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Molecular characterization of inherited medium-chain acyl-CoA dehydrogenase deficiency

(fatty acid oxidation/genetic defect/sudden death/mRNA splicing/mitochondrial proteins)

DANIEL P. KELLY*, ALISON J. WHELAN†, MOIRA L. OGDEN*, RUTH ALPERS†, ZHIFANG ZHANG†, GARY BELLUS‡, NIELS GREGERSEN§, L. DORLAND¶, AND ARNOLD W. STRAUSS†‡

Departments of *Internal Medicine, †Biochemistry and Molecular Biophysics, and ‡Pediatrics, Washington University School of Medicine, Saint Louis, MO 63110; ¶Department of Pediatrics, University Children's Hospital, Utrecht, The Netherlands; and §University Department of Clinical Chemistry, Aarhus Kommune Hospital, Aarhus, Denmark

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ABSTRACT **Deficiency of medium-chain acyl-CoA dehydrogenase (MCAD) is a common inherited defect in energy metabolism. Characterization of the mRNA encoding MCAD in a Dutch MCAD-deficient patient revealed an A → G change at nucleotide position 985 of the MCAD mRNA coding region. This point mutation results in the substitution of a glutamic acid for a lysine at amino acid position 304 of the mature protein. The single base change was not found in any wild-type MCAD mRNAs. A mutant allele-specific oligonucleotide probe was used in a hybridization analysis of amplified genomic DNA of MCAD-deficient family members, a carrier, and normal individuals. The hybridization analysis specifically identified individuals who were heterozygotes or homozygotes. In addition to the point mutation, a significant proportion of the index patient's MCAD mRNA contained a variety of deletions and insertions as a result of exon skipping and intron retention. The missplicing occurred in multiple regions throughout the MCAD mRNA. Analysis of the patient's MCAD gene in the regions where the missplicing occurred most frequently did not reveal a mutation in the splicing acceptor or donor sites. Therefore, the molecular characterization of this family revealed a crucial point mutation in the MCAD gene and an unusual abnormality in MCAD pre-mRNA splicing.**

Medium-chain acyl-CoA dehydrogenase [MCAD; acyl-CoA:(acceptor) 2,3-oxidoreductase, EC 1.3.99.3] is a mitochondrial flavoenzyme that catalyzes the initial reaction in fatty acid β-oxidation (1). MCAD deficiency was first described in 1983 and is now recognized as a common inherited metabolic disorder that may be fatal (2–4). Studies from Great Britain indicate that MCAD deficiency occurs at a frequency of 1 in 5000–10,000 births (5). Clinical manifestations of MCAD deficiency are variable and include hypoglycemic coma, hepatic dysfunction, and sudden death. The disorder is often initially misdiagnosed as Reye syndrome or sudden infant death syndrome (SIDS) (6–8). The initial clinical presentation is usually precipitated by a period of fasting during the second year of life, although the onset and frequency of clinical events are variable and unpredictable. MCAD-deficient individuals may die suddenly or remain asymptomatic (8). Familial studies are consistent with an autosomal recessive inheritance (9).

Previous studies showed that the cultured skin fibroblasts of >20 MCAD-deficient patients had a normal-sized immunodetectable MCAD protein (10, 11). These data suggest that a crucial point mutation or small in-frame insertion or deletion is responsible for the catalytically inactive MCAD in these patients. As an initial step in the determination of the molecular defect responsible for MCAD deficiency, we have isolated and characterized clones encoding human MCAD mRNA and the human MCAD gene (12, 13). The MCAD gene has been localized to chromosome 1 (14).

In 1986, Duran *et al.* (8) described a child who died suddenly of MCAD deficiency. Recently, we have shown that the liver tissue of this child contains a markedly diminished steady-state level of MCAD protein and a normal-sized MCAD mRNA (11). We now report the molecular characterization of MCAD deficiency in this patient and family.

MATERIALS AND METHODS

MCAD Enzymatic Activity. MCAD activity of the family members' lymphocytes was measured by a gas chromatographic assay (15).

