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Nonsense-Codon Mutations of the Ornithine Aminotransferase Gene with Decreased Levels of Mutant mRNA in Gyrate Atrophy

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Summary

A generalized deficiency of the mitochondrial matrix enzyme ornithine aminotransferase (OAT) is the inborn error in gyrate atrophy (GA), an autosomal recessive degenerative disease of the retina and choroid of the eye. Mutations in the OAT gene show a high degree of molecular heterogeneity in GA, reflecting the genetic heterogeneity in this disease. Using the combined techniques of PCR, denaturing gradient gel electrophoresis, and direct sequencing, we have identified three nonsense-codon mutations and one nonsense codon–generating mutation of the OAT gene in GA pedigrees. Three of them are single-base substitutions, and one is a 2-bp deletion resulting in a reading frameshift. A nonsense codon created at position 79 (TGA) by a frameshift and nonsense mutations at codons 209 (TAT→TAA) and 299 (TAC→TAG) result in abnormally low levels of OAT mRNA in the patient's skin fibroblasts. A nonsense mutation at codon 426 (CGA→TGA) in the last exon, however, has little effect on the mRNA level. Thus, the mRNA level can be reduced by nonsense-codon mutations, but the position of the mutation may be important, with earlier premature-translation termination having a greater effect than a later mutation.

Introduction

Gyrate atrophy (GA) of the choroid and retina is an autosomal recessive eye disorder involving a progressive loss of vision due to severe chorioretinal degeneration (Valle and Simell 1989). The underlying biochemical defect in GA is in the mitochondrial matrix enzyme, ornithine aminotransferase (OAT). The defect is systemic and leads to hyperornithinemia. The mechanism by which the OAT deficiency and hyperornithinemia lead to the chorioretinal degeneration, however, is not known.

A variety of OAT mutations have been reported in GA patients, by our group and others (Inana et al. 1988, 1989; Mitchell et al. 1988, 1989*b;* Ramesh et al. 1988; McClatchey et al. 1990; Akaki et al. in press), demonstrating a high degree of molecular heterogeneity in this disease. In view of this, a rapid and efficient method of detection of mutations would be useful for the study of GA. Such a method is a combination of denaturing gradient gel electrophoresis (DGGE) and direct sequencing of PCR-amplified (Saiki et al. 1985) gene sequences. DGGE is a gel system developed by Fischer and Lerman (1983) that separates DNA fragments according to their sequence-specific melting properties and that is capable of resolving DNA fragments differing by as little as a single base. Attachment of a GC-rich region (GC clamp) to PCR-amplified genomic DNA fragments has improved the resolution of DNA fragments shown, by DGGE, as containing a single-base substitution (Sheffield et al. 1989). Using these techniques, we have identified three nonsense-codon mutations and one

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nonsense codon–generating mutation of the OAT gene in GA pedigrees. Three nonsense mutations are due to single-base substitutions in codons 209, 299, and 426, and one (codon 79) is caused by a 2-bp deletion at codons 64 and 65, resulting in a reading frameshift. The nonsense-codon mutations result in a variable decrease in the level of steady-state OAT mRNA, with the earlier premature terminations in the coding sequence appearing to have a greater effect than the later ones.

Subjects and Methods

Subjects

Patient 1.—This patient was diagnosed, at the age of 23 years, as having GA. Retinal findings were characteristic, as was the elevation of plasma ornithine. No change in plasma ornithine was noted on pyridoxine therapy. OAT activity was not detectable in cell extracts from skin fibroblasts. The patient was adopted at birth in Canada, and no family information was available.

Patient 2.—The clinical characteristics of this patient have been described elsewhere (patient 4 of Weleber et al. [1982] and Kennaway et al. [1989]; also see fig. 12-5 in Weleber and Kennaway 1988). OAT activity was not detectable in cell extracts from skin fibroblasts. Southern blot analysis and northern blot analysis of this patient and his family have been reported elsewhere (Inana et al. 1988; Hotta et al. 1989). This patient has a partial heterozygous deletion of 1,072 bp, including exon 6, of the functional OAT gene (Akaki et al., in press). Northern blot analysis revealed no detectable OAT mRNA. The patient is of English, Dutch, German, and French ancestry.

