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

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Summary

We have identified two novel mutant alleles in the transacylase (E2) gene of the human branched-chain α-keto acid dehydrogenase (BCKAD) complex in 6 of 38 patients with maple syrup urine disease (MSUD). One mutation, a 2-bp (AT) deletion in exon 2 of the E2 gene, causes a frameshift downstream of residue (−26) in the mitochondrial targeting presequence. The second mutation, a G-to-T transversion in exon 6 of the E2 gene, produces a premature stop codon at Glu-163 (E163*). Transfection of constructs harboring the E163* mutation into an E2-deficient MSUD cell line produced a truncated E2 subunit. However, this mutant E2 chain is unable to assemble into a 24-mer cubic structure and is degraded in the cell. The 2-bp (AT) deletion and the E163* mutant alleles occur in either the homozygous or compound-heterozygous state in the 6 of 38 unrelated MSUD patients studied. Moreover, an array of precise single- and multiple-exon deletions were observed in many amplified E2 mutant cDNAs. The latter results appear to represent secondary effects on RNA processing that are associated with the MSUD mutations at the E2 locus.

Introduction

Maple syrup urine disease (MSUD) or branched-chain ketonuria is an autosomal recessively inherited deficiency in the mitochondrial branched-chain α-keto acid dehydrogenase (BCKAD) complex. The multienzyme complex degrades the α-keto acids derived from the three branched-chain amino acids leucine, isoleucine, and valine (Danner and Elsas 1989). The classical form of the disease is characterized by the rapid onset of severe ketoacidosis at birth, which is associated with seizures, coma, and, if it is untreated, death (Menkes et al. 1954). There is a high incidence of mental retardation in survivors. The residual activity of the BCKAD complex in the classical patient is less than 2% of nor-

mal. Several milder variant forms of MSUD have also been described, which include intermediate, intermittent, thiamine-responsive, and deficiencies of specific subunits (Danner and Elsas 1989).

The mammalian BCKAD complex is a macromolecule consisting of three catalytic components, i.e., a decarboxylase (E1) comprising two α ($M_r = 47,000$) and two β ($M_r = 37,000$) subunits, a transacylase (E2) core consisting of 24 identical lipoate-bearing subunits ($M_r = 46,500$), and a dehydrogenase (E3) that exists as a homodimer (monomer $M_r = 52,000$) and is common to the related pyruvate and α-ketoglutarate dehydrogenase complexes (Yeaman 1989). In addition, the mammalian BCKAD complex contains two regulatory enzymes, a specific kinase and a specific phosphatase, that control the activity of the enzyme complex through a phosphorylation/dephosphorylation cycle (Randle et al. 1984).

Limited proteolysis (Chuang et al. 1985; Hu et al. 1986) and cDNA cloning (Griffin et al. 1988; Wynn et al. 1992) show that each E2 chain has three folded do-

mains, i.e., lipoate-containing, E1/E3-binding, and inner-core domains. The carboxyl inner-core domain of E2 contains the active site and confers the 24-mer cubic structure. The three domains are connected by two flexible hinge regions. The E2 gene (symbol DBT) is located on chromosome 1p31 (Zneimer et al. 1991). The structure of the E2 gene has recently been elucidated. The gene spans 68 kb and contains 11 exons (Lau et al. 1992). Three patients have been described with mutations at the E2 locus. These include a 17-bp intronic insertion, a T-to-G transversion corresponding to F215C substitution (Fisher et al. 1991b), a 124-bp deletion in the coding region (Herring et al. 1991), and a 78-bp deletion as the result of a single base deletion in the 5' splice donor site (Mitsubuchi et al. 1991).

In an effort to identify mutations in the E2 locus that affect the structure and function of the enzyme complex, we have studied six additional MSUD patients who have deficiencies of the E2 subunit. In this communication, we report a novel 2-bp deletion allele and a previously undescribed nonsense mutant allele that occur in MSUD patients as either compound heterozygotes or homozygotes. Moreover, an array of precise single- and multiple-exon deletions were observed in amplified mutant E2 transcripts. The latter results appear to represent unexpected secondary effects that are associated with the MSUD mutations at the E2 locus.

Material and Methods

Cell Lines

Lymphoblast cell line GM-1366 and fibroblast cell line GM-612 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). GM-1366 cells are from a black female with classical MSUD, and GM-612 cells are from a Caucasian female with classical MSUD. Fibroblasts from a black female classical MSUD patient, T.H., were provided by Dr. David Valle (John F. Kennedy Institute, Baltimore). Fibroblasts of a Caucasian male classical MSUD patient, Ech, were provided by Dr. Juan Sotos (Children's Hospital, Columbus, OH). Fibroblasts from a black female classical MSUD patient, L.J., were received from Dr. Jeffery Chinsky (Baylor College of Medicine, Houston). Fibroblasts from a classical MSUD patient of Ashkenazi Jewish descent, M.F., were provided by Dr. Selma Snyderman (New York University Medical Center, New York). An additional 32 MSUD cell lines were studied by allele-specific oligonucleotide (ASO) probing. Among these, 22 were classical MSUD patients and 10 were MSUD variants by clinical criteria. Lymphoblast

cell lines were grown in RPMI-1640 medium (ICN-Flow; Costa Mesa, CA) containing 15% heat-inactivated FBS according to a method described elsewhere (Fisher et al. 1991a). Fibroblast lines were grown according to a method described elsewhere (Fisher et al. 1989).

