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Mutations in the X-Linked $E_1\alpha$ Subunit of Pyruvate Dehydrogenase: Exon Skipping, Insertion of Duplicate Sequence, and Missense Mutations Leading to the Deficiency of the Pyruvate Dehydrogenase Complex

Kathy Chun,[1] Nevena MacKay,[1] Roumyana Petrova-Benedict,[1] Antonio Federico,[2] Alberto Fois,[3] David E. C. Cole,[4] Evelyn Robertson,[5] and Brian H. Robinson[1]

[1]Departments of Paediatrics and Biochemistry, University of Toronto, and the Research Institute, Hospital for Sick Children, Toronto; [2]Department of Neurology, University of Siena, and [3]Department of Paediatrics, University of Siena Children's Hospital, Siena, Italy; [4]Departments of Paediatrics and Pathology, Dalhousie University, and Izaak Walton Killam Children's Hospital, Halifax; and [5]Departments of Paediatrics and Genetics, University of Adelaide, Adelaide

Summary

Human pyruvate dehydrogenase (PDH)–complex deficiency is an inborn error of metabolism that is extremely heterogeneous in its presentation and clinical course. In a study of 14 patients (7 females and 7 males), we have found a mutation in the coding region of the $E_1\alpha$ gene in all 14 patients. Two female patients had the same 7-bp deletion at nt 927; another female patient had a 3-bp deletion at nt 931. Another female patient was found to have a deletion of exon 6 in her cDNA. Two other female patients were found to have insertions, one of 13 bp at nt 981 and one of 46 bp at nucleotide 1078. Two male patients were found to have a 4-bp insertion at nucleotide 1163. The remaining six patients all had missense mutations. A male patient and a female patient both had an A1133G mutation. The other missense mutations were C214T, C615A, and C787G (two patients). Five of these mutations are novel mutations, five have been previously reported in other patients, and two were published observations in other patients in an $E_1\alpha$-mutation summary. In the four cases where parent DNA was available, only one mother was found to be a carrier of the same mutation as her child.

Introduction

The pyruvate dehydrogenase (PDH) complex is a large multienzyme assembly that plays a key role in aerobic energy metabolism, as it delivers the product of glycolysis, pyruvate, to the citric acid cycle in the form of acetyl CoA. This mitochondrial assembly is composed of three catalytic enzymes: PDH (E_1), dihydrolipoamide transacetylase (E_2), and dihydrolipoamide dehydrogenase (E_3) (Reed 1974; Randle et al. 1978). E_1, an $\alpha_2\beta_2$ tetramer, is the main site of regulation for the whole complex. This regulation is achieved by phosphorylation/dephosphorylation of the $E_1\alpha$ subunit, with secondary modulation by nicotinamide adenine dinucleotide and coenzyme A availability (Linn et al. 1969). The actual function of the E_1 component is to catalyze a thiamine pyrophosphate (TPP)–dependent decarboxylation of pyruvate, and thus the role of the α and β subunits of E_1 in binding TPP is central to understanding the active-site mechanism of E_1. While the $E_1\alpha$ subunit has been shown to possess a conserved TPP binding motif (Hawkins et al. 1989), the 2.5-Å resolution of the crystal structure of TPP-requiring transketolase (Lindqvist et al. 1992) allowed us to predict that the PDH $E_1\alpha$ subunit was responsible for pyrophosphate binding while the PDH $E_1\beta$ subunit was responsible for binding the thiazolium ring of TPP (Robinson and Chun 1993).

PDH-complex deficiency is one of the most common causes of primary congenital lactic acidosis (Robinson et al. 1980; Robinson 1989*a*). Human PDH deficiency is extremely heterogeneous, in both clinical presentation and clinical course. Symptoms vary from episodic ataxia to severe fatal neonatal lactic acidosis, although structural CNS damage and psychomotor retardation are prevalent in those who suffer between these two extremes (Robinson et al. 1987; Ho et al. 1989; Robinson 1989*a*; Brown 1992). In most cases, the defect can be localized to the E_1 subunit, with a minority of defects being found in the E_2, E_3, or X components (Robinson et al. 1989, 1990).

A number of mutations in the coding region of the $E_1\alpha$ subunit have been reported (Endo et al. 1989; Dahl et al. 1990; Chun et al. 1991, 1993; Wexler et al. 1992; Matthews et al. 1993*b*), and schemes have been proposed to account for the variability and sex distribution of PDH E_1 deficiency, on the basis of both the expression of the X-linked $E_1\alpha$ gene in males and females and the influence of

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Address for correspondence and reprints: Dr. Brian H. Robinson, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

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X inactivation (Brown 1992; Chun et al. 1993). Initially, all these mutations were different; however, as more patients were studied, some mutations were found to be common despite strong evidence that most mutations were germ line in origin (Dahl et al. 1992; Chun et el. 1993). In the present paper we have studied a further 14 patients (7 females and 7 males) with PDH deficiency and have found five novel mutations (2 patients with the same mutation), five mutations that have been previously reported (1 male patient and 1 female patient with the same mutation), and two mutations that were unpublished observations in an $E_1\alpha$-mutation summary (Dahl et al. 1992).