Patient 3.—This 19-year old male of English and German ancestry had the GA diagnosis made at 19 years of age. Night vision had been poor since early childhood. With $-8.00 + 0.50$ axis 125° he saw 20/80 ±, J3 at near, right eye, and with -4.00 sphere he saw 20/300, J12 at near, left eye. He had normal color vision in the right eye and a moderate color deficiency in the left eye, midperipheral scotomas between 10° and 30° from fixation, and, on Goldmann perimetry, marked decrease of solid angles of peripheral kinetic visual fields (Weleber and Tobler 1986) (IV4e test Isopter was 1.41 steradian [right] and 1.36 steradian [left] [normal 3.59 ± 0.25], and I4e test Isopter was 0.274 steradian [right] and 0.077 steradian [left] [normal 2.621 ± 0.266]). His examination showed moderately advanced posterior subcapsular cataracts bilaterally, typical total vascular atrophy of the peripheral choroid and retina, and a nonaveraged Ganzfeld electroretinogram that was undetectable above noise (<10 μV). Serum ornithine level before treatment with vitamin B_6 was 912 ± 21 μmol/liter and, after treatment with 300 mg/d, was 903 and 912 μmol/liter (normal 83 ± 30 μmol/liter). OAT activity was not detectable in extracts from skin fibroblasts.

Patient 4.—The clinical characteristics of this patient have been described elsewhere (fig. 12-1 of Weleber and Kennaway 1988). OAT activity was 10 ± 3 nmol/mg protein/h (normal 276 ± 111) in cell extracts from skin fibroblasts (patient 8 of Kennaway et al. 1989). The patient is of Italian, Dutch, and Irish ancestry.

Patient 5.—The clinical characteristics of this patient have been described elsewhere (patient 2 of Hayasaka et al. 1981). OAT activity was not detectable in cell extracts from skin fibroblasts. The patient is Japanese. None of the five patients was responsive to vitamin B6 therapy.

Southern Blot Analysis

High-molecular-weight DNA was isolated from the patients' skin fibroblasts according to the method of Blin and Stafford (1976). The DNA was digested with several restriction enzymes, electrophoresed on 0.8% agarose gel, transferred onto nylon membrane, hybridized with the human OAT cDNA, washed, and autoradiographed, as described by Inana et al. (1988).

Northern Blot Analysis

Total RNA was isolated from the patient skin fibroblasts by the guanidine thiocyanate method (Chirgwin et al. 1979), electrophoresed on 0.8% denaturing agarose gel, transferred onto nylon membrane, hybridized with the human OAT cDNA or actin cDNA, washed, and autoradiographed, as described by Inana et al. (1988). Actin probing was performed to check the quality and quantity of RNA present on the blot for each lane. The intensities of the hybridization bands were quantitated by densitometry.

Amplification of Genomic DNA by PCR

Short segments of genomic DNA were amplified by the PCR technique using the *Taq* DNA polymerase (Perkin Elmer Cetus). A pair of primers (22-mer) complementary to the sense and antisense strands were chosen from the intron sequence flanking each exon.

PCR amplification was carried out for 30 cycles on a DNA thermal cycler (Perkin Elmer Cetus); each round consisted of 1 min at 94°C for denaturation, 1 min at 54–60°C for annealing, and 1 min at 72°C for extension.

DGGE

A 5′ primer, which included the above-mentioned original 5′-primer sequences to which a 40-bp GC-rich sequence (GC clamp) was added, was synthesized (Sheffield et al. 1989). The amplification was performed using the original 3′ primer and the GC-clamped 5′ primer. Each of the amplified sequences was tested with the computer programs MELT, MU, and SQHTX (Lerman and Silverstein 1987) to determine the optimal condition for the gel run.

The denaturing gradient gel apparatus used in this investigation consisted of the PROTEAN® II xi cell (BIO-RAD) system placed in a 60°C water bath (Myers et al. 1987). The denaturing gel is a 6.5% polyacrylamide gel containing a linearly increasing gradient from 20% to 80% denaturant. The 100% denaturant consists of 7 M urea and 40% (v/v) formamide. The PCR-amplified product was electrophoresed at 80 V or 150 V for 6–15 h. After electrophoresis, the gel was stained in ethidium bromide (2 μg/ml) for 15 min and was photographed by UV transillumination with Polaroid type 57 panchromatic film.

Direct Sequencing of PCR products

A sequencing primer located internal to the amplification primer for a PCR-amplified DNA was end labeled with [γ-^{32}P] ATP (3,000 Ci/mmol) by using T4 DNA polynucleotide kinase. Double-stranded amplified DNA (200–300 ng) was sequenced by a direct genomic-sequencing technique using a Sequenase kit (United States Biochemical) containing T7 polymerase, according to a method described by Newton et al. (1988).

Results

Southern and Northern Blot Analysis of the OAT Gene

Southern blot analysis of genomic DNA extracted from skin fibroblasts of patient 2 demonstrated one normal appearing OAT allele and another with a partial heterozygous deletion, as previously reported (data not shown; Inana et al. 1988; Hotta et al. 1989). Southern blot analysis of patients 1 and 3–5 did not reveal any modification of the OAT gene fragments with the OAT cDNA probe, eliminating the possibility of a gross deletion or rearrangement of the OAT gene.

