0031-3998/93/3405-0694$03.00/0
PEDIATRIC RESEARCH
Copyright © 1993 International Pediatric Research Foundation, Inc.
Vol. 34, No. 5, 1993
Printed in U.S.A.

Expression and Characterization of Human Mutant (Glutamic Acid^{304}) Medium-Chain Acyl-Coenzyme A Dehydrogenase in Mammalian Cells

ALISON J. WHELAN, ARNOLD W. STRAUSS, DANIEL E. HALE, NANCY J. MENDELSOHN, AND DANIEL P. KELLY

Departments of Medicine and Pediatrics, St. Louis Children's Hospital and Washington University School of Medicine, St. Louis, Missouri 63110, and Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104

ABSTRACT. Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is a frequent and sometimes fatal inherited metabolic disorder of fatty acid β-oxidation. A eukaryotic expression system was used to study naturally occurring mutations in MCAD. The 1263 nucleotide coding region of human MCAD cDNA was inserted downstream of the SV40 early promoter for high-level expression in Chinese hamster ovary cells. Both normal MCAD cDNA and a mutant MCAD cDNA containing the common, disease-causing A to G transition at position 985 (A985G), which alters a lysine to a glutamic acid (K304E), were inserted into expression vectors. Transient transfection of Chinese hamster ovary cells was performed with the expression constructs. The steady state level of expressed normal MCAD protein antigen was substantially higher (5-fold) than the expressed mutant protein. The MCAD enzymatic activity in protein extracts from cells containing the expressed normal MCAD cDNA was also much higher (6-fold) than the activity in cells expressing the mutant MCAD. Therefore, these data confirm that the common K304E mutation causes MCAD deficiency primarily by decreased protein stability rather than reduction of catalytic activity and, in fact, demonstrate that the K304E mutant protein has a similar sp act against octanoyl CoA substrate as the normal protein. (*Pediatr Res* 34: 694–697, 1993)

Abbreviations

MCAD, medium-chain acyl-CoA dehydrogenase
ACD, acyl-CoA dehydrogenase
PCR, polymerase chain reaction
CHO, Chinese hamster ovary

MCAD (2,3-oxidoreductase, EC 1.3.99.3) is a mitochondrial matrix flavoprotein that catalyzes the initial reaction in fatty acid β-oxidation (1). MCAD is one of three straight-chain ACD with distinct but overlapping fatty acyl substrate specificities (2). MCAD deficiency (3–5) is a relatively common disorder with an estimated frequency of 1 per 10 000–15 000 births in Northern Europe (6–8). Clinical manifestations, generally first noted in early childhood, may include a Reye-like syndrome of recurrent hypoglycemia with coma (9, 10) or sudden death (11, 12). Based upon our characterization of the human MCAD mRNA (13) and gene (14), we analyzed the MCAD mRNA of MCAD-deficient individuals. We and others (15–18) identified an A to G transition at position 985 (A985G) of the MCAD mRNA that causes a lysine to glutamate substitution at residue 304 (K304E) of the mature protein. This mutant allele is present in over 90% of MCAD-deficient individuals in the United States and Europe (8, 19, 20). This common mutation alters a basic residue to a negatively charged acidic residue, a change that could dramatically affect the structure and therefore the catalytic function of the mutant enzyme. However, analysis of the crystal structure of tetrameric pig MCAD suggests that the mutation at amino acid 304 would not directly affect the active site. Rather, this residue lies within the helices forming subunit interfaces and may therefore interfere with proper tetramer assembly (21, 22) and protein stability.

Our initial characterization of the mutant (Glu^{304}) MCAD protein by Western blot analysis in patients' fibroblasts consistently revealed a normal-sized, immunoreactive protein (13) with the levels comparable to normal fibroblasts. However, in postmortem liver tissue, we observed that the mutant protein levels were markedly reduced (13), raising the possibility that the mutant protein was rapidly degraded or that there is some tissue-specific variability of MCAD protein stability. Others initially examined the mutant protein with short-term labeling experiments and found comparable amounts of newly synthesized immunoreactive MCAD protein in normal and MCAD-deficient fibroblasts (23). With pulse-chase experiments, however, levels of K304E mutant protein were markedly decreased after 24 h (24). Although such studies suggest that protein instability is the major cause of MCAD deficiency in patients with the K304E mutation, careful examination of K304E mutant enzyme activity was not performed.