Material and Methods

Cultured skin fibroblasts were grown in α-MEM culture medium from forearm skin biopsies taken with informed consent. The activity of the PDH complex in the native and dichloroacetate-activated state were determined in fibroblast extracts, by the method of Sheu et al. (1981).

mRNA, cDNA, and DNA Preparation

Total RNA was purified from near-confluent cultures of skin fibroblasts of the patient and control cell lines by the method of Maniatis et al. (1982). First-strand cDNA synthesis was carried out using 10 μg of total cellular RNA and an $E_1\alpha$-specific oligonucleotide, αG17′ (5′-TCTAGA-ATTCGTACAAACTGCATGCAATTAC-3′), with M-MLV (Moloney murine leukemia virus) reverse transcriptase (Bethesda Research Labs). The first strand of the $E_1\beta$ cDNA synthesis was carried out using oligo(dT) as the primer. PCR amplification (Saiki et al. 1988) of the cDNAs was carried out as described elsewhere (Chun et al. 1991). In brief, the total coding sequence of the $E_1\alpha$ subunit was amplified in two overlapping fragments, was subcloned into pSP65, and was sequenced using a series of α-specific primers. The β subunit was amplified as a 1.25-kb entity, was subcloned, and then was sequenced with β-specific primers. For both α and β subunits, at least six individual clones were sequenced from three separate PCR reactions, in order to account for both alleles and to discount any errors resulting from *Taq*I polymerase amplification. DNA was prepared from cultured skin fibroblasts of patients, parents, and control cell lines by a modified version of the method of Miller et al. (1988). Genomic DNA sequences of all patients and of the available parents were amplified with the oligonucleotides specified in table 1. After 30 cycles of amplification under the conditions specified, the amplified fragments were visualized on ethidium bromide–stained gels, were extracted, and were subcloned into pSP65. For females, six independent genomic clones were sequenced as stated for the cDNA procedure. All DNA sequencing was performed by the Sanger et al. (1979) dideoxy chain–termination method, on double-stranded templates, using a T7 polymerase sequencing kit (Pharmacia).

Western Blot Analysis

Mitochondrial preparations were made from human cultured skin fibroblasts and lymphoblasts, by the method of Bourgeron et al. (1992). The mitochondrial proteins were resolved on an SDS/10% polyacrylamide gel. This gel was then electroblotted onto nitrocellulose, was blocked with 2% gelatin, and was probed with rabbit anti–bovine heart PDH-complex antibody. Immunoreactive proteins were visualized using goat anti–rabbit IgG and alkaline phosphate staining (Bio-Rad).

X Inactivation Analysis

DNA was prepared as described above, and enzymes were purchased from Boehringer Mannheim. Analysis of the DSX255 locus by using the M27β probe was carried out as described elsewhere (Brown et al. 1990; Brown and Brown 1993). In brief, the *Msp*I and *Hpa*II digestions were carried out overnight and were followed by a second digestion with *Pst*I for 6 h after adjustment of the buffer concentration. Samples were separated by running on a 0.6% agarose gel for 20 h at 10 V. The DNA was transferred to Hybond N^+ nylon membrane (Amersham) and was probed with random-primed ^{32}P-labeled M27β.

Results

Fourteen patients (seven females and seven males) with E_1 deficiency of the PDH complex were investigated at the molecular level. The results of this investigation are summarized in table 2. Mutations found in the $E_1\alpha$ cDNA were all confirmed on genomic DNA by PCR amplification of the corresponding exons, subcloning, and sequencing of the PCR fragments. The sequencing gels of the mutations are found in figure 1. PCR oligonucleotides and the PCR conditions used for amplifying the corresponding exons are summarized in table 1. Sequencing of the $E_1\beta$ cDNA of the patients revealed that the $E_1\beta$ coding region was normal for all the patients (data not shown).

Deletions

All but one of the female patients in this study had either a deletion or an insertion in their mutant cDNA. Patients 4249 and 4202, who died at ages 18 mo and 11 mo, respectively, both had a 7-bp deletion at nucleotide 927. This deletion causes a shift in the reading frame, creating a termination codon at nt 974, thereby resulting in a shorter $E_1\alpha$ protein. This deletion of the first AGTAAGA in the tandem repeat in exon 10 is a mutation that already has been twice reported (Dahl et al. 1990; Chun et al. 1993). Patient 3530, who has psychomotor retardation and microcephaly, also has a deletion in the same region as do patients 4249 and 4202, except that it is a deletion of only 3 bp at nt 931. It is within the segment removed in the 7-bp deletion. As with the 7-bp deletion, the deletion of AAG is part of an AAGAAG tandem repeat.

Table 1

Oligonucleotides Used for the Amplification of PDH $E_1\alpha$ Exons from Genomic DNA

Exon(s) and Oligonucleotide	Sequence[a]	Patient(s)	PCR Conditions
2 and 3:			
Forward	5′-tttgaattcGTCTTTTAAGGCAAGCAGAG-3′	3855	94°C, 1 min
Reverse	5′-ttttctagaTACCACTCACCTGACCATCA-3′		56°C, 1 min
7 and 8:			
Forward	5′-ACTTCtAGaGCCAGATATTCGAAGC-3′	3929	94°C, 1 min
Reverse	5′-AGaaTTcCCTTcccAGATCTAC-3′		49°C, 1 min
8 and 9:			
Forward	5′-tttgaattcATTACCAGGTGGATGGAATGG-3′	2438, 4320	94°C, 1 min
Reverse	5′-ttttctagaGAGCGTACCTGACTCCAGGG-3′		62°C, 1 min
10:			
Forward	5′-tttgaattcCCCCATAGTTACCGTACACG-3′	4249, 4202	94°C, 1 min
Reverse	5′-tattctagaGACTGTACCTTTAGTTCTTCC-3′	3530, 2944	58°C, 1 min
11:			
Forward	5′-tttagGAAtTcGATGTGGAAGT-3′	4224, 2975	94°C, 1 min
Reverse (αG17′)	5′-tctagaattcGTACAAACTGCATGCAATTAC-3′	2653, 2272	48°C, 1 min

[a] Lowercase denotes a linker or substitution; uppercase denotes intronic sequence; and underlining denotes exonic sequence.

Exon Skipping

At the level of transcription, both the normal and mutant transcripts of $E_1\alpha$ for the female patients were detectable by PCR, with the notable exception of patient 2641. Patient 2641, who died of severe neonatal lactic acidosis within the first 2 wk of life, had a deletion of exon 6 in all the cDNAs examined; no normal cDNA could be detected by PCR, despite numerous attempts. Amplification of exon 6 of her genomic DNA revealed two alleles: one normal allele and one with a G-to-A change at nt 523. Western blot analysis revealed that there were no detectable amounts of $E_1\alpha$ and $E_1\beta$ protein (Robinson et al. 1989). Northern blotting of total cellular RNA showed a normal level of an $E_1\alpha$ transcript slightly smaller than the control (Robinson et al. 1989); there was no detectable normal sized transcript.

