An Initiator Codon Mutation in Ornithine-δ-Aminotransferase Causing Gyrate Atrophy of the Choroid and Retina

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Abstract

Gyrate atrophy of the choroid and retina (GA) is an autosomal recessive chorioretinal degeneration caused by deficiency of the mitochondrial matrix enzyme, ornithine-δ-aminotransferase (OAT). To study the molecular basis of the mutations causing GA, we cloned and sequenced the human OAT cDNA and determined the intron-exon arrangement of the structural gene. Using the cDNA template, we synthesized antisense RNA probes and performed RNase A protection experiments with RNA from four Lebanese GA patients. We found a probe-target mismatch at the 5′ end of the first coding exon and amplified this region of the patients' genomic DNA using the polymerase chain reaction. Sequence analysis showed a G → A transition, changing the initiator ATG (methionine) codon to ATA. This mutation segregates with the GA allele in both pedigrees. Initiation of translation at the closest in-frame methionine codon would truncate OAT by 138 amino acids, eliminating the entire mitochondrial leader sequence and 113 amino acids of the mature peptide.

Introduction

Gyrate atrophy of the choroid and retina (GA)[1] is an autosomal recessive chorioretinal degeneration leading to blindness in early adult life (1). It is caused by a deficiency of ornithine-δ-aminotransferase (OAT) (E.C. 2.6.1.13) (1), a mitochondrial matrix enzyme.

As part of our study of OAT and its role in GA, we wished to define the OAT mutations causing GA. To this end we cloned a human liver OAT cDNA (2, 3) and determined the organization of the human OAT structural gene (4). Multiple restriction digests of genomic DNA of probands from 23 pedigrees were normal. Northern blot analysis of fibroblast RNA of patients in 32 sibships have been normal (81%) or shown a quantitative reduction in OAT mRNA (19%). Thus, neither of these approaches allowed us to precisely delineate the mutation(s) causing GA. We therefore coupled the techniques of RNase A protection (5–9) and polymerase chain reaction (PCR) (10–12) to define a deleterious point mutation in the OAT structural gene, without passing through a stage of genomic cloning.

Methods

cDNA cloning. We isolated a nearly full length human liver cDNA clone from the λgt11 library provided by Savio Woo (13). The clone includes 60 bp of 5′ nontranslated sequence, the complete 1,317 bp open reading frame including a 75 base pair mitochondrial leader sequence (14), and the entire 636 bp 3′ nontranslated region. The sequence agrees with that of previously reported human OAT cDNA clones (15, 16).

RNA probe synthesis. The probes used in these experiments are shown in Fig. 1. The indicated fragments were subcloned into the pGEM 4 plasmid vector (Promega Biotech, Madison, WI). Antisense RNA was prepared (17) using 1 μg of linearized plasmid, 10 U T7 polymerase, and ATP, TTP, and CTP (Promega Biotech), each 500 mM. [^{32}P]GTP (New England Nuclear, Boston, MA) was present at the following concentrations and specific activities: for R1B, 200 μM and 78 Ci/mmol; for R1X and XP, 500 μM and 78 Ci/mmol. The reaction mixtures were incubated at 40°C in a 10-μl volume for 90 min, digested at 37°C with 20 U of RNase-free DNase (Worthington Biochemicals, Freehold, NJ) for 10 min, extracted with phenol-chloroform, and precipitated with ethanol.

RNase protection analysis. RNase A protection analysis was performed as described (5, 9) by annealing to 1–5 × 10^6 cpm of radiolabeled probe with the indicated amount of whole cell RNA overnight in a total volume of 100 μl. The reaction mixture was passed through oligo-T paper (Hybond mAP paper; Amersham Corp., Arlington Heights, IL) and the bound material was eluted and digested with RNase A (Sigma Chemical Co., St. Louis, MO), 10 μg/ml for 30 min at 25°C. The reaction was stopped with SDS-proteinase K digestion, extracted with phenol and chloroform, precipitated in ethanol and run on an 8% polyacrylamide-50% urea gel. RNA was isolated by guanidium thiocyanate extraction (18) from a variety of sources. Patients' RNA was isolated from confluent cultures of skin fibroblasts. Control human liver RNA was obtained from a surgical specimen. Rat liver RNA was isolated from a 460-g Sprague-Dawley rat fed a 60% protein diet and served as a control for the completeness of probe digestion in the presence of RNA with known mismatches. (The rat and human OAT mRNA sequences are 85% homologous over the coding region.)

