JCI The Journal of Clinical Investigation

Maple syrup urine disease caused by a partial deletion in the inner E2 core domain of the branched chain alpha-keto acid dehydrogenase complex due to aberrant splicing. A single base deletion at a 5'-splice donor site of an intron of the E2 gene disrupts the consensus sequence in this region.

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J Clin Invest. 1991;87(4):1207-1211. https://doi.org/10.1172/JCI115120.

Research Article

We have studied the molecular bases of maple syrup urine disease by analyzing the activity, subunit structure, mRNA sequence, and the genome of the affected enzyme. The branched chain alpha-keto acid dehydrogenase (BCKDH) activity in the patient was 4.2-4.5% of the control level. Immunoblot analysis revealed that the E2 subunit of BCKDH (Mr 52,000) was absent and another protein band with an Mr of 49,000 was present. We amplified the cDNA of the E2 subunit obtained from the patient's cell using the polymerase chain reaction method, then sequenced the amplified cDNA, in which a 78-bp deletion was identified. The consanguineous parents and a sister had two species of mRNA; the one corresponding to the normal E2 subunit and the other with a 78-bp deletion, whereas findings in a brother were normal. The molecular size of the translation products as deduced from the abnormal mRNA sequence was compatible with an abnormal protein band (Mr 49,000) detected in the patient's cells by immunoblot analysis. Analysis of genomic DNA of BCKDH-E2 subunit revealed that the 78-bp deletion in the mRNA was caused by an exon skipping due to a single base deletion in the 5'-splice donor site. As a result of the mutation, part of the inner E2 core domain was omitted. The specified region of the inner E2 core domain was highly […]

Maple Syrup Urine Disease Caused by a Partial Deletion in the Inner E_2 Core Domain of the Branched Chain α-Keto Acid Dehydrogenase Complex due to Aberrant Splicing

A Single Base Deletion at a 5'-Splice Donor Site of an Intron of the E_2 Gene Disrupts the Consensus Sequence in This Region

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Abstract

We have studied the molecular bases of maple syrup urine disease by analyzing the activity, subunit structure, mRNA sequence, and the genome of the affected enzyme. The branched chain α-keto acid dehydrogenase (BCKDH) activity in the patient was 4.2–4.5% of the control level. Immunoblot analysis revealed that the E_2 subunit of BCKDH (M_r 52,000) was absent and another protein band with an M_r of 49,000 was present. We amplified the cDNA of the E_2 subunit obtained from the patient's cell using the polymerase chain reaction method, then sequenced the amplified cDNA, in which a 78-bp deletion was identified. The consanguineous parents and a sister had two species of mRNA; the one corresponding to the normal E_2 subunit and the other with a 78-bp deletion, whereas findings in a brother were normal. The molecular size of the translation products as deduced from the abnormal mRNA sequence was compatible with an abnormal protein band (M_r 49,000) detected in the patient's cells by immunoblot analysis.

Analysis of genomic DNA of BCKDH-E_2 subunit revealed that the 78-bp deletion in the mRNA was caused by an exon skipping due to a single base deletion in the 5'-splice donor site. As a result of the mutation, part of the inner E_2 core domain was omitted. The specified region of the inner E_2 core domain was highly homologous to the region of the E_2 subunit of pyruvate dehydrogenase and α-ketoglutalate dehydrogenase. These observations imply the biological importance of the region in the inner E_2 core domain of BCKDH to maintain normal function of the activity. (*J. Clin. Invest.* 1991. 87:1207–1211.) Key words: inherited metabolic disease • branched chain amino acid • polymerase chain reaction • mRNA • mutation