Insertions

Two of the remaining three female patients each had a different insertion in their mutant cDNA. Patient 2944, who was psychomotor retarded and died at the age of 4 years, had a 13-bp insertion at nt 981. This is a duplication of the 13 bp immediately before, resulting in a tandem repeat. As the insertion is 13 bp, there is a frameshift in the reading frame, and a premature termination codon at nt 1014 results in a shorter protein (−47 amino acids). Western blotting does not detect the shorter $E_1\alpha$ protein, and there are slightly decreased levels of $E_1\alpha$ and $E_1\beta$ protein as compared with that in the control (data not shown). Patient 4224, who died at age 15 mo of Leigh disease, had a 46-bp insertion at nt 1078. As with patient 2944, this insertion is a duplication of the 46 nt immediately previous to nt 1078, creating a tandem repeat. Electrophoresis of the PCR products of exon 10 amplification of the genomic DNA revealed a double band: one normal allele of 143 bp (including the PCR primers) and a mutant allele of 189 bp (fig. 2). Again, this is a frameshift mutation and results in a premature stop codon at the seventh nucleotide of the repeat. The mutant protein is 28 amino acids shorter than the normal protein. Western blot analysis reveals a distinct shorter $E_1\alpha$ protein directly above the $E_1\beta$ (fig. 3). In fact, there seems to be more mutant $E_1\alpha$ protein than normal $E_1\alpha$ protein.

In two fairly mildly affected male patients (patients 2975 and 2653), we found the same mutation in the $E_1\alpha$ gene: an AAGT duplication at nt 1163, resulting in the formation of a tandem repeat. This insertion creates a premature stop codon such that the mutant protein is shortened by two amino acids. Western blot analysis of patient 2653 (Robinson et al. 1989) showed barely detectable levels of the $E_1\alpha$ and $E_1\beta$ proteins in cultured skin fibroblasts. The mutant $E_1\alpha$ band is just slightly lower than that of the control, which is expected.

Missense Mutations

The remaining five males and one female studied in this investigation had missense mutations. The most severely affected male patient (patient 2272) died of Leigh disease and lactic acidosis at age 9 mo. Nucleotide 1133 was changed from an A to a G, resulting in an arginine-to-histidine substitution at amino acid 349 in exon 11. Patient 5570, who has the same mutation as does patient 2272, is a developmentally delayed 2-year-old girl diagnosed with Leigh disease and lactic acidosis. This same mutation was first reported, also in a male, by Hansen et al. (1991).

Table 2

Correlation of Clinical Presentation, PDH-Complex Activity, and $E_1\alpha$ Mutations

Patient	Clinical Presentation	PDH-Complex Activity[a]	Western Blot[b]	$E_1\alpha$ Mutation	Northern Blot[c]
Female:					
2641	Died at age 12 d; severe neonatal lactic acidosis	3.5%	−	G523A in exon 6; deletion of exon 6 in cDNA	Smaller transcript
4249	Alive at age 3 years: severe hydrocephaly	7.5%	+	7-bp deletion at bp 927 in exon 10; AGTAAGA tandem repeat	ND
4202	Died at age 11 mo: enlarged ventricles, Leigh disease	8.3%	+	7-bp deletion at bp 927 in exon 10, 974 stop; AGTAAGA tandem repeat	ND
3530	Alive at age 7 years; psychomotor retardation, microcephaly	10.8%	+	3-bp deletion at bp 931 in exon 10; removes R282	ND
5570	Alive at age 2 years: Leigh disease, developmental delay	44.7%	+	G1133A in exon 11; R349H	ND
2944	Died at age 4 years: psychomotor retardation, microcephaly, agenesis of the corpus callosum	61.2% (skin fibroblasts), 19.5% (liver), 84.4% (skeletal muscle), 24.6% (brain)	+	13-bp repeat at bp 981 in exon 10; premature termination	ND
4224	Died at age 15 mo; Leigh disease, dilated ventricles, cortical thinning	100%	Normal band, lower-molecular-weight band	46-bp repeat at 1078 in exon 11; premature termination	ND
Male:					
2975	Alive at age 5 years: ataxia, mental retardation	8.5%	+, lower-molecular-weight band	AAGT duplication at bp 1163 in exon 11; premature termination	N
2653	Alive at age 7 years: ataxia, mental retardation	13.7%	+, lower-molecular-weight band	AAGT duplication at bp 1163 in exon 11; premature termination	N
2438	Alive at age 7 years: ataxia, mental retardation	16.0%	+	C787G in exon 8, R234G	N
3855	Alive at age 8 years: ataxia, mental retardation	19.8%	ND	C214T in exon 3, R43C	ND
2272	Died at age 9 mo: Leigh disease, lactic acidosis	22.4%	+	G1133A in exon 11, R349H	N
3929	Alive at age 3 years: Leigh disease, psychomotor retardation	35.5%	ND	C615A in exon 7, F176L	ND
4320	Alive at age 8 years: ataxia, mental retardation, comatose episodes	48.5%	+	C787G in exon 8, R234G	ND

[a] Expressed as a percentage of the activity in controls, when both are fully activated by dichloroacetate (Sheu et al 1981).

[b] A plus sign (+) denotes presence of a normal appearing $E_1\alpha$ band; and a minus sign (−) denotes absence of an $E_1\alpha$ band. ND = not done.

[c] ND = not done; and N = normal appearance of $E_1\alpha$ band.

