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Molecular characterization of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency: identification of a lys^{329} to glu mutation in the MCAD gene, and expression of inactive mutant enzyme protein in *E. coli*

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Summary. A series of experiments has established the molecular defect in the medium-chain acyl-coenzyme A (CoA) dehydrogenase (MCAD) gene in a family with MCAD deficiency. Demonstration of intra-mitochondrial mature MCAD indistinguishable in size (42.5-kDa) from control MCAD, and of mRNA with the correct size of 2.4 kb, indicated a point-mutation in the coding region of the MCAD gene to be disease-causing. Consequently, cloning and DNA sequencing of polymerase chain reaction (PCR) amplified complementary DNA (cDNA) from messenger RNA of fibroblasts from the patient and family members were performed. All clones sequenced from the patient exhibited a single base substitution from adenine (A) to guanine (G) at position 985 in the MCAD cDNA as the only consistent base-variation compared with control cDNA. In contrast, the parents contained cDNA with the normal and the mutated sequence, revealing their obligate carrier status. Allelic homozygosity in the patient and heterozygosity for the mutation in the parents were established by a modified PCR reaction, introducing a cleavage site for the restriction endonuclease *Nco*I into amplified genomic DNA containing G^{985}. The same assay consistently revealed A^{985} in genomic DNA from 26 control individuals. The A to G mutation was introduced into an *E. coli* expression vector producing mutant MCAD, which was demonstrated to be inactive, probably because of the inability to form active tetrameric MCAD. All the experiments are consistent with the contention that the G^{985} mutation, resulting in a lysine to glutamate shift at position 329 in the MCAD polypeptide chain, is the genetic cause of MCAD deficiency in this family. We found the same mutation in homozygous form in 11 out of 12 other patients with verified MCAD deficiency.

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Introduction

Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is an inherited defect of fatty acid β-oxidation (Gregersen 1985; Roe and Coates 1989). The disease is characterized by episodes of hypoketotic hypoglycemia, lethargy and excretion of C_6–C_{10}-dicarboxylic acids and other metabolites, especially hexanoylglycine, phenylpropionylglycine and suberylglycine (Gregersen 1985; Rinaldo et al. 1988). The first patient was described in 1976 (Gregersen et al. 1976), and defective MCAD was independently determined in several patients in 1982–1983 by three groups (Kølvraa et al. 1982; Rhead et al. 1983; Stanley et al. 1983). The number of reported cases has rapidly increased, especially among children suffering from sudden infant death syndrome (SIDS). In England, MCAD deficiency accounted for 2%–3% of SIDS cases (Bennett et al. 1990).

MCAD is one of three straight-chain acyl-CoA dehydrogenases that catalyze dehydrogenation of fatty acyl-CoA esters in mitochondrial β-oxidation (Schultz 1990; Ikeda and Tanaka 1990a). Human MCAD is encoded in the nucleus from the ACADM (acyl-CoA dehydrogenase, medium chain) locus on chromosome 1 (Matsubara et al. 1986) as a precursor protein with a 25-amino-acid leader sequence and a mature enzyme protein of 42–43 kDa (Kelly et al. 1987). This pre-MCAD protein is post-translationally translocated to the mitochondrial matrix (Strauss et al. 1990) with cleavage of the leader peptide. The monomeric enzyme protein is assembled to

a homotetrameric active enzyme, with flavin adenine nucleotide (FAD) attached to each subunit (Ikeda and Tanaka 1990b).

The first approach to elucidating the molecular defect in MCAD deficiency was undertaken by Ikeda and coworkers, who analyzed mutant protein from 13 patients (Ikeda et al. 1986). They showed that both the precursor and the mature mitochondrial MCAD protein in all cell lines were produced and indistinguishable in size from those in control cells. They concluded that the lack of enzyme activity in these patients was most probably the result of point-mutations.

A cDNA encoding MCAD was first isolated and sequenced by Kelly et al. (1987). In the 2.4-kb mRNA, they identified a 1263-bp coding region and a 738-bp 3′-untranslated region. Analysis of a cDNA library from the liver of a Dutch patient with MCAD deficiency indicated that mis-splicing of MCAD pre-mRNA was associated with the disease (Strauss et al. 1990).

