Am. J. Hum. Genet. 55:297–304, 1994

Molecular Basis of Maple Syrup Urine Disease: Novel Mutations at the E1α Locus That Impair E1($\alpha_2\beta_2$) Assembly or Decrease Steady-State E1α mRNA Levels of Branched-Chain α-Keto Acid Dehydrogenase Complex

Jacinta L. Chuang,[1] Carolyn R. Fisher,[1] Rody P. Cox,[2] and David T. Chuang[1]

Departments of [1]Biochemistry and [2]Internal Medicine, University of Texas Southwestern Medical Center, Dallas

Summary

We report the occurrence of three novel mutations in the E1α (BCKDHA) locus of the branched-chain α-keto acid dehydrogenase (BCKAD) complex that cause maple syrup urine disease (MSUD). An 8-bp deletion in exon 7 is present in one allele of a compound-heterozygous patient (GM-649). A single C nucleotide insertion in exon 2 occurs in one allele of an intermediate-MSUD patient (Lo). The second allele of patient Lo carries an A-to-G transition in exon 9 of the E1α gene. This missense mutation changes Tyr-368 to Cys (Y368C) in the E1α subunit. Both the 8-bp deletion and the single C insertion generate a downstream nonsense codon. Both mutations appear to be associated with a low abundance of the mutant E1α mRNA, as determined by allele-specific oligonucleotide probing. Transfection studies strongly suggest that the Y368C substitution in the E1α subunit impairs its proper assembly with the normal E1β. Unassembled as well as misassembled E1α and E1β subunits are degraded in the cell.

Introduction

Maple syrup urine disease (MSUD) or branched-chain keto aciduria is an autosomal recessive disorder in the degradation of branched-chain α-keto acids derived from leucine, isoleucine, and valine (Dancis et al. 1960). The classical form of the disease is characterized by the rapid onset of severe keto acidosis within the first week of life. This is accompanied by a maple-syrup odor in urine and, clinically, by seizures, coma, and death, if untreated. There is a high incidence of mental retardation in survivors. Several milder forms of MSUD have also been described, which include intermediate, intermittent, thiamin responsive, and E3 deficient (Chuang and Shih, in press). MSUD is a rare metabolic disorder, but in certain consanguineous populations such as Mennonites the incidence is as high as 1 in 176 live births (Marshall and DiGeorge 1981).

The enzyme deficient in MSUD patients, the mitochondrial branched-chain α-keto acid dehydrogenase (BCKAD) complex, consists of three catalytic components and two regulatory enzymes (Yeaman 1989). The subunits of the BCKAD complex are encoded by nuclear genes, synthesized in the cytosol and imported into mitochondria where assembly occurs (Lindsay 1989). The E1 component is a decarboxylase comprising two α (Mr = 47,000) and two β (Mr = 37,000) subunits. The human E1α precursor contains 445 amino acids and is processed into a mature polypeptide of 400 amino acids. The E1β has a precursor of 392 amino acids, and the mature peptide has 342 amino acids. The E2 component, a transacylase, is the core of the enzyme complex consisting of 24 identical lipoate-bearing subunits (calculated monomer Mr = 46,518) that form a cubic structure with octahedral symmetry. The E3 component is a dehydrogenase that exists as a homodimer (monomer Mr = 52,000), and it is common to the pyruvate and α-ketoglutarate dehydrogenase complexes. The two regulatory enzymes of the BCKAD complex are a specific kinase and a specific phosphatase, which regulate the activity of the enzyme complex through a phosphorylation (inactivation)/dephosphorylation (activation) cycle (Randle et al. 1984).

MSUD is genetically heterogeneous, as demonstrated by mutations in E1α, E1β, E2, and E3 loci of the BCKAD complex (Chuang and Shih, in press). The molecular mechanisms underlying MSUD are complex. Mutations in one of the subunits can disrupt the macromolecular assembly and, therefore, the function of the BCKAD complex. We have shown elsewhere that the homozygous Tyr-393–to–Asn (Y393N) mutation in the E1α subunit of Mennonite patients impairs assembly with E1β, which causes degradation and loss of catalytic activity of the E1 component (Fisher et al. 1991*a*, 1991*b*). The Y393N mutation was initially reported to occur in a compound-heterozygous non-Mennonite patient (GM-649) with classical MSUD (Zhang et al. 1989). The other allele in this patient was postulated

Received December 29, 1993; accepted for publication April 18, 1994.

Address for correspondence and reprints: Dr. David T. Chuang, Department of Biochemistry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-9038.

0002-9297/94/5502-0010$02.00

to be a regulatory mutation, since the E1α mRNA level for this allele was reduced (Zhang et al. 1989). In the present study, we have elucidated the mutation in the second allele of this patient. We have also studied another MSUD patient with an intermediate phenotype (Gonzales-Rios et al. 1985) and low E1α mRNA and subunit levels (Fisher et al. 1989). Here we report three novel mutations in these two patients, as well as the molecular mechanism responsible for the MSUD phenotype.