Polymerase chain reaction. Using 500 ng of patient genomic DNA

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1. *Abbreviations used in this paper:* GA, gyrate atrophy of the choroid and retina; OAT, ornithine-δ-aminotransferase; PCR, polymerase chain reaction.

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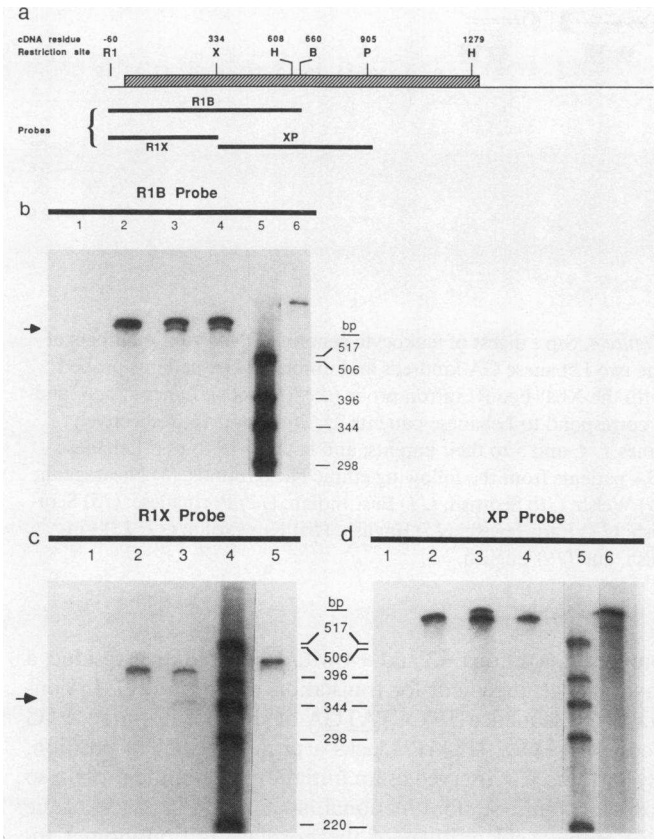

Figure 1. (*a*) Antisense OAT RNA probes used in RNase protection experiments, shown in relation to the OAT cDNA. Residue +1 is the adenine of the initiator (ATG) codon. The hatched box represents the cDNA coding sequence. Important restriction enzyme sites are marked. Abbreviations: B, Bam HI; H, Hinf I; P, Pst I; R, Eco RI; X, Xba I. (*b–d*) RNase A protection analysis of patient and normal RNAs with the indicated probes and target RNAs. (*b*) The R1B probe: (*1*) rat liver, 200 μg; (*2*) control human liver, 200 μg; (*3*) fibroblasts, patient 7, 100 μg; (*4*) fibroblasts, patient 26, 100 μg; (*5*) size markers; (*6*) undigested R1B probe. The cleavage fragments in lanes *3* and *4* are indicated by an arrow. (*c*) The R1X probe: (*1*) rat liver RNA, 200 μg; (*2*) control human liver RNA, 100 μg; (*3*) patient 26 fibroblast RNA, 100 μg; (*4*) size markers: (*5*) undigested probe. The cleavage fragment in lane *3* is indicated by an arrow. A small amount of undigested probe remains in lane *2*. (*d*) The XP probe: (*1*) rat liver RNA, 200 μg; (*2*) control human liver RNA; (*3*) patient 26 fibroblast RNA, 100 μg; (*4*) patient 7 fibroblast RNA, 100 μg; (*5*) size markers; (*6*) undigested probe. A small amount of undigested probe is present in lanes *1* and *3*.

and *Thermus aquaticus* (Taq) DNA polymerase (a gift from Cetus Corp.), 30 amplification cycles were performed according to the manufacturer's specifications. To assess the procedure, 30% of the reaction mixture was run on a 3% Nusieve agarose (FMC Bioproducts, Rockville, ME) −1% agarose (Sigma A-6013) gel, stained with ethidium bromide and photographed.

