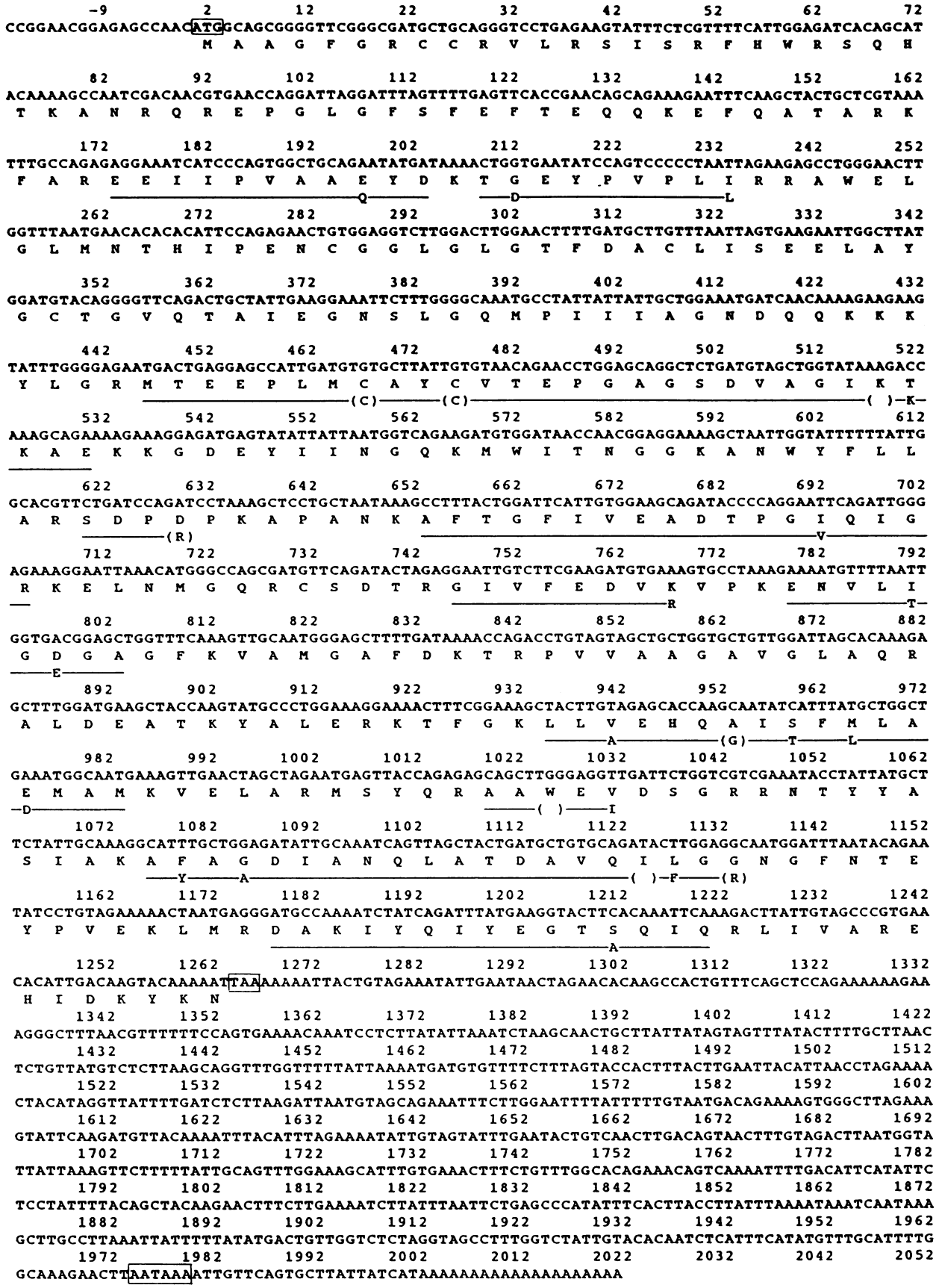

FIG. 3. Nucleotide sequence of human MCAD mRNA. The numbering is from the 5′ end to the poly(A) tract with corresponding predicted amino acid sequence. Amino acids are designated by the single-letter code. Underlined regions represent the matching porcine MCAD sequence obtained by automated Edman degradation of the peptides with substitutions as indicated. Parentheses indicate equivocal amino acid assignments. Boxes contain the initiation codon, stop codon, and polyadenylylation signal sequence. Only 20 of the >50 nucleotides of the poly(A) tract are shown.

positive clones, sequence analysis of one (M-2) confirmed that it encoded MCAD and that it contained the remaining MCAD 5'-nucleotide coding region (Fig. 2). Comparison of the DNA sequence of M-2 with that of M-3 revealed complete identity in the 350 nucleotides analyzed in the overlap regions as shown in Fig. 2. This identity supports the existence of identical MCAD proteins in the two tissues.