Patients, Material, and Methods

MSUD Patients and Cell Lines

Skin fibroblasts from a classical MSUD patient (GM-649, a 9-mo-old Caucasian male) and the heterozygous mother (GM651) and father (GM650) (Chuang et al. 1982; Zhang et al. 1989) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Fibroblasts of a classical MSUD patient (CQ, a 7-mo-old Caucasian female) and her mother (DQ) were provided by Dr. A. E. Chudley, (University of Saskatchewan, Saskatoon). CQ at 5 mo of age had the following plasma levels: leucine, 31.4 mg/dl; isoleucine, 5.8 mg/dl; and valine, 6.8 mg/dl. She responded to dietary therapy. Lymphoblasts of an intermediate-MSUD patient (Lo) (Gonzales-Rios et al. 1985) were supplied by Dr. Dean Danner (Emory University, Atlanta). Normal lymphoblast cultures were established by standard methods from peripheral blood lymphocytes by infection with Epstein-Barr virus (Miller and Lipman 1973). Lymphoblast cell lines were grown in RPMI-1640 medium (ICN-Flow) containing 15% heat-inactivated fetal bovine serum as described elsewhere (Fisher et al. 1991*a*). Fibroblast lines also were grown as described elsewhere (Fisher et al. 1989).

Cloning and Sequencing of Amplified Genomic DNA

Genomic DNA was isolated (Davis et al. 1986) and was amplified by PCR in 50 μl of the following reaction mixture: 1 μM each of the sense primer and the antisense primer, 200 μM each of dNTPs, 1.25 units of *Taq* DNA polymerase (Promega), 5 μl of 10 × reaction buffer (Promega), and 200 ng of template. The reaction was cycled at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for a total of 35 cycles. Primers used were as follows: 5′-TGCTTCTGATGCAGGTGGTCTC-3′ (intronic) (sense) and 5′-GTGGAGGCACAGATAGACGT-3′ (intronic) (antisense) for exon 2, 5′-TCGTGCATGTTC-CTTATCTCAGC-3′ (intronic) (sense) and 5′-GTCAGTGCT-GTGGGGATGCT (intronic) (antisense) for exon 7, and 5′-TAGCCTGCCCACTGCCCCATGT-3′ (intronic) (sense) and 5′-TCTCGGGGTACCTGAGGATGG-3′ (antisense) (bases 1408–1388) for exon 9 (with a G-to-C change at base 1397, to create a *Kpn*I site. The nucleotides in the human E1α gene and cDNA were numbered according to the system described elsewhere (Chuang et al. 1993). PCR-generated fragments for exon 2 (320 bp), exon 7 (240 bp), and exon 9 (243 bp) were purified by agarose gel electrophoresis and were sequenced directly, by using the fmol DNA sequencing kit (Promega). For heterozygous alleles, purified PCR fragments were subcloned by blunt-end ligation into pBluescript SK+ plasmids (Stratagene) previously digested with *Sma*I. Positive clones were selected for double-strand sequencing (Chen and Seeburg 1985).

Allele-specific Oligonucleotide (ASO) Probing of Amplified cDNA

cDNAs from MSUD cell line Lo and normal cell lines were prepared by reverse transcription of the total cellular poly $(A)^+$ RNA isolated from each cell line (Chirgwin et al. 1979). Each reaction mixture (in 50 μl) contained 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 5 μg of poly $(A)^+$RNA, 125 μM each of dNTPs, 5 μM oligo $(dT)_{17}$, 24 units of RNasin (Promega), and 500 units of Moloney murine leukemia virus reverse transcriptase (RNase H^-) (GIBCO/BRL). mRNA was denatured at 95°C for 5 min, the reaction components were added, and the reaction was carried out at 45°C for 120 min. The reaction mixture was held at −20°C until amplification. The primers used for amplification were as follows: 5′-GGGCACCACAGCACCAGTGAC-3′ for bases 1027–1047 (sense) and 5′-TCTCGGGGTACCTGAGG-ATGG-3′ for bases 1408–1388 (antisense), which generated a 382-bp fragment. The first round of amplification was performed in 50 μl of a reaction mixture containing the standard PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1 μM $MgCl_2$, and 0.1% Triton-X) with the following components: 10 μl of cDNA, 1 μM each of the primers, 200 μM each of dNTPs, and 1.25 units of *Taq* DNA polymerase (Promega). The reactions were cycled 35 times through the following series of reaction temperatures: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min and then were held at 4°C. One microliter of the product of the first-round amplification was used as template for a second-round amplification under identical conditions.

cDNA fragments amplified by PCR were electrophoresed on a 1.2% agarose gel and were blotted onto Genescreen Plus (DuPont/NEN). Blots were probed sequentially with end-labeled oligonucleotides containing the Y368C mutation (5′-GACGTGTGTCAGGAG-3′) and the normal sequence (5′-GACGTGTATCAGGAG-3′).

Construction of EBO Expression Vectors and Transfection Studies

The Epstein-Barr virus–based expression vector EBO-pLPP was provided by Dr. Robert Margolskee (Roche Institute of Molecular Biology, Nutley, NJ). The characteristics of the vector and the insertion of a full-length normal human E1α cDNA have been described elsewhere (Fisher et al. 1991*a*). The EBO vector contains the hygromycin phosphotransferase sequence, which confers antibiotic resistance. To construct a recombinant EBO vector expressing the Y368C mutant E1α, a *Bpm*I-*Kpn*I fragment (bases

1192–1402) that contained the A-to-G transition at base 1256 was obtained from the amplified E1α cDNA from Lo cells (see description of cDNA amplification). An *Xba*I-*Bpm*I fragment (1,322 bp) that contained the mitochondrial targeting sequence and the remainder of the human E1α coding sequence was obtained from the normal hE1α/EBO plasmid (Fisher et al. 1991*a*). The two restriction fragments were ligated into the EBO vector previously digested with *Xba*I and *Kpn*I.