Northern blot analysis of skin fibroblast RNA from the patients with standardization, by actin mRNA hybridization and densitometric analysis, of quantity and quality of RNA present in each lane demonstrated a variable decrease in the level of OAT mRNA in five patients (fig. 1). The steady-state levels of total OAT mRNA, which were densitometrically determined and normalized, are shown in table 1. Presumed allele-specific mRNA levels, which had a range of 0%–100%, were estimated from the results of the complete analysis, as described in the Discussion. The analysis was repeated three times to confirm the results.

DGGE and Direct Sequencing of PCR-amplified OAT Gene

In patient 1, DGGE revealed two separate exon 3 bands and four exon 6 bands, which represents a pattern for a heterozygote (fig. 2). In exon 6, the bottom band migrating with the normal band represents the normal allele, the second band up from the bottom represents the mutant allele, and the top two bands represent the heteroduplex DNA fragments produced by PCR as artifacts. This is a typical DGGE pattern for heterozygosity. In exon 3, the lower band is made up of the normal allele and a comigrating mutant allele, making it broader, and the upper band contains heteroduplex DNA fragments that are comigrating, resulting in an atypical heterozygous pattern. Direct sequencing analysis of the PCR-amplified exon 3 region revealed a 2-bp deletion (AG) (fig. 3*A*) in codons 64 and 65, which results in a reading frameshift and a premature stop codon at codon 79 (T GAC→TGA C) in exon 4. Direct sequencing of the exon 6 region revealed a C→A nucleotide substitution in the second nucleotide of codon 199, replacing a Pro with a Gln (data not shown).

Patient 2 was found to have a single exon 6 band that did not migrate with the normal band (fig. 2). This single-band pattern in DGGE normally represents a pattern of a homozygous mutation or failure to obtain a PCR product from one of the alleles. The latter, in fact, was the case in this patient, and the pattern represents a compound heterozygote, because the other allele has been shown to have a partial deletion of the gene, including exon 6 (Inana et al. 1988; Akaki et al., in press). Direct sequencing of the exon 6 region showed a T→A nucleotide substitution in codon 209 (fig. 3*B*). This alteration results in a nonsense mutation, replacing a Tyr with a TAA stop codon. The