Restriction enzyme digestion. Eco RI, Hind III, and Ssp I restriction enzymes were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, and used according to the manufacturer's directions.

Sequence analysis of the subcloned amplified fragment. 50% of the amplified fragment was digested with Eco RI and Hind III and cloned into pGEM 4 plasmid vectors. After alkaline denaturation of the plasmids, the insert was sequenced using the dideoxynucleotide method (19).

Southern blot analysis. Leukocyte genomic DNA (5 μg) was digested with restriction enzyme electrophoresed in a 1% agarose gel, transferred to a Genescreen Plus membrane (New England Nuclear) and hybridized to a probe radiolabeled by the random oligonucleotide primed method (20). Probes used corresponded to the entire cDNA sequence, and in Fig. 4 to a 750 bp Xbal − Eco RI intron fragment situated 2 kb 3′ to the end of the first coding exon. (The OAT cDNA probe detects pseudogenes (4) and yields a complex pattern including a 2.6 kb Ssp I fragment that complicates the interpretation of this digest.)

Results and Discussion

We studied two GA sibling pairs (cases 7,16, and 25,26, reference 21) of Lebanese Maronite descent. Their clinical presentations (21) were typical of GA; plasma ornithine values on a normal diet were 745–876 μmol/liter (normal, 75±25 μmol/liter) and their fibroblast OAT activity was undetectable (<1% of normal) by a radioisotopic assay (1). On Southern blot analysis of genomic DNA digested with several enzymes and probed with our OAT cDNA, we detected no alteration in the OAT structural gene of these patients. Northern analysis of fibroblast RNA from each patient showed normal sized OAT message in amounts within the control range (2).

These results suggested that the patients had a point mutation or a genomic rearrangement too small to detect on Southern blots. Therefore, we used the more sensitive RNase A protection assay (5–9) to detect the mutation. When an antisense RNA probe corresponding to residues 608–1283 of the OAT cDNA was annealed to patient RNA and digested with RNase A, the probe was completely protected indicating that this region was normal. In contrast, an antisense probe spanning residues −60 to +660 (probe R1B, Fig. 1) was partially cleaved to fragments of about 660 base pairs and 60 base pairs in length (Fig. 1 *b*, lanes *3* and *4*). Identical cleavage patterns were observed for the R1B probe with RNA from all four Lebanese patients, but not with control human liver RNA (Fig. 1 *b*), nor with fibroblast RNA from a non-Lebanese GA patient (not shown).

To orient the site of cleavage in the R1B probe, two antisense probes were synthesized, spanning the 5′ and the 3′ ends of the R1B probe, respectively (Fig. 1 *a*). A cleavage fragment ~ 60 nucleotides shorter than full length was observed with the 5′ probe (R1X), localizing the site of cleavage to the 5′ end of the coding sequence (Fig. 1 *c*, lane *3*).