Transient transfection experiments were carried out as described elsewhere (Fisher et al. 1991*a*). Cells at a concentration of 5×10^7 ml in RPMI 1640 medium (JRH Biosciences) containing 15% heat-inactivated FCS and antibiotics (kanamycin, 0.1 mg/ml; penicillin, 50 U/ml; and streptomycin, 50 mg/ml) were incubated with EBO plasmid DNA (100 μg/ml) and sheared salmon sperm DNA (400 μg/ml) at room temperature for 10 min with gentle inversions. Electroporation of 1-ml aliquots of cell/DNA mixtures were carried out as described elsewhere (Fisher et al. 1991*a*). Hygromycin B (200 μg/ml) selection of transfected cells was initiated 48 h after electroporation. Viable cells were separated from nonviable cells by Ficoll gradient at 10 d. Intact untransfected and transfected lymphoblasts were assayed for decarboxylation rates by using α-keto [1-^{14}C] isovalerate as described elsewhere (Chuang et al. 1982). Decarboxylation assays were carried out on triplicate dishes for each untransfected and transfected cell line.

Results

Identification of the Second Mutation in Classical MSUD Patient GM-649

As described above, GM-649 was previously shown to be a compound heterozygote at the E1α locus (Zhang et al. 1989). One allele carried the Y393N mutation, and the second allele was postulated to be a regulatory mutation, on the basis of a reduced mRNA level for this allele. To study the possible regulatory defect, we amplified the promoter regulatory region corresponding to bases +27 to −320 and bases +27 to −909 of the E1α gene from patient GM-649. Direct sequencing revealed that the patient is heterozygous for an A-to-T transversion at base −284. The mother, whose mRNA level was approximately half of normal levels (Zhang et al. 1989), is also heterozygous for this base substitution. The A-to-T transversion is within the region for optimal basal gene expression, between bases +1 and −320, as previously determined by promoter mapping (Chuang et al. 1993). The significance of this base alteration for promoter activity was investigated by using the luciferase reporter assay. The constructs containing the A-to-T conversion, when transfected into HepG2 cells, showed normal luciferase activity (data not shown). The results excluded the possibility that this base change in the promoter region was responsible for the reduced mRNA level and the MSUD phenotype. We concluded that the A(−284)-to-T transversion in the E1α gene was a normal polymorphism.

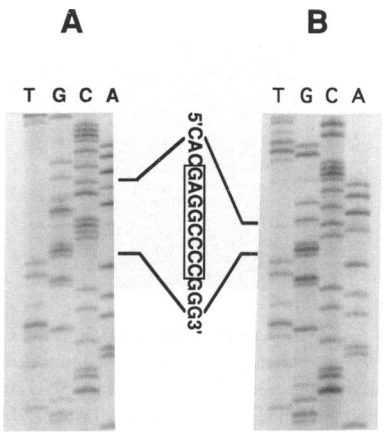

Figure 1 Nucleotide sequencing showing the 8-bp deletion in exon 7 of the E1α gene. Genomic DNAs were prepared from normal fibroblasts (*A*) and MSUD fibroblasts (patient GM-649) (*B*). The region encompassing exon 7 was amplified (for the primers used, see Patients, Material, and Methods) The amplification products (240 bp) were phosphorylated and subcloned into pBS plasmids and were sequenced using the sense primer. The boxed sequence (5′ to 3′) indicates the 8-bp deletion corresponding to bases 887–894 of the human E1α cDNA (Chuang et al. 1993).

To detect the mutation in the second allele of GM-649, each of the nine exons of the E1α gene of this patient were amplified and directly sequenced. These sequences revealed that the patient was heterozygous for an alteration in exon 7 (data not shown). This region was subcloned, and the sequencing of the subclones indicated that an 8-bp deletion occurred between bases 887 and 894 in one allele of GM-649 (fig. 1*B*), when compared with a normal sequence (fig. 1*A*). The results established, at the genomic level, that the second mutation in GM-649 was the 8-bp deletion at the E1α locus.

The 8-bp deletion produces a nonsense codon 255 of the mature E1α subunit. The same deletion abolishes a *Sma*I restriction site in exon 7 of the E1α gene. This alteration in the restriction was used to study segregation of the 8-bp deletion in this family. As shown in figure 2, uncut amplified exon 7 DNA was 240 bp in size (lane 1). Digestion of the amplified DNA from normal subjects by *Sma*I generated two smaller fragments, 169 and 71 bp in size (lane 2). Two identical fragments were obtained with amplified DNA from the father (GM-650), who does not carry the deletion (lane 4). The patient (GM-649) had one allele with the 8-bp deletion, which produced an amplified exon 7 DNA that was not cut by *Sma*I (lane 3). The other allele contained the *Sma*I site and was cut into two fragments by the restriction enzyme. Thus, a total of three fragments is observed in lane 3. The mother (GM-651), who was heterozygous for the 8-bp deletion, produced the

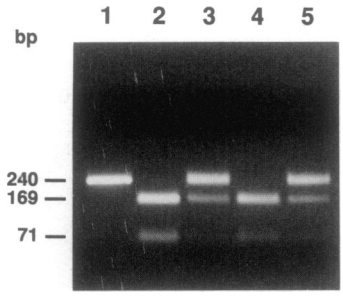

Figure 2 Segregation of the 8-bp deletion in the GM-649 family by restriction-enzyme analysis. The region containing exon 7 was amplified from fibroblasts from normal cells (lane 2), MSUD patient GM-649 (lane 3), the mother (GM-651) (lane 5), and the father (GM-650) (lane 4), as described in fig. 1. The amplified products were digested with *Sma*I restriction enzyme. The digests were separated on 2% agarose gels, and the DNA fragments were stained with ethidium bromide. The DNAs of normal cells and of cells from the father contained wild-type exon 7 with the *Sma*I site and were cut into two fragments, of 169 and 71 bp. The patient and the mother were heterozygous for the 8-bp deletion, which abolishes the *Sma*I site. As a result, both the uncut PCR product and two cut normal fragments are present. Lane 1 is an undigested 240-bp PCR product from the normal cells.