After expression of the mutant enzyme in *Escherichia coli*, we noted readily detectable MCAD on protein immunoblots, but no enzymatic activity was detected (17). However, most of the expressed MCAD protein was aggregated in inclusion bodies and improperly folded, making interpretation of the results problematic. Moreover, because bacteria lack mitochondria, neither the effect of the mutation on mitochondrial import nor the stability of the mutant protein in its normal mitochondrial environment could be examined. To characterize adequately the mutant enzyme, we used expression of cDNA encoding MCAD in a eukaryotic system and report herein the effect of the K304E mutation on MCAD activity and stability.

MATERIALS AND METHODS

Construction of normal and Glu^{304} mutant expression plasmids. RNA isolation, cDNA synthesis, PCR amplification, and

Received February 4, 1993; accepted June 1, 1993.
Correspondence and reprint requests: Arnold W. Strauss, M.D., St. Louis Children's Hospital, 1 Children's Place, St. Louis, MO 63110.
D.P.K. is the recipient of a Lucille P. Markey Scholar Award. A.J.W. is a Fellow of the American Heart Association, Missouri Affiliate. Supported by grants from the Lucille P. Markey Charitable Trust, the March of Dimes Foundation, and the National Institutes of Health (AM20407).

subsequent subcloning into plasmid vectors were performed as described previously (15). Sequencing of MCAD cDNA clones from a patient with MCAD deficiency and normal controls resulted in the identification of one clone containing normal MCAD cDNA without any PCR errors (WTHT26) and one clone containing MCAD cDNA diverging only by the single A to G point mutation at position 985 (KJ1). The normal MCAD cDNA from subclone WTHT26 and mutant 985G MCAD cDNA from subclone KJ1 were inserted into the *Bam*HI site of the mammalian expression vector PSG5 (Promega, Madison, WI) and subjected to DNA sequence analysis to confirm proper insertion and orientation.

Cell transfection. The cell line CHO-K1 was maintained in an atmosphere of 5% CO_2 in growth medium (Dulbecco's modified Eagle's medium supplemented with 10% FCS). The cells were plated at a density of approximately 1.5×10^6 cells per 60-mm dish. The next day, the cells were refed with growth medium for 3 h, and transfections were performed by the calcium phosphate coprecipitation method (25). Each precipitate contained 15 μg of test plasmid and 5 μg of pMSV βgal to normalize for transfection efficiency and cell number. Forty-eight h later, the cells were harvested, pooled, and divided into aliquots for Western blot analysis, RNA analysis, protein activity, and β-galactosidase activity (26).

RNA analysis. Total RNA was extracted from cell pellets using the RNAzol B (Tel-test, Inc., Friendswook, TX) technique. RNA (10 μg total RNA per lane) was separated by formaldehyde/agarose gel electrophoresis, and the conditions for preparation of the RNA blots were as described previously (13).

Western blot analysis. Extracts from the transfected cells were prepared by three cycles of freezing and thawing in 250 μL of 0.25 mM Tris, pH 7.8, followed by sonication. Protein extract (25 μg) from each of the transfection experiments was separated on a 12.5% SDS polyacrylamide gel. The proteins were transferred to Immobilon (Millipore Corp., Bedford, MA) and incubated with anti-porcine MCAD (15). An alkaline phosphatase calorimetric detection system (Promega) was used for visualization of the proteins.

ACD activity assay. The enzymatic assays were performed as previously described (27) on cell pellets from each transfection using electron transfer flavoprotein as the electron acceptor.