We now report an A to G point mutation at position 985 in the coding region of the MCAD cDNA. This A to G mutation causes decreased enzyme activity in recombinant mutant MCAD protein expressed in *E. coli*. We also report mis-spliced MCAD mRNA, resulting from exon-skipping, which seems to be more pronounced in patients than in control fibroblasts.

Materials and methods

MCAD deficient family and controls

MCAD deficiency was diagnosed in the index case (RB) in 1982 (Kølvraa et al. 1982). β-Oxidation in cultured fibroblasts of the consanguinous parents (HB and AB) and a healthy brother (TB) have now been measured by a recently developed tritium-release assay using ^{3}H-myristic acid (Amersham, Buckinghamshire, UK) as substrate, essentially as described by Manning et al. (1990). For genetic analysis, cDNA from normal fibroblasts, monocytes and T-lymphocytes, together with genomic DNA from 26 persons were used as controls.

Cell material

Skin fibroblasts were grown in RPMI 1640 medium (Biochrom, Berlin, FRG) containing 10% fetal calf serum. Cell pellets containing 2×10^7 cells were frozen in liquid nitrogen and stored at −80°C.

MCAD protein analysis

Protein extracts for Western blot analysis were obtained from frozen cells by repeated freeze-thawing (4 times) in liquid nitrogen. After centrifugation, the supernatant solution was electrophoresed by SDS polyacrylamide gel electrophoresis (PAGE) (Burnette 1981). The proteins were blotted onto nitrocellulose membranes by semi-dry electroblotting. Membranes were treated with 30% ethanol before MCAD monomeric protein was visualized with an anti-porcine MCAD antibody (Kelly et al. 1987), and ^{125}I Protein-A (Amersham).

Isolation of RNA

Total RNA was isolated from 2×10^7 cells by the guanidinium thiocyanate procedure (Chomczynski and Sacchi 1987). The isolated RNA was further purified by precipitation in 0.3 mol/l sodium acetate (pH 6.0) with ethanol. The pellet was washed with 70% ethanol and stored in 70% ethanol at −20°C. The yield was 40–60 µg RNA/batch.

MCAD mRNA analysis

RNA blot analysis (Northern blotting) was performed by formaldehyde/agarose gel electrophoresis with 8 µg total RNA by the method of Fourney et al. (1988). Hybridization was according to Gregersen et al. (1987) at 42°C with an MCAD cDNA probe (Kelley et al. 1987), labelled with ^{32}P-dCTP (Amersham) and autoradiographed for 1–2 days at −70°C.

First strand cDNA synthesis

First strand cDNA was synthesized with an Invitrogen kit (Invitrogen, Calif., USA) from 8 µg total RNA. The MCAD specific primer used was a 27-mer oligonucleotide: 5′-GCAGTTGCTTAGATTTAATATAAGAGG (pri1366) derived from positions 1366–1392 in the MCAD cDNA (Kelly et al. 1987). cDNA was redissolved in distilled water and 1/5 of the total used for each polymerase chain reaction (PCR) amplification.

Amplification of first strand cDNA

Amplification of single-stranded cDNA involved two sets of primers. One set covered 646 bp of the 5′-end of the cDNA, and the other generated a full-length (1339 bp) cDNA, covering the coding sequence of the mature MCAD (position 76 to 1263).

5′-end set. Sense strand (pri-18): 5′-CCGGGGATCCGAGCCAACATGGCAGCGGGG, covering position −18 to +12 in the cDNA, and containing a *Bam*HI restriction site (GGATCC). Antisense strand (pri609): 5′-CTGGATCAGAACGTGCCAAT, covering position 609 to 628.

Full length set. The sense strand primer is identical to the sense strand primer above, covering position −18 to +12. Antisense strand (pri1292): 5′GGAGAAGCTTCAGTGGCTTGTGTTCTAGTT, covering position 1292 to 1321 and including a *Hin*dIII restriction site (AAGCTT).

