

Splicing Error in *E1 α* Pyruvate Dehydrogenase mRNA Caused by Novel Intronic Mutation Responsible for Lactic Acidosis and Mental Retardation*

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An intronic point mutation was identified in the *E1 α PDH* gene from a boy with delayed development and lactic acidosis, an X-linked disorder associated with a partial defect in pyruvate dehydrogenase (PDH) activity. Protein analysis demonstrated a corresponding decrease in immunoreactivity of the α and β subunits of the PDH complex. In addition to the normal spliced mRNA product of the *E1 α PDH* gene, patient samples contained significant levels of an aberrantly spliced mRNA with the first 45 nucleotides of intron 7 inserted in-frame between exons 7 and 8. The genomic DNA analysis found no mutation in the coding regions but revealed a hemizygous intronic G to A substitution 26 nucleotides downstream from the normal exon 7 5'-splice site. Splicing experiments in COS-7 cells demonstrated that this point mutation at intron 7 position 26 is responsible for the aberrant splicing phenotype, which involves a switch from the use of the normal 5'-splice site (intron 7 position 1) to the cryptic 5'-splice site downstream of the mutation (intron 7 position 45). The intronic mutation is unusual in that it generates a consensus binding motif for the splicing factor, SC35, which normally binds to exonic enhancer elements resulting in increased exon inclusion. Thus, the aberrant splicing phenotype is most likely explained by the generation of a *de novo* splicing enhancer motif, which activates the downstream cryptic 5'-splice site. The mutation documented here is a novel case of intron retention responsible for a human genetic disease.

Accurate pre-mRNA splicing is vital for the conversion of nascent transcripts into mRNA templates for protein synthesis. In humans, this involves the removal of 7–8 introns on average from each nascent transcript (1). Accurate splicing depends upon the recognition of the 5'-splice site, branch site, and polypyrimidine tract/3'-splice site at the boundaries of each intron. However, the splice sites, which are short and highly degenerate, do not contain sufficient sequence information for their accurate recognition (2). Consequently, the se-

quences outside of but in the vicinity of the splice sites play highly important roles in modulating the strengths of the natural splice sites. Exonic and intronic splicing enhancers (ESEs¹ and ISEs, respectively) exert positive roles, whereas exonic and intronic splicing silencers exert negative roles. In constitutive splicing, these sequence elements favor not only the recognition of the authentic splice sites but disfavor the recognition of cryptic splice sites. Splicing control elements are also highly important in specifying alternative splicing events. Alternative splicing generates remarkable proteomic diversity in multicellular organisms and can give rise to tissue-specific or developmentally controlled production of protein isoforms from a single gene.

The recognition of splicing control elements is normally accomplished by two types of protein families, the heterogeneous nuclear ribonucleoprotein, which are frequently involved in negative control, or by the SR splicing factors, which are involved in splicing enhancement through the use of ESEs. SR splicing factors are RNA-binding proteins containing a carboxyl terminal effector domain that is rich in serine arginine dipeptides. SR splicing factors are thought to promote splice site recognition by engaging in protein-protein interactions with subunits of the essential splicing factors, U2AF and U1 small nuclear ribonucleoprotein.

Pyruvate dehydrogenase (PDH) defects are major causes of lactic acidosis and Leigh's encephalomyelopathies in infancy and childhood (3, 4). The PDH complex is responsible for the conversion of pyruvate to acetyl-CoA. The majority of the molecular defects has been localized in the coding regions of *E1 α PDH* gene at chromosome Xp22.1 (gene symbol *PDHA1*, MIM catalog 312170) (5). Also, some rare cases are because of mutations in another gene, the *Hs-PDX1* coding for the E3-binding protein of the PDH complex, and most of them are splicing site mutations (6). Recently, two exonic mutations leading to aberrant splicing in the *E1 α PDH* gene and the excision of exon 6 have been described previously (7). To date, there is no evidence for developmentally regulated alternative splicing events or tissue-specific control of the expression of this gene. This paper describes a new PDH deficiency explained by a novel intronic splicing mutation of the *E1 α PDH* gene in a patient with an encephalopathy and lactic acidosis. A large number of inherited human diseases involve splicing errors caused by mutations within the natural splice sites or within