Densitometric analysis. An LKB ultrascan XL laser densitometer (LKB Instruments, Gaithersburg, MD) was used to quantify data from Western and RNA blots. All densitometer measurements were within the linear response range of the instrument.

RESULTS

Expression of normal (Lys³⁰⁴) and mutant (Glu³⁰⁴) MCAD in CHO cells. cDNA encoding precursor MCAD containing either an adenine (A985) or a guanine (G985) at position 985 were inserted into the PSG5 expression vector downstream of the SV40 early promoter (Fig. 1). The normal (PSG5-A985), mutant (PSG5-G985), or vector without any insert (PSG5-0, as a control) plasmids were transfected into CHO cells. An SV40-β-Gal plasmid was cotransfected to correct for transfection efficiency. To confirm appropriate transcription of the MCAD inserts and to compare levels of endogenous MCAD mRNA with those of transfected cells, total RNA was isolated from cell pellets from each of the three transfection experiments (PSG5-A985, PSG5-G985, and PSG5-0). The transfection efficiencies with the three constructs were equivalent. Equal amounts of total RNA were analyzed by RNA blot analysis with a human MCAD cDNA probe (Fig. 2). RNA from both the normal (PSG5-A985) and mutant (PSG5-G985) transfected cells demonstrated a single band corresponding in size to the expected expressed MCAD mRNA (~1.9 kb). Densitometric analysis of the autoradiogram revealed that the amounts of expressed normal and mutant MCAD mRNA were equivalent, demonstrating equally efficient transcription of both normal and mutant MCAD plasmids (data not shown). In contrast, no band was detected in the lane containing RNA from the PSG5-0 (vector alone) transfected cells, demonstrating that endogenous MCAD mRNA levels are very low in these cells.

To assess the relative levels of the expressed normal and mutant MCAD proteins in comparison with endogenous MCAD protein, immunoblot analysis with a polyclonal rabbit MCAD antibody was performed using total protein extracted from pooled cell pellets from each of the transfection experiments (Fig.

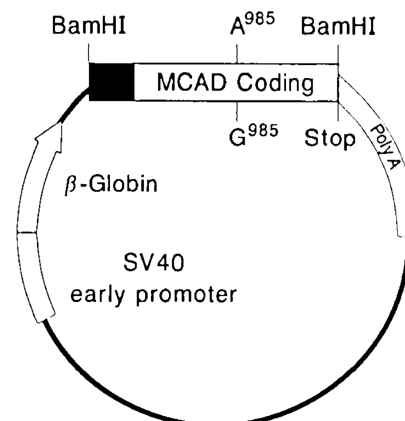

Fig. 1. Diagram of the MCAD expression vector. The MCAD cDNA, containing the entire coding region with the mitochondrial transit peptide (*shaded*) and either an A (A985) or a G (G985) at coding position 985, was inserted into the PSG5 vector at the *Bam*HI site as illustrated. The positions of the SV40 early promoter, β-globin intron sequence, and polyadenylation sites are indicated.

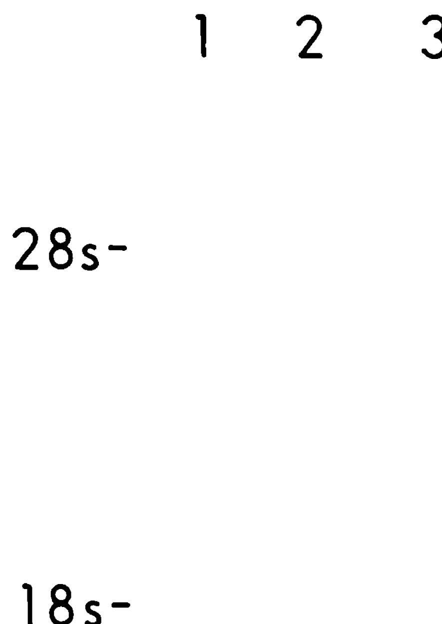

Fig. 2. RNA blot analysis of MCAD mRNA expressed in transfected CHO cells. Cells were harvested 48 h after transient transfection. Ten μg of total RNA were loaded per lane. Positions of migration of ribosomal RNA, identified with ethidium bromide staining, are indicated as *18s* and *28s*. *Lane 1* contains RNA from cells transfected with PSG5-0, the vector containing no insert, as a control. *Lane 2* contains RNA isolated from cells transfected with PSG5-A985 (normal) MCAD cDNA. *Lane 3* contains RNA from cells transfected with PSG5-G985 (mutant) MCAD cDNA.