The PCR was performed according to the Perkin-Elmer/Cetus procedure. In the first PCR cycle, denaturation was performed at 94°C for 8 min in the Cetus-Perkin-Elmer Thermal Cycler, before annealing of the primers at 59°C for 5 min. Two units of *Taq* polymerase was added and polymerization was performed at 74°C for 5 min. In the subsequent 49 cycles, denaturation was for 1 min, annealing for 2 min and polymerization for 4 min. *Taq* polymerase (Cetus Co., Conn., USA) was added after the 20th and 40th cycles. PCR products were analysed by agarose gel electrophoresis (Fig. 1).

Cloning and sequencing of PCR produced MCAD cDNA

Following electrophoresis of the PCR products, the band of the expected length (see Fig. 1) was extracted and purified (GENECLEAN, Bio 101 Inc., La Jolla, Calif., USA). After digestion with appropriate restriction enzymes, the DNA was purified and ligated into pGEM4Z (Promega Co., Madison, Wis., USA). Transformation and selection in *E. coli* J109 cells were performed by standard techniques (Sambrook et al. 1990). Plasmid preparations for sequencing were performed by the modified alkaline lysis method (Promega 1989).

For double-stranded sequencing, the Reagent kit for DNA sequencing using Sequenase (USBC, Cleveland, USA) with ^{35}S-dATP (Amersham) was employed with both commercial primers (SP6 and T7) and synthetic internal primers.

Analysis for G^{985} in genomic DNA

Genomic DNA was isolated from whole blood or fibroblasts by conventional methods. Primers for amplification by PCR of a 199-bp piece of genomic DNA around position 985 were designed based upon the MCAD gene structure (Z. Zhang and A. W. Strauss, un-

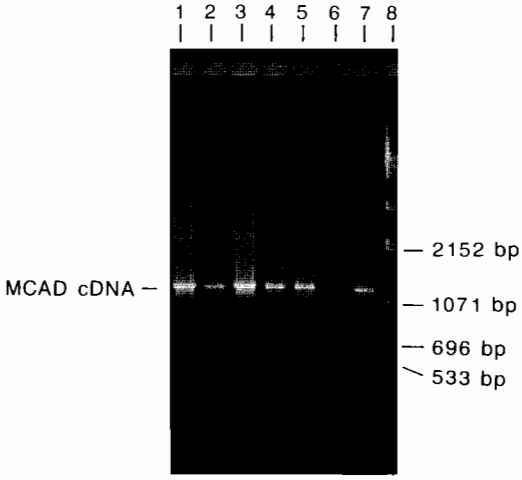

Fig. 1. Ethidium-bromide-stained 2% agarose mini-gel (BioRad, Richmond, Calif.) of PCR-amplified cDNA, produced with primers pri-18 and pri1292. PCR mixture (8 µl) was loaded onto the gel. *Lane 1* index patient, RB; *lane 2* father, AB; *lane 3* brother, TB; *lane 4* mother, HB; *lane 5* control fibroblasts; *lane 6* control monocytes; *lane 7* control T-lymphocytes; *lane 8* size marker, lambda C_I857, digested with *Dra*I

published data). The sense primer (5'-TTTATGCTGGCTGAAATGGCCATG) covers nucleotides 961 to 984 of the cDNA and contains a C:T mismatch at position 981, introducing a *Nco*I restriction site (CCATGG) when the mutant sequence is copied, but not when normal sequence (CCATGA) is produced by PCR. The antisense primer (5'-CAGGATATTCTGTATTAAATCCATGGCCTC) (bp 1130 to 1159 of the cDNA) introduces a G:A mismatch at position 1135 and creates another *Nco*I site, which functions as an internal control of the enzyme cleavage efficiency.

PCR of genomic DNA was performed as described above, except that the annealing temperature was 61°C, the polymerization time was 2 min, and 30 cycles were run.

After PCR, DNA was extracted by phenol/chloroform and precipitated with ethanol. After *Nco*I restriction endonuclease (Promega) treatment, the analysis was performed by 12% PAGE, and the bands were visualized by ethidium bromide staining.

