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Splicing Defect at the Ornithine Aminotransferase (OAT) Locus in Gyrate Atrophy

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Summary

Gyrate atrophy (GA), a recessive eye disease involving progressive vision loss due to chorioretinal degeneration, is associated with the deficiency of the mitochondrial enzyme ornithine aminotransferase (OAT), with consequent hyperornithinemia. We and others have reported a number of missense mutations at the OAT locus which result in GA. Here we report a GA patient of Danish/Swedish ancestry in whom one OAT allele produces an mRNA that is missing a single 96-bp exon relative to the normal mRNA. Polymerase-chain-reaction amplification and sequencing revealed a 9-bp deletion covering the splice acceptor region of exon 5, resulting in the absence of exon 5 sequences from the mRNA with no disruption to the reading frame. This mutation, which was not present in 15 other independent GA patients, adds to the array of allelic heterogeneity observed in GA and represents the first example of a splicing mutation associated with this disorder.

Introduction

Ornithine aminotransferase ([OAT] ornithine; oxo-acid aminotransferase, E.C.2.6.1.13) is a pyridoxal phosphate–dependent mitochondrial matrix enzyme that catalyzes the interconversion of ornithine and α-ketoglutarate to pyrroline-5-carboxylate and glutamate. Human OAT cDNAs have been cloned from both liver and retinoblastoma cells (Inana et al. 1986; Ramesh et al. 1986; Mitchell et al. 1988*b*). The OAT structural gene (*OAT*) was first assigned to chromosome 10 on the basis of expression of the human enzyme in somatic cell hybrid lines (O'Donnell et al. 1988). Hybridization with cDNA probes mapped *OAT* to band 10q26 but revealed a cluster of highly related sequences (*OATL1*) on the X chromosome (Barrett et al. 1987; Ramesh et al. 1987).

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The *OAT* structural gene on chromosome 10 spans 21 kb of DNA (Mitchell et al. 1988*b*) encoding a transcript of 2.2 kb in 11 exons. The 5′-regulatory region of OAT has elements homologous to Spl binding sites, a TATA box, an estrogen-responsive element, and sequences homologous to the promoter regions of urea-cycle enzymes. Translation of OAT begins from exon 3. Exon 2, whose existence was indicated by its presence in a processed pseudogene from the X chromosome, has been absent from all cDNAs, implying that it is spliced out of the mature OAT transcript (Mitchell et al. 1988*b*). However, a very recent study on the regulation of OAT in retinoblastomas has demonstrated the presence of exon 2 in one of two different mature OAT mRNAs from two strains of retinoblastoma lines (Fagan et al. 1989).

In humans, deficiency of OAT activity is associated with gyrate atrophy (GA), an autosomal recessive disorder characterized by progressive vision loss due to chorioretinal degeneration. Phenotypic heterogeneity is evident in GA, where the hyperornithinemia in a subset of patients can be partially alleviated by pyridoxine (vitamin B6) administration (Berson et al. 1978; O'Don-

nell et al. 1978; Shih et al. 1978; Kennaway et al. 1980). We and others have reported six different missense mutations in OAT mRNAs from GA patients, demonstrating allelic heterogeneity which extends to both B6-responsive and B6-nonresponsive subtypes (Mitchell et al. 1988*a,* 1989*a;* Ramesh et al. 1988*b;* Inana et al. 1989). To facilitate the identification of additional OAT mutations in GA, we are analyzing OAT mRNAs by direct sequencing of cDNA amplified by the polymerase chain reaction (PCR). Here we report the first example of an OAT RNA splicing defect associated with GA. The patient (MGF1139) is of Danish-Swedish ancestry and displays a 9-bp deletion affecting the acceptor splice sequence of exon 5. This deletion results in the absence from the mature mRNA of 96 bp corresponding precisely to exon 5.