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¹ The abbreviations used are: ESE, exonic splicing enhancer; ISE, intronic splicing enhancer; PDH, pyruvate dehydrogenase; SR, specific serine/arginine-rich proteins; RT, reverse transcriptase.

exonic enhancer sequences (8–10). In addition, mutations within introns that generate cryptic splice sites have been shown to result in the aberrant inclusion of intron sequences (11–15). Here we show a novel case in human pathology of a mutation responsible for the generation of a *de novo* splicing control element, most probably an enhancer that favors the recognition of a cryptic 5'-splice site downstream of the mutation.

EXPERIMENTAL PROCEDURES

Case Report—The patient, a boy, was born at term to unrelated parents, but his mother's sister died suddenly at the age of 2–3 months. The patient was first examined at 3 months for hypotonia. At 4 months of age, his general state was impaired. At 9.5 months, seizures appeared. At this time, lactates were 2.8–3.9 and pyruvates were 0.33 and 0.41 mmol/liter before and after meals, respectively, and the lactate in cerebrospinal fluid was 3.2 mmol/liter. The MRI was normal. At 16 months of age, he suffered from ataxia and developmental delay and he was treated by a ketogenic diet (80% lipids) and thiamine (500 mg/day), which improved the neurological and biological features but maintained the mental retardation.

Biochemical Studies—Pyruvate dehydrogenase complex activity was determined by measuring the release of $^{14}\text{CO}_2$ from 0.2 mM [$1\text{-}^{14}\text{C}$]pyruvic acid in fibroblast homogenates and lymphocytes (16).

For Western blot analysis, 7 μg of protein from total fibroblasts was separated on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The blots were probed with anti-PDH complex holoenzyme antibodies. All of the PDH subunits were visualized by use of the ECL detection system (Amersham Biosciences).

Molecular and Genetic Analysis—The DNA of the patient was prepared from lymphocytes and fibroblasts. The DNA of the mother was purified from blood using proteinase K/sodium dodecyl sulfate extraction method. The individual exons and the exon-intron boundaries were amplified (primers available on request). The sequence of the short intron 7 (181 bp) was amplified in a single PCR fragment with exons 7–8 with forward primer 5'-GGC AGA GCA GCA GCT GTT AG-3' and reverse primer 5'-CAG CTT CAG CAG GCA CAT GG-3'.

Total RNA was extracted from cultured fibroblasts and from fresh lymphocytes using High Pure RNA Isolation Kit (Roche Molecular Biochemicals). cDNA was prepared from 2 μg of RNA using Omniscript reverse transcriptase kit (Qiagen) and oligo(dT) primer and amplified in two overlapping PCR fragments (Fig. 2A), GH (from bases –46 to 616) and CD (from bases 528 to 1325). Because of an abnormal CD PCR product, we performed a RT-PCR with primers PDS30–32, which amplify only the exons 6–9 of the cDNA (281 bp).

All of the PCR products were purified with Concert Rapid PCR purification system (Invitrogen) and sequenced with the Big Dye terminator kit on a 3100 ABI Prism sequencer (Applied Biosystems).

Cloning of the Full-length *E1 α* cDNA—Total cDNA from the fibroblasts of the patient (as described in molecular analysis) was submitted to a PCR with primers G and D to obtain the full-length PDH *E1 α* cDNA. The RT-PCR product was cloned into the pGEM-T Easy vector (Promega). The presence of the insert was checked after digestion with *EcoRI*. The positive clones were analyzed by PCR with primers PDH30–32 (17) to differentiate the normal from the mutated clones and sequenced as above.