Introduction

Branched chain α-keto acid dehydrogenase (BCKDH)[1] is a multienzyme complex associated with the mitochondrial inner membrane and catalyzes oxidative decarboxylation of the α-keto acids derived from transamination of the branched chain amino acids, valine, leucine, and isoleucine. The BCKDH consists of three catalytic components; a branched chain α-keto acid decarboxylase (E_1), a dihydrolipoyl transacylase (E_2), and dihydrolipoamide dehydrogenase (E_3) (1–4). The enzyme complex also contains two regulatory enzymes, a specific kinase (5–7) and a specific phosphatase (8, 9) that regulates the enzyme activity by phosphorylation–dephosphorylation. The E_2 subunits form the structural core of the enzyme, to which E_1, E_3, the kinase, and the phosphatase are attached through noncovalent interactions (1, 8, 10). The E_2 subunit has three domain structures; lipoyl-bearing, E_3-binding, and inner core domains. The last domain has the transacylase active site and comprises the carboxy-terminal half of the E_2 polypeptide, assembling into a 24 mer (11).

Impaired BCKDH activity leads to maple syrup urine disease (MSUD) (12). We (13, 14) and others (15) reported that some patients with MSUD showed decreased amounts of E_2 protein. To investigate related molecular mechanisms, we and others isolated a cDNA clone encoding the entire E_2 subunit of the human BCKDH complex (16, 17).

We now report our analysis of a classical case of MSUD, in terms of the activity, subunit structure, mRNA sequence, and genomic sequence of BCKDH. We used the polymerase chain reaction (PCR) to amplify E_2-specific sequences from total RNA and genomic DNA isolated from EB-transformed lymphoblastoid cells derived from an MSUD patient with an abnormal E_2-like protein, detected using immunoblots. Part of the inner core domain of E_2 lacking in the patient was caused by a deletion of a single guanine at the exon–intron splice junction.

Methods

Cell lines. Lymphoblastoid cell lines were established by Epstein-Barr virus–mediated transformation of peripheral blood B lymphocytes from the patient (18). Studies were done on peripheral leukocytes taken from 10 ml of (heparinized) blood from the family members. Cell line GM 1366 was obtained from the Human Mutant Cell Repository, Camden, NJ.

Enzyme assay. Substrate-dependent kinetics of the BCKDH was determined for disrupted lymphoblastoid cells by quantifying the $^{14}CO_2$ released from α-keto [1-^{14}C] isovaleric acid, in the presence of co-factors, as described (13, 14).

Immunoblot analysis. Mitochondrial proteins were resolved by electrophoresis in a 10% polyacrylamide gel in the presence of SDS essentially as described by Laemmli (19). The resolved proteins were electroblotted to nitrocellulose and cross-reacting proteins were detected using affinity-purified anti-bovine BCKDH (E_1 + E_2) rabbit immunoglobulin, as described (13, 14).

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Received for publication 30 May 1990 and in revised form 1 October 1990.

1. *Abbreviations used in this paper:* BCKDH, branched chain α-keto acid dehydrogenase; E_1, branched chain α-keto acid decarboxylase; E_2, dihydrolipoyl transacylase; E_3, dihydrolipoamide dehydrogenase; MSUD, maple syrup urine disease; PCR, polymerase chain reaction.

J. Clin. Invest.

0021-9738/91/04/1207/05 $2.00
Volume 87, April 1991, 1207–1211

Analysis of cDNA. Total RNA was isolated from cultured lymphoblastoid cells of the patient and from the peripheral leukocytes of family members, as described (20). First-strand cDNAs were generated from 30 μg of total RNA, using Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in 50 mM Tris-HCl (pH 8.3), 40 mM KCl, one mM DTT, 6 mM $MgCl_2$, and 0.5 mM dNTPs (dATP, dCTP, dGTP, and dTTP) with specific antisense oligonucleotides primers; 5'-TGAACATGTGCTGGCACAGCTAGGGT-3'. The cDNAs were then subjected to 40 cycles of enzymatic amplification (21). The two sets of sense/antisense oligonucleotides were designed to cover the entire normal human cDNA sequence; 5'-GGCTGCAGTCCGTATGCTGAGAACCT-3'/5'-ATGAGGTATCTTCAGGGCTGCAGACA-3', and 5'-AATGGTCAAGACTATGTCTGCAGCCC-3' / 5'-GGGAGCTCAAAAAGTTCAAGAATGTC-3' (16). The amplified cDNAs were subcloned into the multicloning site of a plasmid vector pUC18, and four independent clones of each amplified cDNA segment were sequenced using a Sequenase kit (United States Biochemical Corp., Cleveland, OH).