We have determined that the first coding exon of the OAT structural gene spans cDNA residues −29 to +199 (residue +1 is the adenine of the initiator ATG codon), and have sequenced the flanking intron regions (4). Using 5′ and 3′ oligomers homologous to the flanking intron sequences as primers, we utilized PCR to amplify a 337 base pair fragment of genomic DNA from patients 7 and 26 (Fig. 2) and cloned the amplified fragments for sequence analysis. Intron primers were chosen so that the entire exon, including its splice junctions, could be analyzed. Furthermore, these intron sequences have no counterpart in OAT-related pseudogenes found on the X chromosome (4). Sequence analysis of the amplified fragment (Fig. 3) revealed a G → A transition at residue +3 of the cDNA, which changes the methionine initiation codon (ATG) to an isoleucine codon (ATA). This was present in all five clones analyzed, three of which came from a single amplification reaction of DNA from patient 26; two of which came, one

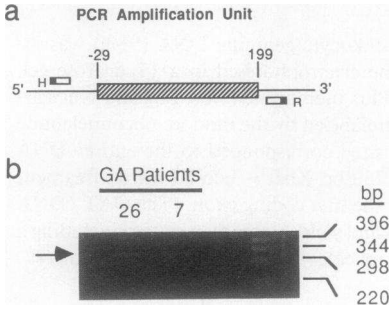

Figure 2. Amplification of patient genomic DNA using the polymerase chain reaction. (*a*) The amplified region of genomic DNA, which includes the entire sequence of the first OAT coding exon (residues −29 to +199 of the OAT cDNA, indicated by the hatched box) and its flanking intron sequences. The 5′ oligomer contains a cloning Hind III site (*H*, shown in black) followed by a 20-bp region of perfect homology to the intron, and ends 37 bp 5′ to cDNA residue −29; the 3′ oligomer contains a cloning Eco RI site (*R*, shown in black) and a 21-bp region of perfect homology to the noncoding DNA strand and ends 18 bp 3′ to cDNA residue +199. (*b*) The products of amplification are shown. The arrow indicates the 337-bp amplified fragment.

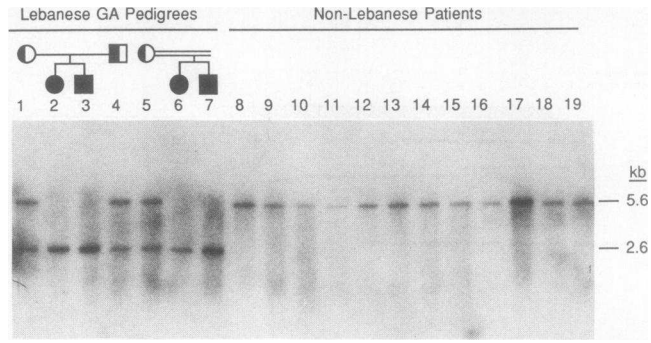

Figure 4. Ssp I digest of leukocyte genomic DNA from members of the two Lebanese GA kindreds and unrelated GA patients probed with the Xbal-Eco RI intron probe (see Methods). Lanes *2, 3, 6,* and *7* correspond to Lebanese patients 25, 26, 7, and 16, respectively; lanes *1, 4,* and *5* to their parents; and lanes *8–19* to non-Lebanese GA patients from the following ethnic backgrounds: (*8*) Nicaraguan; (*9*) Welsh; (*10*) Scottish; (*11*) East Indian; (*12*) Portuguese; (*13*) Scottish; (*14*) Iraqi Jewish; (*15*) English; (*16*) Hungarian; (*17, 18*) Finnish, and (*19*) English.

each, from separate amplification reactions performed with DNA from patient 7. Thus, the same mutation was found in all amplified fragments from patients in both kindreds.

The nucleotide substitution creates a restriction site for Ssp I (AATGTT → AATATT). To confirm the presence of this mutation, we digested genomic DNA from these patients and their parents with Ssp I and performed Southern blot analysis (Fig. 4). The four patients lacked the 5.6-kb fragment present in all controls and, in contrast, had a 2.6-kb fragment not present in controls. Obligate heterozygotes had both fragments. The presence of a unique Ssp I fragment that segregates with the GA allele is consistent with the sequence alteration detected by PCR. We performed Ssp I genomic digests of 17 other GA patients from 12 ethnic backgrounds. 12 of these digests are shown in Fig. 4. A normal 5.6-kb fragment was observed in all non-Lebanese patients studied, indicating that this mutation is present only in the Lebanese GA pedigrees and that there is molecular heterogeneity in the mutations causing GA.

