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Pyruvate Dehydrogenase Deficiency Caused by Deletion of a 7-bp Repeat Sequence in the E1α Gene

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Summary

A 7-bp deletion in the X-chromosomal pyruvate dehydrogenase (PDH) E1α gene was characterized in a female patient with the "cerebral" form of PDH deficiency. The mutation was localized using the chemical cleavage method and further characterized by application of the polymerase chain reaction and DNA sequencing. This 7-bp sequence is found in the normal gene as a direct tandem repeat. The deletion causes a change in the reading frame. Results have shown that the level of normal sized PDH E1α in the fibroblast sample was approximately 30% of that of normal controls. This is consistent with normal transcription from the X chromosome carrying the nonmutated form of the E1α subunit, as this chromosome is active in approximately 30% of this patient's cells. The severity of PDH E1α deficiency in affected females is to a large extent dependent on the X-chromosome inactivation pattern in the brain. The clinical picture might therefore vary significantly between patients with the same mutation. We show that the 7-bp deletion must be a de novo mutation, because it is not present in the parent's X chromosomes. Furthermore, the deletion was not detected in chorionic villus samples in two subsequent pregnancies.

Introduction

The pyruvate dehydrogenase (PDH) complex catalyzes the oxidative decarboxylation of pyruvate to acetylCoA and thereby plays an essential role in aerobic energy metabolism (Reed 1974). The enzyme complex is located in the mitochondrial matrix and is composed of multiple copies of pyruvate decarboxylase (E1; E.C.1.2.4.1), dihydrolipoamide acetyltransferase (E2; E.C.2.3.1.12), dihydrolipoamide dehydrogenase (E3; E.C.1.8.1.4), E1 kinase (E.C.2.7.1.99), phospho-E1 phosphatase (E.C.3.1.3.43), and protein X, a protein of unknown function. The E1 enzyme is composed of two α and two β subunits (Barrera et al. 1972). Decarboxylation of pyruvate, catalyzed by the E1 component, is the rate-limiting step in the overall activity of the PDH complex. The main mechanism for regulation of PDH activity is phosphorylation/dephosphorylation of serine residues on the E1α subunit (Linn et al. 1969).

PDH deficiency is the major cause of lactic acidosis in children. In the majority of cases the defect is located in the E1α subunit of the complex (McKay et al. 1986; Brown et al. 1987; G. K. Brown, unpublished data). The clinical spectrum of PDH deficiency is extremely broad, ranging from fatal lactic acidosis in the newborn period to chronic neurological dysfunction without severe lactic acidosis ("cerebral" lactic acidosis [Brown et al. 1988]).

We and others have recently reported the complete cDNA sequence coding for the PDH E1α subunit (Dahl et al. 1987; De Meirleir et al. 1988; Koike et al. 1988; Ho et al. 1989). In addition we have mapped the functional gene in somatic cells to the X chromosome, band Xp22.1 (Brown et al. 1989*b*), and determined the structure of this gene (Maragos et al. 1989). This has allowed us to study the mutations affecting the PDH E1α subunit and correlate the mutation with the clinical picture seen in patients with PDH E1α deficiency. The effect of X-chromosome inactivation, the shortage of functional reserve of this subunit, and the dependency of

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the brain on PDH activity must be taken into account when interpreting the results. Here we report the characterization of a mutation in a heterozygous female with early onset of neurological problems, gross cerebral changes, and elevated levels of pyruvate and lactate in cerebrospinal fluid. It is interesting that the mutation is a deletion of a 7-bp direct repeat sequence in exon 10 of the PDH E1α gene.

Material and Methods

DNA, mRNA, and cDNA Preparation

The method for DNA isolation from fibroblasts and chorionic villus cell cultures was as published elsewhere (Dahl et al. 1987). Poly(A)+ RNA was purified from patient (LA-31a) and normal control skin fibroblasts by using oligo(dT) cellulose as described by Dahl et al. (1989) First-strand cDNA was synthesized using approximately 1 μg mRNA and a cDNA synthesis kit (Amersham International plc).