3). Extracts from cells containing either normal or mutant (G985) MCAD cDNA demonstrated a single signal identical in size to the endogenous MCAD protein in control (PSG5-0) transfected CHO cells. The levels of both the expressed normal MCAD and the expressed mutant (Glu^{304}) MCAD proteins were substantially higher than the levels of endogenous MCAD protein present in the control (PSG5-0) transfected cells. Furthermore, the level of expressed normal MCAD protein was much higher than the expressed mutant MCAD protein. To quantify the relative, steady state amounts of normal and mutant immunodetectable protein, densitometric analysis of the immunoblot (Fig. 3) was performed. The level of immunodetectable normal MCAD expressed was 5-fold greater than the expressed mutant (Glu^{304}) protein (data not shown).

ACD enzyme activity measurements of expressed normal and mutant (Glu^{304}) MCAD. ACD activities were determined in extracts from each of the three transfection experiments (PSG5-A985, PSG5-G985, and PSG5-0) using a fluorometric assay with electron transfer flavoprotein as the electron acceptor (Table 1). Mean MCAD activity, as determined by the activity with octanoyl-CoA substrate, was 7-fold higher in the extract derived from cells transfected with normal MCAD cDNA (PSG5-A985) (72.3 ± 14.6 nmol/min/mg) compared with the activity in the extract from the control (PSG5-0) transfected cells (10.4 ± 1.0 nmol/min/mg). The extract from cells transfected with mutant MCAD cDNA (PSG5-G985) had modest (2- to 3-fold) but reproducibly increased activity compared with the PSG5-0 transfected cells. The MCAD activity in the cells transfected with normal MCAD cDNA (PSG5-A985) was much higher than that in the mutant cDNA (PSG5-G985) transfected cells (72.3 ± 14.6 *versus* 20.4 ± 3.3 nmol/min/mg protein). After subtraction of the endogenous MCAD activity, the expressed normal MCAD had 6-fold higher activity than the expressed mutant MCAD. This degree of reduced MCAD activity in the cells with expressed mutant MCAD, as compared with the normal, was similar to the 5-fold reduction in immunodetectable MCAD antigen (Fig. 3). Thus, calculated sp act of the mutant MCAD is similar to that of normal MCAD (Table 1).

DISCUSSION

MCAD deficiency is a common and frequently fatal inherited metabolic defect. Although population and genetic studies are quite convincing that the "common mutation" (an A to G transition at nucleotide 985 that results in a lysine to glumatic acid change at position 304 of the protein) is indeed a disease-causing mutation, the results (13, 23, 24) regarding the basis of reduced enzyme activity in individuals homozygous for this mutation, which have used patients' fibroblasts, are incomplete and contradictory. We therefore chose to overexpress normal MCAD and the K304E mutant MCAD in CHO cells to better delineate whether the mechanism responsible for MCAD deficiency is increased degradation rate of MCAD protein, a decrease in MCAD sp act, or a combination of both. The results demonstrate equal levels of mRNA, indicating no differences in synthesis (plasmid transcription) or degradation between mutant and normal MCAD mRNA. The increased levels of normal-sized mature MCAD protein in cells containing expressed normal MCAD compared with control cells indicate that normal translation, mitochondrial import, and cleavage of the leader or transit peptide can occur in this eukaryotic expression system. Because cells containing the mutant MCAD cDNA also have increased amounts of mature MCAD protein compared with control cells, we conclude that the mutant MCAD protein can also be appropriately expressed, transported into mitochondria, and processed to the mature form. Moreover, this mutant protein is sufficiently long-lived to allow detection of an increased amount of mutant protein compared with control cells. Similar results were obtained by Jensen *et al.* (28) after transfection of wild-type and mutant MCAD into COS cells.