Protein Blot Analysis. Cultured skin fibroblasts of the family were donated by M. Duran and S. K. Wadman (University Children's Hospital, Utrecht, The Netherlands) and W. J. Kleijer (Erasmus Universiteit, The Netherlands). Protein immunoblot analysis was performed with anti-porcine MCAD antibody and ^{125}I-labeled staphylococcal protein A (16).

cDNA Cloning of Mutant MCAD. The liver tissue of the index patient was provided by M. Duran and S. K. Wadman. The MCAD enzymatic activity in the patient's liver tissue was determined previously by W. J. Rhead (8). Total liver RNA was isolated from the index patient's liver by the guanidium isothiocyanate technique (17). Poly(A)$^+$ RNA was selected by separation on an oligo(dT) column. cDNA synthesized from the poly(A)$^+$ RNA was used to construct a cDNA library in the *Eco*RI sites of the vector λgt10 (18). *Eco*RI methylation was performed prior to ligation of the double-stranded DNA to the *Eco*RI linkers.

Patient MCAD cDNA clones were isolated with human MCAD cDNA probes by standard screening protocol. cDNA probes were prepared by labeling to high specific activity with [α-^{32}P]dCTP (19). The clones were subcloned into pGEM plasmid vectors (Promega Biotec) and subjected to DNA sequence analysis by the dideoxy chain-termination technique (20).

Polymerase Chain Reaction (PCR) Cloning of MCAD. Total RNA was isolated (17) from confluent monolayers of the cultured skin fibroblasts and from human liver and heart tissue (provided by the Mid-America Organ Donor Center at Washington University Medical School). Fibroblast and control heart and liver cDNA was synthesized from 10 μg of total RNA. The cDNA of the index patient was synthesized from 1 μg of poly(A)$^+$ liver RNA. The cDNA of the family members and normal human tissues was synthesized from 10 μg of total RNA. For full-length MCAD mRNA coding-region amplification, 25% of the total cDNA was amplified

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Abbreviations: MCAD, medium-chain acyl-CoA dehydrogenase; PCR, polymerase chain reaction.

using oligonucleotide primers: 5′ primer, nucleotide positions −44 to −20; 3′ primer, positions +1299 to +1311. DNA amplification was performed with *Thermus aquaticus* (*Taq*) polymerase in a Perkin–Elmer/Cetus programmable thermocycler by standard protocol (21). A second amplification was performed with a set of primers that were nested within the first set. The second set of primers contained *Bam*HI restriction sites. The amplified DNA was subcloned into the *Bam*HI restriction sites of pGEM-3Z plasmid vectors. PCR amplification of the 3′ portion of the MCAD mRNA was accomplished using a 5′ primer (positions 557–576 of the MCAD mRNA coding region), the 3′ primer described above, and a cDNA template. The 3′ primer contained a *Bam*HI restriction site. The amplified DNA was subcloned into pGEM-3Z vectors by using the 3′ primer *Bam*HI site and the internal *Eco*RI site [base pair (bp) 706, ref. 12]. Size analysis of the full-length subcloned DNA inserts was performed by electrophoresis in a 1.2% agarose gel.

Colony Hybridization. Individual subclones were grown in TB_1 cells on Immobilon (Millipore) nitrocellulose filters (22). The filters were hybridized with 18-bp oligonucleotide probes specific for (*i*) exon 2, (*ii*) exon 5, (*iii*) the 12-bp intron insertion of mutant clone 1, and (*iv*) the 4-bp intron insertion of mutant clone 9. The probes were end-labeled with [γ-^{32}P]ATP. The hybridization was performed for 16 hr at 55°C in 6× SSC/1× Denhardt's solution/0.1% SDS. The filters were washed in 2× SSC/0.1% SDS at 40°C. (SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7; Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin.)

Cloning of the Index Patient's MCAD Gene. DNA was isolated (23) from the MCAD-deficient liver and digested with *Eco*RI. DNA fragments of 3000–6000 bp were used to construct a library in the vector λgt11. The genomic library was screened with normal human genomic MCAD probes, and two independent MCAD genomic clones were plaque-purified and subcloned into the plasmid vector pGEM.

Genomic DNA was amplified by PCR across exons 1, 2, and 3. The oligonucleotide primers (30-mers) were designed to amplify a region of DNA that included the entire exon and ≈100 bp of flanking intron DNA. The 5′ end of the normal exon 1 has not been determined and therefore this region was not studied. The amplified DNA was subcloned into pGEM via the *Bam*HI or *Hin*dIII restriction sites contained in the PCR primers. The PCR-generated clones were sequenced in their entirety.