Amplification of DNA

cDNA and genomic DNA was amplified in the polymerase chain reaction (PCR) (Saiki et al. 1988). Conditions were as recommended by the manufacturer of the *Taq* polymerase (Cetus Perkin-Elmer). Synthetic oligonucleotide primers were PDH-M (5′-ACTGCCTGTGCTTCATGAG-3′), PDH-O (5′-AAGTCAGGCAGACCTCATC-3′), PDH-P (5′-GATTGCTCTAGCCTGTAAG-3′), PDH-E (5′-TGCATGCAATTGCTACCTGC-3′), PDH-Z1 (5′-TTACCGTACACGAGAAGAAA-3′), and PDH-Z2 (5′-CTTTAGTTCTTCCACACTGG-3′). A cycle consisted of denaturation at 93°C for 105 s, annealing at 61°C (for cDNA) or 63°C (for genomic DNA) for 150 s, and extension at 72°C for 180 s. Usually 35 cycles were performed. Amplified fragments were visualized on ethidium bromide–stained NuSieve (FMC Bioproducts) agarose gels.

Asymmetric PCR using the primers PDH-Z1 and PDH-Z2 was based on the method described by Gyllensten and Erlich (1988). The concentration of dCTP in the PCR reaction was reduced to 5 μM, and the amount of primer PDH-Z2 was reduced 100-fold to a final concentration of 90 ng/ml. After 10 cycles 70 μCi α-^{32}P dCTP (3,000 Ci/mmol; NEN DuPont) was added, and another 25 cycles were performed. The products were analyzed on a 5% denaturing urea-polyacrylamide gel followed by autoradiography.

The Chemical Cleavage Reaction

Heteroduplex formation was carried out in a volume of 50 μl containing 5 μl PCR-amplified cDNA fragments (or approximately 0.1 μg cDNA), 5 ng labeled probe, 110 mM Tris/HCl (pH 7.5), 825 mM NaCl, 5 mM EDTA. After denaturation at 100°C for 5 min, the mixture was annealed at 65°C for at least 1 h and was ethanol precipitated. Conditions for modification of mismatches in the heteroduplexes with hydroxylamine or osmium tetroxide, treatment with piperidine, and product analysis on denaturing urea-acrylamide gels were as described elsewhere (Dahl et al. 1989).

DNA Sequence Analysis

Single-stranded DNA was prepared from M13 clones and was sequenced using the Sequenase system (U.S. Biochemical). The sequence of the PDH E1α–specific primer PDH-L was 5′-GGAATGGATATCCTGTGC-3′.

Analysis of X-Chromosome Inactivation

Identification of the parental origin of the patient's X chromosomes and analysis of the pattern of X-chromosome inactivation was performed using the DXS255 locus. Within this locus is a hypervariable tandem repeat unit which is flanked by *Msp*I sites. As the copy number of the repeat unit is different on the two X chromosomes of most human females, digestion of genomic DNA with *Msp*I will generate different-sized fragments which will identify the individual X chromosomes in most cases. The fragments which are generated can be detected with probe M27β (Fraser et al. 1989).

In addition, the cytosine residues at the *Msp*I sites are methylated on active X chromosomes and are therefore resistant to digestion with the *Msp*I isoschizomer, *Hpa*II (Fraser et al. 1989). The proportion of each X-chromosome band which is susceptible to *Hpa*II digestion provides a semiquantitative measure of the fraction of cells in the sample in which each X chromosome is inactive. Restriction-enzyme digestion and Southern blot analysis using the M27β probe have been described in detail elsewhere (Brown et al., in press).

Results

Clinical and Biochemical Findings

Some of the clinical and biomedical details of the patient have been reported elsewhere (Chow et al. 1987; Brown et al. 1988). She was the first child of unrelated parents and was born at 39 wk gestation following a

normal pregnancy and delivery. Birth weight was 2,690 g, and a number of mild dysmorphic features, including a high forehead and palate, facial asymmetry, a single simian crease, and a high-pitched cry, were apparent.

