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Identification of a Splice-Site Mutation in the Aldolase B Gene from an Individual with Hereditary Fructose Intolerance

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Summary

Hereditary fructose intolerance (HFI) is a potentially fatal autosomal recessive disease of carbohydrate metabolism. HFI patients exhibit a deficiency of fructose 1-phosphate aldolase (aldolase B), the isozyme expressed in tissues that metabolize fructose. The eight protein-coding exons, including splicing signals, of the aldolase B gene from one HFI patient were amplified by PCR. Dot-blot hybridization of the amplified DNA with allele-specific oligonucleotide (ASO) probes revealed a previously described A149P mutation in one allele from the proband. The mutation in the other allele was identified by direct sequencing of the double-stranded PCR-amplified material from the proband. The nucleotide sequence of exon 9 revealed a 7-base deletion/1-base insertion ($\Delta 7 + 1$) at the 3′ splice site of intron 8 in one allele. This mutation was confirmed by cloning PCR-amplified exon 9 of the proband and determining the sequence of each allele separately. ASO analysis of 18 family members confirmed the Mendelian inheritance of both mutant alleles. The implications of this unique splice-site mutation in HFI are discussed.

Introduction

Hereditary fructose intolerance (HFI) is an autosomal recessive disease which causes patients to experience severe abdominal pain, vomiting, and hypoglycemia following fructose ingestion (Chambers and Pratt 1956). The condition becomes apparent—and may be fatal—on weaning, when affected infants are exposed to sugars other than lactose (Gitzelmann et al. 1989). Chronic intake of fructose can lead to liver and kidney damage, growth retardation, coma, and death (Schulte and Lenz 1977). Children with HFI, however, often learn to avoid the offending sugars, and a presumptive diagnosis can be made on the basis of eating habits (Odiévre et al. 1978). Intravenous fructose loading tests have been recommended to establish the diagnosis but can be dangerous. Definitive diagnosis has been by direct measurement of aldolase B activity in liver biopsy samples (Lameire et al. 1978). Treatment, to which patients respond dramatically, is through a diet free of fructose.

HFI results from the catalytic deficiency of aldolase B (fructose 1-phosphate aldolase), the isozyme expressed in tissues (liver, kidney, and intestine) that metabolize fructose (Hers and Joassin 1961). The human gene for aldolase B is located on chromosome 9 (Henry et al. 1985; Lebo et al. 1985). The 14.5-kbp gene has been sequenced and consists of nine exons which code for 363 amino acids (Rottmann et al. 1984; Tolan and Penhoet 1986). Studies of patients from Europe have shown that there is a small set of mutations in the aldolase B gene that are responsible for a majority of HFI cases. The three most common European mutations are missense mutations: A149P (Ala149→Pro) (Cross et al. 1988) and A174D (Ala174→Asp) (Cross et al. 1990*a*), both in exon 5, and N334K (Asn334→Lys) in exon 9 (Cross et al. 1990*b*). These gene defects would result in enzymes with single amino acid substitutions. Other, less common mutations that would result in truncated or internally deleted proteins have been described elsewhere (Cross and Cox 1990; Cross et al. 1990*a;* Dazzo and Tolan 1990; Kajihara et al. 1990).

In the present investigation we report a splice-

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Table 1

Primers for Amplification of Aldolase B Exons

Exon and Primers	Sequence (5′ to 3′)	Size (bp)	[$MgCl_2$] (mM)
2:			
E2+500	CCACAGAATAGAGAGACAGT	505	1.25
E2-	GTTGTTATATGATGAGACTG		
3:			
E3	CTAGGCCACCTGAGAGCAACCA	540	2.50
E3rc.2	TCTCTGTGGGAAGATGACGA		
4/5:			
E4	GATGCAAACTGTTAGTTAG	1,215	1.50
PCR-2	GGTCCATTTGTAGTTATAGT		
6/7:			
PCR6-5	CAACAATTTATACCAATTGC	~1,400	2.50
E7rc	GCTTGGTATTCTGAAGTG		
8:			
E8	CTCAAGCAGGGTATATAAG	417	1.00
E8rc	CTCAATCCTCATACTGACCTC		
9:			
E9	TTCCCATGAGAGGCAGA	711	1.00
E9-3	GACCTTTACTGTTGAAACCC		

junction mutation in a compound heterozygous American patient. This mutation, the first described that is unique to an American patient, is interesting in both the nature of the deletion and its possible consequences.