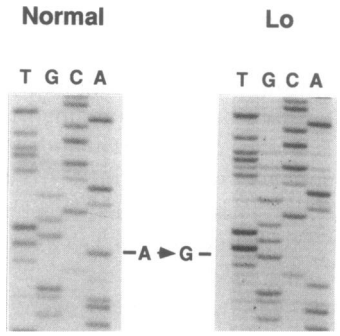

Figure 3 Nucleotide sequencing showing the A-to-G transition (mutation Y368C) in amplified E1α cDNA from intermediate-MSUD patient Lo. mRNA prepared from normal and Lo lymphoblasts was reverse transcribed, and the region corresponding to bases 1027–1408 of the E1α cDNA was amplified (for the primers used, see Patients, Material, and Methods). The PCR products (382 bp) were subcloned into PCR-100 vector (Invitrogen). Plasmid DNA prepared from selected colonies was sequenced using the fmol sequencing kit (Promega). The A-to-G transition at base 1265 is indicated.

same three fragments (lane 5) as were seen in the patient (lane 3).

Identification of Mutations in an Intermediate-MSUD Patient (Lo)

In an earlier report we had shown that the E1α mRNA level was reduced in an intermediate-MSUD patient (Lo) (Gonzales-Rios et al. 1985), whereas the E1β mRNA level was normal (Chuang et al. 1990). The levels of E1α and E1β subunits were both reduced in cells from this patient (Fisher et al. 1989). The results suggested that patient Lo has a mutation in the E1 component. E1α cDNAs prepared from normal and Lo cells were amplified, and subclones were sequenced. Figure 3*A* shows a single A-to-G transition in the E1α cDNA from Lo, which results in the Tyr-368–to–Cys (i.e., Y368C) mutation in the E1α subunit. Amplification followed by direct sequencing of exon 9 of the E1α gene from Lo indicated that the patient was heterozygous for the A-to-G conversion.

To identify the second mutation, the nine exons of the E1α gene from Lo were amplified and directly sequenced. Comparison with normal genomic sequences revealed a single C nucleotide insertion in exon 2 of the patient (fig. 4). This resulted in a frameshift at codon 4 of the mature E1α subunit, with the generation of a premature stop codon (TGA) at base 174 of the E1α mRNA.

The amplified normal DNA fragment containing exon 2 is 320 bp in size and has three *Bsl*I sites with a specificity for the CCN_7GG sequence. The digestion of the amplified normal DNA produced four fragments, of 171, 60, 48, and 29 bp, that were separated by PAGE (fig. 5, lanes 2 and 6). The single C insertion mutation created an additional *Bsl*I site within the 171-bp fragment, resulting in two smaller fragments, of 126 and 41 bp. As shown in lane 3 of figure 5, the 126-bp fragment was readily detectable in amplified DNA from Lo and was diagnostic for this mutation. The 41-bp fragment migrated close to the 48-bp and was difficult to discern. Since Lo was heterozygous for the single C insertion, the normal 171-bp fragment was also present (lane 3). An identical restriction-fragment pattern was observed in classical patient CQ (lane 5) and the mother DQ (lane 4). The result suggested that both the patient and the mother were heterozygous for the single C insertion. This was confirmed by direct sequencing of amplified DNA from these subjects.

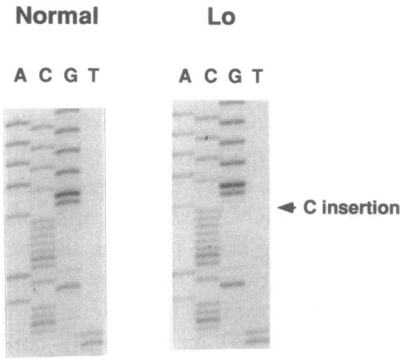

Figure 4 Nucleotide sequencing showing a single C nucleotide insertion in exon 2 of the E1α gene. Genomic DNAs were prepared from lymphoblasts from a normal control and intermediate-MSUD patient Lo. The region containing exon 2 was amplified (for primers used, see Patients, Material, and Methods). The amplified products (320 bp) were subcloned and sequenced as described in fig. 1. The arrow shows the single C insertion at base 137.