Cloning and Sequencing of cDNA and Genomic DNA

cDNA from MSUD cell lines GM-612 and Ech and normal cell lines M7 and M9 was 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 (Bethesda Research Laboratories, Gaithersburg, MD), 5 µM random hexamers (Pharmacia, Piscataway, NJ), 24 units of RNasin (Promega, Madison, WI), and 800 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). mRNA was denatured at 95°C for 5 min, the reaction components were added, and the reaction was carried out at 42°C for 120 min. The reaction mixture was held at −20°C until amplification. The primers used for amplification were as follows: 5'-CGCTGCAGTCCG-TATGCTGA-3' for bases 17–36 (sense) and 5'-CTAG-TAGCATAACAGCTGGGT-3' for bases 1433–1453 (antisense), which generated a 1,436-bp fragment. The amplification reaction was performed in a 50-µl reaction mixture containing standard PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.4, 1.5 mM MgCl$_2$, and 100 µg of gelatin/ml) with the following components: 10 µl of cDNA, 1 µM each of the primers, 200 µM each of dNTP, and 1.25 units of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). The reactions were cycled through the following series of reaction temperatures: 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. The reaction was cycled 30 times and then held at 4°C.

The reaction mixtures were treated with 60 units of T4 polynucleotide kinase (Harrison and Zimmerman 1986). The products were ligated into Bluescript SK II+ (Stratagene, La Jolla, CA) plasmid digested with SmaI. The resulting plasmids were used to transform XL1-Blue (Stratagene, La Jolla, CA) cells that were plated on indicator plates. A random-primed (Feinberg and Vogelstein 1983) labeled bovine cDNA containing only the coding sequence (Lau et al. 1988) was used to select positive clones. Plasmids containing cDNA were sequenced using the dideoxy-chain termination method (Chen and Seeburg 1985).

Genomic DNA was amplified by the PCR using the

following conditions: 1 µM of the sense and antisense primer, 50 µM each of dNTP, 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI), 5 µl of 10 × reaction buffer (Promega, Madison, WI), and 1 µg template in a 50-µl volume. The reaction was cycled at 94°C for 30 s, 54°C (for exon 6) or 52°C (for exon 2) for 15 s, and 72°C for 30 s, for a total of 30 cycles. Primers used were as follows: 5'-ACTGTCAGTTGATCAAATTCC-3' (intronic) (sense) and 5'-CTACTACTGAGGTAGCT-TCC-3' (intronic) (antisense), for exon 6; and 5'-TTGT-GTTCGCTATTTTC-3' for bases 68–84 (sense), and 5'-CAGCAGTTGTTTTCAGGA-3' for bases 173–190 (antisense), for exon 2. The 374-bp fragment generated by the PCR for exon 6 was subcloned using the TA Cloning system (Invitrogen, San Diego) according to the manufacturer's directions. Positive colonies were selected, and plasmid DNA was isolated for sequencing.

ASO Probing

Genomic DNA fragments amplified by the PCR were electrophoresed on a 1.5% agarose gel and were blotted onto GeneScreen Plus (DuPont/NEN, Boston). Blots for exon 2 were probed sequentially with end-labeled oligonucleotides containing the 2-bp deletion (5'-CAAACGTGGTAATGTT-3') and the normal sequence (5'-TTCAAACATGTGGTA-3'). Blots for exon 6 were probed sequentially with end-labeled oligonucleotides containing the E163* mutation (5'-CAAAGTTT-AAATTAT-3') or the normal sequence (5'-CAAAGTT-GAAATTAT-3').

Construction of EBO Expression Vectors

The Epstein-Barr virus–based expression vector EBO-pLPP was provided by Dr. Robert Margolskee (Roche Institute of Molecular Biology, Nutley, NJ). The EBO vector contains an *oriP* origin of replication and a sequence that encodes *trans*-acting nuclear antigen EBNA-1 (Margolskee et al. 1988). These two elements allow replication of the plasmid in Epstein-Barr virus–transformed lymphoblasts. The expression of the cDNA insert is driven by the SV40 early promoter. The vector also encodes a hygromycin phosphotransferase that provides for selection with this antibiotic.

The cDNA insert encoding the E163* E2 precursor was constructed using the following four restriction fragments: *Sal*I-*Xba*I (bases 30–386) and *Xba*I-*Eco*RV (bases 387–626) fragments were derived from the hE2-1 cDNA clone (Lau et al. 1988). Base numbers are according to the complete E2 gene structure, beginning at the transcription start site (Lau et al. 1992). The *Sal*I-

*Xba*I fragment contains sequences that encode the 56 amino acids of the mitochondrial targeting peptide. An *Eco*RV-*Apa*I fragment (bases 627–1281) was derived from 1,436-bp amplified E2 cDNAs derived from GM-612 cells. The primers used in amplification have been described in the Cloning and Sequencing of cDNA and Genomic DNA subsection above. The fragment from GM-612 contained the G-to-T mutation at base 685. The fourth restriction fragment, *Apa*I-*Not*I (bases 1282–1866), was from a normal E2 cDNA clone containing the 3' coding sequence. The clone was obtained by amplification of E2 cDNA from a normal cell line (see below) and subcloning into Bluescript plasmids. The primers used were 5'-GGATAGCAATGGA-TACTG-3' for bases 1049–1066 (sense) and 5'-GGCCAGGTTTCACCATGT-3' for bases 1849–1866 (antisense). The four restriction fragments were ligated into the EBO vector previously digested with *Sal*I and *Not*I.

To construct the normal cDNA insert, the 5' terminus of the presequence was extended to include a Met- (−61). Amplification of a 5'-extended sequence was carried out using as a template an E2 cDNA containing the transcription start site, which is located 14 bp 5' to the initiation ATG. The latter E2 cDNA clone was obtained by the rapid amplication of cDNA ends (RACE), according to a method described elsewhere (Lau et al. 1992). The sense primer used was 5'-GAGCTCGTCGAC*TCCGGGGTAAGATGGCTG*-3', with the underlined sequence corresponding to bases 4–21 of the full-length E2 cDNA. The remainder of the sequence contained *Sac*I and *Sal*I sites and was not present in the E2 mRNA. The antisense primer was 5'-CGAACACAAATCAGCTTCC-3', spanning bases 76–58. The amplified fragments were digested with *Sal*I and *Bsm*I. The resulting restriction fragments were ligated to a *Bsm*I-*Eco*RV fragment from the hE2-1 cDNA (Lau et al. 1988). The *Sal*I-*Eco*RV fragment containing the Met- (−61) residue was ligated to the remaining restriction fragments described above, except that the *Eco*RV-*Apa*I fragment was derived from the normal cDNA. The normal and mutant fragments generated by PCR were confirmed by nucleotide sequencing (Chen and Seeburg 1985).