Subject and Methods

Subject

The proband Caucasian male of northern European, Cherokee, and Karok Indian extraction was born at term after a normal pregnancy and delivery. He was well on breast milk until the age of 2 mo, when he was admitted to the local hospital pale, hypotonic, and hypoglycemic. Several more episodes followed, resulting in an increase in liver size, with laboratory results showing high partial thromboplasin time (46.5 s), aminoaciduria, high total bilirubin (4.9 mg/dl), and high alkaline phosphatase (1,160 IU/liter), symptoms consistent with liver dysfunction. He was placed on a fructose-free diet, and the episodes of hypoglycemia, the liver size and the liver enzymes soon normalized. He has continued on this diet, and at the age of 15 years was at the 50th percentile for height and weight.

PCR

Genomic DNA was obtained from peripheral leukocytes by a proteinase K (Sigma) digestion, phenol-extraction/isopropanol-precipitation method (Orkin et al. 1978). Oligonucleotides (table 1) were synthesized as described elsewhere (Dazzo and Tolan 1990). Aldolase B exons 2, 3, 4 + 5, 6 + 7, 8, and 9 including splicing signals were amplified using *Taq* DNA polymerase (Perkin Elmer—Cetus) and the PCR (Saiki et al. 1985). The amplification profile was as follows: 94°C for 5 min to denature; 5 cycles of 94°C for 1.5 min, either 42°C for 1.5 min or 50°C for 1.0 min, and 72°C for 1.5 min, followed by 20 or 30 cycles of 94°C for 1.5 min, either 42°C for 1.5 min or 50°C for 1.0 min, and 72°C for 3.0 min. For most pairs of primers, specificity of the reaction was significantly increased by annealing at 50°C instead of at 42°C.

Direct Sequence Determination of Amplified Aldolase B Exons

Sequence determination was as described by Dazzo and Tolan (1990). In brief, radiolabeled oligonucleotide primers (10 pmol; table 2) whose binding sites were within the PCR product were used with 200 ng amplified DNA. The DNA was purified using either a Bio-gel P-6DG (Bio-Rad) gel-filtration column (Sambrook et al. 1989) or a Centricon-30 filtration device (Amicon), as directed by the manufacturers. The latter purification method was used preferentially, as it consistently yielded twofold more DNA.

Subcloning and Sequence Determination

Ends of crude PCR-amplified DNA were made blunt using the Klenow fragment of DNA polymerase

Table 2

Sequencing Primers

Exon and Primer	Sequence (5′ to 3′)	Direction
2:		
E2 + 200	GAGCCACCCATGGTTCTGTG	5′ to 3′
2-	GTTGTTATATGATGAGACTG	3′ to 5′
3:		
E3sq	CCTGGCTTGCTCCTTATGC	5′ to 3′
E3rc	TGTTCAGAGTGTTGGCCCTG	3′ to 5′
4:		
E4.2	GTTCAAGAGTTCTGCTTGTG	5′ to 3′
E4rc	GCCTTCATTTCTAGCTTAC	3′ to 5′
5:		
PCR-1	GGATGGATCAGGTACAAAGG	5′ to 3′
PCR-2	GGTCCATTTGTAGTTATAGT	3′ to 5′
6:		
E6sq	GGATAACAGTGAGCACAGC	5′ to 3′
E6rc	AGATTTTTCAACTAGAATTG	3′ to 5′
7:		
E7	CTGCAGTGTAAATGTGCCAA	5′ to 3′
E7rc.2	GCTAAGACCTTGAGTTAGAG	3′ to 5′
8:		
E8sq	GAGTGAAATGGCCTTCTCTC	5′ to 3′
E8rc.2	AAATGTGAACATCATCAA	3′ to 5′
9:		
E9.2	ACATTTACTCTAACCAGTC	5′ to 3′
E9rc.3	GCTGTGTAAGAGCGACTGGGTG	3′ to 5′

I (Boehringer Mannheim) or T4 DNA polymerase (New England Biolabs). This DNA was purified from a 5% nondenaturing polyacrylamide gel and was ligated into M13mp19, and the sequence was determined (Tolan and Penhoet 1986).

Allele-specific Oligonucleotide (ASO) Hybridization

Amplified DNA (50 ng) was denatured and bound to a nylon membrane (ZETA probe; BioRad) by using a dot-blot apparatus (BioRad) as instructed by the manufacturer. Blots were prehybridized and hybridized according to a method described by Angelini et al. (1986), except without dextran sulfate. Hybridization of duplicate blots was at 50°C overnight with radiolabeled ASOs (table 3). High-stringency wash was in 6 × SSC/0.1% SDS for 2 min at the discriminatory temperatures specified for each ASO (table 3) or as described elsewhere (Cross et al. 1990*a*, 1990*b*).