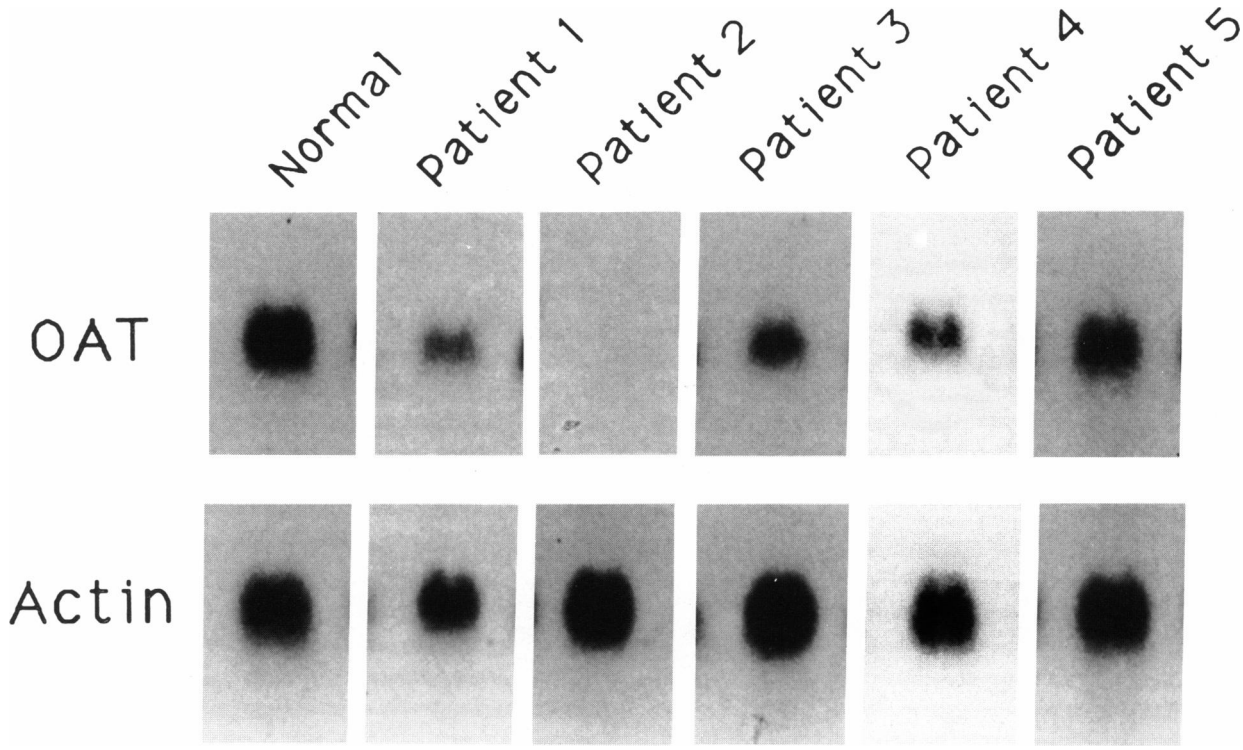

Figure 1 Northern blot analysis of OAT and actin mRNA in five GA patients with nonsense-codon mutations. The quantity and quality of RNA present in each lane was standardized by actin mRNA hybridization and densitometric analysis, and the normalized OAT mRNA levels relative to those in the control are given in table 1.

deletion including exon 6, in the other allele, also results in a premature stop codon in exon 7, because of a reading frameshift (data not shown; Akaki et al., in press).

Patient 3 had three separate exon 6 bands by DGGE, indicating heterozygosity (fig. 2). The bottom band migrated in a manner identical with that shown by patient 2's fragment and represents the mutant allele. Direct sequencing analysis of exon 6 showed, in addition to the normal T nucleotide, T→A nucleotide substitution in codon 209 (Tyr to stop codon), identical to that in patient 2, which is consistent with a pattern for a heterozygote (Fig. 3*B*). Direct sequencing of the exon 10 region, where the other mutation was detected by DGGE, revealed a G→A nucleotide substitution in the second nucleotide of codon 373, replacing a Gly with a Glu (data not shown).

Patient 4 had three separate bands in exons 6 and 8, by DGGE, indicating heterozygosity in these regions (fig. 2). A heterozygous C→G nucleotide substitution in codon 299 in exon 8 was identified by direct sequencing analysis (Fig. 3*C*). This mutation results in a nonsense mutation, replacing a Tyr with a TAG stop codon. Direct sequencing of the exon 6 region revealed a heterozygous G→C nucleotide substitution in the second nucleotide of codon 180, replacing an Arg with a Thr (data not shown). A homozygous G↔C nucleotide substitution is reported to be indistinguishable by DGGE (Traystman et al. 1990). Thus, in this case, G↔C mutations were detected by DGGE, because of their heterozygosity and the presence of heteroduplex bands.

Patient 5 was found, by DGGE, to have a single exon 11 band that did not migrate with the normal band, a result that represents either a pattern for a homozygous mutation or failure to obtain a PCR product from one of the alleles (fig. 2). A homozygous C→T nucleotide substitution in codon 426 was identified by direct sequencing analysis (Fig. 3*D*). This mutation results in a nonsense mutation, replacing an Arg with a TGA stop codon.

Discussion

By analysis of genomic DNA, using PCR in combination with DGGE and direct sequencing, we have

Table 1

Mutations in OAT Gene (439 Codons) in GA

Patient	Total OAT-mRNA Level[a] (%)	Mutations	Estimated Allele-specific OAT mRNA Level[b] (%)
1.....	50	Codon 64, 65, AGA GGA (2-bp deletion)→frameshift→stop codon (codon 79[c], exon 4)	0–10
		Codon 199 CCA (Pro)→CAA (Gln)	90–100
2.....	0	Codon 209 (exon 6) TAT (Try)→TAA (stop codon)	0
		Partial deletion of gene including exon 6→frameshift→stop codon (codon 192,[c] exon 7)	0
3.....	50	Codon 209 (exon 6) TAT (Try)→TAA (stop codon)	0
		Codon 373 GGA (Gly)→GAA (Glu)	100
4.....	60–70	Codon 299 (exon 8) TAC (Tyr)→TAG (stop codon)	20–40
		Codon 180 AGG (Arg)→ACG (Thr)	100
5.....	80–90	Codon 426 (exon 11, homozygous) CGA (Arg)→TGA (stop codon)	80–90

[a] Determined by densitometric analysis of northern blots (fig. 1), with normalization of RNA quantity and quality by actin mRNA hybridization, expressed as percentage of averaged normal level.

[b] Estimated on the basis of both codominance of expression of the OAT gene alleles and available information on the mRNA expression of specific alleles. See Discussion. Expressed as percentage of averaged normal level for an allele, which is half of the total level.

[c] Newly generated codon resulting from a reading frameshift mutation.

identified four premature-termination codon mutations in the OAT gene of five GA patients: three from point mutations and one from a frameshift mutation due to a 2-bp deletion. In one of these cases another premature-termination mutation—which results from a 1,072-bp deletion, elimination of exon 6, and a reading frameshift (Akaki et al., in press)—was also known. Northern analysis of OAT mRNA in these cases demonstrated a variable decrease in their steady-state levels.