Partial restriction analysis and nucleotide sequencing strategy for clones M-3 and M-2 are shown in Fig. 2. The nucleotide and predicted amino acid sequences are shown in Fig. 3. The coding region contains 1263 nucleotides starting with the putative initiation codon as part of the consensus initiation sequence (CCAACATG) described by Kozak (28). The 3'-nontranslated region begins with the stop codon at position 1264 and ends in a poly(A) tract 23 bp downstream of the polyadenylylation signal sequence (AATAAA). A second potential polyadenylylation signal sequence is present at nucleotide number 1859, 149 residues prior to the poly(A) tract. The 421 amino acids derived from the cDNA sequence are displayed (Fig. 3) with corresponding amino acids from the porcine MCAD tryptic and cyanogen bromide peptides. The M_r of the precursor protein predicted by our MCAD cDNA is 46,588. The M_r of the transit peptide of MCAD is ≈4000 (16). Therefore, the predicted mature protein M_r is 42,000–43,000, consistent with the M_r of the signal present in the protein blot analysis of human tissue (Fig. 1).

Within the 30 NH_2-terminal amino acids predicted by the cDNA sequence repeating arginine residues are found, but acidic amino acids are absent. These properties are characteristic of the transit peptides described for other mitochondrial proteins (29). The COOH-terminal amino acid of the transit peptide has not been determined because the NH_2 terminus of the mature protein is blocked (see above). A likely reason for this would be cyclization of a glutamine residue, such as those found at positions 23 and 30 of the MCAD precursor. Thus, we suggest that the transit peptide ends just prior to one of these glutamines.

All 13 peptides of porcine MCAD for which chemical sequence was determined were matched with the amino acid sequence predicted by the human MCAD cDNA. In this comparison, only 17 differences were observed, resulting in 88% sequence identity on the amino acid level. As shown in Fig. 3, the majority of amino acid substitutions between species were conservative. This marked similarity is present throughout the available protein sequence, which suggests a selective pressure to maintain the entire structure. Comparison of the cDNA-derived MCAD amino acid sequence with amino acid sequences in the FAD-binding folds of other flavoproteins such as lipoamide dehydrogenase and glutathione reductase (30) shows similarity with MCAD amino acids 164–184. This region contains the amino acid sequence Gly-Ala-Gly and other essential glycine residues conserved in the α_A and β_A units of bacterial and mammalian flavin-binding regions (30). It thus may represent the FAD-binding site of this enzyme. Computer search of the National Institutes of Health protein and nucleotide sequence data banks did not reveal any significant sequence identity.

RNA Blot Hybridization Analysis. To investigate the levels of mRNA encoding MCAD in a variety of tissues, an RNA blot analysis was performed using a 571-bp cDNA probe derived from the 5' end of clone M-3. As shown in Fig. 4, a single band (2.4 kb in human and 2.2 kb in rat) is present in each tissue. The mRNA encoding MCAD is present in greatest abundance in liver in both species. The hybridization with RNA from cultured skin fibroblasts from K.J., a patient with MCAD deficiency, is also shown in Fig. 4. The mutant fibroblast MCAD mRNA is present and similar in size as compared to the MCAD mRNA in normal human liver and colon. The mutant fibroblast MCAD mRNA is also similar in size to normal control fibroblast MCAD mRNA, as shown in Fig. 5.

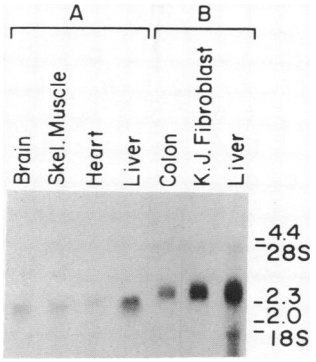

FIG. 4. RNA blot analysis of rat (*A*) and human (*B*) tissues. Ten micrograms of total RNA was separated on a 1.5% denaturing agarose gel and transferred to a GeneScreen membrane. Hybridization was performed with a ^{32}P-labeled MCAD cDNA fragment (5' *Eco*RI/*Eco*RI fragment of clone M-3, 571 bp). The lanes are as labeled; "K.J. fibroblast" RNA was isolated from cultured skin fibroblasts from a patient with MCAD deficiency. Size markers (*Hin*dIII-digested λDNA, *Hae* III-digested φX174 DNA, and ribosomal RNA) are indicated on the right.