Results

Screening the proband and immediate family members for the known missense mutations by ASO hybridizations of amplified DNA revealed an A149P mutation in one allele (fig. 1). DNA samples from the

Table 3

ASO Probes

Allele	Sequence (5′ to 3′)	Discriminatory Temperature (°C)
A149 wt ..	AAGTGGCGTGCTGTGCTGA	65
A149P	AAGTGGCGTCCTGTGCTGA	65
Δ7 + 1 wt	TTGTCTTCTAGGCTAACT	52
Δ7 + 1	TTGTCTTCTACTGCCAGG	52

proband and the father hybridized with both the A149P and wild-type probes, indicating heterozygosity for this mutation. DNA samples from the mother and sister showed hybridization only to the wild-type probes in this experiment, as well as in experiments using other ASOs (data not shown). When similar ASO hybridizations with the A149P and wild-type probes were performed on DNA from three generations of the extended family, the paternal grandmother and great uncle were shown to be heterozygous for the A149P mutation in exon 5; no other family members possessed this allele (data not shown).

The mutation in the proband's maternal aldolase B allele was identified by direct sequence determination of PCR-amplified DNA encoding exons 2–9 (Dazzo

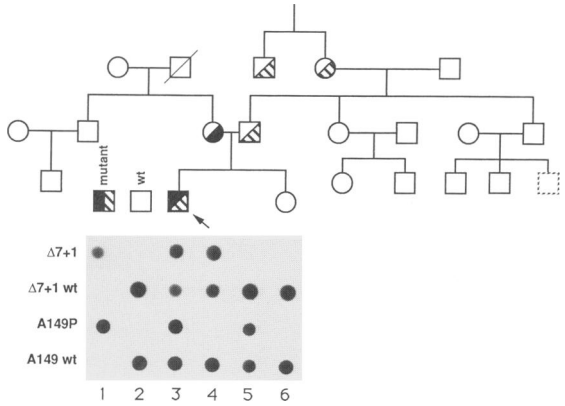

Figure 1 Proband pedigree analysis using hybridization of PCR-amplified DNA from exons 5 and 9 with ASO probes: Δ7 + 1; Δ7 + 1 wt; A149P; and A149 wt. The arrow indicates the proband. Completely unmarked and completely marked symbols represent the normal and mutant genotypes, respectively, and half-unmarked/half-marked symbols represent heterozygotes (◪ = A149P; ◪ = Δ7 + 1). The deceased maternal grandfather (◪) and a cousin (⬚) were not tested. Lane 1, DNA from clone of Δ7 + 1 allele or from unrelated A149P homozygote. Lane 2, Exon 9 or exon 5 DNA from clone of unrelated normal individual. Lane 3, DNA from proband. Lane 4, DNA from mother. Lane 5, DNA from father. Lane 6, DNA from sister.

and Tolan 1990). By this method, the nucleotide sequence only in exons 5 and 9 showed changes from the normal gene sequence. As expected, at position 149 the sequence for exon 5 showed the wild-type G in one allele and the mutant C in the other (data not shown), confirming the ASO hybridization results and the A149P heterozygosity of the proband. The nucleotide sequence of exon 9 revealed a deletion (or an insertion) at the intron 8/exon 9 boundary in one allele (fig. 2*A* and *B*). Because the proband is heterozygous at this site, the sequence is clear until the point of deletion (or insertion); after that point the two sequences are superimposed. Sequence determination of the other strand confirmed these observations.

The apparent complexity of the mutational event(s) was confirmed by the nucleotide sequence of the cloned mutant allele (fig. 2*C*), which clearly shows a 7-base deletion/1-base insertion ($\Delta 7+1$) mutation. This mutation includes deletion of both guanines of the 3′-AGG splice site of intron 8, thereby likely precluding normal splicing of exon 9. Several PCR amplification and cloning experiments further confirmed this result. In addition, the $\Delta 7+1$ mutation creates a *BalI* site that is not present in the wild-type allele; results of *BalI* digestion of exon 9 from the proband were consistent with the sequence at the $\Delta 7+1$ site (data not shown).