Other Methods

Genomic DNA was prepared from cultured cells according to a method described elsewhere (Davis et al. 1986, pp. 44–46). Transfections were performed according to a method described elsewhere (Fisher et al.
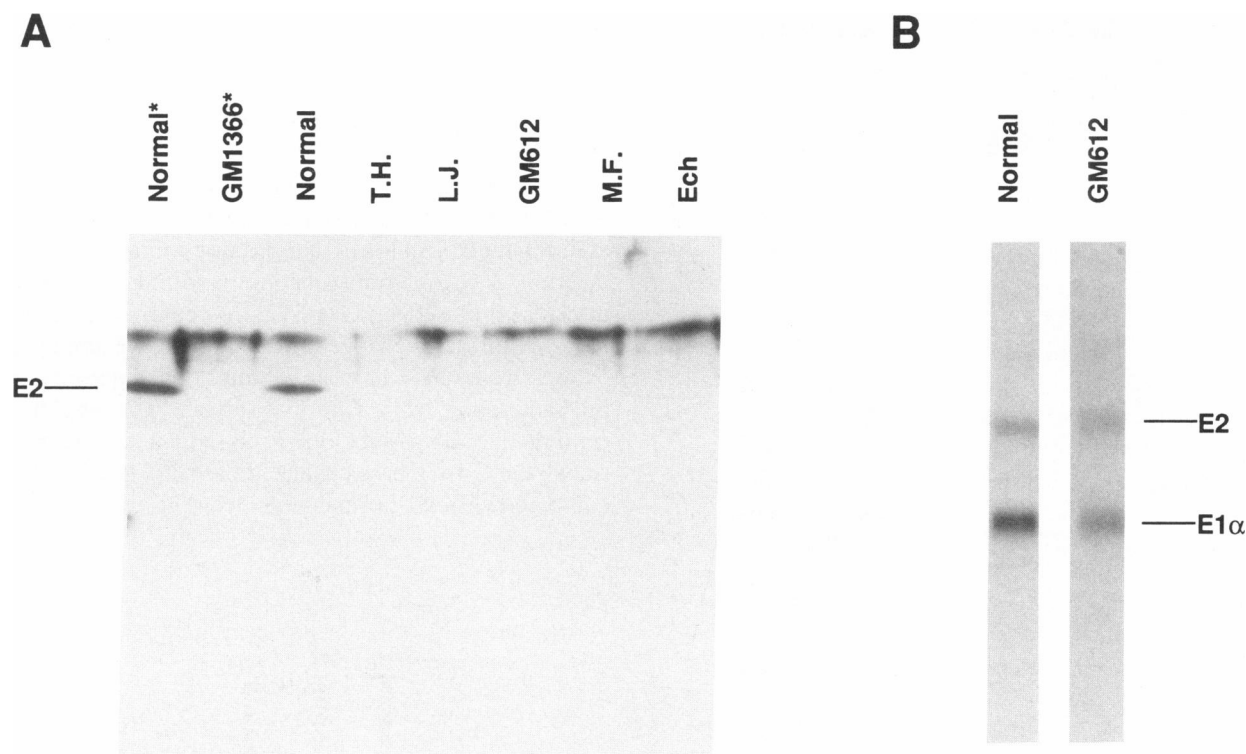
A

B

Figure I Deficiencies in the E2 subunit in cultured cells from MSUD patients. *A,* Western blotting with normal and MSUD mutant cells. Lysates (200 µg/lane) prepared from cultured fibroblasts or lymphoblasts (indicated by asterisks) were subjected to SDS-PAGE. Proteins were electrotransferred to Immobilon-P membranes and were probed with anti-bovine E2 and ^{125}I-protein A. GM-1366, T.H., L.J., GM-612, M.F., and Ech are classical MSUD patients. The result with Ech has been reported elsewhere (Fisher et al. 1989) but is included here for the completeness of the study. The level of the E1α subunit is normal in MSUD mutant cells when anti-E1α is used as a probe (data not shown). *B,* Northern blotting with poly(A)$^{+}$ RNA isolated from cultured normal and GM-612 fibroblasts. A mixture of nick-translated human E1α and E2 cDNAs was used as the probe (Fisher et al. 1989). Each lane contains 5 µg of poly(A)$^{+}$ RNA.

1991*a*). Transfected cell lines were maintained in medium containing 200 µg hygromycin B/ml, after selection was completed. Decarboxylation assays with intact viable cells were performed according to a method described elsewhere (Fisher et al. 1991*a*). Northern and western blotting also were performed according to methods described elsewhere (Fisher et al. 1989). For western blotting, enhanced chemiluminescence (ECL) was also carried out using anti-rabbit IgG-horseradish peroxidase and an enhancer, according to the manufacturer's (Amersham, Arlington Heights, IL) procedure.

Results

Identification of a 2-bp Deletion at the E2 Locus

As described above, the BCKAD is a multienzyme complex encoded by at least six genetic loci. To determine the genetic locus affected, cultured cells from six MSUD patients were subjected to northern and west-