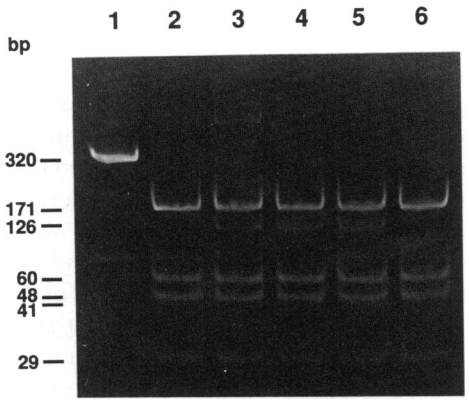

Figure 5 Screening of the single C nucleotide insertion by restriction-enzyme analysis. The 320-bp PCR products were digested with *Bsl*II, and the fragments were separated by 12% PAGE in 1 × TBE buffer. The gel was stained with ethidium bromide. The normal sequence contains three *Bsl*II sites, at bases 175, 208, and 260, which produce four fragments, of 29, 48, 60, and 171 bp (lanes 2 and 6). The single C insertion generates an additional *Bsl*II site, which results in further cleavage of the 171-bp fragment, into 41- and 126-bp fragments. This is observed in the compound heterozygotes Lo (lane 3), classical MSUD patient CQ (lane 5), and her carrier mother DQ (lane 4). Lane 1 shows the undigested PCR product from normal cells.

Characterization of the Y368C Mutation and the 8-bp Deletion

The E1α cDNA containing the A-to-G transition (Y368C) was inserted into an EBO expression vector for transient-expression studies. The plasmids carrying the normal and the mutant E1α cDNAs were transfected into E1α-deficient lymphoblasts derived from a Mennonite patient (LMK). LMK cells are homozygous for the Y393N substitution in the E1α subunit (Fisher et al. 1991*b*). Intact untransfected and transfected lymphoblasts were used to measure the rate of decarboxylation of α-keto [1-^{14}C] isovalerate. As shown in figure 6, untransfected LMK cells (lane 2) and LMK lymphoblasts transfected with the EBO vector without insert (lane 3) exhibited trace or no decarboxylation activity. Transfection of LMK cells with vector carrying a normal E1α cDNA (lane 4) fully restored decarboxylation activity to the levels observed in untransfected normal lymphoblasts (lane 1). LMK cells transfected with vector containing E1α cDNA with the Y368C substitution (lane 5) were unable to decarboxylate the α-keto acid. The results established that the Y368C mutant subunit was a defective polypeptide, which failed to restore decarboxylase activity and therefore was the cause of MSUD.

The lysates prepared from untransfected and transfected lymphoblasts were subjected to western blotting. Antibody against either E1α or E1β subunits was used as a probe. Both in untransfected LMK cells (lane 2) and in those transfected with EBO vectors without inserts (lane 3), the E1α-subunit level was reduced compared with the normal level (fig. 7, *top*), whereas E1β subunit was present

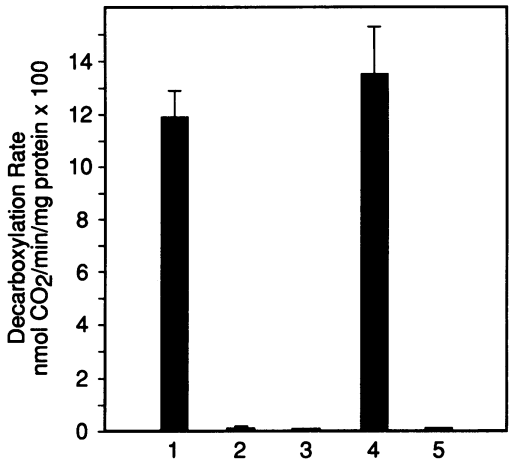

Figure 6 Decarboxylation rates of α-keto [1-^{14}C] isovalerate by intact E1α-deficient (patient LMK) lymphoblasts transfected with the EBO vector carrying the normal or Y368C E1α cDNA. The intact-cell assay with α-keto [1-^{14}C] isovalerate as substrate was carried out as described in Patients, Material, and Methods. The results are expressed as means ± standard error of the mean, of three separate dishes. The rates of decarboxylation of [1-^{14}C] pyruvate were in the normal range in both untransfected and transfected cells (data not shown). Lane 1, Untransfected normal cells. Lane 2, Untransfected LMK cells. Lane 3, LMK cells transfected with EBO vector without insert (control). Lane 4, LMK cells transfected with the EBO vector carrying the normal E1α cDNA. Lane 5, LMK cells transfected with the vector containing the Y368C E1α cDNA.

in trace amount (fig. 7, *bottom*). Transfection of the LMK cell with vector harboring normal E1α cDNA (lane 4) increased the E1α-subunit level so that it was similar to normal levels (lane 1), with concomitant appearance of a smaller proteolytic fragment (fig. 7, *top*). The E1β subunit

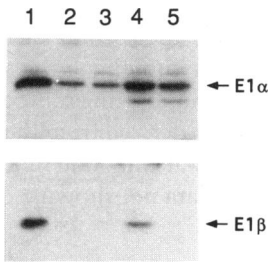

Figure 7 Western blotting of lysates from normal and transfected LMK lymphoblasts. Cultured cells were transfected with EBO vectors as described in the legend to fig. 6. Cell lysates were subjected to SDS-PAGE at 200 μg/lane. Separated proteins were electrotransferred to polyvinylidene difluoride membranes. The filters were probed with either E1α (*top*) or E1β (*bottom*) polyclonal antibodies radiolabeled by coupling with ^{125}I-protein A (Fisher et al. 1989). Lane 1, Untransfected normal cells. Lane 2, Untransfected LMK (E1α-deficient MSUD) cells. Lane 3, LMK cells transfected with the EBO vector without insert (control). Lane 4, LMK cells transfected with the EBO vector carrying the normal E1α cDNA. Lane 5, LMK cells transfected with the vector carrying the Y368C E1α cDNA.