Analysis of genomic DNA. A human genomic DNA library was screened for clones carrying the BCKDH E_2 subunit gene, using the plaque hybridization technique (22). The library was constructed from Sau 3A partial digests of human leukocytes DNA (a kind gift from Dr. M. Mori, Kumamoto University). Approximately 1×10^6 EMBL 4 phages of total DNA library were screened, using the human BCKDH E_2 subunit cDNA insert as a probe. Phage DNAs of positive clones were characterized by restriction mapping and Southern hybridization analysis. Subclones were constructed with plasmid pUC18 as a vector, and the nucleotide sequences were determined. Genomic DNA for analysis was purified from cultured lymphoblastoid cells according to Maniatis et al. (22), but with some modification. The specific amplification of a genomic region that included an exon corresponding to the deletion in the mRNA of BCKDH-E_2 subunit in the patient was attempted with oligonucleotide A and B as primers (see Fig. 5). Primer A (5'-CATTGGCAGTAATGTGAC-3') is complementary to the antisense DNA strand of the intron, 141–158 nucleotides upstream of the exon. Primer B (5'-GGTACAATAGAAGTCTCT-3') is complementary to the sense DNA strand of the intron, 67–84 nucleotides downstream of the exon. The amplified DNAs were subcloned into the multicloning site of pUC18, and four independent clones of each amplified DNA segment were sequenced.

Clinical features of the MSUD patient. The patient T.H. (female) was the progeny of second cousin parents. A severe ketoacidosis associated with convulsion and unconsciousness occurred on the 10th postnatal day. Elevated levels of blood leucine became apparent at 11 d of age and exchange transfusion and peritoneal dialysis were initiated. After this treatment, she was put on a diet restricted for protein and branched chain amino acids (400–500 mg/d as leucine). Under these conditions, she experienced frequent attacks of severe ketoacidosis for the next 5 yr, and the attacks were usually preceded by an infection. The clinical phenotype was categorized as a classical case of MSUD.

Results

The disrupted normal lymphoblastoid cells showed a hyperbolic Michaelis-Menten kinetics over the substrate range tested, 0.05–1.0 mM. The V_{max} and apparent K_m values were 13–15 nmol/h per milligram of protein and 0.053–0.056 mM, respectively. Cells from the patient (T.H.) showed a significantly reduced enzyme activity, in that the V_{max} and apparent K_m in the patient were 1.2 nmol/h per milligram of protein and 0.103 mM, respectively. Another cell strain related to E_2 deficiency (GM 1366) was analyzed as a reference (13, 14) (Fig. 1).

Immunoblot analysis of BCKDH revealed that three main immunogenic peptides, the E_2 (M_r 52,000), $E_1\alpha$ (M_r 46,000), and $E_1\beta$ (M_r 37,000) were present in the normal control (lane *1*). In the two samples (GM 1366 in lane *2* and patient T.H. in lane 3), the E_2 subunits (M_r 52,000) were absent and a protein band (M_r 49,000) was present in the latter sample (lane 3, *arrow*).