Expression of mutant MCAD in E. coli

The ApaI/*Hin*cII fragment of the MCAD expression plasmid pWTMCAD-2 (Bross et al. 1990) with the coding sequence of mature MCAD and the ribosome binding site was introduced between the *Apa*I and *Sma*I sites in the polylinker of pBluescript KS- (Stratagene, La Jolla, Calif., USA) placing the gene under control of the lac promoter (pBMCK2(−)) (Fig. 2). In order to introduce the A to G mutation at position 985 of the MCAD cDNA into pBMCK2(−), an *Eco*RI/*Bam*HI fragment of cloned PCR-amplified cDNA carrying this mutation and a *Bam*HI restriction site in pri1292' was introduced instead of the corresponding wild-type sequence. The resulting mutant plasmid was called p985-41 (Fig. 2). The relevant part of p985-41 was sequenced to ensure that there were no additional mutations introduced because of PCR artifacts.

For expression of wild-type and 985-mutant MCAD, cells transformed with the respective plasmid were grown in LB (Luria-Bertani) medium supplemented with ampicillin and IPTG (Isopropyl-β-D-thiogalactopyranoside). Aliquots were withdrawn for Western blotting and the remainder of the culture was harvested and disrupted by sonication. After centrifugation, the supernatant was precipitated by addition of 80% ammonium sulfate. The precipitate was dissolved in potassium-phosphate buffer (50 mmol/l potassium phosphate, 0.3 mmol/l EDTA, pH 7.4). Measurement of MCAD activity and Western blot analysis were performed as described by Bross et al. (1990).

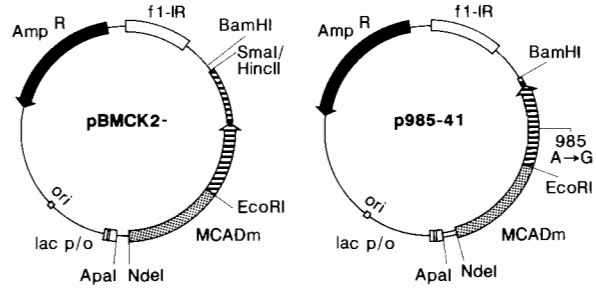

Fig. 2. Plasmid maps of pBMCK2- and p985-41. Relevant restriction sites, locations and orientations of the genes and the position of the 985 mutation in the MCAD gene are indicated

Results

Diagnosis of index patient

A precondition for the validity of the genetic analyses is that the diagnosis of MCAD deficiency is unambiguous. Previously, the determination of specific activity of MCAD in crude fibroblast homogenate from fibroblasts from the patient RB (Kølvraa et al. 1982) established MCAD deficiency in this patient. A low myristic acid β-oxidation activity of 2.3 pmol/min per mg fibroblast protein in whole fibroblasts from RB compared with a control value of 16.9 ± 5.5 pmol/min per mg protein ($n = 19$) supports the diagnosis. The β-oxidation activity in fibroblasts from the father (AB) and mother (HB) were 12.6 and 10.8 pmol/min per mg protein, respectively. These activities are in the lower control range. The very wide control range indicates an overlap between normal and heterozygous activities. Thus, the activities in the parents are compatible with their obligate carrier status. The activity of 12.1 pmol/min per mg protein in the brother's cells indicates that he is also heterozygous for a mutant allele. In addition, the patient exhibited clinical symptoms and urinary excretion patterns characteristic for MCAD deficiency (Gregersen et al. 1980; 1983).

Because the parents in this family originate from a common ancestor five generations back, it is likely that the patient is truly homozygous for the disease-causing mutation.

Protein and mRNA analyses

To investigate the enzyme protein in the index patient, immunoblot analysis was performed with anti-porcine MCAD antiserum and protein from cultured fibroblasts. Figure 3 demonstrates that no MCAD protein antigen of an incorrect size was present in mutant fibroblasts from the patient.

Northern blotting analysis (Fig. 4) of total fibroblast RNA was performed with a full-length MCAD cDNA probe. Only mRNA of the correct length (2.4 kb) was present in RNA from both patient and all three family members.