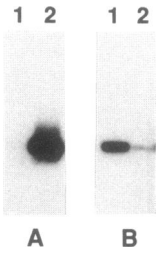

Figure 8 ASO probing of amplified cDNA fragments derived from normal and MSUD (patient Lo) lymphoblasts. The amplified 382-bp cDNA fragments (see fig. 3) from normal and Lo cells were electrophoresed on 1.2% agarose gel and were transferred onto GeneScreen Plus membrane. The filters were sequentially probed with end-labeled normal (5′-GACGTGTATCAGGAT-3′) (*B*) and mutant (5′-GACGTGTGTC-AGGAG-3′) (*A*) oligonucleotides. The A-to-G transition is underlined in both the normal and mutant sequences. Lanes 1, Normal cells. Lanes 2, Lo cells.

in transfected LMK cells (lane 4) was restored to ~50% of that in normal cells (fig. 7, *bottom*). A similar increase in the E1α level was observed in LMK cells transfected with the Y368C E1α cDNA, but the E1β subunit remained in trace amount (lanes 5).

The abundance of the mutant E1α mRNA carrying the single C insertion was determined by ASO probing. As described above, Lo was a compound heterozygote carrying a Y368C substitution in one allele and a single C insertion in the second. cDNA prepared from Lo cells was amplified. The amplified region corresponded to the C-terminal segment of the E1α subunit, which carried the Y368C substitution (an A-to-G transition). The amplified cDNA, when probed with the A-to-G mutant probe (fig. 8*A*), showed a positive signal for Lo cells (lane 1) and a negative signal for normal cells (lane 2). Reprobing of the same filter with a normal ASO (fig. 8*B*) showed that the signal in Lo cells (lane 1) was far less than 50% of that in normal cells (lane 2), as expected for the second allele. This indicated that the mutant mRNA containing the single C insertion was in low abundance. Supporting this conclusion was the sequencing of subclones derived from the amplified cDNA of Lo, which showed that only one in five clones carried the single C insertion (data not shown).

Discussion

The present study identifies three novel MSUD mutations in the E1α gene that either decrease the steady-state mRNA level or impair E1 assembly of the human BCKAD complex. Our study of the E1α promoter region of the compound-heterozygous MSUD patient GM-649 was prompted by the earlier suggestion, based on low abundance of the E1α mRNA (Zhang et al. 1989), of a second regulatory mutation. The region between bases +1 and −320 contains two AP-2 elements that were shown to be essential for basal transcription of the E1α gene (Chuang et al. 1993). The presence of an A-to-T transversion at base −284 in both GM-649 and the mother (GM-651) suggested that this base change occurs in the second allele of the patient. However, normal promoter activity of this allele indicates that base −284 is not a critical base for promoter function. This result also suggested that the second mutation in GM-649 was in the coding region of the E1α gene. The putative second mutation was not previously detected by cDNA amplification and sequencing, presumably as the result of low abundance of the mutant mRNA (Zhang et al. 1990). In the present study, an alternate approach, i.e., amplification of exonic DNAs, led to the detection of a heterozygous 8-bp deletion in exon 7 of the E1α gene of the patient (GM-649) and the mother (GM-651).

The association of nonsense codons with a reduction in the mRNA level had previously been observed in two MSUD mutant alleles. One was the nonsense allele E163* in human E2 (Fisher et al. 1993), and the second was the Q(-6)* in bovine E1α (Zhang et al. 1990). In the present study, we show that nonsense codons 4 and 255 of the mature E1α subunit were generated by a single C insertion and an 8-bp deletion, respectively, of the E1α gene. Occurrence of these premature stop codons also resulted in low abundance of the mutant E1α mRNA. The decreased level of mRNA caused by nonsense mutations has been documented in other eukaryotic mRNAs, including mRNAs for β-globin (Baserga and Benz 1988), arginosuccinate synthetase (Dennis et al. 1989), dihydrofolate reductase (Urlaub et al. 1989), triosephosphate isomerase (Daar and Maquat 1988), ornithine aminotransferase (Mashima et al. 1992), and muscle phosphorylase (Tsujino et al. 1993). It has been suggested that nonsense mutations promote ribosome dissociation from the mRNA, thus prematurely subjecting the naked mRNA to endogenous nuclease degradation (Daar and Maquat 1988). Both of the nonsense codons produced by the C insertion and the 8-bp deletion are located within the first three-quarters of the coding region of the E1α (445 amino acid residues). It has been proposed that a nonsense codon may impair the transport of the nascent triosephosphate isomerase mRNA from the nucleus to the cytoplasm (Belgrader et al. 1993). The same study also indicates that tRNA and ribosomes may function in concert in the recognition of nonsense codons, which triggers degradation of the mutant mRNA in the nucleus.

The decarboxylation of the α-keto [1-^{14}C] isovaleric acid in E1α-deficient host lymphoblasts (patient LMK) was restored by transfection with the EBO vector containing a normal E1α cDNA, but not with the vector harboring the Y368C mutation. The results provide direct evidence that this mutation disrupts the function of the BCKAD complex. The host cells were derived from a Mennonite MSUD patient (LMK) who carries the homozygous Y393N mutation in the E1α subunit (Fisher et al. 1991*b*).