The segregation of the $\Delta 7+1$ mutation in the proband's family was determined through ASO hybridizations (fig. 1). The proband's DNA hybridized, as did the DNA of the mother, with both the wild-type and $\Delta 7+1$ probes, confirming heterozygosity at this site. DNA from the father and sister hybridized only with the wild-type probe. Thus, the proband inherited the A149P mutation from his father and the $\Delta 7+1$ mutation from his mother; the sister inherited wild-type alleles from both parents. Furthermore, the maternal grandmother was free of the $\Delta 7+1$ allele, and the

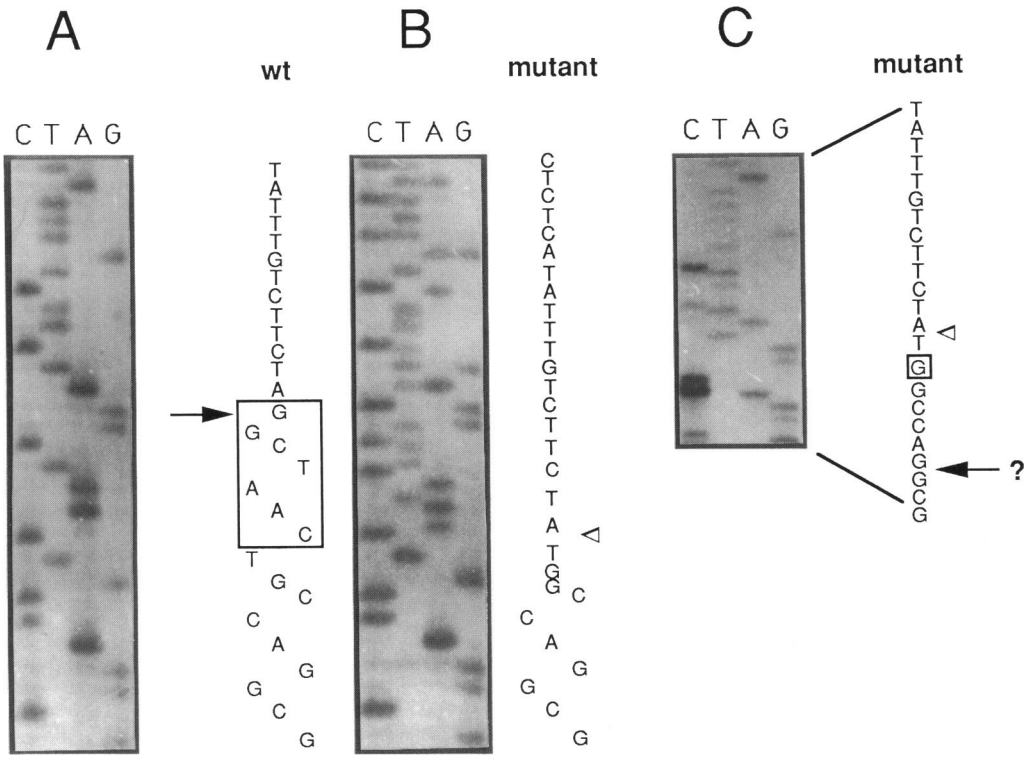

Figure 2 Radioautograph of intron 8/exon 9 boundary sequence from aldolase B gene. The nucleotide sequence of the antisense strand was determined; the sequences depicted at the left and right sides of the figure are those of the sense strands. The boxed nucleotides depict the deleted (GGCTAAC) and the inserted (G) sequences. The triangles indicate the point of the deletion in the mutant allele. The arrow on the left indicates the intron/exon boundary in the normal allele; the arrow on the right indicates the putative intron/exon boundary if splicing occurs at the downstream AGG. *A* and *B*, Direct sequence determination with radiolabeled primer Erc9.3 (table 2) of PCR-amplified DNA from a clone of a wild-type individual (*A*) and from proband (*B*). *C*, Sequence determination of M13 clone of amplified exon 9 from proband.

genotype of the maternal grandfather was undetermined.

ASO hybridizations with the Δ7 + 1 probes were performed on 11 independent alleles from nine unrelated HFI patients with unknown genotypes. This analysis revealed that these patients did not carry the Δ7 + 1 mutation (data not shown).

Discussion

The present investigation describes a novel splice-junction mutation, Δ7 + 1, in one allele of the aldolase B gene of an American HFI patient. The mutation in the other allele is the common European A149P mutation. The Δ7 + 1 mutation at the boundary of intron 8 and exon 9 is a 7-base deletion and a 1-base insertion, resulting in deletion of both intron and exon guanines of the AGG acceptor consensus splice site of intron 8 (Mount 1982). Splice-junction mutations involving the 3′ splice site have been documented in the globin genes of thalassemia patients (Bank and Dobkin 1987; Wong et al. 1989), as well as in patients with other genetic disorders (Cladaras et al. 1987; Kerem et al. 1990; Kobayashi et al. 1990). In HFI this type of defect is relatively uncommon in the cases so far examined. The only other case was one allele where both the first guanine of exon 3 and three other bases of the aldolase B gene were deleted (Cross and Cox 1990). The effect on splicing at this site is not known, because the possibility for normal splicing at the resultant AGC 3′ splice site remains (Padgett et al. 1986).