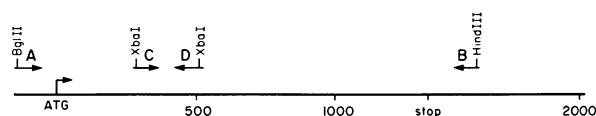

Figure 1 Schematic representation of OAT mRNA, showing oligonucleotide primers used for PCR. Primer sets A (bp −40) and B (bp 1390) flank the coding sequence; C (bp 340) and D (bp 510) contain synthetic *Xba*I sites for subcloning and lie within exons 4 and 5, respectively, bridging the small (100-bp) intron and the splice sites.

Material and Methods

Southern Blot Analysis

High-molecular-weight DNA was prepared from skin fibroblasts of either the patient or normal subjects according to a method described elsewhere (Gusella et al. 1979). The DNA was digested with a panel of restriction enzymes including *Eco*RI, *Hin*dIII, *Pst*I, *Bgl*II, *Bam*HI, and *Msp*I; the separation of this DNA by agarose gel electrophoresis, DNA transfer, hybridization, and autoradiography were performed according to methods described elsewhere (Ramesh et al. 1986).

Northern Blot Analysis

Isolation of total cellular RNA, electrophoresis, transfer, and hybridization were performed as described by Ramesh et al. (1986).

cDNA Synthesis

First-strand cDNA synthesis employed 20 μg total cellular RNA obtained from skin fibroblasts of the patient by oligo dT_{12-18} (Pharmacia) primer and Moloney murine leukemia virus reverse transcriptase under the conditions supplied by the manufacturer (BRL). After phenol-chloroform extractions single-stranded cDNA was ethanol precipitated.

Amplification of cDNA by PCR

The products of the cDNA synthesis from 20 μg total liver RNA were dissolved in 20 μl Tris-EDTA buffer, and 1 μl was subjected to 30 cycles of amplification employing a thermal cycler (Perkin Elmer–Cetus, South Plainfield, NJ) (Mullis and Faloona 1987). Primers corresponding to human OAT cDNA (HOAT1) nucleotides −36 to −16 (fig. 1, A) and complementary to nucleotides 1355 to 1385 (fig. 1, B) were used to amplify the entire coding sequence (see fig. 1 for the location of primers). Each amplification cycle included a denaturing step at 94°C for 1 min and an annealing step at 55°C for 2 min followed by an extension step at 72°C for 3 min. About 1.25 units of *Taq* polymerase (Cetus) was used for each reaction. The amplified product was analyzed on a 0.8% agarose gel.

Amplification of Genomic DNA by PCR

One microgram of genomic DNA isolated from skin fibroblasts was amplified using primers corresponding to nucleotides 331 to 353 (fig. 1, C) of HOAT1 and residues complementary to nucleotides 470 to 487 with *Xba*I linkers attached (fig. 1, D), using the same conditions as above. Prior to the amplification step the genomic DNA was sheared through a 26-gauge needle, and an initial denaturing step was carried out at 94°C for 2 min before the addition of *Taq* polymerase to the reaction mix.

DNA Sequencing

For direct sequencing of the PCR-amplified product, the DNA was cleaned by spin dialysis using a Sepharose CL6B column and was ethanol precipitated. Sequencing primers (50 ng) end labeled with γ-^{32}P-ATP and T4 polynucleotide kinase were annealed to 200 ng of the template after the strands were heat denatured at 95°C for 3 min, and the sequencing reactions were carried out using the Sequenase kit (U.S. Biochemical) and following the conditions of Yandell et al. (1989). PCR-amplified products were also cloned into either the *Bam*HI-*Hin*dIII sites or the *Xba*I site of pUC19 for which corresponding sites are present in the oligonucleotides (fig. 1). DNA sequencing was also carried out on supercoiled plasmid DNA described by Chen and Seeburg (1985).