These data suggest that at least one allele of the mutant MCAD gene is transcribed and translated into full-

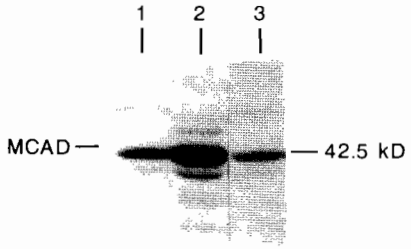

Fig. 3. Western blot of MCAD in disrupted skin fibroblasts from a normal individual (*lane 1*), the index patient RB (*lane 3*), and from normal liver tissue (*lane 2*)

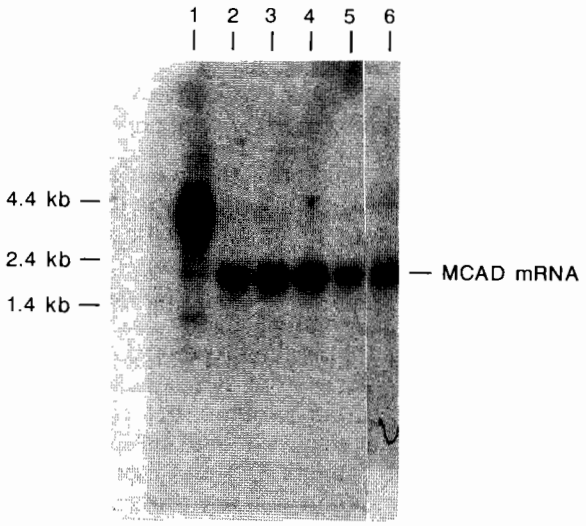

Fig. 4. Northern blotting analysis of mRNA from the index patient RB (*lane 2*), family members (*lane 3* brother TB; *lane 4* father AB; *lane 5* mother HB), and normal fibroblasts (*lane 6*). *Lane 1* represents a size-marker

length protein, thus indicating that a point-mutation or small in-frame insertion or deletion in at least one allele is the cause of the disease.

cDNA analysis for point-mutations

To characterize the sequence of the MCAD mRNA coding for the mature intramitochondrial protein, a primer (pri1366) located in the 3'-untranslated region of the mRNA was used for first-strand cDNA synthesis. The cDNA was then amplified directly by PCR with primers (pri-18 and pri1292) encompassing the entire coding region of the mature protein. Analysis of the PCR products (Fig. 1) revealed a major product of the same size as that generated from RNA derived from normal fibroblasts, monocytes and T-lymphocytes. The products are, however, not homogeneous. Small amounts of products of shorter lengths were present. After cloning, clones with inserts of the expected size for full-length cDNA sequence (1339 bp) and clones with shorter inserts were found (results not shown).

Because of the high error rate for *Taq* polymerase (see Discussion), several clones with full-length inserts from the index case and family members were subjected to complete nucleotide sequence analysis. The characterization of shorter cDNA inserts will be described below.

Clones ($n = 13$) from the patient RB were analyzed, with 9 sequenced through the whole strand. The only consistent point substitution in these 9 clones, compared with the wild-type cDNA sequence, was the shift from A to G at position 985. This changes a lysine codon to a glutamate codon at position 329 in the polypeptide chain. The same mutation was identified in 4 additional clones of the patient's cDNA, strongly suggesting homozygosity for the G.

To support this conclusion, MCAD cDNA clones derived from RNA of fibroblasts from family members were sequenced around position 985. Figure 5 shows the pedigree with the total number of identified clones of each variant; 9 cDNA clones from the father (AB), 7 from the mother (HB), and 6 from the brother (TB) all contain either an A or a G at position 985. This indicates that they are heterozygous for the variant allele. On the other hand, only A at position 985 was found in clones derived by PCR from normal fibroblasts, monocytes and T-lymphocytes (Fig. 5).