Mutant Allele-Specific Hybridization Analysis. DNA was isolated (23) from cultured skin fibroblasts. Two micrograms of genomic DNA was used for the amplification of a portion of MCAD exon 11 and 3′ flanking intron with the PCR primers described above. The amplified DNA was extracted with phenol/chloroform and precipitated with ethanol. The DNA pellet was resuspended in 50 μl of H_2O and 350 μl of 0.4 M NaOH, and 0.025 M EDTA was added. Samples (100 μl) of this mixture were applied to a GeneScreen (NEN) nylon membrane under vacuum in a Schleicher & Schuell DNA blotting manifold. Oligonucleotides (15-mers) containing either a guanine (5′-GGCAATGGAAGTTGA-3) or an adenine (5′-GGCAATGAAAGTTGA-3) corresponding to position 985 of the MCAD mRNA were end-labeled with [γ-^{32}P]ATP for use as probes. Prehybridization was performed in 3 M tetramethylammonium chloride (Me_4NCl)/6× SSC/6× Denhardt's solution/0.1% SDS/0.1 M sodium phosphate, pH 6.8/1 mM EDTA at 50°C for 2 hr. Hybridization was done in 3 M Me_4NCl/6× Denhardt's solution/0.1% SDS/0.1 M sodium phosphate, pH 6.8/1 mM EDTA at 50°C for 16 hr. The membranes were washed in 3 M Me_4NCl/50 mM Tris, pH 8.0/0.2% SDS twice at 40°C and subsequently in 2× SSC/0.1% SDS at room temperature (15 min per wash).

Table 1. MCAD activities in lymphocytes of family members, normal controls, MCAD-deficient persons unrelated to the index patient, and carriers

Person(s)	Activity*
Mother	1.35
Father	0.22
Sibling 1	0.08
Sibling 2	0.23
Normal control	3.46 ± 0.18 (n = 15)
MCAD-deficient	0.08–0.23 (n = 10)
Carrier	1.91 ± 0.41 (n = 15)

*Nanomoles of 3-hydroxyoctanoate produced per min per mg of soluble protein. Values shown for the controls and carriers are the mean of the assays for n individuals. The range of assay values is shown for the MCAD-deficient individuals. SD of the mean of the measurements on controls and carriers are also shown.

RESULTS

MCAD Activity and Protein in the Index Patient and Surviving Family Members. The MCAD enzymatic activity in the liver tissue of the index patient was 14% of normal liver tissue control as described in a previous report (8). To define the phenotype of the surviving family members, the MCAD activity in their lymphocytes was determined (Table 1). The MCAD activity of the father's lymphocytes was 6% of normal control. The MCAD activities of the siblings' lymphocytes were 2% and 7% of control. The mother's lymphocytes had 39% of control activity. Therefore, by enzymatic criteria, the index patient, his father, and surviving siblings are presumed to be homozygous MCAD-deficient and the mother is presumed heterozygous for MCAD deficiency. The clinical history of the family members and the profile of their excretion of dicarboxylic acids have been described in detail (8). All surviving family members are healthy and have no history of clinically significant hypoglycemia or liver dysfunction. An older sibling died at age 19 months with clinical manifestations similar to those of the index patient (8).

The index patient's liver tissue has a very low steady-state level of immunodetectable protein and the MCAD mRNA is of normal size (ref. 11 and unpublished results). To compare the MCAD protein size and steady-state levels in the index patient's liver tissue with those of the family members, protein blot analysis was performed with polyclonal anti-porcine MCAD and protein extracts of the index patient's MCAD-deficient liver and the cultured skin fibroblasts of the surviving family members (Fig. 1). The fibroblasts from each surviving family member contain a normal-sized, immunodetectable MCAD protein. Therefore, the mutation responsible for MCAD deficiency in this family results in a normal-sized, catalytically inactive enzyme that appears to be unstable in liver tissue.