Detection of an Aberrant Splicing *In Vitro*—The PCR fragment containing exon 7, intron 7, and exon 8 was performed from the genomic DNA of the patient and a control using the following primers: sense, 5'-GCT-GTT-AGA-GAT-GAT-GAA-GCC-TCG-AGA-AAG-AAG-3', and antisense, 5'-GGG-AGG-CAG-TCT-TTG-AAC-TTC-TAG-AGA-TAA-TG-3'. These primers introduced sites for *XhoI* and *XbaI* restriction enzymes in the 5' and 3' ends of the PCR product, respectively, that were used for the insertion of the PCR fragment into the pET01 Exon Trap vector (Mobictec). After digestion with *XhoI* and *XbaI*, this fragment was 494-bp long: 45 bp of intron 6, exon 7 (156 bp), intron 7 (181 bp), and exon 8 (72 bp) and 40 bp of intron 8.

Three independent transfections were carried out in COS-7 cells using LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was obtained with RNA-PLUSTM extraction solution (Quantum-Bioprobes). 2 μg of RNA was used for RT-PCR with the First Strand cDNA Synthesis kit (Amersham Biosciences) using the specific primer (cDNA primer number 01) from the Exon Trap kit. The PCR was performed with the 5'-PCR primer 02 and 3'-primer 03 also from the kit. With this construction and PCR primers, the

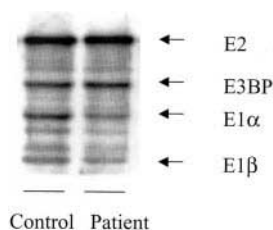


FIG. 1. Western blotting of total fibroblasts with the anti-PDH holoenzyme. Proteins (7 μg) from a control and from the patient were separated on a 10% SDS-polyacrylamide gel under reducing conditions, transferred to a nitrocellulose membrane, and probed with anti-PDH holoenzyme.

expected size of the normal product is 477 bp. The PCR fragment was visualized on a 1.5% agarose gel.

SR Protein ESE Motif Analyses (18, 19) in Normal and Mutated Intron 7—The nucleotide sequences of normal and mutated intron 7 were searched with the ESEfinder program using the default settings (exon.cshl.org/ESE/).

RESULTS

The patient was diagnosed as having a defect in PDH activity because of neonatal hypotonia, seizures, and mental retardation associated with hyperlactacidemia and a normal lactate/pyruvate ratio. The assays of enzymatic PDH activity confirmed a partial pyruvate dehydrogenase defect in cultured fibroblasts (0.50 nmol/mg protein/min; normal control range of 0.64–1.01) and in isolated lymphocytes. Respiratory chain complex activities were normal in muscle biopsy.

Immunochemical analysis using an antibody to the whole complex revealed that there was a slight decrease of both immunoreactive *E1 α* and *E1 β* subunits with no evidence for any form with altered size (Fig. 1).

Direct DNA sequencing was performed for all 11 exons including their exon-intron boundaries of the *E1 α* PDH gene with the result that no mutation was found.

RT-PCR was performed by using RNA obtained from fibroblasts and lymphocytes (patient and control) for sequencing analysis. The product GH (5' end) was normal (Fig. 2A); however, the analysis of fragment CD (3' end) displayed a superposition of two sequences in both directions between exons 7 and 8. This prompted us to perform a new RT-PCR with primers PDH30–32, which amplified from a part of exon 6 until the middle of exon 9 (281 bp). These results showed the same abnormal RT-PCR pattern in both tissues, an elongated band together with the normal band in smaller amounts (Fig. 2B, lanes 1 and 2). The full-length PDH *E1 α* cDNA was cloned from the patient sample for sequencing in pGEM-T Easy. The presence of the insert was checked with *EcoRI* digestion (data not shown). We distinguished the plasmids containing the normal from the mutated cDNA by PCR with primers PDH30–32 (Fig. 2B, lanes 3–5). The mutated sequence was found to have an insertion of 45 bases between exons 7 and 8. The insertion corresponded to the genomic region of the intron 7, giving a longer mRNA coding region for 15 new amino acids. This is an in-frame insertion that generates a protein of 405 rather than 390 residues.