ern analysis. In previous studies, GM-612 cells were shown to be deficient in the E2 subunit (Eisenstein et al. 1991; J. L. Chuang, R. P. Cox, and D. T. Chuang, unpublished results). Figure 1*A* confirms that the E2 protein is absent in this cell line. The E2 subunit is also absent in cultured cells from GM-1366, T.H., L.J., M.F., and Ech (fig. 1*A*), indicating that the E2 locus is affected in these MSUD patients. The diffused "bands" above E2 are nonspecific background frequently observed with the anti-E2 polyclonal antibody (fig. 1*A*). Figure 1*B* shows that the size and amounts of E2 and E1α mRNA in GM-612 are similar to those in the normal control. Elsewhere we showed that the E2 mRNA is also present and is nearly normal, in size and abundance, in Ech (Fisher et al. 1989). These findings suggest that the mutation in E2 may be a small insertion/deletion or a base change. To investigate these possibilities, we amplified and sequenced the cDNA prepared from two normal cell lines and from mutant cell lines GM-
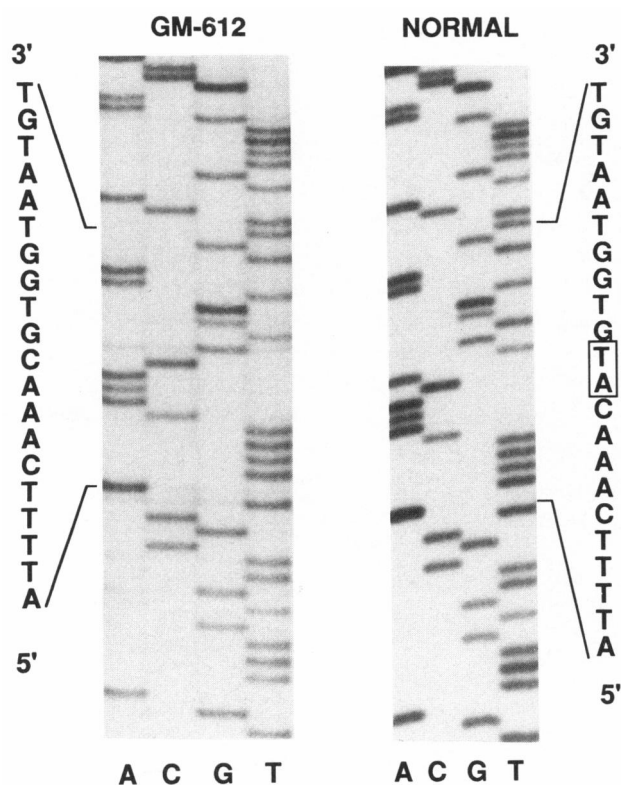
Figure 2   Nucleotide sequencing showing a 2-bp deletion in cDNA clones from GM-612. Poly(A)$^+$ RNA isolated from normal and GM-612 fibroblasts was reverse transcribed and amplified by the PCR. Amplified cDNA was subcloned into Bluescript plasmids. Positive clones were purified and sequenced. The boxed AT nucleotides are the 2-bp deletion in a GM-612 clone corresponding to bases 89 and 90 in normal E2 cDNA.

612 and Ech. Figure 2 shows a 2-bp (AT) deletion in exon 2 of GM-612, at bases 89 and 90, compared with normal cell lines. This deletion results in a frameshift after residue (−26) in the mitochondrial targeting pre-sequence. The same mutation was detected in a cDNA clone from Ech (data not shown). To determine the frequency of this mutant allele, genomic DNA (exon 2) was amplified from 36 cell lines of unrelated MSUD patients including GM-612. The amplified products were probed with normal and mutant ASOs. Figure 3A shows that amplified DNA from three MSUD patients —GM-612, M.F., and Ech—hybridize with the mutant ASO probe containing the 2-bp deletion. The normal ASO probe hybridizes with DNA from GM-612 and Ech but not with that from M.F. (fig. 3B). The results demonstrate that the mutation occurs in the gene and indicate that both GM-612 and Ech are heterozygotes for the 2-bp deletion. M.F. is homozygous for this dele-

tion, on the basis of its failure to hybridize with the normal ASO probe.

Detection and Expression Analysis of the E163* Mutation

To identify a putative second mutant allele in GM-612, we amplified and sequenced from this cell line additional cDNA clones that did not contain the 2-bp deletion. A G-to-T transversion was found at base 685 in cDNA clones from GM-612. To confirm the occurrence of this base change in the E2 gene, we amplified exon 6, from GM-612, that contains this mutation and the same exon from a normal subject. The amplified genomic DNA was subcloned and sequenced. Figure 4 shows the G-to-T base change at base 685 in GM-612, compared with the normal sequence. This creates a ter-
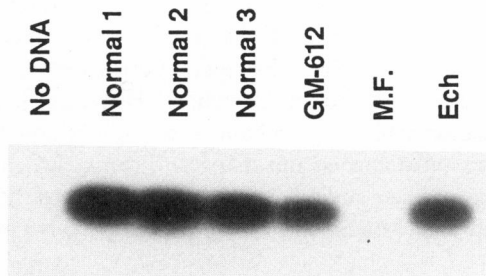
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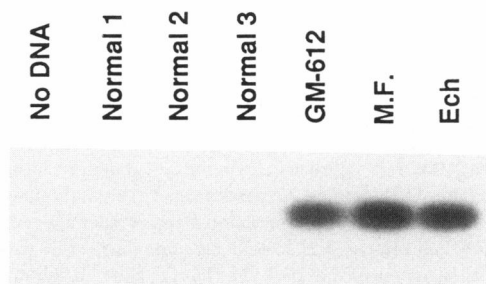
B