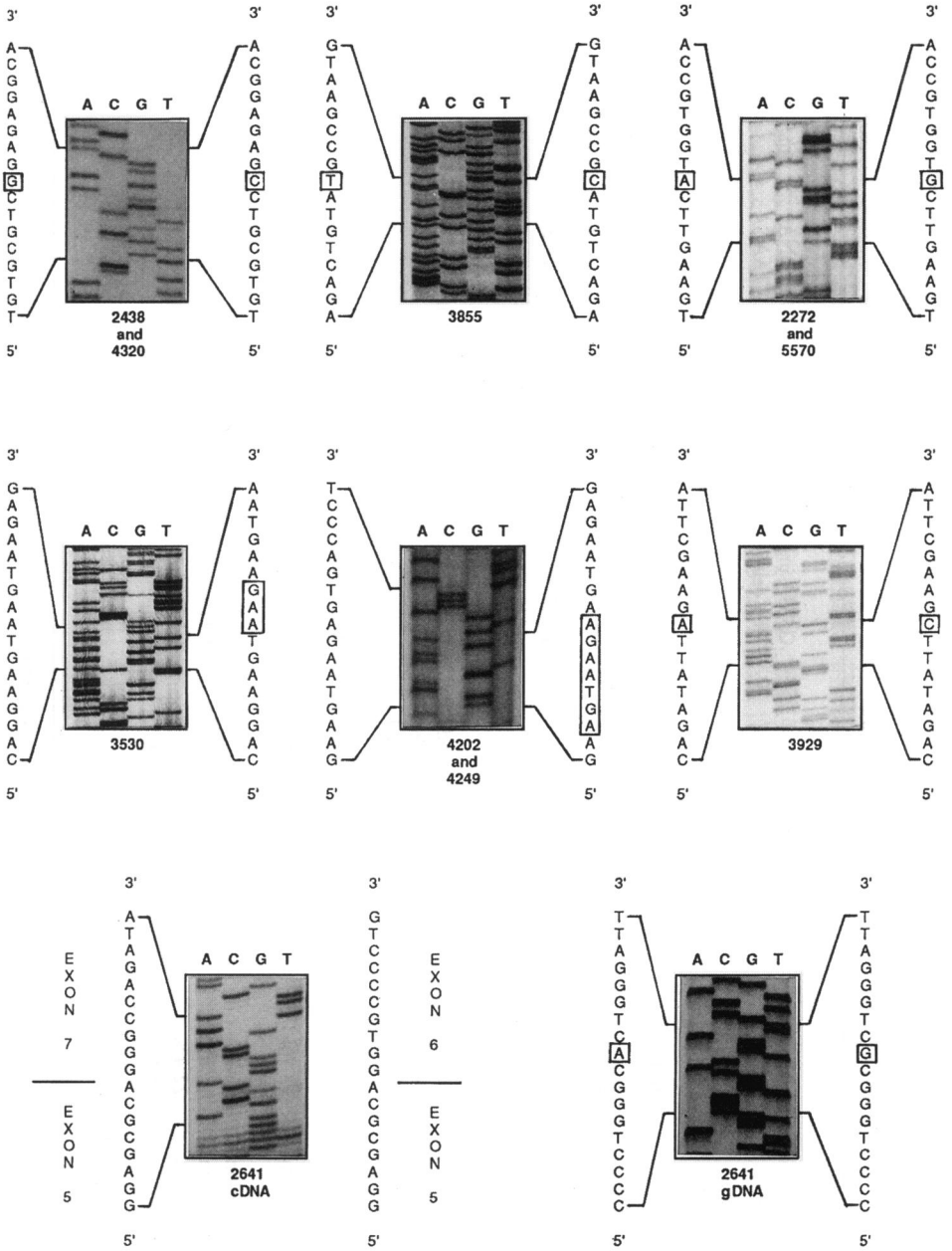

Figure 1 Sequence abnormalities in the $E_1\alpha$ cDNA, detected by dideoxynucleotide sequencing in 10 patients (*above*) and in 3 patients (*opposite page*). The sequencing ladders showing the mutations are shown for 13 of the 14 patients in this study. At the left of each ladder is the sequence obtained from patient cDNA, and on the right is the sequence obtained from control cDNA. All mutant sequences were subsequently confirmed by amplification and sequencing of genomic DNA. The patient-cell-line number appears under each sequencing ladder.

Patient 3855 is an 8-year-old male who is fairly mildly affected with ataxia and mental retardation. In exon 3 of his $E_1\alpha$ gene, a C-to-T transition was found at nt 214. Another patient with this arginine-to-cysteine substitution at amino acid 72 was reported in a PDH-mutation update (Dahl et al. 1992). A more severely affected male patient (patient 3929) is a 3-year-old with Leigh disease and psychomotor retardation. His $E_1\alpha$ mutation was found to be a C-to-A transition at nt 615. This phenylalanine-to-leucine substitution at amino acid 176 in exon 7 has also been reported previously in a male who died at age 18 mo, with evidence of Leigh disease at autopsy (Dahl and Brown 1994).

Patient 2438 (9 years old) and patient 4320 (9 years old) are two males with ataxia and mental retardation. Patient 4320 also suffers from comatose episodes. Our investigation revealed that both patients 2438 and 4320 have a C-to-G change at nt 787. This arginine-to-glycine substitution

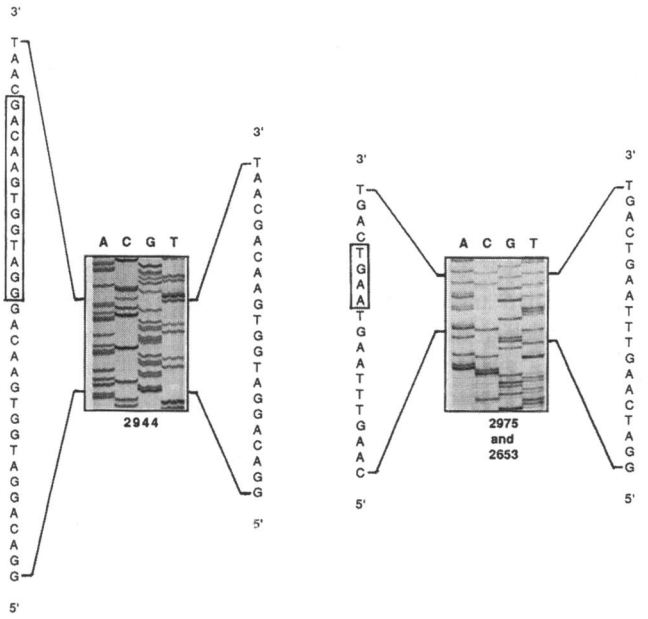

Figure 1 (continued)

at amino acid 234 in exon 8 has been reported elsewhere (Kerr et al. 1988; Wexler et al. 1992; Chun et al. 1993). Wexler et al. (1992) found this C787G mutation in two brothers with tissue-variable expression of PDH; the affected C787G patient described by Chun et al. (1993) was also male. Hence, this mutation has, to date, been shown to affect five males, two of whom were brothers.