There were some difficulty in establishing respiration, and from birth the baby was markedly hypotonic and showed no interest in feeding. Cerebral ultrasound revealed gross hydrocephalus involving the lateral and third ventricles, with some suggestion of cystic spaces in the floor of the lateral ventricle.

Metabolic acidosis was not a significant clinical problem, with the blood lactate concentration being 2–5 mmol/liter and the pyruvate concentration being 0.17–0.34 mmol/liter. By contrast, the concentrations of lactate and pyruvate in the cerebrospinal fluid were significantly elevated in the range 5–7 mmol/liter and 0.46–0.57 mmol/liter, respectively. There was no biochemical response to trials of thiamine and biotin supplementation.

The baby's condition progressively deteriorated, and she died at 1 mo of age. At postmortem examination, the most striking abnormalities were found in the central nervous system. In the brain there were extensive structural changes in addition to the cerebral atrophy and hydrocephalus demonstrated by ultrasound. These structural changes included developmental anomalies such as agenesis of the corpus callosum, absence of the medullary pyramids, and ectopic foci of the inferior olives. The pathological changes have been described in detail elsewhere (Chow et al. 1987).

In cultured fibroblasts from the patient, overall PDH complex activity was 25%–30% of that in normal controls, and there was a corresponding reduction in the amount of E1α immunoreactive protein (Brown et al. 1988, and in press). PDH E1α immunoperoxidase studies of the fibroblasts by using an affinity-purified anti-E1α antibody demonstrated mosaicism, with about 30% of cells containing normal amounts of PDH E1α protein while the remainder were completely deficient (Brown et al., in press).

Both parents are clinically normal, and their cultured fibroblasts have normal PDH activity and E1α immunoreactive protein. In addition, the fibroblasts from the mother are uniformly E1α-immunoperoxidase positive (results not shown).

Characterization of the Mutation in Patient LA-31a

The coding region for the X chromosome–linked PDHE1α gene spans approximately 1,500 bp (Maragos et al. 1989). The gene is expressed in fibroblasts, and it is therefore relatively easy to locate a structural mutation by applying the chemical cleavage method (CCM) to cDNA made from skin fibroblasts and amplified using the PCR (Dahl et al. 1989). The coding region of the cDNA was PCR amplified in two overlapping sections to facilitate more accurate localization of the mutation (fig. 1). Primers PDH-M/O and P/E were used to amplify the two sections of the PDHE1α gene from cDNA prepared from patient LA-31a and a control. The same primers were used to make a randomly labeled ^{32}P probe by amplification of the sequence in the plasmid pPDH1c (Dahl et al. 1987). Probes were annealed to the corresponding fragments from patient and control, treated with hydroxylamine or osmium tetroxide, subjected to cleavage with piperidine, and analyzed on a urea-acrylamide gel as described in Material and Methods. No mismatches in the patient heteroduplex DNA were seen in the 5′ region covered by primers PDH-M/O. Analysis of the 3′ half of the coding region amplified with primers PDH-P/E identified specific bands after both hydroxylamine and osmium tetroxide treatment (results not shown). To precisely locate the mutation, a *Ban*I fragment from the 3′ end of the coding region of pPDH1c was end labeled by the kinase reaction and annealed to PCR-amplified DNA, and the CCM reactions were repeated. Specific cleavage of the LA-31a heteroduplex was again observed in both the hydroxylamine and the osmium tetroxide reactions, suggesting the presence of a deletion (fig. 2). A 217-bp band is seen in all lanes, including the 0-min timepoint, suggesting that it is an artifact generated during probe preparation. We have sequenced the relevant region in control, LA-31a, and probe DNA, and the sequences are in all cases identical to those published. In appropriately designed experiments such background bands are easy to identify, and they do not invalidate the CCM technique.