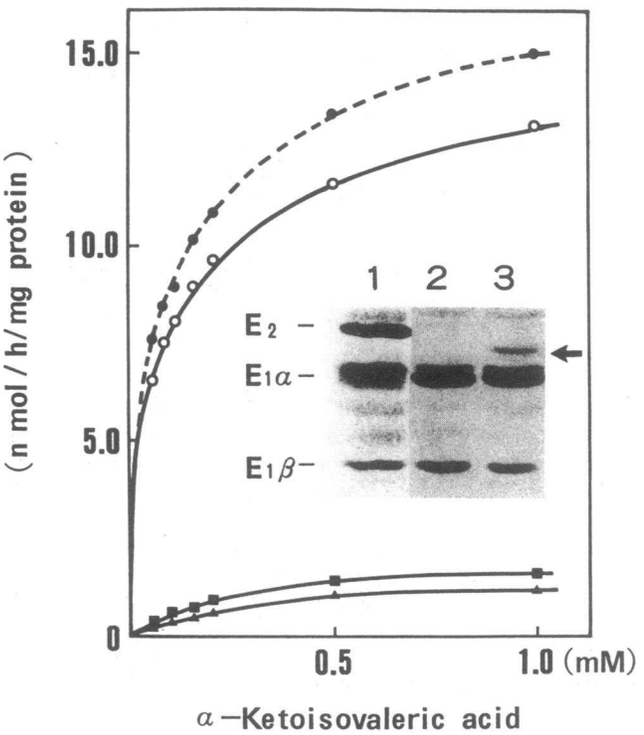

Figure 1. Activities of BCKDH and immunoblot analysis of BCKDH proteins in disrupted lymphoblastoid cells from disease-free and MSUD subjects. The rate of the overall reaction catalyzed by the multienzyme complex was measured as described in Methods. The cell lines from disease-free and MSUD subjects are: control 1 (●); control 2 (○); the patient (T.H.) (▲); and GM 1366 (■). BCKDH immunoreactive proteins were detected by the immunoblot technique, using affinity-purified antibody. The BCKDH (E_1 + E_2) peptides were, in decreasing size: E_2, dihydrolipoyl transacylase (M_r 52,000); $E_1\alpha$, the α-subunit of branched-chain α-keto acid decarboxylase (M_r 46,000); $E_1\beta$, the β subunit of branched-chain α-keto acid decarboxylase (M_r 37,000). (Lane *1*) Disease-free cell line (control 1); (lane *2*) MSUD patient (GM 1366); (lane *3*) a cell line from MSUD patient (T.H.). The arrow indicates the abnormal protein band.

To define the gene mutation in the patient (T.H.), we cloned and sequenced the cDNA of the E_2 subunit of BCKDH by reverse transcription of RNA followed by PCR. Two sets of sense–antisense primers were designed, based on the normal human BCKDH-E_2 cDNA sequence (Fig. 2). Thus, the complete coding region of the mature BCKDH-E_2 was amplified as overlapping two segments, and these were subcloned into pUC18 for DNA sequencing.

The amplified segment corresponding to the 5' region of cDNA was within a normal length and there was no change in the nucleotide sequence. Another amplified segment corresponding to the 3' region of cDNA was shorter than the normal one. We found a 78-bp in-frame deletion in the cDNA sequence, as shown in Fig. 3 *a*. The abnormal cDNA encoded 451 amino acids with the lack of the 26 amino acids inner E_2 core domain (Fig. 3 *b*).

The molecular size of the translation products deduced from the abnormal mRNA sequence was compatible with the

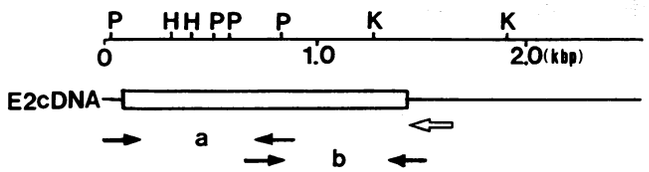

Figure 2. A feature of the human BCKDH-E_2 subunit precursor cDNA and locations of the primary PCR primers. The open box and the line depict coding and noncoding regions, respectively. Restriction sites are indicated above the cDNA. Restriction enzymes used were: HindIII (*H*); KpnI (*K*); and PstI (*P*). The white arrow indicates the orientation and the region of primer used for the synthesis of the first strand cDNA. The solid arrows indicate the orientation and the region of primers used for PCR amplification. (*a*) Segment of 5′ region. (*b*) Segment of 3′ region.