The codominance of the expression of the OAT gene alleles has been amply demonstrated in the past, at both the protein level and the mRNA level (Valle et al. 1977; Hotta et al. 1989). Each allele appears to express its share of OAT independently of the other, be it at a normal or a subnormal level. This was clearly demonstrated, at the mRNA level, in obligate heterozygous carriers in a GA family in which the proband carried two null alleles (Hotta et al. 1989). Thus, one should be able to make a reasonable estimate of allele-specific OAT-mRNA levels if information is available both for the total and for one of the two alleles. Estimation of the allele-specific mRNA levels, on the basis of this principle and other information (discussed below), indicated that these nonsense mutations result in a variable decrease in the level of steady-state OAT mRNA.

Our patient 1 was a compound heterozygote with a 2-bp deletion producing a premature-termination codon in one allele and a missense mutation in codon 199 in the other allele, identical to the missense mutation reported by Kaufman et al. (1990). Since no abnormality of mRNA level was mentioned for the codon 199 missense mutation, the allele with the premature-termination codon (new codon 79) appears to lead to nearly a 100% decrease in the level of *its* OAT mRNA, resulting in a net total decrease of approximately 50% (table 1). Patient 2, whose northern blot analysis revealed no detectable OAT mRNA, had compound heterozygous mutations in the OAT gene,