Characterization of the Mutant MCAD mRNA of the Index Patient and Surviving Family Members. A cDNA library was constructed with mRNA isolated from the index patient's liver

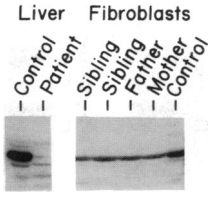

FIG. 1. Protein immunoblot analysis of family members. The blot was probed with anti-porcine MCAD after transfer of the proteins from an SDS/12.5% polyacrylamide gel. Prior to transfer each lane contained 100 μg of protein from extracts of liver tissue of the patient, cultured skin fibroblasts of the surviving family members, and normal controls.

tissue and screened with human MCAD cDNA probes. Six mutant partial MCAD cDNA clones were isolated and sequenced. In the two clones (1J and 3B) containing the 3′ end of the MCAD mRNA, an A → G substitution was present at position 985 of the coding region (relative to the adenine of the start codon, designated as 1; ref. 12) (Fig. 2). The other four partial clones contained 5′ coding region and terminated at bp 706 of the coding region, which is the location of the internal *Eco*RI site (12). The cDNA synthesized from the index patient's liver RNA served as a template for PCR amplification of the entire MCAD mRNA coding region. DNA sequence analysis of the entire coding region of four independent PCR-derived MCAD clones revealed that each contained the A → G change at bp 985. In addition, the A → G change was present in 10 additional independent subclones, which were partially sequenced in a region containing bp 985. No other point mutations were found in these clones. The A → G change predicts a Lys^{304} → Glu substitution in the mature protein. Therefore, all the MCAD mRNAs in the index patient contained a single base change that caused the substitution of a positively charged amino acid for a negatively charged one in the catalytically inactive mutant protein.

To determine whether the point mutation cosegregates with MCAD deficiency as defined by enzymatic assay, we analyzed the MCAD mRNA and MCAD gene of family members and tissues from normal individuals. cDNA was synthesized from total RNA isolated from the family members' skin fibroblasts and from normal human fibroblast, heart, and liver derived from different individuals. A 592-bp region of the MCAD mRNA that contains nucleotide position 985 was amplified by PCR and the DNA was subcloned into plasmid vectors. In addition, genomic DNA from fibroblasts of each family member served as the template for PCR amplification of the region of exon 11 that contained the point mutation. The amplified genomic DNA was also subcloned into plasmid vectors. The independent genomic and cDNA clones were subjected to DNA sequence analysis. All clones encoding MCAD in the father and both siblings contained guanine at position 985. The clones derived from the mother contained either an adenine or a guanine at position 985; thus she is heterozygous for the point mutation. In addition, all clones from normal individuals contained an adenine at position 985. Thus, all MCAD-deficient family members are homozygous for G^{985}, and G^{985} is not present in any of the normal individuals.

To develop a screening method for the point mutation, we employed 15-base oligonucleotide probes that contained either the G^{985} point mutation or the A^{985} normal sequence. The probes were end-labeled with ^{32}P and hybridized with DNA amplified from exon 11 of the MCAD gene (which contains the single base change). DNA isolated from family members, a known heterozygote, and normal human liver was examined (Fig. 3). The G^{985} probe gave a positive signal with the DNA samples from individuals who were homozygous and

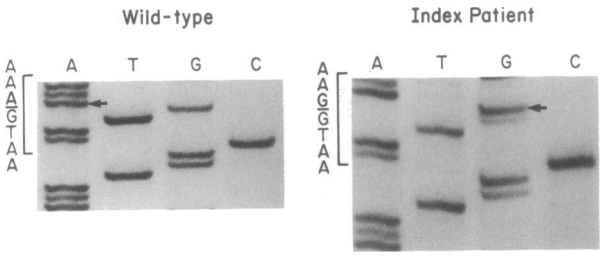

FIG. 2. DNA sequence analysis of wild-type and mutant MCAD cDNA clones. The DNA products were separated in a urea/6% polyacrylamide gel. The DNA sequence of the wild-type and index patient's cDNA is shown with the difference nucleotide at bp 985 underlined. The difference band is denoted by an arrow.