X Inactivation Analysis

The relative activities of the two X chromosomes in the fibroblasts of patients 2641 and 4224 were analyzed from the methylation status at locus DXS255. Patient 2641 was heterozygous for the VNTR polymorphism at this locus (fig. 4). One allele (7.1 kb) is almost completely active, while the other (5.7 kb) remains almost completely inactive. This is in agreement with the very low enzyme activity, northern blot, and cDNA analysis, where only the mutant mRNA and cDNA could be detected. Patient 4224 was homozygous, at the DXS255 locus, for *Pst*I, *Hin*dIII, and *Bam*HI, and thus the relative activity of the two X chromosomes could not be determined.

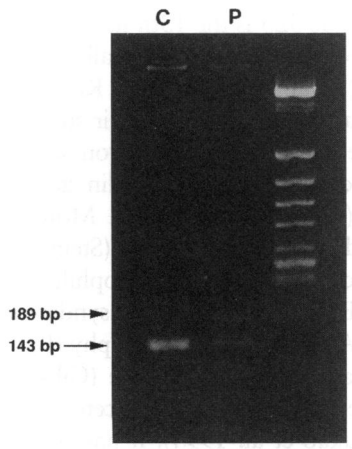

Figure 2 Analysis for the presence of a 46-bp insertion in exon 10 of patient 4224. The PCR reaction was performed using oligonucleotide primers E10F and E10R and subsequently was run on a 2% agarose gel. The sizes of the PCR products of the normal gene (143 bp) and the mutant gene (189 bp) are shown on the left. Lane C, Control DNA. Lane P, Patient DNA. A DNA size marker is shown in the third, rightmost lane (unmarked).

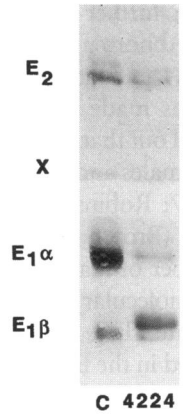

Figure 3 Western blotting of cultured skin fibroblast mitochondrial extracts from patient 4224, with anti–PDH-complex antibody. Details of this method can be found in the Material and Methods section. The left-hand lane is the control cell line; and the right-hand lane is the cell line from patient 4224, who has a second $E_1\alpha$ band directly above the $E_1\beta$ band.

Discussion

Elucidation of the sequence of PDH $E_1\alpha$ and $E_1\beta$ cDNAs in PDH-deficient patients has led to an increased understanding of the disease. Here we have examined the $E_1\alpha$

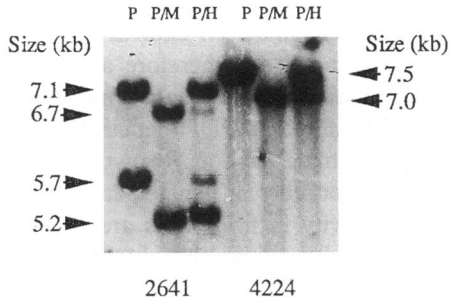

Figure 4 X inactivation analysis of patients 2641 and 4224 at the DXS255 locus. The DXS255 locus of patients 2641 and 4224 was probed using the M27β probe (Fraser et al. 1989; Boyd and Fraser 1990). Patient 2641 is heterozygous at this locus, and one allele remains preferentially active (7.1 kb) while the other remains preferentially inactive (5.7 kb). Patient 4224 is homozygous at this locus, and therefore it is not possible to determine the extent of X inactivation at this locus. P = *Pst*I; M = *Msp*I; and H = *Hpa*II.

and $E_1\beta$ cDNAs of a further 14 E_1-deficient patients, 7 females and 7 males. Abnormalities were found only in the $E_1\alpha$ sequence of all 14 patients; subsequent confirmation on genomic DNA was made for all the individuals. We previously had pointed out that in our studies we had found an equal number of females and males with PDH deficiency (Robinson et al. 1987; Robinson 1989*a*). Since $E_1\alpha$ was shown to be X linked (Brown et al. 1989; Robinson et al. 1989), a greater number of male patients might have been expected. However, molecular evidence confirms our earlier observation: to date, there are 26 female and 23 male $E_1\alpha$ mutations reported in the literature, including the present paper (Endo et al. 1989, 1991; Dahl et al. 1990; Chun et al. 1991, 1993; De Meirleir et al. 1991; Hansen et al. 1991, 1993, 1994; Ito et al. 1992; Wexler et al. 1992; Matthews et al. 1993*a*, 1993*b*, 1994; Takakubo et al. 1993*a*, 1993*b*; Awata et al. 1994; Dahl and Brown 1994). The female patients carry one normal X chromosome and one X chromosome with a mutation on the $E_1\alpha$ gene. Nonrandom inactivation of the normal X chromosome results in an affected female (Brown et al. 1989; Dahl et al. 1990). On the basis of their low residual PDH activity (table 2), five of the seven female patients seem to be predominantly expressing the abnormal gene in their cultured fibroblasts. A special case in point is patient 2641, in whom only the mutant exon 6–deleted transcript could be detected, in both northern analysis and PCR analysis. Rare cases do exist in which the normal gene is predominantly expressed in fibroblasts, and patient 4224 is a prime example. This is almost certainly not the case in the brain of the patient where the thinned cortex and expanded ventricles suggest that cells adversely lyonized have died while those expressing the normal $E_1\alpha$ allele survive. Since males have only one X chromosome and hence only one copy of $E_1\alpha$, $E_1\alpha$ mutations with little or no resultant enzyme activity may cause the fetus to die in utero and their deficiency to not come to light. Thus, PDH $E_1\alpha$ deficiency could be classified as both an X-linked dominant and X-linked lethal disease, depending on the mutation in question. Female fetuses, on the other hand, can express their normal $E_1\alpha$ to a certain extent and can therefore at least survive to term. This is probably why there are approximately equal numbers of female and male PDH-deficient patients, rather than an excess of male patients. Brown (1992) has proposed that a large number of female patients with normal PDH-complex activity in fibroblasts are in fact $E_1\alpha$ mutants, on the basis of the similarity of symptoms to known cases of $E_1\alpha$ deficiency in females.