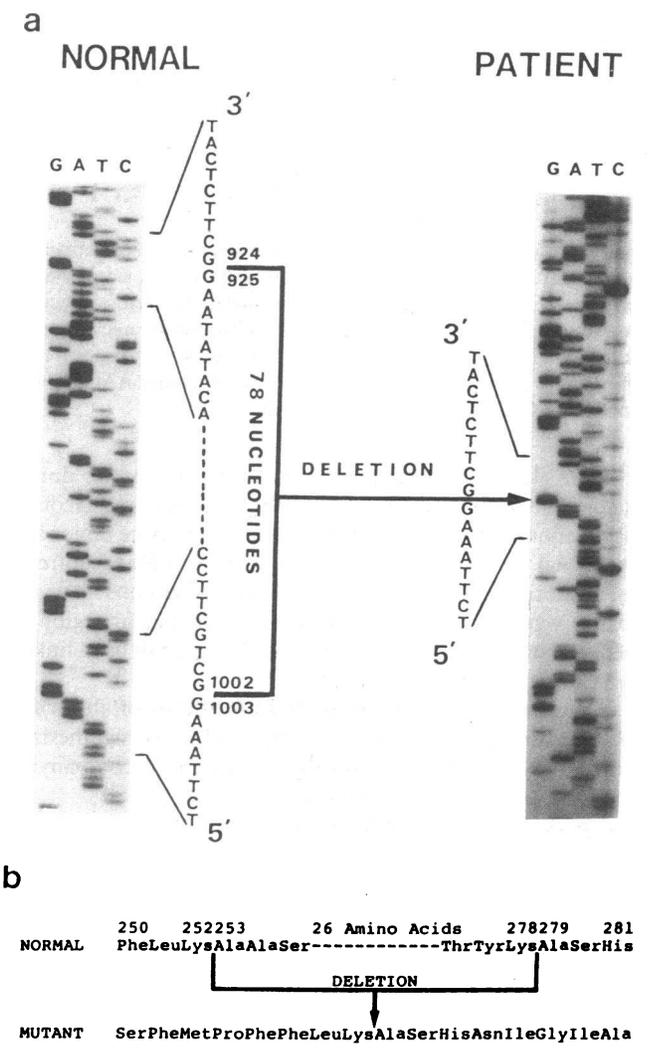

Figure 3. (*a*) Sequence analysis of cDNAs synthesized and amplified from RNAs obtained from cultured cells from the patient and the control. The results of nucleotide sequencing of the normal cDNA and the mutant cDNA are shown. The 78-bp deletion was present in the patient. The base numbers for the breakpoints are indicated. (*b*) Amino acid sequence of a region of E_2 of normal and mutant proteins predicted from the cDNA sequences. In-frame deletion of 78 bp resulted in elimination of 26 amino acid residues from the normal sequence in the mutant E_2.

abnormal protein band (M_r 49,000) found in the patient's cells by immunoblot analysis. To demonstrate that this change was not due to an amplification or cloning artifact, the PCR of this region was repeated four times with different batches of cDNA prepared from total RNA of EB-transformed lymphoblastoid cells and peripheral leukocytes from the patient; the deletion appeared in all cases. PCR amplification of the segment of 3′ region of the cDNA from the family members was done similarly; the PCR products were digested with PstI–KpnI and hybridized with the normal cDNA sequence.

As shown in Fig. 4, the parents had two species of cDNA fragments, one with a smaller size that was compatible with the abnormal mRNA with a deletion of 78 nucleotides, and one with a normal size. Only the brother carried the fragment of a normal size. The sister had two fragments, one normal and one the mutant cDNA, similar to findings in the parents. These data indicated that the parents and sister were heterozygotes for the mutant allele and normal allele, and the brother as a homozygote for the normal allele.