An ATG codon is essential for the initiation of translation in higher eukaryotes and in most prokaryotic genes (22). The ATA codon has negligible initiation activity in prokaryotic (23) and lower eukaryotic systems (24). Therefore, in transcripts containing the Lebanese initiator codon mutation, translation will start at an abnormal site, or not at all. There are no ATG triplets 5′ to the +1 residue in the OAT cDNA but five are present in the first 500 bp downstream. The first four, at residues 122–124, 149–151, and 212–215, and 296–298 are not in frame. The codon at residues 149–151 is flanked by purines at positions −3 and +4 with respect to its A residue, a favorable arrangement for translation initiation (22). In contrast, the sequence TTCCTATGA at the first in-frame ATG codon (residues 415–417) lacks a purine in the −3 position. Even if this ATG served as an initiation codon, the protein so formed should be nonfunctional, as it would lack 138 of the 439 amino acid residues of the normal OAT monomer including the mitochondrial leader (14). Thus it is likely that this mutation causes OAT deficiency and leads to GA in these patients.

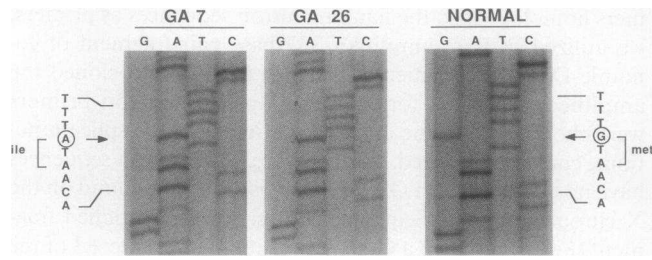

Figure 3. Sequence analysis of the coding strand of the amplified mutant DNAs. The normal G residue at position +3 is circled and the arrow indicates its position on the right panel; the mutant A residue is circled and indicated on the two left panels by the arrow.

We know of two other examples of initiation codon mutations in man. Pirastu and colleagues (25) found an AUG → ACG transition in the α^2-globin gene to be a common cause of α-thalassemia in Sardinia; and Liebhaber and co-workers have discovered an AUG → GUG transition in the α^1-globin gene of a patient with mild α-thalassemia (26).

Certain genetic diseases occur at high frequency in Lebanon, in part because several Lebanese ethnic groups have small, isolated gene pools (27). We would expect that a single or small number of mutant alleles, possibly unique to that population, would be found for each of these disorders. For example, Lehrman et al. (28) have recently described a mutant allele of the low density lipoprotein receptor gene in Lebanese Arab patients with familial hypercholesterolemia. Our finding of a mutant OAT allele in affected members of two Lebanese Maronite pedigrees, not present in GA patients from 12 other ethnic backgrounds, is also consistent with this expectation.

Only a small percentage of mutations cause restriction enzyme site changes detectable by Southern blot analysis (29, 30) and Northern blot analysis is of little help in localizing mutations (31). In contrast, the RNase A protection assay allows examination of the entire mature mRNA. Its sensitivity is dependent upon the degree of cleavage of a mismatch, which in turn depends upon the nonpaired nucleotides, the surrounding nucleotide sequences and experimental conditions (5–8). The OAT mutation that we describe leads to a C:A mismatch with cytosine in the labeled strand and results in ~ 25% cleavage. Isolation of probe bound to poly A RNA with oligo-dT paper as described by Gibbs and Caskey (5) reduced background and aided the detection of this subtle cleavage. Detection was also

enhanced by selection of patients likely to be true homozygotes rather than genetic compounds.

By combining RNase A protection studies with PCR, we characterized this OAT mutation without genomic cloning. Veres et al. coupled these methods to identify a point mutation causing murine ornithine transcarbamylase deficiency (9). To our knowledge, the initiator OAT mutation is the first human mutation to be discovered by this combination of techniques, and also represents the first molecular characterization of a mutation causing gyrate atrophy of the choroid and retina.

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