Results

The patient reported here (MGF 1139) is a 42-year-old male of Danish/Swedish ancestry who has a proved diagnosis of GA on the basis of fundus appearance and elevated serum ornithine levels of 1,189 μmol/liter. The OAT activity in the patient's fibroblast line was undetectable (<2% of normal) and did not show an increase in activity when excess pyridoxal phosphate was added in the assay mixture, placing this patient in the B6 "nonresponsive" category. On Southern blot analysis of genomic DNA digested with a number of enzymes and probed with HOAT1 cDNA, we detected no major gene deletion or rearrangement. Northern analysis of fibroblast RNA revealed an apparently normal sized OAT mRNA with levels comparable with those in the control (Ramesh et al. 1986). These results suggested that the patient had a subtle sequence alteration whose detection was beyond the resolution of our standard Southern and northern analyses.

RNA from the patient's fibroblasts was amplified using primers flanking the OAT cDNA coding sequence (see fig. 1 for primer locations). The primers were designed such that they could cover both a unique *Bgl*II site at the 5′ end of the cDNA upstream from the initiation codon and a *Hin*dIII site just beyond the termination codons. This facilitated the cloning of the entire coding sequence into *Bam*HI-*Hin*dIII sites of pUC19. On directly sequencing the amplified material, we observed a clean readable sequence identical to the normal mRNA—until bp 424, at which point an obvious mixture of sequences was apparent. Amplified products from several independent PCR reactions were then cloned, and individual clones were sequenced. Of the eight clones sequenced, three differed from the wild-type OAT cDNA sequence by the absence of 96 bp corresponding precisely to exon 5 of the OAT gene. This result could be explained by a partial gene deletion or by a RNA splicing mutation that efficiently removed the coding sequences of one exon.

The patient's genomic DNA was then amplified using primers spanning exons 4 and 5 (see fig. 1 for primer locations), and the amplified product was cloned in the *Xba*I site of pUC19. Two amplification products were detected by visual examination on agarose gels. In addition to the predicted 260 bp amplification product, we also observed a fragment of 160 bp. Cloning and sequencing of this lower-molecular-weight species revealed an intronless OAT sequence with 95% homology to the cDNA sequence. This probably represents a processed OAT pseudogene sequence from the X-chromosome *OATL1* locus.

For the larger PCR product, the 260-bp band ex-

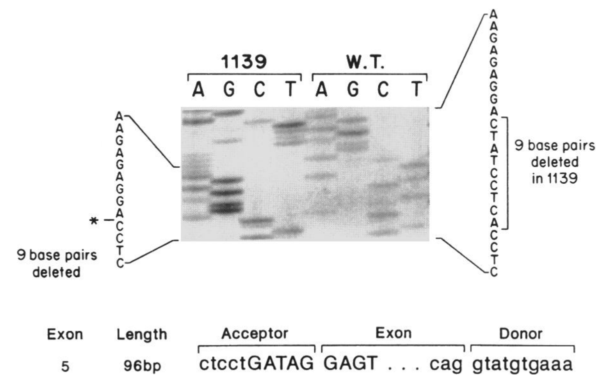

Figure 2 Comparison of genomic OAT sequence from patient MGF 1139 with that of a normal individual (W.T.), showing the deletion of 9 bp from MGF1139. The sequence of the antisense strand is shown, and the deletion is shown in capital letters at the bottom.

pected from the known structure of the chromosome 10 *OAT* structural locus, we sequenced four independent clones. Two were identical to the wild-type OAT gene sequence, whereas two revealed a 9-bp deletion covering the 3′ splice acceptor region of intron 4 (fig. 2). This 9-bp deletion has resulted in the loss of exon 5 while maintaining the codon reading frame of the mRNA. The abnormally spliced mRNA is thus generated by efficient exon skipping from the last codon of exon 4 to the first codon of exon 6. The loss of the 3′ acceptor site results in the absence of exon 5 from the mature mRNA, but because the exon is 96 bp in size the reading frame of the remainder of the mRNA is maintained intact. Exon 5 begins with the second base of codon 142 (Gly G/GA) and ends with the first base of codon 174 (Ala G/CT) (Mitchell et al. 1988*b*). Thus, the codon produced in the mRNA at the junction point of the deletion is GCT and continues to specify an ALA residue such that no novel amino acid residue is introduced by the deletion.