The Δ7 + 1 mutation deletes the AGG at the 3′ end of intron 8; thus, normal splicing of exon 9 is likely precluded. However, a possible AGG splice site is located 13 bases downstream from the normal one in the wild-type gene. If this site were used, then the resultant mRNA would be in the correct reading frame, and a mutant protein, rather than an unstable message and no protein, would be predicted. This mutant enzyme would have an Ala-Asn-Cys-Gln (333–336) deletion, the first four amino acids coded by exon 9. Such a mutant protein may be interesting, since the carboxyl-terminal region, in addition to being highly variable in both length and sequence among aldolases (Kelley and Tolan 1986; Rottmann et al. 1987), is thought to be involved in substrate specificity (L. Berthiaume and J. Sygusch, personal communication). The presence of another mutation (N334K) in this same region (Cross et al. 1990*b*) may indicate that residues in this area are important for fructose 1-phosphate cleavage.

The Δ7 + 1 mutation, which at first glance appears to have arisen through two mutational events, may have arisen through only one (fig. 3). The Δ7 + 1 allele differs from the wild-type allele by the deletion of seven bases and the insertion of one base. Such a complex mutation may have arisen through a single event of template-directed intrastrand misalignment (Golding and Glickman 1985). Formation of a secondary structure (fig. 3*A*) may be directed by a quasi-palindrome located nine bases downstream from the 3′ end of the deletion site. DNA repair enzymes, directed by the intrastrand template, may have removed the excluded sequence of seven bases and inserted a guanine by template-directed replication, to generate a more stable structure (fig. 3*B–D*). Models for such sequence-

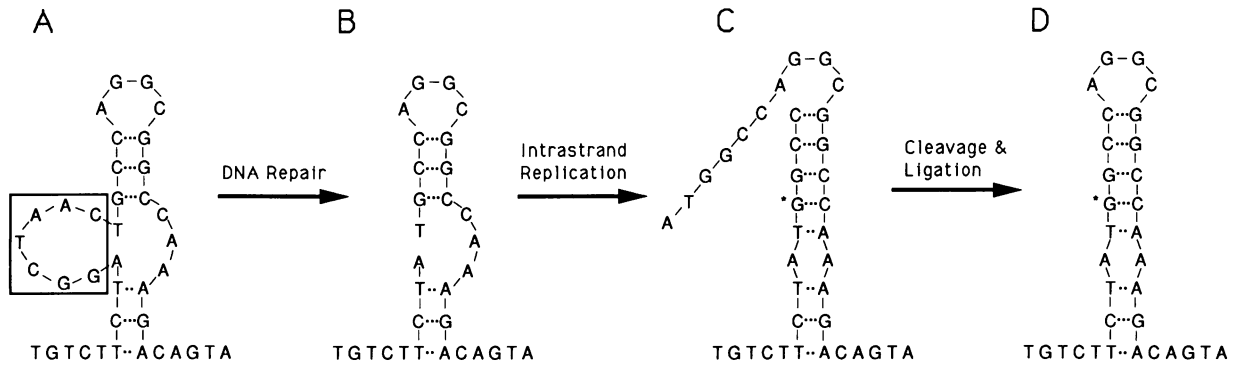

Figure 3 Proposed mechanism for generation of Δ7 + 1 mutation identified in exon 9 of aldolase B gene of proband. *A*, Sequence of normal allele showing inverted repeat which may have resulted in Δ7 + 1. This sequence includes seven nucleotides which can form correct basepairs and is only nine bases downstream from the deletion site. The boxed nucleotides are deleted in the Δ7 + 1 allele. *B*, DNA repair enzymes which remove seven bases that are looped out. *C*, Replication occurring with use of an intrastrand template. *D*, Cleavage and ligation which result in sequence of mutant allele. The asterisk denotes the inserted guanine.

directed mutational events have been proposed for complex mutations, in both prokaryotes (Glickman and Ripley 1984) and eukaryotes (Golding and Glickman 1985). The mechanism depicted in figure 3, however, is not entirely satisfying, and other possibilities must be considered.

Relatively few genotypes have been determined for American patients with HFI, and this genotype analysis represents the first case of an American HFI patient who is a compound heterozygote. The described $\Delta 7 + 1$ allele may be a truly American allele; the maternal grandfather is of Cherokee descent, and, although his genotype was undetermined, Mendelian segregation would indicate that he was likely heterozygous for the $\Delta 7 + 1$ allele if this mutation did not arise in the mother. This may indicate that this allele could be common in those of Cherokee descent, although additional study of this population will be required to determine this.

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