Quite a novel PDH $E_1\alpha$ mutation can be found in patient 2641. Only one transcript could be detected by PCR of reverse-transcribed mRNA, a transcript that lacked precisely exon 6. The normal allele seemed not to be transcribed, as no transcript could be detected in either northern blot or PCR analysis. It would seem that in skin fibroblasts patient 2641 is completely adversely lyonized. This was nicely demonstrated when analysis of the X inactivation pattern in patient 2641 was carried out, at the DXS255 locus, with the M27β probe (Fraser et al. 1989; Boyd and Fraser 1990) (fig. 4). A VNTR flanked by *Msp*I sites and its isoschizomer *Hpa*II allows the two X chromosomes to be distinguished in >90% of the females (Boyd and Fraser 1990). These restriction sites are methylated on active X chromosomes and therefore are resistant to *Hpa*II digestion, but they are unmethylated on inactive X chromosomes (Boyd and Fraser 1990). In females, the proportion of each allele at this locus that is resistant to *Hpa*II digestion provides a direct measure of the X inactivation pattern.

The *Pst*I digestion results in two bands: one of 7.1 kb and one of 5.7 kb. Parents' DNA was not available to determine which allele was from which parent. Further digestion with *Hpa*II revealed that one allele (7.1 kb) remained preferentially active and that the other allele (5.7 kb) remained preferentially inactive. This correlated well with the very low enzyme activity, northern blot, and cDNA analysis, where only the mutant mRNA and cDNA could be detected. Western blot analysis (Robinson et al. 1989) revealed no detectable $E_1\alpha$ or $E_1\beta$ protein.

Mutations in splice donor and acceptor sites are anticipated to affect splicing of mRNA molecules. However, results of PCR and subsequent sequencing of intron/exon boundaries on either side of exon 6 were completely normal. PCR and sequencing of introns 5 and 6 were also found to be normal. PCR and sequencing of exon 6 in the genomic DNA revealed a G523A mutation in one of the $E_1\alpha$ alleles; the other allele was completely normal. G523A could be hypothesized to be a benign polymorphism. However, we and others have never seen this in the normal population, and the absence of any transcripts of mRNA carrying the G523A sequence strongly suggests that it is responsible in some way for the transcript with the missing exon. Nucleotide 523 is the 13th nucleotide in from the 5′ end of exon 6, a position not normally associated with the acceptor site (Senapathy et al. 1990; Krawczak et al. 1992).

The association of single-base-pair substitutions, not involving splice consensuses, with exon skipping was originally reported for the thyroglobulin gene in Afrikander cattle goiter (Ricketts et al. 1987). More recently, it has been detected in the HPRT gene (Steingrimsdottir et al. 1992), the factor VIII gene in hemophilia A (Naylor et al. 1993), the FBN1 gene in Marfan syndrome (Dietz et al. 1993), the OAT gene in gyrate atrophy (Dietz et al. 1993), the Fanconi anemia group C gene (Gibson et al. 1993), and the T2 gene in mitochondrial acetoacetyl-CoA thiolase deficiency (Fukao et al. 1994). It has been proposed that point mutations in an exon could lead to disruption of secondary structure and to aberrant mRNA splicing (Steingrimsdottir et al. 1992). We used the MULFOLD computer program (Jaeger et al. 1989*a*, 1989*b*; Zuker 1989), which predicts RNA secondary structure by free-energy minimization, to analyze exon 6. Figure 5 shows that the predicted

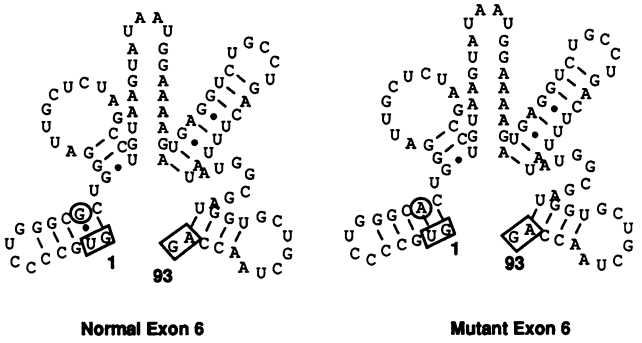

Figure 5 Predicted secondary structure of the normal and mutant exon 6 of patient 2641. The secondary structures of normal and mutant exon 6 were predicted using the computer program MULFOLD (Jaeger et al. 1989*a*, 1989*b*; Zuker 1989). Boxed sequences are the dinucleotides of the 5′ and 3′ consensus splice sites. Circled nucleotides are the 13th nucleotides of normal and mutant exon 6.

mRNA structure of exon 6 is not altered by the G523A mutation. In fact, the structure is more stabilized by the change, as the bond that the 13th nucleotide makes with the conserved U of the GU consensus sequence changes from a G-U to an A-U bond, thus making exon 6 less susceptible to normal exon splicing.