As described above, the latter mutation impairs proper assembly of E1α with E1β, resulting in the degradation of both subunits (Fisher et al. 1991*a*). Western blotting (fig. 7) showed that the E1β subunit in the LMK host cells was only partially restored by introduction of a normal E1α cDNA. The inability of the normal E1α to increase the E1β subunit in LMK cells to a normal level remains perplexing. Two possible mechanisms may explain this observation. First, the normal mRNA was overexpressed in transfected E1α-deficient lymphoblast (Fisher et al. 1991*a*), but the E1α-subunit level was comparable to that in untransfected normal cells. This indicated that only a portion of the recombinant E1α was properly folded and able to assemble with E1β. Elsewhere we have shown that chaperon proteins are essential for the proper folding and assembly of E1 heterotetramers (Wynn et al. 1992). The availability of endogenous chaperons in lymphoblasts may limit the production of properly folded E1α. Unassembled E1α and E1β were degraded in the cells. The smaller species of E1α in western blotting (fig. 7, *top*, lane 4) probably represented intermediate proteolytic products. Alternatively, the host LMK cells are homozygous for the Y393N mutation (Fisher et al. 1991*b*). In transfected LMK cells, the recombinant E1α may have competed with Y393N E1α for assembly with E1β, resulting in a low level of assembled normal E1. Our recent pulse-chase experiments in an *Escherichia coli* expression system indicated that the Mennonite Y393N mutant E1α was capable of limited and inefficient assembly with E1β (J. R. Davie, W. Wynn, J. L. Chuang, R. P. Cox, and D. T. Chuang, unpublished data). The reduced E1β subunit in LMK cells transfected with the normal E1α suggested that the properly assembled E1 was less than normal. It was therefore surprising that the full decarboxylation rate was present in transfected cells, compared with normal cells (fig. 6). This may indicate a possible excess of E1 in normal cells, with a portion not bound to E2. In support of this possibility is evidence that the "activator" protein of the BCKAD complex in mitochondria is the unbound E1 component (Fatania et al. 1982; Yeaman et al. 1984).

The failure of the Y368C E1α to rescue E1β subunits (fig. 7, *bottom*) is similar to the effect of the Y393N mutation in Mennonite patients. The Y393N E1α mRNA was overexpressed by the EBO vector in host cells, to a level similar to that of the recombinant normal E1α mRNA (Fisher et al. 1991*a*). Thus, in the present transfection studies, the normal and the Y368C E1α mRNAs were expected to be similarly expressed. This was supported by the comparable levels of normal and Y368C E1α subunits in transfected LMK cells (fig. 7, *top*). However, only a small fraction of Y368C E1α subunit was able to assemble with E1β, as determined by the pulse-chase experiment (data not shown). The latter result and the immunologic absence of E1β in Y368C transfected cells strongly suggest that this mutation impairs proper folding and subsequent assembly of E1α with E1β. Unassembled as well as misassembled Y368C E1α and normal E1β are degraded in the cell.

Studies of MSUD mutations have implicated Tyr-368 (present study) and Tyr-393 (Fisher et al. 1991*a*) of the E1α subunit as important residues for E1 assembly. Tyr-368 is completely conserved in the E1α subunits of human, rat, bovine, and *Pseudomonas* BCKAD complexes, and Tyr-393 is present in all three mammalian E1α subunits (Wexler et al. 1991). The results strongly suggest that the carboxy-terminal region is critical for proper assembly of E1α with E1β. This region is distal from the putative thiamin pyrophosphate–binding motif (residues 182–229) (Hawkin et al. 1989) and the two phosphorylation sites (Ser-292 and Ser-302) (Fisher et al. 1989) and is distinct from the proposed subunit interaction sites (residues 199–255) (Wexler et al. 1991). The role of Tyr-368 and Tyr-393 is uncertain, but they may be structural in that their side chains provide hydrogen bonding that is required for stable subunit interactions. Hydrogen-bonding networks involving Tyr residues are shown to exist around the active site of phospholipase A2 (Küipers et al. 1990). The precise molecular mechanisms that impair E1 assembly involving the Tyr residues must await the solution of the E1 structure.

Acknowledgment

This work was supported by NIH grant DK-26758 and March of Dimes Birth Defects Foundation grant 1-1149.

References

Baserga SJ, Benz EJ (1988) Nonsense mutations in the human β-globin gene affect mRNA metabolism. Proc Natl Acad Sci USA 85:2056–2060

Belgrader P, Cheng J, Maquat LE (1993) Evidence to implicate translation by ribosomes in the mechanism by which nonsense codons reduce the nuclear level of human triosephosphate isomerase mRNA. Proc Natl Acad Sci USA 90:482–486

Chen EY, Seeburg PH (1985) Supercoiled sequencing: a fast and simple method for sequencing plasmid DNA. DNA 4:165–170

Chirgwin JM, Praybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299

Chuang DT, Ku LS, Cox RP (1982) Detection of heterozygotes in maple syrup urine disease: measurement of branched-chain α-keto acid dehydrogenase and its components in cell cultures. Am J Hum Genet 34:416–424

Chuang DT, Shih VE. Disorders of branched-chain amino acid and keto acid metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, 7th ed. Vol 1. McGraw-Hill, New York (in press)

Chuang JL, Cox RP, Chuang DT (1990) Molecular cloning of the mature E1b-β subunit of human branched-chain α-keto acid dehydrogenase complex. FEBS Lett 262:305–309

——— (1993) Characterization of the promoter-regulatory region and structural organization of the E1α gene (BCKDHA)

of human branched-chain α-keto acid dehydrogenase complex. J Biol Chem 268:8309–8316