Figure 3   ASO probing for the 2-bp deletion, with amplified genomic DNA from MSUD patients. Genomic sequences for exon 2 of the E2 gene were amplified by the PCR in normal and MSUD patients. The amplified genomic DNA was electrophoresed, transferred to GeneScreen Plus, and probed with the mutant ASO containing the 2-bp deletion (5′-CAAACGTGGTAATGTT-3′) (A) or the normal ASO (5′-TTCAAACATGTGGTA-3′) (B).
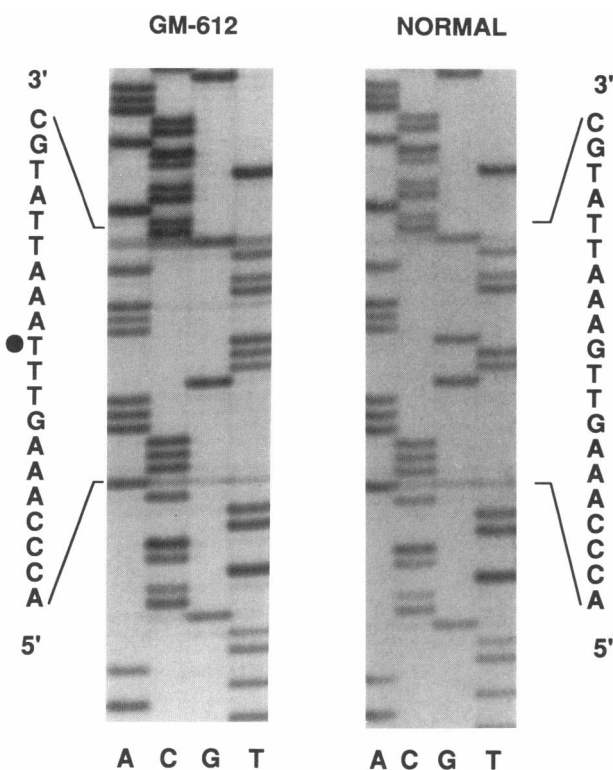
Figure 4 Nucleotide sequencing of genomic clones, showing the E163* mutation. Genomic sequence for exon 6 of the E2 gene from normal and GM-612 cells was amplified. The amplified products were subcloned using the TA-cloning system. Positive clones were selected, and plasmid DNA was isolated and sequenced. The T nucleotide marked by the black dot shows the G-to-T transversion at base 685 in GM-612.

mination codon TAA and should result in a premature translation termination at glutamate 163 (E163*). The expression of this mutant E2 was studied. Normal E2 cDNA and mutant cDNA containing the G-to-T transversion were inserted into the EBO vector. The lymphoblast cell line GM-1366 deficient in the E2 subunit was transfected with the vector containing normal or mutant cDNA. Figure 5A shows that the vector containing the normal E2 cDNA produces the mature polypeptide in the E2-deficient host. Restoration of BCKAD-complex activity was obtained by this transfection (data not shown). In contrast, the EBO vector containing the G-to-T transversion produced a markedly truncated polypeptide corresponding to the E163* premature termination (fig. 5B, arrow). This polypeptide does not contain the inner-core domain and is catalytically inactive. These results are consistent with the inability to restore BCKAD-complex activity by the mutant vector in transfected lymphoblasts (data not shown).

ASO Probing for the E163* Mutation

Genomic DNA (exon 6) from 38 unrelated MSUD patients including GM-612 was amplified by the PCR and was probed with normal and mutant (i.e., G-to-T) ASOs. Figure 6A shows that amplified DNA from four MSUD patients (GM-612, T.H., GM-1366, and L.J.) hybridizes with the mutant ASO probe. This result demonstrates that the G-to-T mutation is present in the E2 alleles of these patients. The normal ASO probe hybridizes with the normal control, GM-612, and L.J. (fig. 6B). These findings show that GM-612 and L.J. are compound heterozygotes and that T.H. and GM-1366 are homozygotes for the E163* mutation. The second mutation in L.J. is currently not known.

Single- and Multiple-Exon Skipping in Amplified E2 cDNAs

Sequencing of the amplified cDNA from cultured cells of MSUD patients showed unexpectedly that E2 cDNAs have large internal deletions. Figure 7 shows that several of the amplified mutant transcripts of GM-612 have internal deletions occurring precisely at the intron-exon junction. Specifically, there are two mutant transcripts with exon 6 skipped, two with exon 4 deleted, and two with large deletions of exons 2–8. Four undeleted GM-612 transcripts containing either the 2-bp deletion or the E163* mutation are also present. Figure 7 also shows amplified transcripts from another E2-deficient patient, Ech, who has the 2-bp (AT) deletion and a second 2-bp (gt) deletion at the 5' splice junction of intron 5 (J. L. Chuang, D. T. Chuang, and R. P. Cox, unpublished observations). Exon skipping is prominent in Ech, in all but one transcript that contains the exonic 2-bp deletion. Single, multiple, and tandem deletions of exons occur. Sequencing of eight amplified transcripts from two normal cell lines (M7 and M9) shows a single exon 9 skipped in one transcript (fig. 7).

To determine whether exon skipping in GM-612 is related to mutations at splice-site junctions, exon 6 and its 5' and 3' flanking intronic sequences from normal and GM-612 cells were amplified. The amplified exon 6 containing fragments was subcloned and sequenced. There were no base changes in the splice junctions of exon 6 in GM-612, compared with the normal cell lines (data not shown).