The alternate allele of *OAT* in this patient does not carry this deletion mutation. To date, sequencing of the cloned PCR cDNA products that do contain exon 5 has not yet revealed the probable missense mutation carried on the alternate allele in this patient. We have also sequenced the exon 5 region from 15 other independent GA patients representing different ethnic origins—including one patient of Swedish/Finnish ancestry—and none of these has revealed the same splice defect.

Discussion

GA is a relatively rare disorder, but patients are not confined to a single ethnic group. The genetic hetero-

geneity observed in this disease is reflected by a diverse array of mutations at the *OAT* locus. We and others have reported at least six different missense mutations in the *OAT* gene in GA patients (Mitchell et al. 1988*a*, 1989*a;* Ramesh et al. 1988*b;* Inana et al. 1989). The present case is the first splicing mutation reported in GA. The 96 bp absent from the OAT mRNA, spanning positions 425 to 520 of the OAT cDNA sequence, corresponds precisely with exon 5 of the structural gene. This difference of 96 bp was apparently too small to resolve on our standard northern gels. Only one of the reported missense mutations in GA occurs in exon 5 (Mitchell et al. 1989*b*). The contribution of the peptide specified by this exon to the OAT enzymatic activity is not known, although it shows strong evolutionary conservation, with 19 amino acid identities and five conservative changes, when compared with OAT from *Saccharomyces cerevisiae.* Exon 5 does not contain the pyridoxal phosphate binding site which spans exons 8–10, but the substrate binding residues have not yet been defined for this enzyme (Mitchell et al. 1988*b*).

Haplotyping of 19 independent GA patients with six OAT RFLPs has revealed that a single common haplotype is present on the majority of GA chromosomes but that at least six other haplotypes are also associated with OAT deficiency (Ramesh et al. 1988*a*). Patient MGF1139 is heterozygous at the *OAT* locus, carrying both the common haplotype associated with GA and a rarer haplotype. Although we have not been able to establish which of the two background haplotypes harbors the 9-bp deletion, the fact that the patient is heterozygous supports the conclusion that the alternate chromosome 10 carries a different mutation.

RNA splicing mutations are known to occur in a number of disorders. The majority of these mutations seem to involve the conserved GT dinucleotide at the 5′ donor site. For example, in β-thalassemia, a G-to-A substitution at the 5′ donor site of intron 2 results in skipping of exon 2 in mature β-globin mRNA (Treisman et al. 1982). Other examples include phenylketonuria, where a G-to-A substitution at the 5′ splice donor site of intron 12 of the phenylalanine hydroxylase gene results in absence of exon 12 from the mRNA (Marvit et al. 1987), and the hamster dihydrofolate reductase gene, where a GT-to-CT transition at the splice donor site of intron 5 results in skipping of exon 5 (Mitchell et al. 1986). However, a recent report of a splice mutation in the pro α2(I) collagen gene implicates the 3′ consensus splice site. Here a single base mutation (AG to GG) at the 3′ end of intervening sequence 27 eliminated exon 28 from the mRNA, without changing the codon reading frame of mRNA (Tromp and Prockop 1988). From the various reports describing splice mutations, it appears that a point mutation or a deletion affecting the 3′ splice acceptor site to produce exon skipping without changing the codon reading frame is a relatively rare phenomenon. The case reported here is predicted to produce an OAT protein from which 32 amino acids are missing. It may be of interest to trace the fate of this protein within the cell, to determine what effect changes in conformation may have on both transport into the mitochondria and overall stability.

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