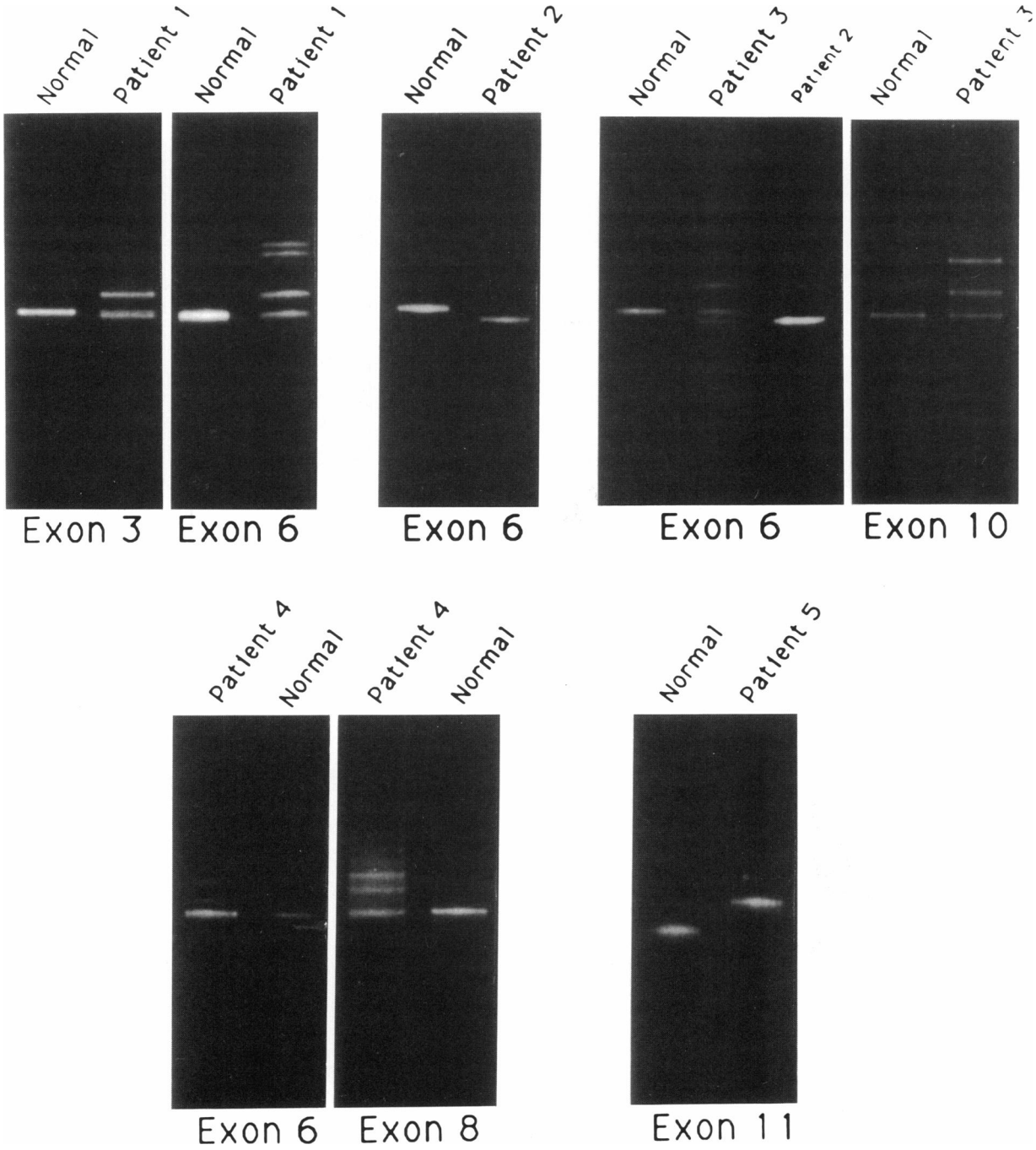

Figure 2 Detection of mutations in exons of the OAT gene, by DGGE, in five GA patients. GC-clamped, PCR-amplified fragments of each exon were electrophoresed in a denaturing gradient gel and were screened for mutations. Patients 1, 3, and 4 demonstrate a heterozygous pattern of mutations with heteroduplexes, and patients 2 and 5 demonstrate a homozygous pattern with only homoduplexes on the gel. Patients 2 and 3 have the same mutation in exon 6, demonstrated by a mutant allele showing identical migration in both patients and confirmed by sequencing.