Direct sequencing of the genomic DNA of the patient revealed that intron 7 of the *E1 α* PDH gene contained a hemizygous G to A substitution at +26 (759 + 26G→A) (Fig. 3A). Furthermore, the mutation was positioned within the 45-nucleotide insertion found in the aberrant mRNA (Fig. 4). Direct sequencing of the genomic DNA of the mother showed a heterozygous pattern (Fig. 3B). On screening for this mutation by PCR in genomic DNA samples from 30 controls, all showed the wild type sequence.

To clarify whether this intronic mutation was responsible for

FIG. 2. A, primers used for the study of the PDH *E1 α* cDNA. B, PCRs of exons 6–9 with primers PDH30–32. Lane 1, direct RT-PCR from lymphocytes cDNA; lanes 2–5, the full-length PDH-*E1 α* cDNA was obtained from fibroblasts by RT-PCR with primers G and D and cloned into pGEM-T Easy; and lane 2, this cDNA was reamplified with primers PDH30–32; and lanes 3–5, PCR products with primers PDH30–32 of three of the eight clones of pGEM-T Easy-PDH-*E1 α* .

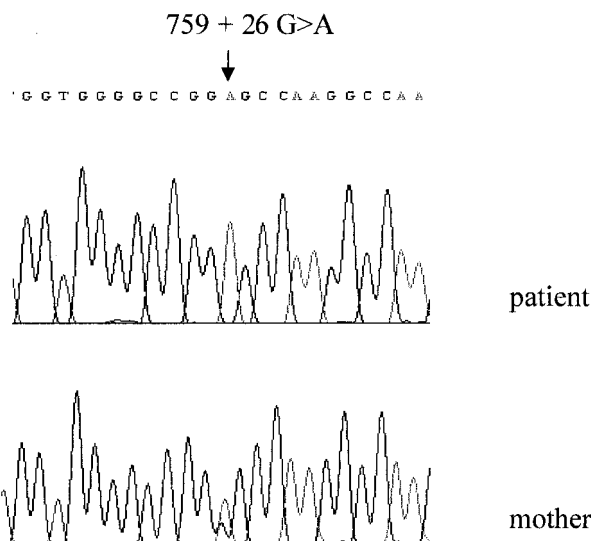
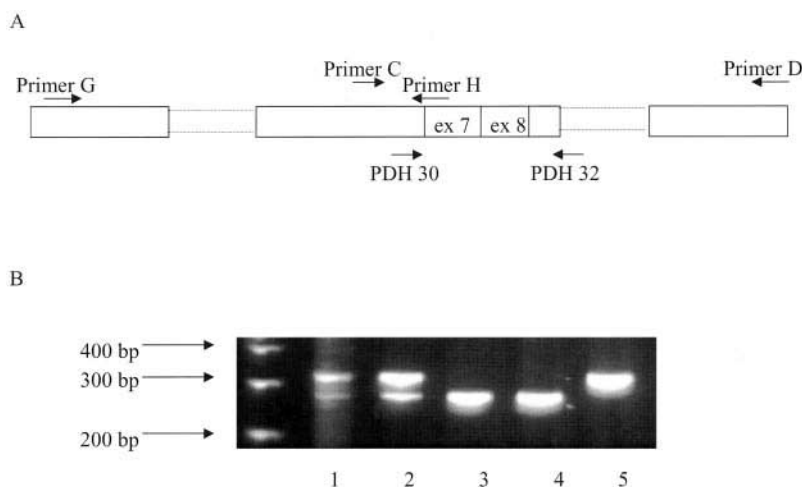


FIG. 3. Direct sequencing of the intron 7 from genomic DNA. The patient showed the G>A substitution at positions 759 + 26 in a hemizygous pattern. The mother is heterozygous for this mutation.