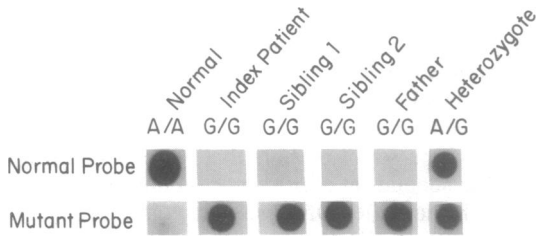

FIG. 3. Detection of the point mutation by hybridization with allele-specific oligonucleotide probes. MCAD oligonucleotide probe containing G^{985} (mutant probe) or A^{985} (normal probe) was hybridized with amplified genomic DNA from the index patient, family members, a known heterozygote, and normal control. The analysis could not be performed with the mother's DNA due to difficulty in maintaining her fibroblasts in culture.

heterozygous for the point mutation, but the normal control DNA was negative. The A^{985} probe was positive with normal or heterozygous DNA but negative with homozygous G^{985} DNA. Therefore, this hybridization analysis specifically identified homozygous MCAD-deficient, heterozygous, and homozygous normal individuals.

In addition to the single base change, three of the six MCAD clones (clones 1, 9, and 1J) isolated from the MCAD-deficient liver cDNA library had deletions and insertions. Mutant clone 1 contained a 12-bp insertion. Clone 9 showed an 88-bp deletion and a 4-bp insertion located 98 bp downstream from the deletion. Clone 1J contained a 491-bp insertion (data not shown). Comparison of these abnormalities with the structure of the human MCAD gene (13) revealed that each of the insertions and deletions resulted from defective splicing of the MCAD pre-mRNA. The insertions in clone 1, 9, and 1J are the result of partial retention of introns 1, 3, and 11, respectively. The deletion in clone 9 is secondary to the skipping of exon 2 during splicing. Thus, a significant proportion of the cDNA clones encoding MCAD from the index patient contained misspliced regions.

To analyze further the heterogeneity of the mutant MCAD mRNA, additional full-length clones containing the entire coding region of the MCAD mRNA in the MCAD-deficient liver were generated by PCR amplification of cDNA synthesized from RNA isolated from the propositus' liver. The amplified DNA was subcloned into plasmid vectors and the misspliced mRNAs were detected by size analysis of the DNA insert and colony hybridization using intron- and exon-specific oligonucleotide probes. All clones containing misspliced regions were subjected to DNA sequence analysis. This approach revealed exon deletions involving many exons in multiple locations throughout the mRNA coding region (Fig. 4). Deletions involving exon 2 were most common. The exon deletions occurred singly and in combination, resulting in a variety of contiguous and noncontiguous deletions. Retention of the entire third intron was found in clone 35. All of these clones also contained the A^{985} → G change. The majority of proteins predicted from the misspliced mRNAs are truncated nonsense proteins (Fig. 4). The mRNAs containing exon 2 deletions encode proteins that lack >50% of the mitochondrial transit peptide and thus cannot be imported into the mitochondria. The wild-type splicing donor and acceptor sites were used in all the deletion mutants with the exception of clones 2 and 13, in which a cryptic acceptor site within exon 11 was employed. The cryptic site was located at position 1075 of the coding region, which is 90 bp downstream from the location of the point mutation. Thirteen of 45 (29%) independent full-length PCR-derived MCAD clones were misspliced. Thus, in addition to a crucial point mutation, a significant proportion of the MCAD mRNA in the index patient's liver tissue was misspliced.