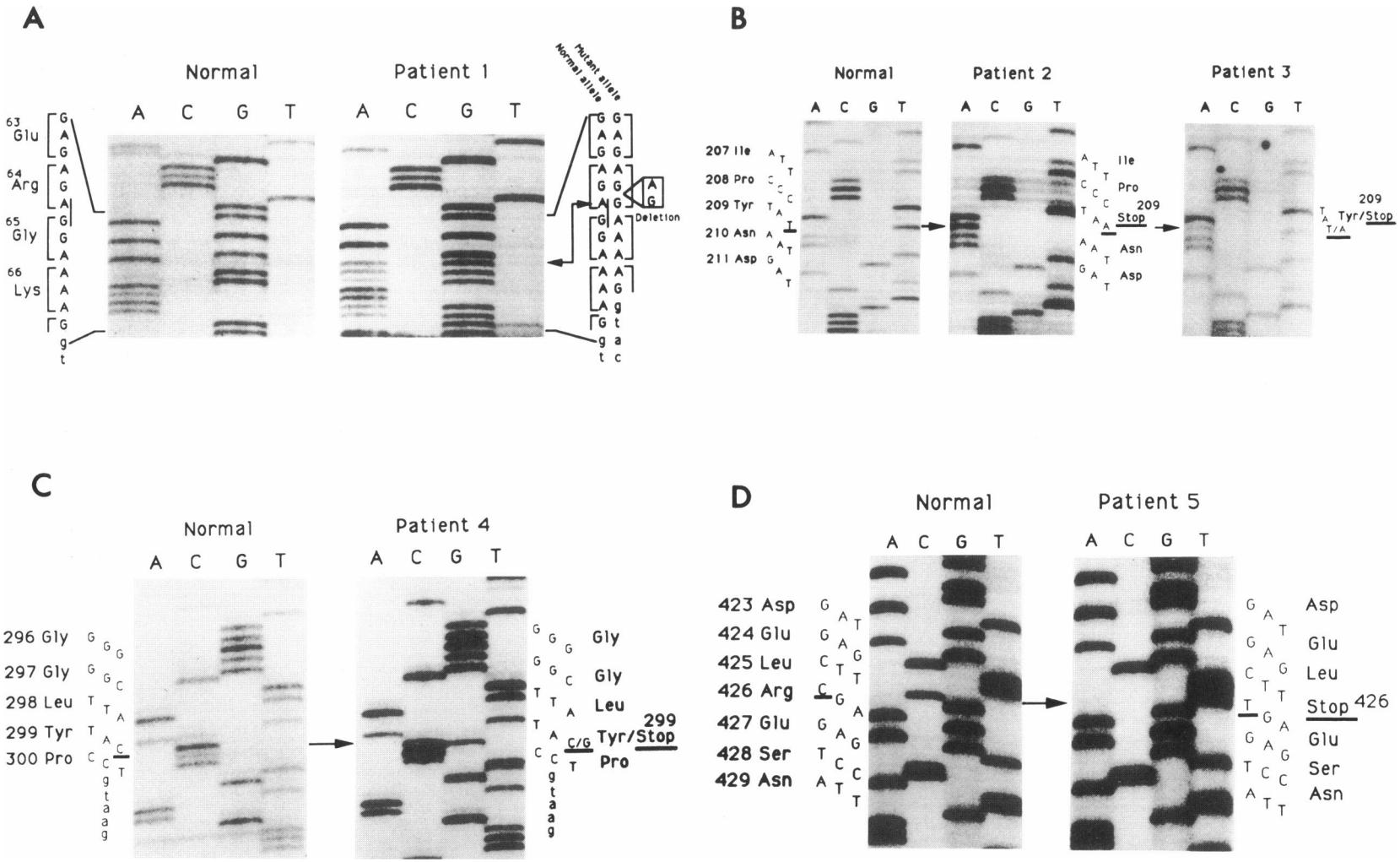

Figure 3 Direct sequencing of PCR-amplified exon sequences identified, by DGGE analysis, as containing a mutation. In panel *A*, patient 1 shows the normal and 2-bp deleted exon 3 sequence at the third nucleotide (A) of codon 64 and the first nucleotide (G) of codon 65. Both the normal and the mutant sequences are present starting at the third nucleotide (←→) of codon 64. In panel *B*, patients 2 and 3 show a T→A nucleotide substitution in the third nucleotide of codon 209, homozygously and heterozygously, respectively. In panel *C*, patient 4 shows a heterozygous C→G nucleotide substitution in the third nucleotide of codon 299. In panel *D*, patient 5 shows a homozygous C→T nucleotide substitution in the first nucleotide of codon 426. Lowercase letters represent intron sequences. The 5′ to 3′ direction is top to bottom.

consisting of a nonsense mutation in codon 209 in one allele and a partial deletion (including exon 6) of the gene, producing, in the other allele, a reading frameshift and a premature translation termination at a new codon 192 in exon 7. Since no mutation was detected in the 5′-flanking promoter region of the OAT gene by direct sequencing analysis, the lack of mRNA expression in both alleles is likely due to the premature-termination codons (table 1). That the abnormal, truncated mRNA from the partially deleted allele could also be unstable and rapidly degraded cannot be ruled out. Absence of mRNA also explains the negligible amount of immunoreactive OAT protein previously found in fibroblast mitochondria from this patient (Kennaway et al. 1989). Patient 3 was also a compound heterozygote with a codon 373 missense mutation and a codon 209 nonsense mutation identical to that in patient 2. In patient 3, as in patient 2, the allele with the codon 209 nonsense mutation is predicted not to express OAT mRNA, such that the missense allele is the only expressing allele, resulting in a net total OAT mRNA level of 50% (table 1), which is in agreement with the result from the northern analysis. Patient 4 was another compound heterozygote with a codon 299 nonsense mutation, a codon 180 missense mutation, and an mRNA decrease. Since the northern blot analysis of a GA patient with a homozygous codon 180 missense mutation was reported to show normal mRNA expression (Mitchell et al. 1989*b*), the decreased level of OAT mRNA must have resulted from the codon 299 nonsense mutation. The codon 299 nonsense mutation appears to lead to nearly a 60%–80% decrease in the level of expressed OAT mRNA (or expression of 20%–40% of normal), resulting in a net total decrease of 30%–40% (or net total expression of 60%–70% of normal) (table 1). This is consistent with our previous demonstration, by immunoblotting, of an appreciable level of normal size OAT protein, presumably expressed by the missense allele in fibroblast mitochondria from this patient (Kennaway et al. 1989). In patient 5, who has a homozygous codon 426 nonsense mutation, the mutation appears to result in only a slight, equal decrease (10%–20%) in the level of OAT mRNA for each of the alleles, which result in a net total decrease of the same amount (or net total expression of 80%–90% of normal) (table 1). There is a small possibility that this patient is actually a compound heterozygote but that only one allele in the exon 11 region could be PCR amplified and examined. Even if this were the case and even if all of the mRNA decrease were due to the codon 426 nonsense mutation, however, the estimated decrease (20%–40%) would still be the least among all the nonsense codons examined here.

Thus, the level of mRNA can be affected by a mutation that affects translation, as shown by the five premature-translation-termination codon mutations described. In the past, two nonsense-codon mutations in the OAT gene have been reported. One was a nonsense mutation in the OAT-coding exon 11, codon 396 (CGA→TGA), but the level of OAT mRNA was not mentioned by Mitchell et al. (1989*c*). The other was a splicing mutation in intron 3, causing a reading frameshift that produced a premature-termination codon (Mitchell et al. 1989*a*). In this case, the level of OAT mRNA was reduced to about 10% of normal.