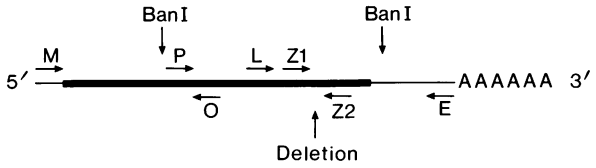

Figure 1 Schematic diagram showing the localization of oligonucleotide primers, the restriction-endonuclease *Ban*I cleavage sites, and the 7-bp deletion.

This result located the mutation in the region of nucleotide 1035 (nucleotide numbering is as published in Maragos et al. 1989), so the DNA fragment generated in the PCR reaction with primers P and E was sub-

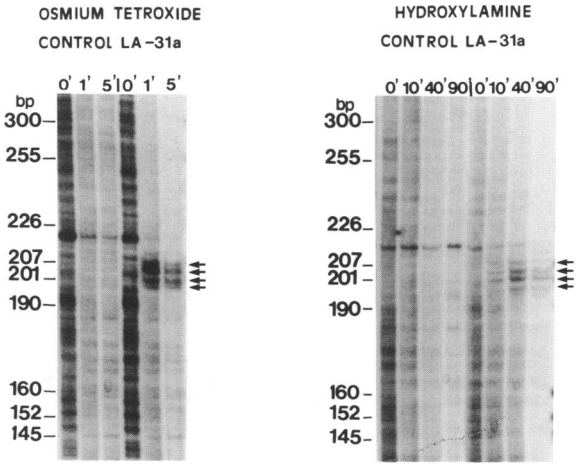

Figure 2 Detection of the mutation in patient LA-31a by using the CCM method. The two panels show the results of osmium tetroxide and hydroxylamine treatment of the heteroduplex formed between the end-labeled *Ban*I fragment and the control DNA or patient LA-31a DNA. Treatment times are shown above the lanes, the sizes of molecular-weight markers are shown to the left, and the arrows to the right indicate the bands generated by the deletion.

cloned into phage M13 and was sequenced using the sequencing primer L. The DNA from several independent clones was sequenced and shown either (*a*) to be identical to the normal control DNA or (*b*) to have a 7-bp deletion starting at nucleotide 1032 (fig. 3), implying heterozygosity at this locus.

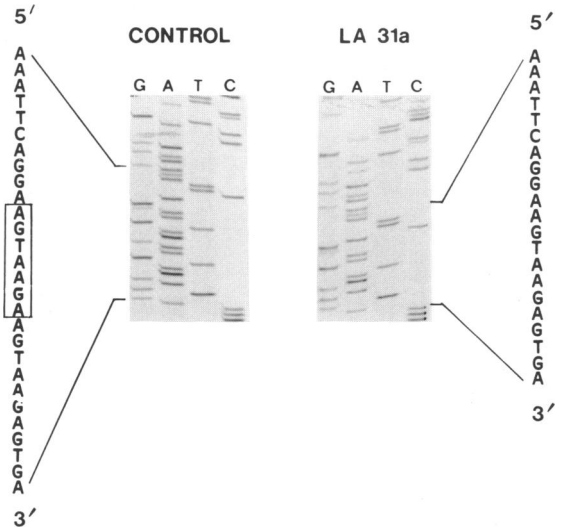

Figure 3 DNA sequence analysis of the mutation in patient LA-31a. The DNA sequence of a normal control is shown at the left, and the sequence of the deleted gene in patient LA-31a is shown to the right. The 7-bp deletion is boxed in the control sequence.