Discussion

This communication describes two novel mutations, i.e., a 2-bp (AT) deletion and an E163* termination, that occur in the E2 locus of six MSUD patients from
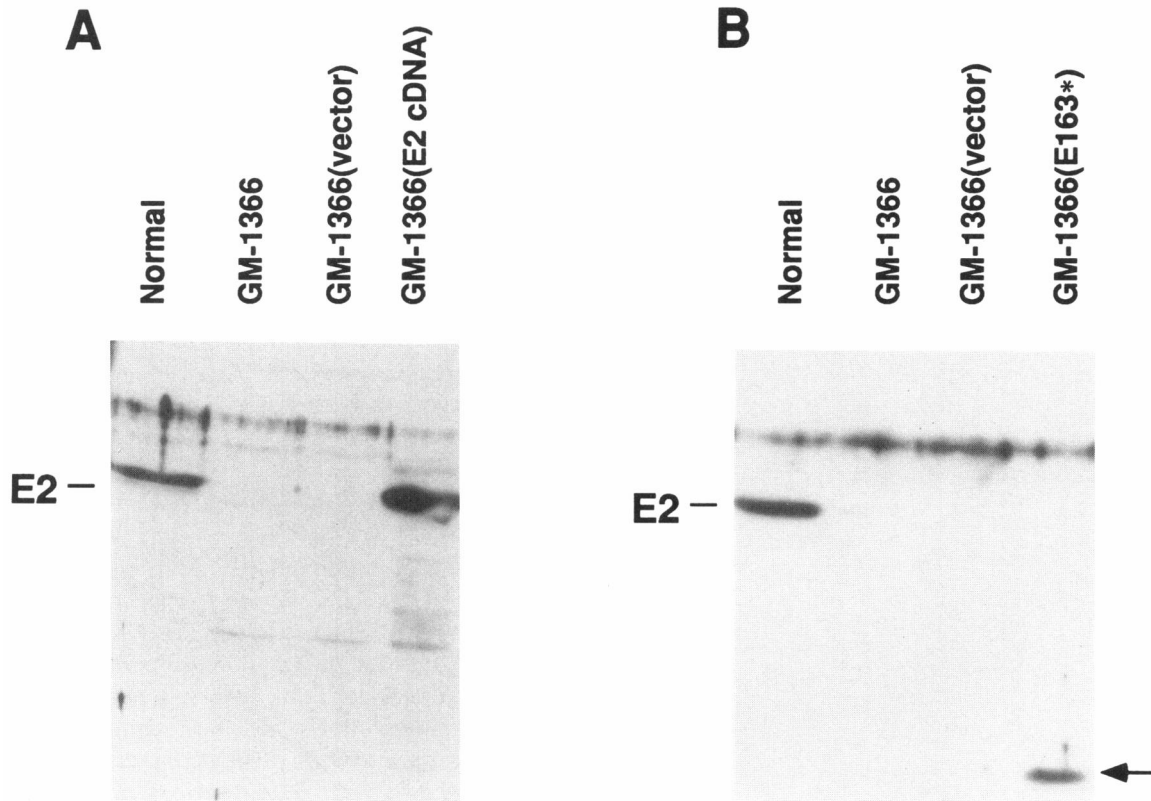
Figure 5   Western blotting of lysates prepared from E2-deficient GM-1366 cells transfected with the EBO vectors carrying normal or E163* E2 cDNAs. Cultured lymphoblasts from E2-deficient GM-1366 were transfected with the EBO vector without insert or with the full-length E2 cDNA containing normal or E163* sequences. Cells transfected with the vector were selected in medium containing hygromycin (200 µg/ml). Lysates prepared from transfected and untransfected cells (normal and GM-1366) were subjected to western blotting using antibody to E2 as a probe. A, Transfection with the normal E2 cDNA. E2 proteins were detected by the ECL label. B, Transfection with the E163* E2 cDNA. E2 proteins were identified by autoradiogram with ^{125}I-protein as a label. The arrow indicates the truncated E163* polypeptide.

diverse ethnic groups. Both mutations were initially identified in GM-612 by the PCR and DNA sequencing. The 2-bp deletion as one allele was detected in Ech by the same methods. The presence of these mutant alleles in four additional patients was found by ASO screening of DNA from 36 other MSUD cell lines. The results suggest that these two mutant alleles may be a relatively common cause of the disorder. They account for 10 of the 76 alleles coding for the E2 subunit that were studied. The two mutations exist in both compound-heterozygous and homozygous states in the six MSUD patients. The additional two alleles that contain mutations other than the two described here are in Ech and L.J. All of the six patients with these two mutations exhibit the classical MSUD phenotype. It is of interest that three (GM-1366, T.H., and L.J.) of the four patients who carry the E163* allele are black. The fourth

patient (GM-612), a compound heterozygote, is a Caucasian. The significance of the apparent association of the E163* allele with MSUD patients of African descent is presently unknown and will require further studies.

The 2-bp deletion in exon 2 of the E2 gene causes a frameshift after residue (−26) in the mitochondrial targeting presequence (fig. 8). This accounts for the absence of the mature E2 subunit in patient M.F., who appears to be homozygous for this mutation (fig. 1A). As shown in figure 8, the E2 subunit consists of three folded domains, i.e., lipoyl bearing, E1/E3 binding, and inner-core domain (5′ to 3′). The inner-core domain contains the homologous binding sites for E2 subunits (Chuang et al. 1985). The E163* mutation leads to a premature chain termination immediately downstream of the E1/E3 binding domain (fig. 8). This results in the synthesis of a mutant E2 comprising the lipoyl-bearing
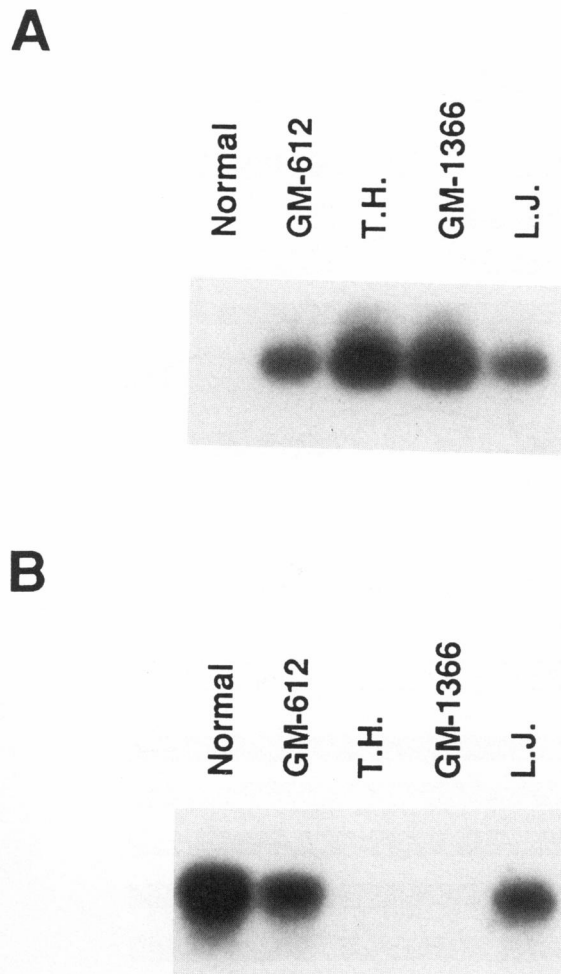
A