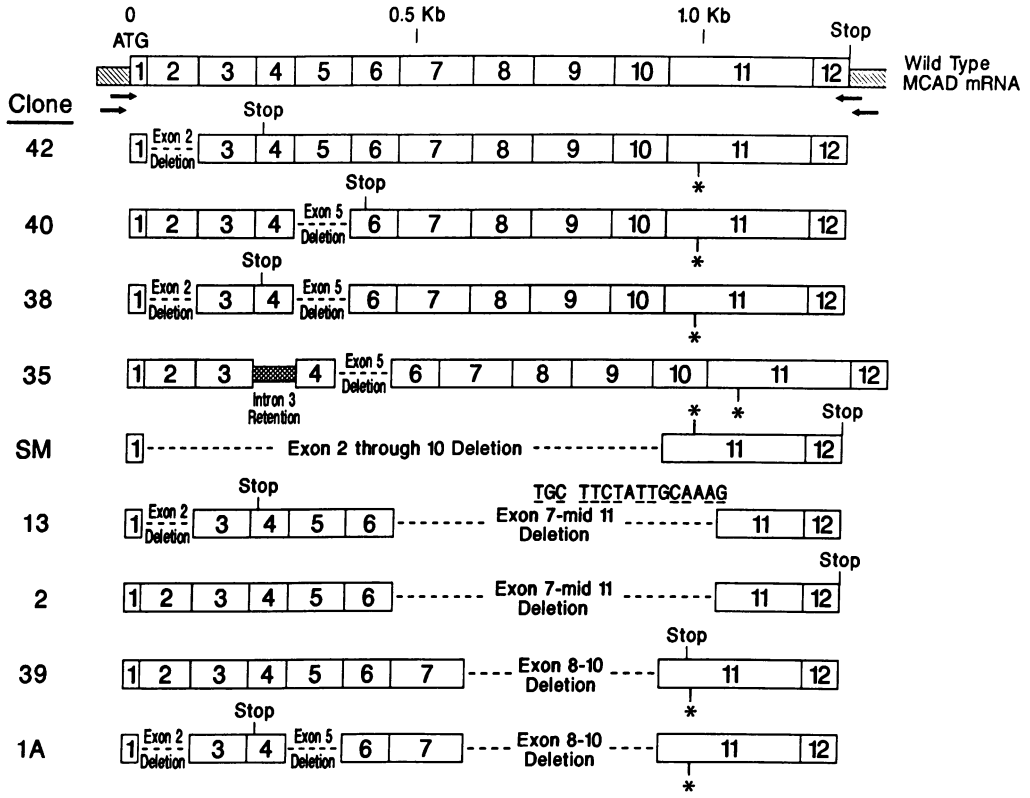

FIG. 4. PCR-derived cDNA clones encoding misspliced MCAD mRNA of the index patient. Numbered boxes indicate the exons that encode the corresponding regions of MCAD mRNA. PCR-derived, full-coding-length clones are shown below the wild-type MCAD mRNA. Arrows indicate the positions of the two sets of primers used to amplify the cDNA. Nucleotide sequence shown in clone 13 is the cryptic acceptor splicing site within exon 11. Underlined nucleotides match the consensus splicing acceptor sequence at the respective positions. Asterisks denote the single base substitution. Wild-type and premature stop codons are indicated.

To further assess the relationship between the point mutation and the MCAD pre-mRNA missplicing, MCAD mRNA isolated from the homozygous MCAD-deficient father and from several normal individuals was analyzed. The clones were derived by PCR and analyzed using the approach described above for the index patient. Ten of 40 (25%) independent clones derived from the father's skin fibroblast RNA were misspliced in the region of exon 2. Analysis of RNA isolated from the tissues of three normal individuals revealed that only a few clones from heart (4/39, 10%), liver (1/10, 10%), and skin fibroblasts (1/13, 8%) were misspliced. Therefore, the homozygous G^{985} state is associated with a high rate of MCAD pre-mRNA missplicing, and a lower but significant degree of missplicing occurs in normal tissues.

Although the splicing abnormalities occur throughout the MCAD mRNA, a predominance of abnormalities are located in the region of exon 2. Therefore, we determined the DNA sequence of the splicing donor, acceptor, and branch-point regions flanking exons 1, 2, and 3 in the index patient's gene. The DNA sequence in these regions is identical to normal except for a single G → C change at bp −32 upstream from exon 2 in the patient's MCAD gene and in a few normal clones, indicating that this represents a polymorphism in the gene. In addition, the sequences of the intron insertion in clones 1, 1J, and 35 are normal. Thus, in contrast to other known splicing disorders, the missplacing is apparently not due to a mutation in the prototypical splicing consensus regions in the patient's MCAD gene.

DISCUSSION

The cultured skin fibroblasts of all MCAD-deficient patients characterized to date contain a normal-sized, immunodetectable protein, indicating that the gene encoding MCAD in these patients contains a crucial point mutation or small in-frame insertion or deletion that results in a catalytically inactive protein (10, 11). In this report, we have shown that a single base substitution in exon 11 of the MCAD gene most likely causes MCAD deficiency in a Dutch family. The MCAD-deficient individuals in this family appear to be homozygous for the G^{985} mutation, since all clones derived from their MCAD mRNA contained the point mutation. The A^{985} → G substitution was not found in the MCAD mRNA from any normal individual. In addition, we have recently determined that all MCAD mRNAs in an MCAD-deficient American child and two unrelated patients from northern Europe contain the G^{985} mutation (unpublished results). Thus, the point mutation appears to be responsible for MCAD deficiency in these families.