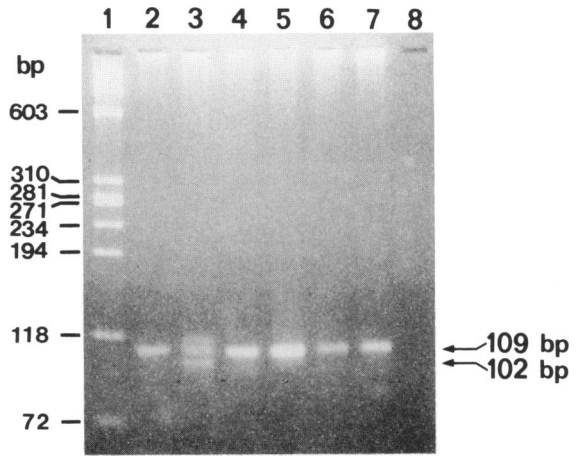

Figure 4 Analysis for the presence of the 7-bp deletion in the PDH E1α gene is the immediate family of LA-31a. The PCR reaction was performed using primers PDH-Z1 and PDH-Z2 and was analyzed as described in Material and Methods. Lane 1, DNA size markers. Lane 2, Father. Lane 3, LA-31a. Lane 4, Sibling CV9. Lane 5, Sibling CV16. Lane 6, Mother. Lane 7, Control DNA. Lane 8, No DNA. The sizes of the DNA markers are shown at the left. The sizes of the PCR product from the normal gene (109 bp) and from the mutant gene (102 bp) are shown on the right.

In order to exclude the possibility that the deletion was an artifact created during the PCR reaction, synthetic oligonucleotide primers Z1 and Z2 flanking the region of the deletion were synthesized. When these primers were used in PCR reactions, an expected 109-bp band was detected in amplified DNA from controls and from the parents of LA-31a. However, when DNA from patient LA-31a was included, two additional bands were seen (fig. 4). The lower band migrates as a 102-bp band, the larger as an approximately 114-bp band. This raised the possibility that the patient was a mosaic, having three different X chromosomes: a normal X, an X containing a 7-bp deletion in the PDH E1α gene, and an X with a 7-bp insertion into the gene. The three bands from the amplified LA-31a DNA were therefore isolated and sequenced. These results showed that the 102-bp band is a homoduplex of strands containing the 7-bp deletion, that the 109-bp is the homoduplex of two normal strands, and that the "114-bp band" is the result of heteroduplex formation between a deleted and a normal DNA strand, thus accounting for its aberrant migration in the gel. In addition, asymmetric PCR amplification in which the final product is mainly single-stranded DNA (Gyllensten and Erlich 1988) did not produce a band of the size that would be expected (116 bp) if an insertion existed (fig. 5). We therefore con-

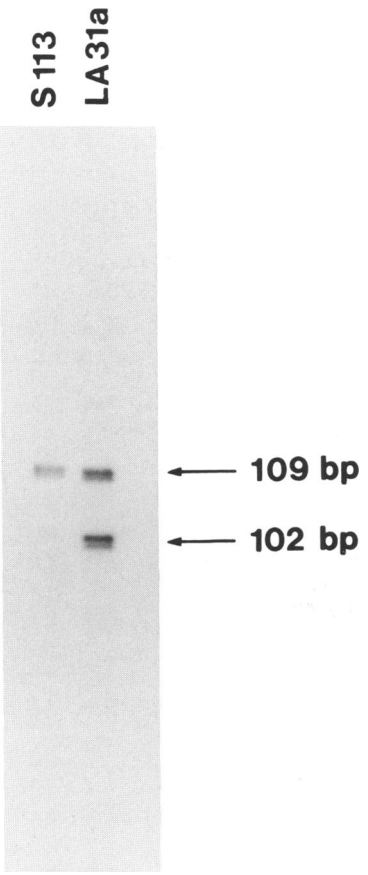

Figure 5 Analysis of the mutation in LA-31a by asymmetric PCR. The reaction was performed with primers PDH-Z1 and PDH-Z2 as described in Material and Methods. Lane S113, Normal control DNA. Lane LA-31a, fibroblast DNA from the patient. The size of a marker fragment is shown on the left, and the size of the PCR products is shown on the right.