Several important conclusions can be drawn from the activity data. The marked increase in activity with octanoyl-CoA substrate seen in cells containing wild-type (PSG5-A985) MCAD cDNA compared with control (PSG5-0) cells confirms that the increased MCAD protein seen on Western blot is catalytically active, and that this is an efficient and functional expression system. Furthermore, the increase in activity with octanoyl-CoA substrate in cells containing mutant MCAD cDNA compared with control cells demonstrates that the mutant protein has substantial enzymatic activity. In fact, the difference (6-fold) in activities of extracts from normal and mutant cells with octanoyl-CoA substrate very closely correlates with the difference in the relative amounts of MCAD protein on the Western blot (5-fold). Thus, the mutant MCAD protein has a very similar sp act (*i.e.* activity per μg MCAD antigen) *versus* C8-CoA substrate to the normal protein. In this assay of crude extracts, mutant MCAD is therefore as active as normal MCAD.

Taken together, our results demonstrate that the amount of mutant MCAD protein is reduced in these transfected cells, even though both mRNA are present in equal amounts. The most likely explanation for this result is that the mutant protein is more rapidly degraded than the normal MCAD. This conclusion is similar to that of Coates *et al.* (24), who used fibroblasts from patients with the K304E mutation in pulse-chase experiments, and that of Jensen *et al.* (28) in transfection experiments similar to ours. The apparent discrepancies with our earlier work (13) in mutant fibroblasts and the short-term labeling experiments of Ikeda *et al.* (23) suggest that MCAD protein half-life may vary with differing experimental conditions. However, these previous studies (13, 24, 28) did not estimate the relative sp act of the normal and mutant proteins, such that no conclusions concern-

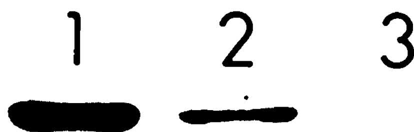

Fig. 3. Protein immunoblot analysis of cell extracts from transiently transfected CHO cells. Cells were harvested 48 h after transient transfection and protein extracts prepared. The blot was probed with anti-MCAD after transfer. *Lane 1* contains 5 μg of protein from cells transfected with PSG5-A985 (normal). *Lane 2* has 5 μg of protein from cells transfected with PSG5-G985 (mutant). *Lane 3* contains 5 μg of protein from the transfection with PSG5-0, the vector without any insert.

Table 1. *Acyl CoA enzyme activity in transfected CHO cells**

	Substrate		
	Palmitoyl-CoA	Octanoyl-CoA (sp act)	Butyryl-CoA
Control vector (PSG5-0)	14.9 ± 2.6	10.4 ± 1.0	24.0 ± 8.1
Normal (PSG5-A985)	42.2 ± 6.0	72.3 ± 14.6 (53.7)	27.5 ± 5.6
Mutant (PSG5-G985)	42.8 ± 6.9	20.4 ± 3.3 (45.5)	33.9 ± 12

* Enzyme activity is expressed as nmol of electron transfer flavoprotein reduced per min per mg of total protein. The SD of multiple assays (n = 3–10) performed on each protein extract is also shown. Sp act is defined as activity with octanoyl-CoA substrate in transfected cells minus activity in control cells divided by the density in arbitrary units of the expressed immunoreactive MCAD band in transfected cells.

ing residual activity of the mutant MCAD were made. Therefore, our demonstration of normal sp act of the mutant MCAD enzyme is crucial in proving that an increased rate of protein degradation, and not reduced enzymatic activity, is the cause of the MCAD deficiency state.