the splicing error, we conducted an exon trapping experiment. COS-7 cells were transiently transfected with a splicing reporter plasmid (pET) containing the genomic sequence from the end of intron 6 to the beginning of intron 8 with either the normal or the +26G>A intron 7. After harvesting RNA from the cells, RT-PCR was used to identify the spliced mRNAs with primers 02 and 03 from the Exon Trap kit. These results show that only the normal spliced product of 477 bp is produced in the control sample, which contains the normal DNA sequence (Fig. 5, lane 1). However, in the cells transfected with the mutant splicing reporter, both the normal and abnormal spliced products (477 and 522 bp) were produced (Fig. 5, lane 2). Moreover, the abnormally spliced product was predominant.

We next performed a search of intron 7 sequences (normal and mutated) of the *E1 α* PDH gene to identify binding motifs of known splicing enhancer proteins. Binding motifs that predict functional ESEs for four of the most well characterized SR proteins, SF2/ASF, SC35, SRp40, and SRp55, were found in the 5' portion of intron 7 for the control and patient sequence (see Table I). Remarkably, this analysis identified one new high affinity SC35 binding motif in the patient but not the control sequence that overlaps with the point mutation at +26. This sequence is shown in boldface in Table I, and the mutated nucleotide is underlined. Note that the new SC35 binding motif also overlaps with an SF2/ASF motif (also highlighted in boldface in the Table

I). Thus, one or both of these newly generated splicing enhancer motifs in the patient DNA may be responsible for the aberrant splicing phenotype because of its close proximity to the cryptic 5' splice site at nucleotide 46 of intron 7.

DISCUSSION

PDH deficiency appears to be a frequent inborn error of energetic metabolism in children with neurological disorders. The beginning of the disorder appears during fetal development of the brain, and the clinical symptoms vary considerably and range from early neonatal lactic acidosis and with mental retardation or intermittent ataxia. Many different mutations have been described in the coding regions of the *E1 α* PDH gene. Only one publication (5) with a new case of the PDH defect described two exonic mutations leading to aberrant splicing in the *E1 α* PDH gene and excision of exon 6. In many patients with typical PDH deficiencies as with our patient described here, the *E1 α* PDH gene is found normal without any mutation in the exons or the exon-intron boundaries. In a few cases of PDH deficiency, mutations have been identified in another gene, the *Hs-PDX1* gene, which encodes the E3-binding protein of the PDH complex (6). In all of these cases, the E3-binding protein (also called protein X) was absent as determined by immunoblotting. In this study, no elongated forms of the *E1 α* subunit were detected with the exception of a slight decrease of *E1 α* and *E1 β* immunoreactivity compared with control samples. The abnormal protein is probably unstable, and the amount of *E1 α* protein corresponds to the translation of the normal *E1 α* cDNA detected by RT-PCR. The gene was abnormally spliced in two tissues, fibroblasts and lymphocytes. Together with the normal product, one aberrant product appeared corresponding to a mRNA with partial intron 7 retention. The unusual feature of this mutation is that the G to A change at position 26 does not alter an authentic splice site nor does it generate a cryptic splice site. Rather, the mutation activates a cryptic 5'-splice site, which lies 20 nucleotides downstream of the mutated site within the same intron. The score for the newly generated SC35 binding motif (3.62) compares favorably with the highest possible score for a SC35 binding motif so far reported in the literature (3.95). The score is a measure of the match of each nucleotide position to the consensus SC35 sequence motif.