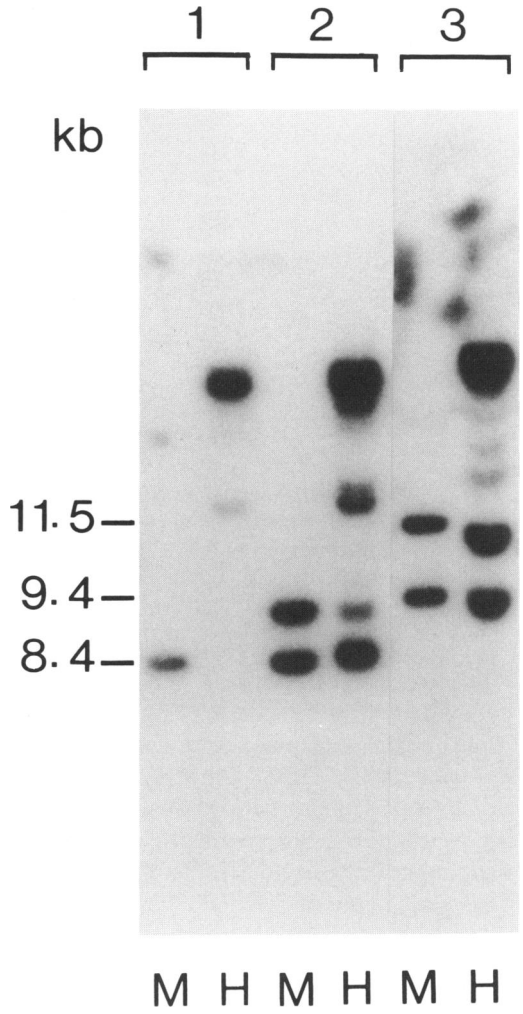

Figure 6 Analysis of the X-chromosome inactivation pattern with the M27β probe. Fibroblast DNA samples were digested with either *Msp*I or *Hpa*II, followed by a second digestion with *Bam*HI. The *Msp*I/*Bam*HI and *Hpa*II/*Bam*HI tracks are labeled M and H, respectively. Tracks 1, DNA from the father. Tracks 2, DNA from the affected daughter. Tracks 3, DNA from the mother.

clude that the patient carries a 7-bp deletion in one of her X chromosomes.

Origin and Expression Pattern of the X-Chromosome Carrying the 7-bp Deletion

Because of the nature and genetics of PDH deficiency, most observed cases are likely to be the result of new mutations. PCR analysis of DNA from maternal and paternal fibroblasts samples by using oligonucleotide primers flanking the 7-bp deletion showed no evidence of the mutation (fig. 4). We also found normal levels of PDH activity and of E1α-immunoreactive protein in the fibroblasts, and maternal fibroblasts stained uniformly positive with E1α-immunoperoxidase. The validity of using these results to exclude the heterozygosity for the mutant gene in the mother was established by demonstrating that the X chromosome which carried the mutant gene in LA-31a was of maternal origin and active in 60% of the mothers fibroblasts. Both the origin of the two X chromosomes in the patient and the pattern of X-chromosome inactivation in fibroblasts from both the patient and her mother were determined using the probe M27β to analyze polymorphism and methylation at the DXS255 locus (Brown et al., in press). The results are shown in figure 6. The single X chromosome from the father is represented by a band of 8.4 kb (track 1M). This is methylated and therefore resistant to digestion with *Hpa*II (track 1H). The two

X chromosomes of the mother give rise to bands of 9.4 and 11.5 bp (track 3M), and, on the basis of the relative proportions in the *Hpa*II track (track 3H), the chromosome represented by the 9.4-kb band is active in approximately 60% of her fibroblasts.

The two X chromosomes in the patient are the paternal chromosome and the maternal chromosome represented by the 9.4-kb band (track 2M). On the basis of the proportion of the two bands resistant to *Hpa*II digestion, the maternally derived chromosome is active in approximately 70% of fibroblasts from LA-31a (track 2H). This correlates well both with the biochemical data (30% residual PDH activity and E1α-immunoreactive protein) and with the observations that 70% of amplified PCR products contained the mutant sequence, and it suggests that the disease in LA-31a arose by a new mutation in her maternal X chromosome.

From the X-chromosome inactivation studies, it is also possible to confirm that the mother does not carry the PDH E1α mutation in her somatic cells. In the maternal fibroblast sample, the two X chromosomes are active in the proportion of approximately 60:40. If either X chromosome carried the mutant PDH E1α gene, the resulting deficiency of PDH activity and E1α-immunoreactive protein would be readily detectable.