Genomic analysis for G^{985}

Allelic analysis of genomic DNA in this family was performed (Fig. 6) to confirm the cDNA data. The patient was homozygous for the allele containing G^{985} in genomic DNA. Two alleles with, respectively, A^{985} and G^{985}

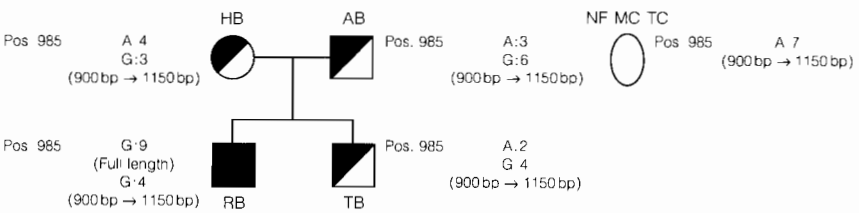

Fig. 5. cDNA sequencing results from the family and from normal fibroblasts, monocytes, and T-lymphocytes. For the patient RB, full-length sequencing of 9 clones with the finding of G (*G:9*) at position 985 in the MCAD cDNA (pos. 985) is indicated. An additional 4 clones from the patient were partially sequenced from position 900 to 1150, and the number with G at position 985 is shown. The number of partially sequenced clones with A or G at position 985 is indicated for family members and for normal fibroblasts (*NF*), monocytes (*MC*) and T-lymphocytes (*TC*)

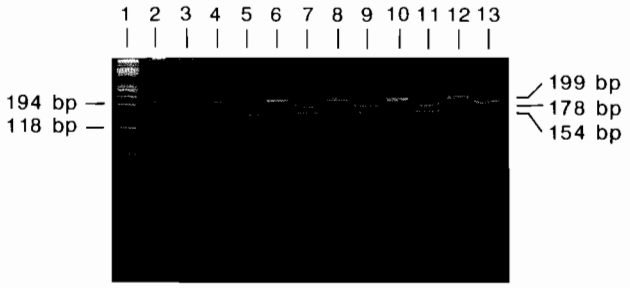

Fig. 6. Ethidium-bromide-stained polyacrylamide gel (PAGE) showing genomic analysis for G^{985} in the index patient, family members and control individuals. PCR products were either loaded directly (*lanes with even numbers*), or digested with *Nco*I before electrophoresis (*lanes with uneven numbers*). *Lane 1* size marker, mixture of HindIII-digested lambda C_I857 DNA and *Hae*III-digested ΦX-174 DNA (Pharmacia, Uppsala, Sweden); *lanes 2, 3* control; *lanes 4, 5 patient RB; lanes 6, 7* father AB; *lanes 8, 9* mother HB; *lanes 10, 11* brother TB; *lanes 12, 13* control

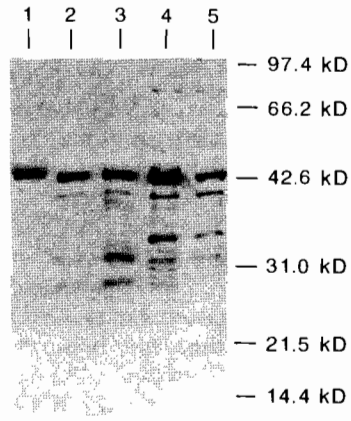

Fig. 7. Western blotting analysis of extracts from *E. coli* JM109 transformed with pBMCK2- and p985-41. Cells were harvested and disrupted by boiling with sample buffer, or extracts were prepared by sonication followed by ammonium sulfate precipitation of the supernatant. *Lane 1* purified recombinant wild-type human MCAD; *lane 2* JM109/pBMCK2- cells disrupted by boiling; *lane 3* JM109/p985-41 cells disrupted by boiling; *lane 4* JM109/pBMCK2- extract; *lane 5* JM109/p985-41 extract. The position and molecular mass of co-electrophoresed marker proteins is shown on the *right*

were present in genomic DNA from the mother, father and brother. DNA from 26 normal individuals was homozygous for the allele containing A at position 985. The A to G mutation is thus strongly associated with the disease, and the likely cause of the disease. Furthermore, in 11 out of 12 other patients with verified MCAD deficiency, the same mutation was found in homozygous form (results not shown).