Nonsense mutations (Atweh et al. 1988; Baserga and Benz 1988; Daar and Maquat 1988; Lim et al. 1989; Urlaub et al. 1989; Kadokawa et al. 1990) and frameshift mutations that produce a nonsense codon (Baumann et al. 1985; Fojo et al. 1988; Lim et al. 1989) have been shown to decrease mRNA levels. Other mutations shown to decrease mRNA levels have included a deletion that removes both the promoter and the first exon of a gene (Hobbs et al. 1987) and a single mutation in the polyadenylation signal sequence (Higgs et al. 1983). Reversion of mRNA-decreasing nonsense mutations to either the wild-type or missense sequence resulted in normalization of the RNA levels (Urlaub et al. 1989). Three major mechanisms have been proposed to explain the mRNA decrease due to nonsense mutations. Nonsense mutations have been found to decrease mRNA levels by increasing mRNA's turnover rate without lowering its instantaneous rate of synthesis (Lim et al. 1989). This increase in turnover is thought to be mediated through ribosomal interaction. When a premature-translation-termination codon is encountered, ribosomes detach from the mRNA. The lack of ribosomal binding to a large section of mRNA may expose it to endogenous nuclease degradation, thus affecting mRNA half-life and turnover (Daar and Maquat 1988). This hypothesis would predict a positional effect of nonsense-codon mutations, such that an earlier premature termination would have a more deleterious effect on the mRNA than would a later one. The other two proposed mechanisms are (1) that the loss of mRNA from nonsense mutations results from a block in ribosome-mediated nuclear-to-cytoplasmic transport and/or (2) the effect that nonsense mutations have on mRNA metabolism in the nucleus (Humphries et al. 1984; Takeshita et al. 1984; Baserga and Benz 1988).

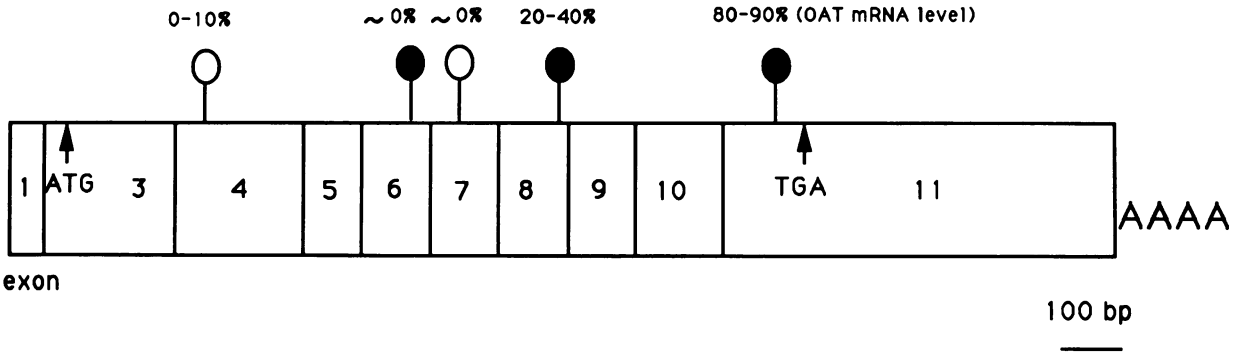

Figure 4 Schematic representation of nonsense-codon mutations in the OAT gene, and their effects on the OAT mRNA level. The numbered boxes represent the OAT gene exons; blackened ovals represent the premature terminations due to point mutations; and unblackened ovals represent the premature terminations due to reading frameshifts. ATG = translation initiation codon; and TGA = translation-termination codon. Estimated allele-specific OAT mRNA levels are indicated above each premature-termination mutation. The premature-termination codon generated in exon 7 by a reading frameshift because of a deletion in the exon 6 region (Akaki et al., in press) is included. The position of this codon in the mutant mRNA is where exon 6 is in the normal mRNA.

The mRNA-decreasing effect of the nonsense-codon mutations appeared to be variable. The degree to which the mRNA level was affected by the nonsense mutations appeared to depend on the position of the mutation in the coding sequence (fig. 4). It has been reported, for genes such as triosephosphate isomerase, that the correlation of mRNA levels with the position of premature-translation-termination mutations is not linear (Daar and Maquat 1988) but also that nonsense codon mutations in the last exon have little effect on the level of mRNA, whereas nonsense mutations in the first half of the mRNA result in decreased levels of mRNA (Baumann et al. 1985; Daar and Maquat 1988; Urlaub et al. 1989). The latter phenomenon was observed in our five GA patients with the nonsense-codon mutations. A significant decrease in OAT mRNA appeared to occur with premature terminations in codons 79 (exon 4), 192 (newly generated in exon 7), and 209 (exon 6), while very little decrease of OAT mRNA occurred with a nonsense codon at 426 (exon 11) (fig. 4). The normal length of OAT is 439 amino acids. There was a suggestion of linearity of the positional effect in our cases, in that the codon 299 (exon 8) nonsense mutation appeared to show an intermediate level of OAT mRNA decrease (60%–80%) (fig. 4). Our results support both (1) the importance of the position of the premature-termination mutation, with respect to its effect on mRNA, and (2) the ribosomal detachment hypothesis of the mechanism of mRNA decrease (Daar and Maquat 1988). Finally, the prevalence of compound heterozygosity (patients 1–4) and the diversity of the mutations (eight different mutations in five patients) confirmed the enormous genetic heterogeneity present in this disease.

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