It has been estimated that 15% of inherited human diseases involve splicing errors because of mutations in splice sites or in splicing control sequences (20). Most splicing mutations are distributed within the coding exons or in the adjacent 5'- and 3'-splice sites, which frequently lead to exon skipping. In human disease genes, there are numerous mutations in ESE control sequences that have been documented to cause aber-

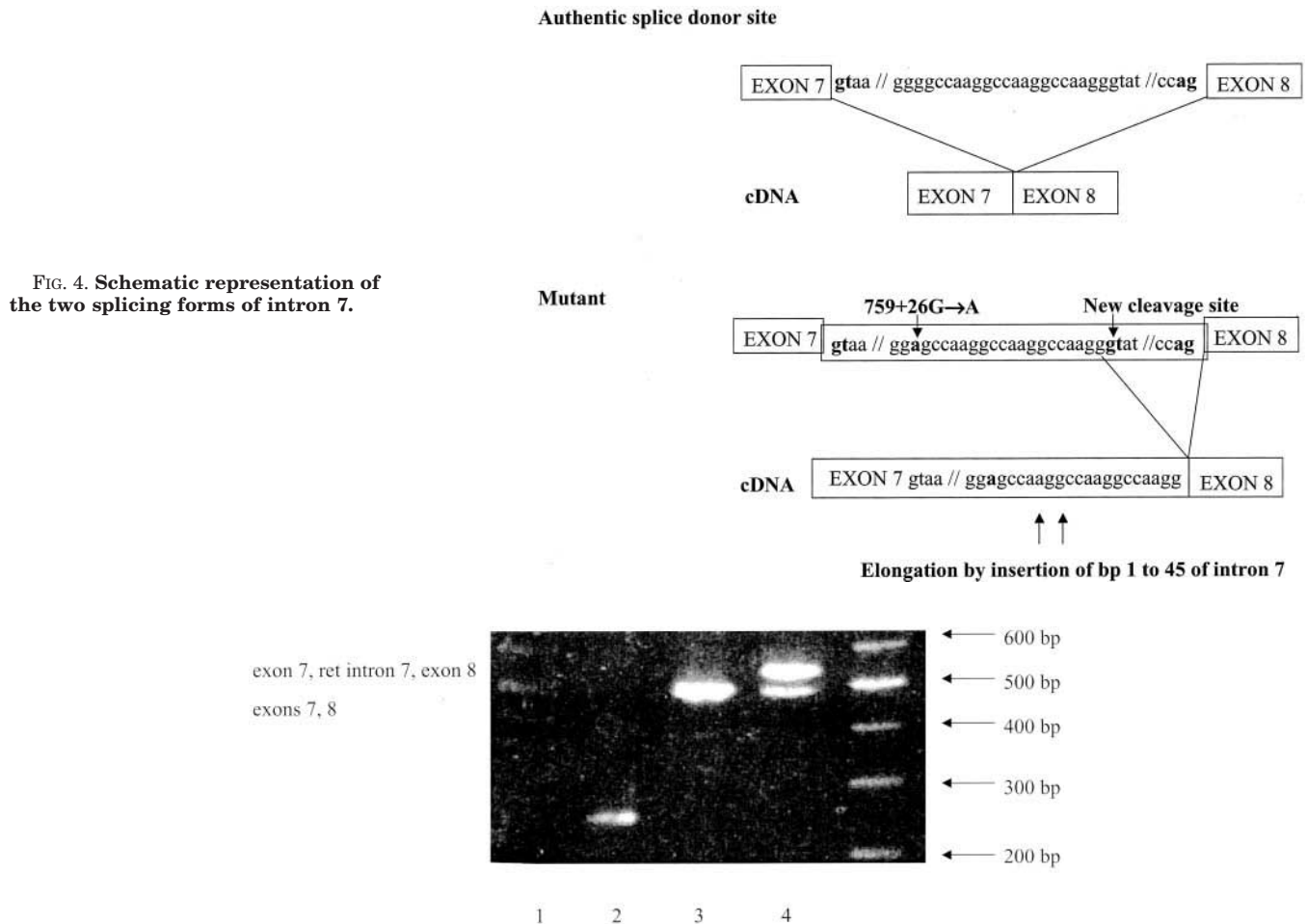


FIG. 5. RT-PCR analysis of transient expression of normal and mutated minigenes. Lane 1, untransfected cells; lane 2, cells transfected with empty vector (249 bp); lane 3, cells transfected with the vector containing the wild type intron 7 (477 bp); and lane 4, cells transfected with the +26 G>A intron 7 (477 and 522 bp). The intronic mutation is responsible for an abnormal splicing pattern with the normal form in a small amount and the partial intron retention in a larger amount.