To examine the putative mutation in the E_2 gene, Southern blot analysis was made of the genomic DNA. Genomic DNA from the patient was digested with five different restriction enzymes (EcoRI, BamHI, HindIII, KpnI, and PstI) and probed with a KpnI–PstI restricted cDNA fragment, including the region of the deletion. No major rearrangement of the gene was detected (data not shown).

We then attempted to isolate genomic DNA fragments containing the nucleotide sequence deleted from the mRNA. The exon related to the deletion was identified by Southern blot analysis of the phage DNAs, using cDNA fragments obtained from the control and from the patient. A 0.6-kb EcoRI fragment that gave a positive signal with the normal cDNA and a negative signal with the abnormal cDNA was identified and was subcloned into a plasmid pUC18, and the nucleotide sequence determined. The EcoRI fragment from the genomic library contained a single exon composed of the 78 bp deleted from the mRNA. A part of the nucleotide sequence is shown in Fig. 5. The results of Southern blot analysis and the sequencing

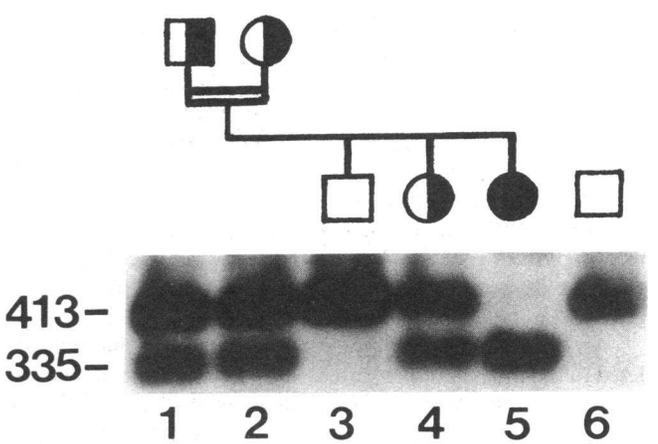

Figure 4. Partial pedigree of the family and PCR analysis of the cDNA. All samples were amplified by PCR for the segment of the 3′ region of cDNA. PCR products were digested with PstI–KpnI and hybridized with a 413-bp, PstI–KpnI fragment derived from normal sequence cDNA. This fragment includes the region of the 78-bp deletion. Fragment sizes (bp) are indicated. (*1*) Father; (*2*) mother; (*3*) brother; (*4*) sister; (*5*) proband; (*6*) control.