Expression of mutant 985 in E. coli

To investigate whether mutation A to G at position 985 alters MCAD enzyme activity, mutant mature protein was produced in *E. coli*. Two derivatives of the MCAD expression plasmid pWTMCAD-2, which we have previously employed to study artificial mutants of MCAD (Bross et al. 1990), were constructed (Fig. 2).

Western blotting analysis of MCAD protein from cells transformed with pBMCK2- or p985-41 revealed that p985-41 produced MCAD protein of the correct size. Figure 7 shows a representative blot. However, mutant MCAD is more susceptible to proteases than the wild-type, and cells disrupted by boiling with sample buffer display additional degradation bands (Fig. 7, lanes 2 and 3). Furthermore, the mutant protein in the extracts prepared from cells disrupted by sonication appears to contain even lower amounts of MCAD antigen (Fig. 7, lane 5) compared with the amounts in the extract from cells with wild-type MCAD (Fig. 7, lane 4). MCAD activity in extracts containing the expressed wild-type was 20 mU/mg soluble protein, whereas no activity (< 5 mU/mg protein) could be detected in extracts containing MCAD with the G^{985} mutation, indicating an impaired enzyme function.

cDNA analysis for deletions

As mentioned above, the products of PCR amplification with the full-length primers were heterogeneous, with smaller bands noted (see Fig. 1). We sequenced a number of shorter clones produced both with the full-length

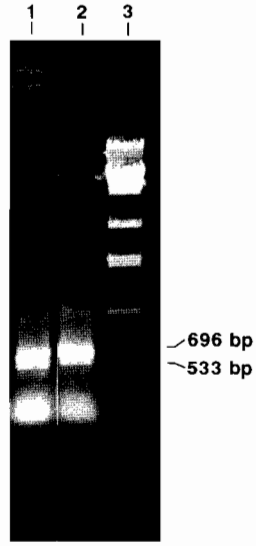

Fig. 8. Ethidium-bromide-stained agarose gel loaded with PCR product produced by primers pri-18 and pri609, covering 646 bp of the 5′-end of the MCAD coding sequence. *Lane 1* patient RB; *lane 2* normal fibroblasts; *lane 3* size marker

primers and with primers for the 646-bp piece from the 5′-end of the cDNA. The analysis (results not shown) showed that many of the shorter clones from the patient contained cDNA with deleted exons 2, 5 and 8. An unexpected and very interesting finding was that control fibroblasts also contained cDNA with similar abnormalities. By cloning PCR-generated cDNA from the electrophoretic bands separately (see Fig. 8), we demonstrated that the band near the 533-bp DNA marker contained exon 2 deletions. Similar PCR analysis of 4 other patients (results not shown) indicates that the amount of shorter PCR product is larger in the patients than in controls.

Discussion

The human MCAD gene has been localized to the short arm of chromosome 1, at 1p31, locus ACADM (Matsubara et al. 1986). Because obligate heterozygous parents of patients suffering from MCAD deficiency are known not to have symptoms, MCAD deficiency can be considered as an autosomal recessive disorder. Therefore, affected children must possess two mutant alleles. Our index patient has low enzyme activity in two assays and exhibits clinical and biochemical symptoms of MCAD deficiency. He must, therefore, be either truly homozygous or compound heterozygous. Consequently, the demonstration that both mature MCAD antigen and mRNA are of the correct sizes, can mean either that both alleles are expressed and translated to give inactive MCAD protein, or that this is the case for only one allele; the second mutant allele might not be expressed, or the mRNA or the protein may be unstable and rapidly degraded, escaping detection. Our analytical approach was, therefore, designed to detect point-mutations (or small deletions/insertions that retained the reading frame for the protein). A major product with the correct length was obtained by PCR of first strand cDNA containing the full coding sequence for the mature enzyme protein from fibroblast total RNA. Our cloning and sequence analysis of many full-length clones excluded small deletions or insertions as causes of the deficiency. However, we demonstrated a common base change from A to G at position 985 for all thirteen patient clones compared with the original published cDNA sequence from human placenta/liver (Kelly et al. 1987), and with our own cDNA synthesized from RNA from control fibroblasts, monocytes and T-lymphocytes.