The point mutation results in a Lys^{304} → Glu substitution in the mature protein. Comparison of the amino acid sequences of human (12), rat (24), and porcine (R.A., D.P.K., and A.W.S., unpublished data) MCAD reveals that Lys^{304} is conserved across species. Analysis of the three-dimensional structure of mature porcine MCAD (25) reveals that Lys^{304} is located in the H-helix region and is distant from the active site and flavin binding region. However, this helix appears to be important in subunit–subunit interaction. The Lys^{304} → Glu substitution results in a reversal of charge, which could decrease ionic interaction between subunits and disrupt normal homotetrameric quaternary structure. The perturbation of tetrameric structure could affect stability of the protein and catalytic function. After expression of mature [Glu^{304}]MCAD in bacteria, the mutant MCAD is unstable and catalytically inactive (Peter Bross, Konstanz University, Konstanz, F.R.G., personal communication). The low steady-state level of MCAD protein in the patient's liver suggests that the mutant MCAD protein is unstable *in vivo*. Therefore, these data are consistent with the single amino

acid substitution causing an unstable, catalytically inactive enzyme.

In addition to the point mutation, a significant proportion of the pre-mRNAs encoding MCAD in the index patient were misspliced. Many naturally occurring mRNA splicing mutants have been reported (26–30). However, the missplicing described here differs from previously reported defects in several ways. (*i*) The aberrant splicing is multiple and spread throughout the mRNA. (*ii*) This unusual missplicing is not a result of a point mutation in the donor or acceptor splicing sites. (*iii*) The MCAD mRNA missplicing occurs in normal tissues, but at a frequency lower than that of the index patient. The cause of the high rate of missplicing in the patient is not clear. The higher rate of missplicing was also found in the MCAD-deficient father. In addition, the MCAD mRNA is misspliced at a similar high rate in an unrelated MCAD-deficient American patient also homozygous for G^{985} (unpublished data). Therefore, the high rate of MCAD pre-mRNA missplicing appears to be associated with the homozygous G^{985} mutation. The point mutation is located in exon 11, 40 bp downstream from the intron 10–exon 11 junction. Interestingly, a cryptic splicing acceptor site in exon 11, located 90 bp downstream from the point mutation, was used in some of the deletion mutants in the index patient (clones 2 and 13, Fig. 4). Although the mutation does not involve a splicing acceptor or donor region, it is possible that this single base change is responsible for the high rate of missplicing by altering pre-mRNA secondary structure. Alternatively, the MCAD-deficient state may result in an increase in MCAD pre-mRNA missplicing via secondary mechanisms.

That the point mutation has been found in several patients from Northern Europe and the United States suggests that it is a common cause of MCAD deficiency. Determination of the frequency of this mutation in MCAD deficiency will be an important initial step in defining the molecular heterogeneity of this disorder. The DNA hybridization analysis shown here will allow us to rapidly screen additional MCAD-deficient individuals. Furthermore, if the point mutation proves to be a common cause of MCAD deficiency, the allele-specific hybridization analysis will be useful for screening the family members of known MCAD-deficient individuals and patients suspected to have disorders in fatty acid oxidation.

The extreme clinical variability of inherited MCAD deficiency is exemplified by the family studied here. Two siblings died suddenly in infancy, but the father and surviving siblings, who are homozygous MCAD-deficient by enzymatic assay criteria, are clinically healthy. Our molecular genetic analysis does not reveal any difference between clinically affected and unaffected individuals in this family. Further, the degree of MCAD pre-mRNA missplicing in the father is similar to that of the propositus. However, we have not excluded the possibility that a greater proportion of misspliced, *unstable* MCAD mRNA, was produced in the index patient compared with his father. Alternatively, the variable expressivity of MCAD deficiency may be due to the relative activity of other enzymes involved in fatty acid oxidation, such as the related short- and long-chain acyl-CoA dehydrogenases or peroxisomal enzymes.

In summary, we have shown that the gene and mRNA encoding the defective MCAD in this family contain a point mutation that results in the substitution of a glutamic acid for a lysine and most likely results in an unstable, catalytically inactive protein. In addition, the patient has an unusual defect in the splicing of the MCAD pre-mRNA. Molecular characterization of additional unrelated MCAD-deficient patients will allow us to determine the frequency of the G^{985} point mutation.

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