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     -150       -140       -130       -120       -110
CATTGGCAGTAATGTGACTAGGTACATGTAGACTTGAAATTAAACTCAGC
      primer A
     -100        -90        -80        -70        -60
ATAAATAAGAGTTCTCTCTCTCCCTCCTTTTATACCTCTTATTGTTTCAA
      -50        -40        -30        -20        -10
ATTCTCTATCCTTTCTAAGTTAAAAGTACTAAAATTGCATTCTTTTTCTG
        1         10         20         30         40
TTAAACAGGCTGCTTCCTTGGGATTACTACCATTTCCTATCCTTAACGCT
         AlaAlaSerLeuGlyLeuLeuProPheProIleLeuAsnAla
       50         60         70         80         90
TCTGTGGATGAAAACTGCCAGAATATAACATATAAGGTTGGCTATGCAGA
SerValAspGluAsnCysGlnAsnIleThrTyrLys
      100        110        120        130        140
GTAAAAATGTTCTTATTATTAATAGAAGATGGGGCAAATGTGCTTGTAGA
      150        160        170        180        190
TTAGAGACTTCTATTGTACCTCAAATAGTGATCTTTTTTAGACTAGAATT
           primer B
```

Figure 5. DNA sequence around the exon deleted in the mRNA in the patient. The exons were boxed and deduced amino acids are indicated under the nucleotide sequence. The nucleotide corresponding to the 5′ end of the exon was numbered 1. The exon was composed from 78-bp nucleotides corresponding to the deletion in the mRNA. The arrows indicate the location of primers for PCR amplification. Fragments of 320-bp nucleotides that covered the exon were amplified with primers A and B by PCR.

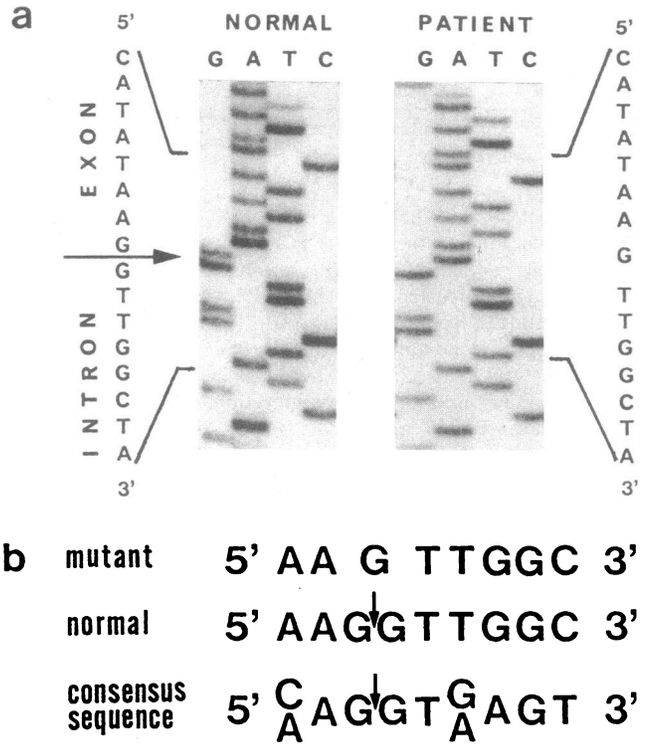

Figure 6. (*a*) Sequence analysis of amplified genomic DNAs obtained from cultured cells from the patient and the control. A single base deletion of G at the 5′ splice donor site is shown. The arrow indicates the exon intron splice junction. (*b*) Comparison of the nucleotide sequences of the mutant, normal, and the consensus sequence for 5′ splice donor site. The sequence in the normal sequence AAGGT was identical to that of the consensus sequence.

of the genomic DNA strongly suggest that the mutation in the patient was related to the splicing of mRNA.

Based on the nucleotide sequence of the introns on the boundary of the exon, we prepared a set of oligonucleotide to amplify the region of the genomic DNA in order to investigate the structure of the gene in the patients (Fig. 5). A DNA fragment of 320 bp was amplified and the amplified DNAs were subcloned and sequenced. The results of the nucleotide sequencing are shown in Fig. 6 *a*. A single guanine from the exon–intron junction was missing in the patient. This mutation did not disrupt the doublet of GT at the 5′ end of the intron; however, the consensus sequence of AAGGT (23) was changed to AAGT (Fig. 6 *b*).

Discussion

The patient with a classical type of MSUD caused by a deficiency of BCKDH-E_2 subunit was found to have a 78-bp deletion in the mRNA of the BCKDH-E_2 subunit. The sequence of the mutated cDNA revealed that the deletion occurred between homologous regions in the cDNA sequences. Therefore, first we speculated that the homologous recombination or slipped mispairing in the region of the E_2 gene seemed to be involved in the putative mutation in the patient (24–26).

Southern blot analysis of the genomic DNA from the patient revealed no major rearrangement in the E_2 gene. For further analysis of gene mutation, we cloned genomic DNAs covering the deleted cDNA sequence, since the structure of the E_2 gene had to be determined.

An exon containing the nucleotide sequence that was deleted in the mRNA of the patient was identified and cloned (by PCR) from the genomic DNA of the patient and the controls. The sequencing of the cloned DNA from the patient revealed a deletion of guanine at the consensus splice site sequence of AAGgt at 5′ end of the intron (Fig. 6 *b*). This region is involved in binding of Ul small nuclear ribonucleoprotein particles and undergoes cleavage in the spliceosome and lariat formation (27). As a result of the mutation, abnormal splicing (exon skipping) of the mRNA occurred and an entire exon of 78 bp was deleted in the mRNA.