Our results do not allow us to determine whether the mutation affects tetramer formation. The structural data (21, 22) demonstrate that subunit interactions occur through the first 15 NH_2-terminal residues, the final 10 COOH-terminal amino acids, the flavin moieties of adjacent subunits, and the H and I helices near the COOH-terminus. If the rate of tetramer formation were retarded, it seems likely that susceptibility to proteolysis would be increased because the most stable conformation of MCAD subunits would not have been achieved. The normal MCAD tetramer is highly ordered and cannot be reformed after denaturation *in vitro*. An alternative to explain increased MCAD degradation rate is that the mutant protein precursor is imported into mitochondria less efficiently than the normal MCAD and is degraded by cytosolic proteases. Because transient transfection systems, such as that used here, do not achieve a prolonged steady state, we believe that stably transfected cell lines will be required to compare MCAD synthetic and degradative rates and to examine the intracellular sites of mutant MCAD breakdown.

In summary, we have used a eukaryotic expression system that should be easily adaptable for the study of mutations of mitochondrial oxidative enzymes including long-chain ACD, short-chain ACD, and additional naturally occurring MCAD mutations. We have also confirmed, at the protein level, that the putative common MCAD mutation does indeed cause MCAD deficiency and that the deficiency state is due primarily to the increased degradation rate of mutant rate MCAD protein rather than to reduced sp act of the mutant enzyme. In fact, the expressed mutant MCAD has a sp act similar to that of the normal protein.

Acknowledgments. The authors thank Moira L. Ogden and Teresa Caira for expert technical assistance.