It was not possible to exclude germinal mosaicism in the mother, but her two subsequent pregnancies (CV9 and CV16) were tested by chorionic villus biopsy and found to have normal levels of PDH activity (results not shown), and there was no evidence of the 7-bp deletion (fig. 4).

Discussion

Heterogeneity in PDH deficiency has recently been reported by several groups (Robinson et al. 1987; Naito et al. 1988; Wexler et al. 1988). By means of the chemical cleavage method, a mutation was located in the PDH E1α gene in a female patient with the "cerebral" form of PDH deficiency (Brown et al. 1988). By DNA sequencing the mutation was shown to be a 7-bp deletion in the coding region. The normal size of the PDH E1α subunit is 390 amino acids, but as a consequence of the change of reading frame we predict the mutant form of the subunit in this patient to be a truncated protein of 322 amino acids. In addition, the sequence of the mutant subunit differs from normal PDHE1α from amino acid 314 to the C terminal end. Protein blots of mitochondrial extract from patient LA-31a were probed with affinity-purified PDH E1α antibody. The level of normal sized PDH E1α in the cells from the patient is approximately 30% of that in control cells, but no truncated protein band was detected (Brown et al. 1988, and in press). This might suggest that the mutant protein is unable to incorporate into the highly structured PDH complex and, as a consequence, is rapidly degraded. A 4-bp deletion in another patient with PDHE1α deficiency recently has been described (Endo et al. 1989). In this case the mutation results in a larger polypeptide, which can be detected on protein blots.

The severity of X-chromosomal PDH E1α mutations depends on the nature of the mutation, the functional reserve of the enzyme, availability of alternative energy sources in different tissues, and, in heterozygous females, the pattern of X-chromosome inactivation. Under normal circumstances, the brain has both little functional reserve of PDH activity and an absolute requirement for aerobic glucose oxidation. As a result, the threshold for manifestation of cerebral symptoms of PDH E1α deficiency is low. Present information suggests that almost all recognized heterozygous females have significant neurological impairment and that variations in clinical severity are determined in large part by different patterns of X-chromosome inactivation (Brown et al. 1989*a*; G. K. Brown, unpublished data).

In fibroblasts from patient LA-31a we detect approximately 30% PDH E1α activity, a result that correlates well with the protein blot data. This means that, as a consequence of the X-chromosome location of the gene, 30% of the cells (namely, those cells that have inactivated the X chromosome carrying the mutated gene) have a normal level of functional PDH E1α and that 70% of the cells have no or very little PDH activity.

Both the mode of inheritance of PDH E1α deficiency and the severity of the illness in the patient make it unlikely that the mutation is present in the somatic cells of her parents. However, the clinical presentation in heterozygous females is a result not only of the nature of the mutation but also of the X-chromosome inactivation pattern in the affected person. It is therefore possible that the clinical presentation in females with the same mutation could vary quite significantly. In order to investigate whether the 7-bp deletion is present in any of the parental—especially the mother's—X chromosomes, genomic fibroblast DNA from the parents were included in the PCR reactions shown in figure 4. The direct analysis of this region of the PDH E1α genes confirmed that the disease was the result of a new mutation, as only the normal sequence was detected. We were unable to determine whether the mother, from whom the mutant X chromosome was inherited, is a germ-line mosaic.

The mutation removes nucleotides 1032–1038, which are part of exon 10 in the PDH E1α gene (Maragos et al. 1989). The deleted sequence is not near the intron-exon junctions. However, it is interesting that it is part of a tandem 7-bp direct-repeat sequence. It appears that repeat sequences are often involved in DNA rearrangements (Chandler 1989), and it is tempting to speculate that this is a result of either unequal chromosome crossing-over or "slipped mispairing." Such mechanisms could generate an insertion in the recipient X chromosome. A larger band was seen in the PCR product from LA-31a, but it was shown by DNA sequence analysis to be a heteroduplex of the normal and the deleted DNA strands.

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