B

Figure 6 ASO probing for the E163* mutation, with amplified genomic DNA from MSUD patients. Genomic sequence corresponding to exon 6 of the E2 gene was amplified from normal and MSUD (GM-612, T.H., GM-1366, and L.J.) cells. Amplified products were electrophoresed and subjected to ASO probing as described in the legend to fig. 3. with (A) the mutant ASO (5'-CAAAGTTTAAATTAT-3') containing the G-to-T transversion and (B) the normal ASO (5'-CAAAGTTGAAATTAT-3').

and E1/E3 binding domains but that is without the distal inner-core domain. The truncated E2 was overexpressed in a homozygous host, GM-1366, by transfection with an EBO vector carrying the E163* mutation (fig. 5). The smaller E2 peptide was readily detected in the transfected cells by western blotting. However, it is not detectable in cultured cells from patients who are homozygous (GM-1366 and T.H.) or compound heterozygous (GM-612 and L.J.) for the E163* allele. We interpret the data to indicate that the E163* peptides are unable to assemble into a 24-mer

structure and are degraded in mammalian cells. The truncated E2 can only be detected in the transfection experiment, where the mutant protein is overexpressed. This differs from the situation in unassembled lipoyl-bearing domains of the related *Escherichia coli* pyruvate dehydrogenase complex. These single domains can be readily expressed in the *E. coli* host as stable proteins (Miles and Guest 1987).

The E2 component is the structural core of the BCKAD complex, the core to which other enzyme components are attached through noncovalent interactions (Yeaman 1989). The absence of E2 subunits in the six MSUD patients studied here prevents the assembly of other enzyme components into a functional multienzyme complex. Unassembled components including E1 and the specific kinase are likely to have markedly reduced catalytic efficiencies. Thus, the E1α subunit from GM-612 cells was shown to be completely dephosphorylated, as opposed to E1α from normal cells, which exists in both phosphorylated and dephosphorylated forms (Eisenstein et al. 1991). These authors proposed that the absence of an assembled BCKAD complex in GM-612 cells precludes the association of the kinase with E1, thereby impeding phosphorylation of the E1α subunit by the kinase. Similarly, unassembled E1 of the bovine pyruvate dehydrogenase complex shows low residual activity for the decarboxylation of pyruvate, compared with the E1 component complexed with E2 and E3 (Roche and Reed 1972). These findings suggest that E1 and E2 interactions are necessary for the high catalytic efficiency of E1. This may explain the deficiency of E1 activity previously observed with E2-deficient GM-612 cells (Chuang et al. 1981).

Exon skipping presented here was observed with PCR-amplified cDNAs derived from the total cellular E2 mRNA. Both normal and mutant E2 mRNAs occur as one predominant species in northern analysis (fig. 1B and Fisher et al. 1989). Therefore, the truncated mRNA with single- or multiple-exon deletions may represent minor species that are beyond the sensitivity of northern blotting and that were preferentially detected by PCR. The precise deletion of one or several exons at the exon-intron junction rules out the possibility that these deletions are PCR artifacts. Exon skipping appears to occur more frequently in amplified mutant transcripts than in normal ones, where a single deletion of exon 9 was observed (fig. 7).

There are several lines of evidence to support the hypothesis that exon deletions are secondary effects of the MSUD mutations. First, two compound-heterozy-
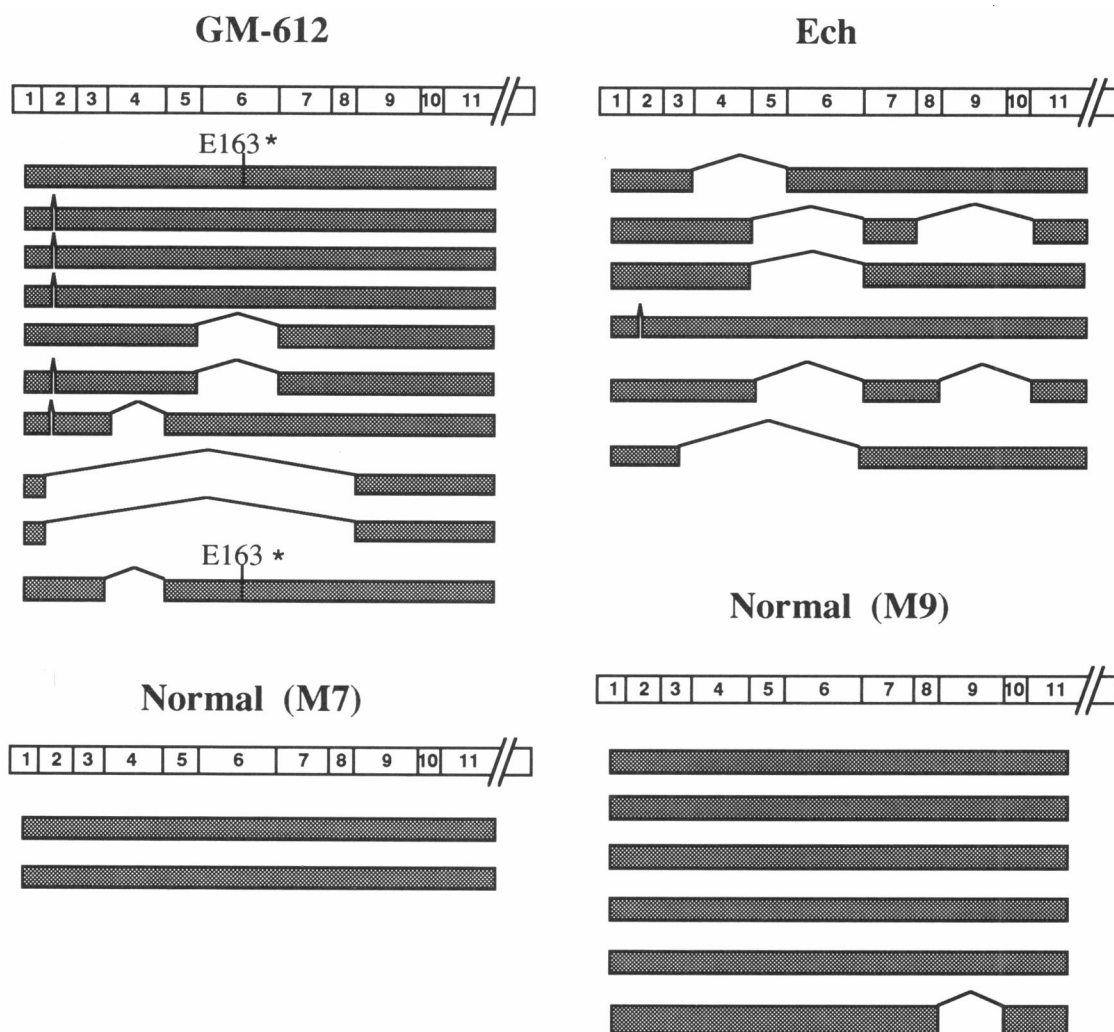
GM-612