TABLE I
Exonic splicing enhancer binding motifs predicted by searching intron 7 sequences with the ESE finder program

SR protein type	SR-protein binding motifs found in control sequence (intron 7, nucleotides 1–52)	SR-protein binding motifs found in patient sequence (intron 7, nucleotides 1–52)	Intron 7 nucleotide positions spanning the predicted ESE motifs
SF2/ASF	GACACCT	GACACCT	6–12
	CACCTGT	CACCTGT	8–14
	GGGCCGG	GGGCCGG	19–25
	GGCCGGG	<u>GGCCGGG</u>	20–26 ^a
SC35	GACACCTG	GACACCTG	6–13
	CACCTGTG	CACCTGTG	8–15
		<u>GGCCGGAG</u>	20–27 ^a
SRp40	CCTGTGG	CCTGTGG	10–16
SRp55	TATGTC	TATGTC	47–52

^a Note that the underlined nucleotides correspond to the point mutation in intron 7 sequence (nucleotide 26 of intron 7) of the patient.

rant exon skipping (10). Although ISEs have also been shown to promote splice site recognition, these sequences do not normally bind to SR splicing factors (21). Using ESE motif prediction tools (18, 19), we found that this mutation in the patient intron generates a new SC35 binding motif in the transcript, which is slightly upstream from the cryptic site (Table I). An ASF/SF2 sequence overlaps the SC35 binding motif. Whether SC35 and ASF/SF2 bind simultaneously or competitively to the patient intron sequence is not known. Moreover, when an “exonic enhancer” type of sequence is introduced in an intronic sequence near a splice site, it normally inhibits splicing (22). Thus, the new SC35 binding site

could inhibit the wild type 5′-splicing site or enhance the cryptic 5′-splice site or both. The SR factors, SC35 and ASF/SF2, have already been described as having antagonistic effects on intronic enhancer-dependent splicing of the β -tropomyosin alternative exon 6A (23).

The intronic mutation identified here is located within a small intron, which may play a role in the partial intron 7 retention mechanism. Although on average most vertebrate introns are much larger than their flanking exons, small introns are not unusual. An examination of splicing phenotypes of other human disease genes indicates that intron inclusion can be observed in genes containing a splice site mutation

within a small human intron. In our case, the mutation (759 +26G→A) was found in a quadruplet of Gs in intron 7 and created an intronic retention. Intron 7 is small (181 bp) and G-rich. Furthermore, the mutation is found in an evolutionarily conserved region in many animal species and yeast. Multiple G triplets and quadruplets are commonly found in intronic sequences near 5'-splice sites, and in some contexts, these sequences function as intronic enhancers (24, 25). Moreover, a recent computational analysis revealed that G triplets are found in most of the highest scoring pentamer sequences in small human introns (2). We consider that GGGG from positions +24–27 of the intron 7 of the *E1 α* PDH gene may function normally as an enhancer of the authentic 5'-splice site, and the G→A mutation in the patient intron may consequently weaken the authentic site. However, this is not a complete loss of recognition, because a small proportion of the patient mRNAs uses the authentic 5'-splice site.

The protein levels of the SR splicing factors vary naturally over a very wide range, and our results support the notion already put forward by other groups (26) that changes in the ratio of these proteins can affect the spliceosome assembly and alternative splicing regulation of a variety of pre-mRNAs *in vivo*. Further studies will be necessary to confirm our hypothesis by performing *in vitro* splicing experiments (27).

In conclusion, this study demonstrates a causal relationship between an inherited intronic mutation in the *E1 α* PDH gene and the activation of a downstream cryptic 5'-splice site with devastating consequences for human brain development.

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