Exon skipping due to a point mutation of 5′-splice donor site has been reported (28–30). Among those single nucleotide substitutions of the dinucleotide of gt at the 5′-splice donor site were usual cases of the abnormal splicing. A unique feature in our mutation was that the dinucleotide of gt consensus sequence was apparently conserved in this particular region of the human E_2 gene. This type of mutation has not been linked as a cause of genetic disorders.

Mutation in the gene resulted in synthesis of abnormal mRNA; subsequently, a mutant subunit of E_2 was synthesized and possibly incorporated into mitochondria accompanying the normal E_1 and E_3 subunits of BCKDH.

The E_2 subunits form the structural core of the enzyme, to which E_1, E_3, the kinase, and the phosphatase are attached through noncovalent interactions (1, 8, 10). The E_2 subunit has three domain structures; lipoyl-bearing, E_3-binding, and inner core domains. The last domain has the transacylase active site (11). The deduced amino acid sequence from the mutated cDNA revealed that the mutant E_2 peptide in the patient, which was weakly visible on the immunoblot, lacks 26 amino acids (Nos. 253–278) in the inner core domain. The BCKDH activity in the patient was decreased to the level seen in another patient with E_2 deficiency (GM 1366), for whom there was no cross-reactive material in the immunoblot. The amount of the mutant E_2 subunit seemed to be decreased in the present patient. Possible interpretations are a decreased synthesis in abnormal mRNA, an impaired incorporation of the precursor protein into mitochondria, or a decrease in the stability of the mutant protein.

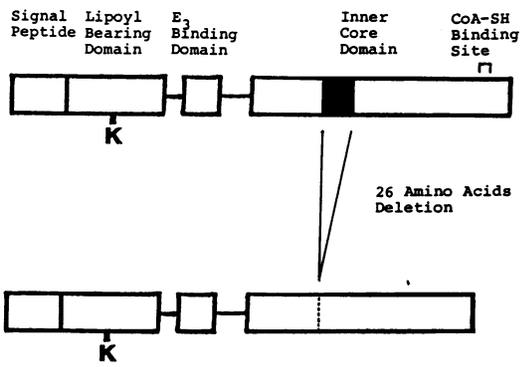

Figure 7. Schematic comparison of domain structures of normal and the mutant E_2 of BCKDH. Open boxes represent the putative leader peptide, lipoyl bearing domain, E_3 binding domain, and inner core domain with the CoA-SH binding site. The solid box represents the region of the deletion in the inner core domain in the patient. K indicates the position of the lipoyllysin residue.

The specific region of the inner E_2 core domain indicates a strong species conservation and is highly homologous to the region of E_2 subunit of pyruvate dehydrogenase, and that of α-ketoglutarate dehydrogenase (16, 17). The region in the inner E_2 core domain of BCKDH, which is deleted in our patient, seems biologically important to maintain normal functions of the subunit protein. This deletion would explain the grossly altered active site of the E_2 subunit (Fig. 7).

Deletion in the mRNA in her family was identified by PCR. The mother, the father, and the sister had two species of mRNA; the one corresponding to the normal E_2 subunit and the other with a 78-bp deletion, thereby indicating that they were heterozygous for this mutation. The brother had a normal size mRNA, hence was homozygous of the normal allele.

Acknowledgments

We are indebted to Dr. Y. Kudo of Kumamoto City Hospital for provision of the peripheral leukocytes from the patient and her family members. Dr. J. Asaka (Shionogi Institute for Medical Science) and Dr. T. Kunisada (Department of Pathology Institute for Medical Immunology, Kumamoto University Medical School) provided the synthetic oligonucleotides. We also thank M. Ohara for helpful comments and M. Hayashi and M. Tsutsui for secretarial services.

This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan (62304042), and from the National Center of Neurology and Psychiatry of the Ministry of Health and Welfare, Japan (2-B).

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