Ech

Normal (M9)

Normal (M7)

Figure 7   Single- and multiple-exon skipping in amplified E2 cDNAs from normal and MSUD cell lines. Poly(A)$^+$ RNA isolated from normal and MSUD (GM-612 and Ech) fibroblasts was reverse transcribed and amplified by PCR as described in Material and Methods. The region amplified corresponds to the coding sequence for mitochondrial targeting signal and the mature E2. Amplified E2 cDNA was subcloned into Bluescript plasmids. Each stippled bar represents one clone that was isolated and sequenced. The numbered, unstippled bar on the top of each set shows the 11 exons, to scale for the E2 gene, that encode the full-length E2 cDNA. The peak in lines indicates single and/or multiple exons that are precisely skipped (shown as gaps), as detected by sequencing each clone. The spike symbols denote the 2-bp deletion in exon 2 (bases 89 and 90). E163* corresponds to the G-to-T transversion (base 685) in exon 6.

gous mutations in the coding sequence of GM-612 are sufficient to result in the absence of both the E2 subunit and the MSUD phenotype. Second, the sequences of the splice-site junctions flanking the skipped exon 6 in GM-612 are normal, excluding the possibility of a primary mutation in these junctions. Third, the gt deletion in the 5' splice site of intron 5 in patient Ech (J. L. Chuang, D. T. Chuang, and R. P. Cox, unpublished observations) explains the consistent skipping of exon 5 in transcripts that do not contain the exonic 2-bp (AT)

deletion (fig. 7). Nevertheless, the skipping of exons other than exon 5 in Ech appears to be secondary to the mutation at the exon 5–intron 5 boundary. Finally, a primary mutation in a branch site within introns (Padgett et al. 1986) cannot account for the tandem array and random nature of multiple-exon deletions.

Exon skipping caused by mutations not involving the splice junction was reported earlier. Mutations in the first exon caused an alteration in downstream splice-site selection, resulting in exon skipping in SV40 late
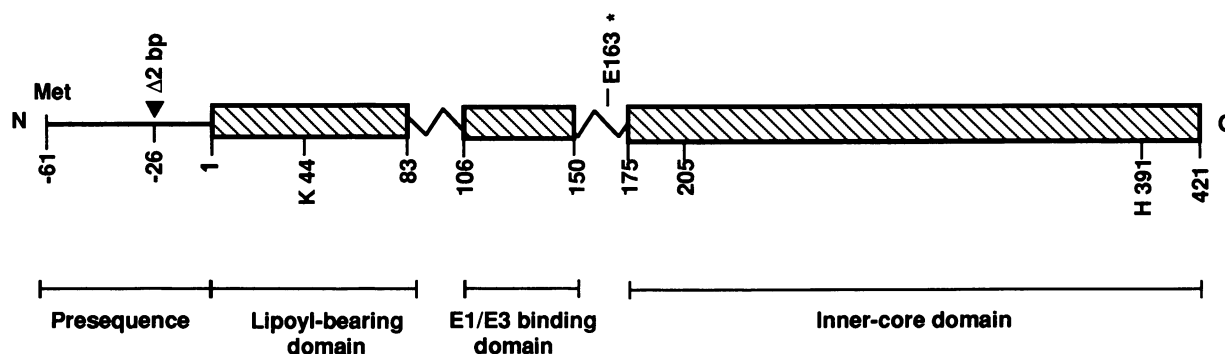
Figure 8   E2 domain structure and locations of the 2-bp deletion and the E163* mutations. Hatched bars represent the three folded domains of the mature E2 subunit (Griffin et al. 1988), which are connected by flexible hinge regions (denoted by zigzag lines). Numbers below the hatched bars indicate the position of amino acid residues. Lysine-44 (K 44) and histidine-391 (H 391) are the lipoic acid–attachment site and the active site, respectively. The initiation Met residue is at position (−61) of the mitochondrial targeting presequence that is removed during the mitochondrial import. Locations of the 2-bp deletion (Δ2bp) and the E163* substitution are shown above the bars.

transcripts (Somasekhar and Mertz 1985). Similar results were observed in the human β-globin gene, in which alterations in exon sequences also affect the pattern of splice-site selection (Reed and Maniatis 1986). However, the number of exons deleted in sequence or in tandem reported here is the most extensive for any gene studied thus far. The mechanism for exon skipping in the mutant E2 transcripts is unknown. Recent reports indicate that the secondary structure may play an important role in pre-mRNA processing. For example, a 52-bp deletion in exon 19 of the dystrophin Kobe gene was shown to abolish a hairpin structure in the exon and to lead to deletion of exon 19 (Matsuo et al. 1992). A primary MSUD mutation in an exon of the E2 gene may alter the global secondary structure of the mutant E2 pre-mRNA. This could cause errors in splicing, leading to deletion of one to several exons, as observed in the mutant E2 transcripts. The extensive exon skipping observed here cautions against interpretation of this as the primary defect. Exon skipping recorded here may also have implications for the general principles underlying RNA splicing.

Acknowledgment

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