To show that these exonic mutations not involving splice consensuses cause exon skipping, in vivo splicing experiments should be carried out. Fukao et al. (1994) did this for the Q272STOP mutation in the T2 gene. They found that this mutation at −13 from the 5′ splice site caused a partial (25%) skipping of exon 8. They proposed that the alteration of the pre-mRNA secondary structure of exon 8 by the Q272STOP mutation reduced access of the splicing machinery to the 5′ splice site of intron 8 and subsequently caused exon 8 skipping in some transcripts (Fukao et al. 1994). We believe this to be the case also with patient 2641, except that the secondary structure is more stabilized rather than altered. However, in patient 2641 the G523A mutation causes a complete skipping of exon 6, as seen from repeated reverse transcription–PCR of her mRNA and by northern blot analysis (Robinson et al. 1989). It is interesting to note that the mutations in the HPRT gene, the OAT gene, the T2 gene, and the PDH $E_1\alpha$ gene all occur at the 13th nucleotide in from the 5′ end of the exon that is spliced out. One of the OAT mutations is also a G-to-A transition in exon 6, just like this $E_1\alpha$ mutation. The role of the 13th nucleotide of the exon in splicing remains to be further investigated.

Two different deletions, AGTAAGA (patients 4249 and 4202) and AAG (patient 3530), were found, and they both occurred at sites of tandem repeats. Krawczak and Cooper (1991) found that 59 of 60 deletions occurred either within or in the vicinity of tandem repeats. The three different insertions—one of 4 bp (patients 2975 and 2653), one of 13 bp (patient 2944), and one of 46 bp (patient 4224)—all created tandem repeats. Our results support the finding that deletions and insertions are almost always associated with tandem repeats. The fact that de novo mutations of this type produce repeat sequence rather than random sequence strongly suggests the involvement of DNA polymerases in the creation of the mutation.

Western blotting of patient 4224's fibroblast protein showed that the 46-bp insertion produced an aberrant $E_1\alpha$ protein of the expected size. Figure 3 shows that the mutant protein of patient 4224 appears to be in greater abundance than the normal protein, which would suggest higher transcription of the mutant mRNA and therefore a lower residual PDH activity. It is also possible that the mutant mRNA may be more stable or more efficiently translated. Alternatively, the mutant protein may be more stable than the normal protein. However, this patient's PDH-complex activity is quite normal in her fibroblasts, even though the disease was severe enough to cause death at age 15 mo. Although the average activity over eight determinations of PDH complex in her fibroblasts was normal, early-passage cells were in fact deficient. The western blot was done with early-passage fibroblasts deliberately to show the presence of the mutant protein. The activity subsequently moved into the normal range after seven to eight passages. Thus some selection of nonadversely lyonized cells does occur in fibroblast culture over a series of generations. Western blotting of the fibroblast proteins of patients 2944, 4249, 4202, and 3530 with PDH-complex antibody showed the same pattern as that in the control (data not shown).

Analysis of the X inactivation pattern in patient 4224 was also carried out at the DXS255 locus (fig. 4). *Pst*I digestion revealed a single band of 7.5 kb, indicating that patient 4224 is homozygous at this locus; the parents' DNA was not available for testing. *Pst*I/*Hpa*II digestion resulted in two bands: 7.5 kb, the active X chromosome; and 7.0 kb, the inactive X chromosome. Unfortunately, because the parents' alleles are identical at this locus, it is impossible to determine the extent of X inactivation with this locus. *Hin*dIII digestion and *Bam*HI digestion at this locus were also not informative (data not shown). Analysis of the PGK (phosphoglycerate kinase) locus (Vogelstein et al. 1987) may provide more information.

Comparison of the clinical presentation of the four known female patients with a 7-bp deletion at bp 927 reveals certain similarities, although each case had different activities of the PDH complex in cultured skin fibroblasts. Hydrocephaly or ventriculomegaly was evident in all four cases, but two had evidence of basal ganglia malformation or necrotization, while two showed cortical atrophy. Two cases also showed agenesis of the corpus callosum. These four cases did not show a good correlation between residual fibroblast activity and severity of symptoms in general. This type of deletion seems not to have any specific bias of lyonization, and, presumably, X inactivation takes place purely on a random basis.

Patients 2975 and 2653 both have the AAGT duplica-

tion at nt 1163 and very low residual PDH activity. Previous western blot analysis of patient 2653 (Robinson et al. 1989) showed barely detectable levels of $E_1\alpha$ and $E_1\beta$ proteins in cultured skin fibroblasts. The mutant $E_1\alpha$ band is just slightly lower than that of the control, which is expected for this frameshift mutation. Clinically, they are also quite similar: they are slightly mentally retarded and suffer from intermittent bouts of ataxia, which are precipitated by increased carbohydrate intake. Both are affected mildly enough that they can attend regular school classes. Interestingly, they are both from the Maritime Provinces in Canada, though unrelated: patient 2975 is from Newfoundland, and patient 2653 is from Nova Scotia. The genomic DNA from the mother of patient 2975 was available for testing. PCR amplification of exon 11, with subsequent subcloning and sequencing, revealed no mutation in either of her $E_1\alpha$ alleles. Therefore, this mutation is likely to be the result of a germ-line mutation. The siting of a mutation close to the 3′ end of the coding sequence is common in males with ataxia syndrome (Dahl et al. 1992).

Patients 2272 and 5570 are the second and third to be reported with the G1133A mutation in exon 11. The patient of Hansen et al. (1991) was extremely similar to patient 2272 described here. Their patient was also a male with 30% PDH activity in his fibroblasts. Clinically, he suffered from Leigh disease and severe lactic acidosis, as patient 2272 did, and died at age 13 mo (patient 2272 died at age 9 mo). Patient 5570 is a 2-year-old female who is not as severely affected clinically as are the two males with the same mutation, presumably because of favorable X inactivation patterns in her tissues. However, she also has been diagnosed with Leigh disease and is developmentally delayed. (A report with a detailed clinical comparison of patients 2272 and 5570 is in preparation.) It is important to note that this is the first case of the same PDH mutation to be found in both a female and a male (who are unrelated). All previous repeated mutations were found to occur in the same sex. Genomic DNAs from both of her parents were tested and proved negative for the G1133A mutation.