Nevertheless, we also found other point-variations in individual clones. This is not unexpected, because the *Taq* polymerase used in the PCR reaction lacks a proofreading exonuclease activity, and therefore exhibits a much higher nucleotide mis-incorporation rate (about 1/10000) than the Klenow polymerase (about 10^{-7}) (Tindall and Kunkel 1988). From the theoretical study of Reiss et al. (1990), it can be assumed that, after 50 cycles, each PCR copy of the parent 1339-bp cDNA would probably contain one or more PCR errors, which will be present in individual clones.

The identification of both normal sequences with A and mutant sequences with G at position 985 in cDNA from both parents in this family strongly indicates that the patient is truly homozygous for the mutation and that he has inherited one mutant allele from each of the parents. The homozygosity is further supported by the existence of a common ancestor 5 generations back. In this family, we have further demonstrated that the brother, who has had no symptoms of MCAD deficiency, is heterozygous for the mutation. Thus, we suggest that the identified A to G shift is disease-causing. The determination in genomic DNA of homozygosity in the patient and heterozygosity in the parents for the G^{985} allele and the exclusive finding of A^{985} in genomic DNA from 26 control persons corroborate this hypothesis.

Because the A to G shift results in an amino acid change at position 329 of the polypeptide chain from basic lysine to acidic glutamic acid, the mutation can be expected to affect the structure and function of the enzyme dramatically. From the three dimensional structure of the MCAD protein from pig liver (Kim and Wu 1988), and from the existing knowledge about active sites in the MCAD protein (Kim and Wu 1988; Powell and Thorpe 1988), it is unlikely that this mutation affects the active sites directly. However, it is localized at a site where it might interfere with the assembly of the monomeric forms to the functional tetrameric form, thus abolishing normal enzyme function.

To investigate this possibility, cDNA encoding the mature form and containing the mutation A to G at position 985 was introduced into the *E. coli* expression system, developed by Bross et al. (1990). MCAD protein of the correct size was detected after induction of plasmid-carrying cells (Fig. 7). The mutant protein is less stable than wild-type MCAD expressed from an analogous plasmid, and more intense degradation bands are present (Fig. 7, lane 2 and 3). In the extracts prepared from the cells, the difference in amounts between wild-type and 985-mutant enzyme is pronounced, indicating that the mutant protein escapes analysis, possibly because of degradation and/or formation of insoluble aggregates.

MCAD activity in the extracts containing the mutant protein was not detectable in the assay used in this study. This means that either the strongly reduced amount of mutant MCAD protein may account for the 'lack' of enzyme activity found or that the mutation renders the protein inactive.

Lys-329 is located within helix H of MCAD, which together with helix I makes the inter-subunit contact (Kim and Wu 1988). Interference with tetramer formation therefore appears to be the most likely reason for defective MCAD activity. Despite the uncertainty about the detailed mechanism resulting in inactivation, the data presented here strongly support the contention that the A to G mutation is disease-causing.

Recently, we analysed 12 more patients with MCAD deficiency (results not shown). The finding of G^{985} on both alleles in 11 of them suggests that this mutation is frequent among patients with MCAD deficiency.

During the investigation of PCR products for point-mutations, we noted cDNAs with exon deletions, most commonly of exon 2, but also of exons 5 and 8. We found these exon-skips in the patient, but exon 2 deletions were also present in cDNA from normal fibroblasts. From the analysis of the PCR products, it seems that the amount of cDNA containing deletions is higher in the patient than in normal persons. Deletion of exon 2 has been described previously by Strauss and coworkers in a Dutch patient, who died during an attack caused by MCAD deficiency (Strauss et al. 1990). The deletions of exons 2, 5 and 8 can only be explained by mis-splicing, which has been observed for other genes (Akeson et al. 1987; Ohno and Suzuki 1988). The relevance for MCAD deficiency is unclear, but it might be speculated either that mutation of residue 985 destabilizes the splice complexes, giving rise to exon skipping, or that the accumu-

lated metabolites affect the precursor mRNA splice processing. In the latter case, this secondary pathophysiological phenomenon might seriously contribute to the course of life-threatening attacks.

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