Daar IO, Maquat LE (1988) Premature translation termination mediates triosephosphate isomerase mRNA degradation. Mol Cell Biol 8:802–813

Dancis J, Hutzler J, Levitz M (1960) Metabolism of the white blood cells in maple-syrup-urine disease. Biochim Biophys Acta 43:342–343

Dennis JA, Healy PJ, Beaudet AL, O'Brien WE (1989) Molecular definition of bovine argininosuccinate synthetase deficiency. Proc Natl Acad Sci USA 86:7947–7951

Fatania HR, Lau KS, Randle PJ (1982) Activation of phosphorylated branched chain 2-oxoacid dehydrogenase complex. FEBS Lett 147:35–39

Fisher CR, Chuang JL, Cox RP, Fisher CW, Star RA, Chuang DT (1991*a*) Maple syrup urine disease in Mennonites: evidence that the Y393N mutation of the E1α impedes assembly of the E1 component of branched-chain α-keto acid dehydrogenase complex. J Clin Invest 88:1034–1037

Fisher CR, Fisher CW, Chuang DT, Cox RP (1991*b*) Occurrence of a Tyr393→Asn (Y393N) mutation in the E1α gene of the branched-chain α-keto acid dehydrogenase complex in maple syrup urine disease patients from a Mennonite population. Am J Hum Genet 49:429–434

Fisher CW, Chuang JL, Griffin TA, Lau KS, Cox RP, Chuang DT (1989) Molecular phenotypes in cultured maple syrup urine disease cells: complete E1α cDNA sequence and mRNA and subunit contents of the human branched-chain α-keto acid dehydrogenase complex. J Biol Chem 264:3448–3453

Fisher CW, Fisher CR, Chuang JL, Lau KS, Chuang DT, Cox RP (1993) Occurrence of a 2-bp (AT) deletion allele and a nonsense (G-to-T) mutant allele at the E2 (DBT) locus of six patients with maple syrup urine disease: multiple-exon skipping as a secondary effect of the mutations. Am J Hum Genet 52: 414–424

Gonzales-Rios MC, Chuang DT, Cox RP, Schmidt K, Knopf K, Packman SA (1985) A distinct variant of intermediate maple syrup urine disease. Clin Genet 27:153–159

Hawkin CF, Borges A, Perham RN (1989) A common structural motif in thiamin pyrophosphate-binding enzymes. FEBS Lett 255:77–82

Küipers OP, Franken PA, Hendriks R, Verheij HM, Haas GH (1990) Function of the fully conserved residues ASP99, Tyr 52 and Tyr 73 in phospholipase A_2. Protein Eng 4:1991–2004

Lindsay JG (1989) Targeting of 2-oxo acid dehydrogenase complexes to the mitochondrion. Ann NY Acad Sci 573:254–266

Marshall L, DiGeorge A (1981) Maple syrup urine disease in the old order Mennonites. Am J Hum Genet Suppl 33:139A

Mashima Y, Murakami A, Weleber RG, Kennaway NG, Clarke L, Shiono T, Inana G (1992) Nonsense-codon mutations of the ornithine aminotransferase gene with decreased levels of mutant mRNA in gyrate atrophy. Am J Hum Genet 51:81–91

Miller G, Lipman M (1973) Release of infectious Epstein-Barr virus by transformed marmoset leukocytes. Proc Natl Acad Sci USA 70:190–194

Randle PJ, Fatania HR, Lau KS (1984) Regulation of the mitochondrial branched-chain 2-oxo acid dehydrogenase complex of animal tissues by reversible phosphorylation. In: Cohen P (ed) Enzyme regulation by reversible phosphorylation-advances. Elsevier, Amsterdam, pp 1–26

Tsujino S, Shanske S, DiMauro S (1993) Molecular genetic heterogeneity of hypophosphorylase deficiency (McArdle's disease). N Engl J Med 329:241–245

Urlaub G Mitchell PJ, Ciudad CJ, Chasin LA (1989) Non-sense mutations in dihydrofolate reductase gene affect RNA processing. Mol Cell Biol 9:2868–2880

Wexler ID, Hemalatha SG, Patel MS (1991) Sequence conservation in the α and β subunits of pyruvate dehydrogenase and its similarity to branched-chain α-keto acid dehydrogenase FEBS Lett 282:209–213

Wynn RM, Davie JR, Cox RP, Chuang DT (1992) Chaperonins groEL and groES promote assembly of heterotetramers ($\alpha_2\beta_2$) of mammalian mitochondrial branched-chain α-keto acid decarboxylase in *Escherichia coli*. J Biol Chem 267:12400–12403

Yeaman SJ (1989) The 2-oxo acid dehydrogenase complexes: recent advances. Biochem J 257:625–632

Yeaman SJ, Cook KG, Boyd RW, Lawson R (1984) Evidence that the mitochondrial activator of phosphorylated branched chain 2-oxoacid dehydrogenase complex is the dissociated E1 component of the complex. FEBS Lett 172:38–42

Zhang B, Edenberg HJ, Crabb DW, Harris RA (1989) Evidence for both a regulatory mutation and a structural mutation in a family with maple syrup urine disease. J Clin Invest 83:1425–1429

Zhang B, Healy PJ, Zhao Y, Crabb DW, Harris RA (1990) Premature translation termination of the pre-E1α subunit of the branched chain α-ketoacid dehydrogenase as a cause of maple syrup urine disease urine disease in Polled Hereford calves. J Biol Chem 265:2425–2427