REFERENCES

1. Beinert H 1963 Acyl coenzyme A dehydrogenases. In: Boyer PD, Hardy H, Myrback K (eds) The Enzymes. Academic Press, New York, pp 447–476
2. Ikeda Y, Ikeda KO, Tanaka K 1985 Purification and characterization of SCAD, MCAD, and LCAD from rat liver mitochondria. J Biol Chem 260:1311–1325
3. Kolvraa S, Gregersen N, Christensen E, Hobolth N 1982 *In vitro* fibroblast studies in a patient with C6-C10-dicarboxylic aciduria: evidence for a defect in general acyl-CoA dehydrogenase. Clin Chim Acta 126:53–67
4. Rhead WJ, Amendt BA, Fritchman KS, Felts SJ 1983 Dicarboxylic aciduria and deficient (1-14C) octanoate oxidation and MCAD in fibroblasts. Science 221:73–75
5. Stanley CA, Hale DE, Coates PM, Hall CL, Corkey BD, Yang W, Kelley RI, Gonzales EL, Williamson JR, Baker L 1983 MCAD deficiency in children with nonketotic hypoglycemia and low carnitine levels. Pediatr Res 17:877–883
6. Howat AJ, Bennett MJ, Variend S, Shaw L, Engel PC 1985 Defects of metabolism of fatty acids in the sudden infant death syndrome. Br Med J 290:1771–1773
7. Blakemore AIF, Singleton H, Pollitt R, Engel PC, Kolvraa S, Gregersen N, Curtis D 1991 Frequency of the G985 MCAD mutation in the general population. Lancet 337:298–299
8. Matsubara Y, Narisawa K, Tada K, Ikeda H, Ye-Qi Y, Danks DM, Green A, McCabe ERB 1991 Prevalence of K329-E mutation in MCAD gene determined from Guthrie cards. Lancet 338:552–553
9. Treem WR, Witzleben CA, Piccoli DA, Stanley CA, Hale DE, Coates PM, Watkins JB 1986 MCAD and LCAD deficiency: clinical, pathological and ultrastructural differentiation from Reye's syndrome. Hepatology 6:1270–1278
10. Bougneres PF, Rocchiccioli F, Kolvraa S, Hadchoel M, Lalau-Keraly J, Chaussain JL, Wadman SK, Gregersen N 1985 The MCAD deficiency in two siblings with a Reye-like syndrome. J Pediatr 106:918–921
11. Duran M, Hofkamp M, Rhead WJ, Saudubray J-M, Wadman SK 1986 Sudden child death and 'healthy' affected family members with MCAD deficiency. Pediatrics 78:1052–1057
12. Kelly DP, Hale DE, Rutledge LS, Ogden ML, Whelan, AJ, Zhang Z, Strauss AW 1991 Molecular basis of inherited MCAD deficiency causing sudden child death. J Inherited Metab Dis 15:171–180
13. Kelly DP, Kim J-J, Billadello JJ, Hainline BE, Chu TW, Strauss AW 1987 Nucleotide sequence of MCAD mRNA and its expression in enzyme-deficient human tissue. Proc Natl Acad Sci USA 84:4068–4072
14. Zhang Z, Kelly DP, Kim J-J, Zhou Y, Ogden ML, Whelan AJ, Strauss AW 1992 Structural organization and regulatory regions of the human MCAD gene. Biochemistry 31:81–89
15. Kelly DP, Whelan AJ, Ogden ML, Alpers R, Zhang Z, Bellus G, Gregersen N, Dorland L, Strauss AW 1990 Molecular characterization of inherited MCAD deficiency. Proc Natl Acad Sci USA 87:9236–9240
16. Yokota I, Indo Y, Coates PM, Tanaka K 1990 Molecular basis of MCAD deficiency. J Clin Invest 86:1000–1003
17. Gregersen N, Andersen BS, Bross P, Winter V, Rudiger N, Engst S, Christensen E, Kelly DP, Strauss AW, Kolvraa S, Bolund L, Ghisla S 1991 Molecular characterization of MCAD deficiency: identification of a Lys329 to glu mutation in the MCAD gene and expression of inactive mutant enzyme protein in *E coli*. Hum Genet 86:545–551
18. Matsubara Y, Narisawa K, Miyabayshi S, Tada K, Coates PM, Bachmann C, Elsas LJ, Pollitt RJ, Rhead WJ, Roe CR 1992 Identification of a common mutation in patients with MCAD deficiency. Biochem Biophys Res Commun 171:498–505
19. Yokota I, Coates PM, Hale DE, Rinaldo P, Tanaka K 1991 Molecular survey of a prevalent mutation 985A-to-G transition and identification of five infrequent mutations in the MCAD gene in 55 patients with MCAD deficiency. Am J Hum Genet 49:1280–1291
20. Curtis D, Blakemore AIF, Engel PC, McGregor D, Besley G, Kolvraa S, Gregersen N 1991 Heterogeneity for mutations in MCAD deficiency in the UK population. Clin Genet 49:1280–1291
21. Kim J-J, Wu J 1988 Structure of the MCAD from pig liver mitochondria at 3-A resolution. Proc Natl Acad Sci USA 85:6677–6681
22. Kim J-J, Wang M, Djordjevic S, Paschke R 1992 The three dimensional structure of MCAD. Prog Clin Biol Res 375:111–126
23. Ikeda Y, Hale DE, Keese SM, Coates PM, Tanaka K 1986 Biosynthesis of variant MCAD in cultured fibroblasts from patients with MCAD deficiency. Pediatr Res 20:843–847
24. Coates PM, Indo I, Young D, Hale DE, Tanaka K 1992 Immunochemical characterization of variant medium-chain acyl-CoA dehydrogenase in fibroblasts from patients with MCAD deficiency. Pediatr Res 31:34–38
25. Gorman L 1985 High efficiency gene transfer into mammalian cells. In: DNA Cloning, Vol II, A Practical Approach, Glover DM (ed) IRL Press, Oxford, UK, pp 143–190
26. Rosenthal NI 1987 Identification of regulatory elements of cloned genes with functional assays. Methods Enzymol 152:704–720
27. Frerman FE, Goodman SI 1985 Fluorometric assay of ACD in normal and mutant fibroblasts. Biochem Med 33:28–44
28. Jensen TG, Andresen BS, Bross P, Jensen UB, Holme E, Kolvraa S, Gregersen N, Bolund L 1992 Expression of wild-type and mutant MCAD cDNA in eucaryotic cells. Biochim Biophys Acta 1180:65–72