The R49C transition now seen in two males with ataxia and mental retardation is unusual in that it occurs in the early part of the $E_1\alpha$ coding sequence. The arginine is not conserved in branched-chain keto acid dehydrogenase $E_1\alpha$ coding sequences and is therefore probably not essential to the mechanism. The 19.8% residual activity in fibroblasts that we report is close to the 30% residual activity reported for L. De Meirleir's laboratory, by Dahl et al. (1992). Though the symptoms are similar, these activities are considerably higher than those reported for the two males with the AAGT duplication at 1163. It may be reasonable to conclude that fibroblast activities in missense mutations in males do not necessarily represent the activity that would be found in the brain. The F176L change in a male with 35% residual activity resulted in a fatal case of Leigh disease at age 9 mo, clearly with more severe effects on the brain but with greater fibroblast residual activity than the 8.5% and 13.7% residual activities shown by the AAGT-duplication patients with ataxia. Different protein-degradation rates therefore may be operative in different tissues, for the same mutation (Robinson 1989*b*).

There have now been five patients—two of whom two were brothers (Wexler et al. 1992)— documented with the C787G mutation (Kerr et al. 1988; Wexler et al. 1992; present study). Three of the four mothers were found to be carriers of the same C787G mutation; the mother of patient 2438 was negative for the mutation. Of the carrier mothers, the mother of patient 4320 was found to be mentally retarded. The carrier mother of patient 2985 (Chun et al. 1993), one of the males with the C787G mutation who previously had been reported by us, is also affected, having suffered from nonfluctuating cerebellar ataxia throughout her life. All carrier mothers showed mildly reduced PDH activity (35%–46% of control values). Lymphocyte and lymphoblast PDH activities were reduced in the four patients for whom they were available. However, there seems to be variable fibroblast PDH activity. The three boys studied in our laboratory all have very similar fibroblast activities—51.5%–55.9% of control values. However, the two siblings reported by Wexler et al. (1992) have very high fibroblast PDH activities—notably patient SH, whose activity was found to be 168.8%. This value seems unusually high. There is difficulty in measuring PDH-complex activity in cultured fibroblasts and lymphoblasts, and we have found that three to five separate measurements give a reliable estimate. Wexler et al. (1992) do not specify how many times the PDH assay was repeated.

Our three C787G-mutation patients presented with ataxia, mental retardation, and some pathology of the basal ganglia, while the two siblings (Wexler et al. 1992) both exhibited persistent weakness, ataxic episodes, hypotonia, and lactic acidemia. One of these two siblings died of respiratory insufficiency at age 2 years, with hemorrhagic infarction of the basal ganglia, while the other sibling remains alive with mental retardation and ataxia at age 9 years. Thus, at least four of the patients with the mutation have followed a similar clinical course, all surviving to age 9 years. The puzzling biochemical findings are the variable activities in fibroblasts and lymphoblasts in patients with the same mutation. As mentioned above, difficulty in measuring PDH in tissues could be a major factor in the variability. Also, one can question whether the C787G mutation really does result in the symptoms seen in these children. Preliminary results of a transfection study in our laboratory indicate that the C787G mutation causes a decrease in PDH activity in normal transformed lymphoblasts. Thus, it is likely that the C787G mutation is responsible for the symptoms in the affected males. The clinical histories of four of the patients are very similar, although the fifth one is somewhat atypical. Perhaps the atypical patient had some further complications. We know

that expression of PDH E_1 defects is extremely variable in tissues. For instance, patient 2272, a male with the G1133A, R349H mutation had 22.4% fully activated PDH complex in fibroblasts but had 18.8%, 13.2%, 5.2%, 4.5%, and 0.8% in skeletal muscle, heart, brain, liver, and kidney, respectively. This can be explained only by differential rates of degradation of a mutant $E_1\alpha$ with partial activity. Notwithstanding, it is still difficult to explain how the C787G mutation brings about the differential effects that it does, in spite of the compelling epidemiological evidence, from five cases, that it is responsible for the symptoms in the affected males.

The genomic DNAs of the parents of patients 5570, 4320, and 2438, as well as the genomic DNA of the mother of patient 4202, were available for testing. PCR amplification, subcloning, and sequencing of the exons containing the mutations were performed on the parents' DNA. Several clones of each were examined, and in only one case was the mutation present in a parent. Only the mother of patient 4320, who was found to be mentally retarded with reduced PDH activity, was heterozygous for the C787G mutation that patient 4320 was found to carry. This is the second case in our laboratory in which we have found a mildly affected mother. Only one other case exists in the literature, and she is the mother of a severely affected daughter; both have the same C904T mutation (Dahl et al. 1992). Nonetheless, most cases of PDH $E_1\alpha$ deficiency seem to be caused by new mutations.

To date, we have reported 18 different mutations in the $E_1\alpha$ gene of 25 PDH-deficient patients. The majority of the mutations cluster at the 3′ end of the gene, mainly in exons 10 and 11, which seem either to be a hotspot for mutations or to produce mutations more compatible with life. Other mutations are scattered throughout the gene, affecting functional portions of the gene, such as the TPP-binding site (exons 6 and 7), the third phosphorylation site (exon 7), and the first phosphorylation site (exon 9). As much as these mutations vary, the clinical presentations and biochemical analyses also greatly vary. The difficulty in analyzing PDH deficiency is in trying to correlate these three aspects. Variation in clinical presentation is due to several factors (Dahl et al. 1992), such as the inactivation pattern of the X chromosome in females, the proportion of cells expressing the normal gene in females, tissue-variable expression of the mutant $E_1\alpha$ protein, the brain's dependency on PDH $E_1\alpha$ activity, the energy requirement of neurological structures during fetal development, and the severity of the mutation. Biochemical analysis is complicated by difficulty in accurately measuring PDH-complex activity in cultured fibroblasts and lymphoblasts, although repeated measurements in most cases give a reliable estimate (Robinson 1989*a*).

Despite all these difficulties, we have made advances in understanding PDH deficiency since the first cloning of the $E_1\alpha$ cDNA (Dahl et al. 1987; De Meirleir et al. 1988) and its gene (Maragos et al. 1989). However, there is still much to learn about this disease. Expression studies must still be carried out to show that these $E_1\alpha$ mutations are indeed the cause of the defective PDH-complex activity and, thus, of the disease phenotype.

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