DISCUSSION

We have isolated cDNA clones predicting the complete human MCAD amino acid sequence. Recently, partial rat and human cDNA clones were isolated and the corresponding gene was localized to human chromosome 1 (31). However, no sequence information was reported. Our MCAD clones were isolated to determine the molecular basis of MCAD deficiency, to analyze the tissue distribution of the acyl-CoA dehydrogenases, and to assist in the study of the structural properties of the acyl-CoA dehydrogenases and other flavoproteins. Our work also characterizes the cDNA and derives the amino acid sequence for an enzyme of mammalian mitochondrial fatty acid oxidation.

In comparing the 137 amino acids determined from porcine MCAD with the cDNA-derived human sequence, an 88% amino acid identity is noted. A high degree of sequence conservation across species is further supported by the RNA blot data using rat tissues (Fig. 4). The similarity is well distributed throughout the MCAD sequence, suggesting selective pressure to preserve sequence information necessary for maintenance of enzymatic activity and flavin binding. Similar interspecies homology has been noted for other mitochondrial enzymes such as malate dehydrogenase (32). Conservation of amino acid sequence has also been demonstrated for other flavoproteins, including lipoamide dehydrogenase (30).

An analysis of the initial 30 NH_2-terminal amino acids predicted by the MCAD cDNA reveals properties typical of

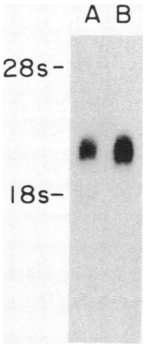

FIG. 5. RNA blot analysis of human fibroblast cells. RNA isolated from the cultured skin fibroblasts of a normal control (lane A) and a patient (K.J.) deficient in MCAD activity (lane B). Ten micrograms of total RNA was loaded in each lane, separated in a 1.5% denaturing gel, and transferred as described above. Hybridization was performed with the same ^{32}P-labeled cDNA probe as in Fig. 4.

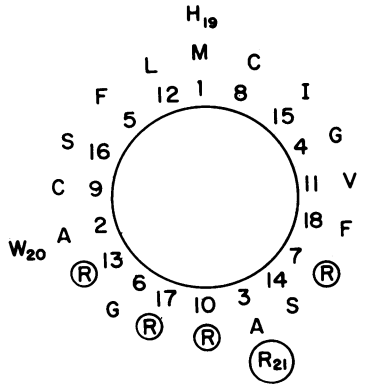

FIG. 6. α-Helical diagram of the MCAD transit peptide. An end-on view of an α-helix composed of the NH_2-terminal amino acids predicted from the cDNA of MCAD. The amino acids are denoted by the single-letter code and are numbered according to the primary protein sequence. The arginines are circled to demonstrate the arrangement of the positively charged residues.

mitochondrial protein transit peptides. The net positive charge and lack of acidic residues are characteristic. Furthermore, if an α-helical secondary structure is assumed, the arginine residues are located in close proximity to one another, forming an amphiphilic molecule capable of interacting at a membrane–water interface such as the mitochondrial outer membrane. This is illustrated in the "helical wheel" (33) diagram shown in Fig. 6. A measure of amphiphilicity, the hydrophobic moment (34), based on a window of 18 residues (five full α-helical turns) is 12.5 for residues 4–21 in the MCAD presequence, higher than any of the maximal hydrophobic moments reported for 23 transit peptide sequences analyzed by von Heijne (35).

The determination of the relative tissue abundance of the acyl-CoA dehydrogenases provides better understanding of their functional importance in normal metabolism and in MCAD-deficient patients. RNA blot analysis revealed mRNA encoding MCAD in each tissue studied (Fig. 4). This is not surprising in view of the essential role of this enzyme in fatty acid β-oxidation. Expression in rat brain suggests that fatty acid catabolism does occur in this tissue, perhaps in the fasting state when glucose availability is limited. The high level of expression of hepatic MCAD mRNA is consistent with the key role of the liver in ketone production. A defect in fatty acid β-oxidation in these tissues is responsible for the clinical features of hypoglycemia, coma, and impaired hepatic ketone production seen in MCAD deficiency. Future studies of the mRNA expression of all three straight-chain acyl-CoA dehydrogenases will aid in the understanding of fatty chain oxidation in the normal and disease state.

MCAD deficiency is currently recognized as a common inherited metabolic defect associated with a significant incidence of childhood mortality. As an initial step in determining the molecular defects in MCAD deficiency, we have shown that the fibroblasts from an MCAD-deficient patient express an mRNA encoding MCAD that is similar in size to that derived from human skin fibroblasts with normal MCAD activity. Our observations support the existence of a crucial point mutation or small deletion as the underlying defect in this patient. Delineation of the molecular defects in patients with immunoreactive, but inactive, MCAD (16) will provide insight into the structural features required for catalytic activity and flavin binding.

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