

Type III 3-Methylglutaconic Aciduria (Optic Atrophy Plus Syndrome, or Costeff Optic Atrophy Syndrome): Identification of the *OPA3* Gene and Its Founder Mutation in Iraqi Jews

Yair Anikster,¹ Robert Kleta,¹ Avraham Shaag,² William A. Gahl,¹ and Orly Elpeleg²

¹Section on Human Biochemical Genetics, Heritable Disorders Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda; and ²Metabolic Disease Unit, Shaare-Zedek Medical Center, Faculty of Medicine of the Hebrew University, Jerusalem

Type III 3-methylglutaconic aciduria (MGA) (MIM 258501) is a neuro-ophthalmologic syndrome that consists of early-onset bilateral optic atrophy and later-onset spasticity, extrapyramidal dysfunction, and cognitive deficit. Urinary excretion of 3-methylglutaconic acid and of 3-methylglutaric acid is increased. The disorder has been reported in ~40 patients of Iraqi Jewish origin, allowing the mapping of the disease to chromosome 19q13.2-q13.3, by linkage analysis. To isolate the causative gene, *OPA3*, we sequenced four genes within the critical interval and identified, in the intronic sequence of a gene corresponding to cDNA clone *FLJ22187*, a point mutation that segregated with the type III MGA phenotype. The *FLJ22187*-cDNA clone, which we identified as the *OPA3* gene, consists of two exons and encodes a peptide of 179 amino acid residues. Northern blot analysis revealed a primary transcript of ~5.0 kb that was ubiquitously expressed, most prominently in skeletal muscle and kidney. Within the brain, the cerebral cortex, the medulla, the cerebellum, and the frontal lobe, compared to other parts of the brain, had slightly increased expression. The intronic G→C mutation abolished mRNA expression in fibroblasts from affected patients and was detected in 8 of 85 anonymous Israeli individuals of Iraqi Jewish origin. Milder mutations in *OPA3* should be sought in patients with optic atrophy with later onset, even in the absence of additional neurological abnormalities.

Introduction

3-Methylglutaconic aciduria (MGA) has been divided into four different disease categories (Gibson et al. 1993). Type I MGA, characterized by mild neurological disease, results from deficiency of 3-methylglutaconyl-CoA hydratase in the leucine-oxidation pathway. Type II MGA, or Barth syndrome, is an X-linked disorder that consists of dilated cardiomyopathy, short stature, and neutropenia and that results from mutations in the tafazzin gene (*G4.5*) (Bione et al. 1996). Type III MGA, or Costeff optic-atrophy syndrome, occurs in Iraqi Jews as a relatively homogeneous neuro-ophthalmologic disorder. In contrast, type IV MGA is extremely heterogeneous, with moderate-to-severe neurological disease, sometimes associated with cardiac, ophthalmic, hepatic, and renal symptoms.

The distinctive phenotype of type III MGA (MIM 258501) and its occurrence in members of a genetic isolate have led to the mapping of the responsible gene

to chromosome 19q13.2-q13.3 (Nystuen et al. 1997). Characteristic clinical manifestations include early-onset bilateral optic atrophy and later-onset spasticity, extrapyramidal dysfunction, ataxia, and cognitive deficit (Costeff et al. 1989, 1993). Urinary excretion of 3-methylglutaconic acid and of 3-methylglutaric acid is increased to variable degrees in all patients and is considered a hallmark of the disease, allowing diagnosis as early as 1 year of age, in infants with optic atrophy. Life span of affected individuals appears normal, and affected adults in the 4th decade of life have been reported (Elpeleg et al. 1994). Neither the basic defect in type III MGA nor the gene causing type III MGA has been identified. In this study, we sought candidate genes within the minimal disease region (between markers *D19S412* and *D19S918*), isolated the gene responsible for type III MGA, and determined its tissue-expression pattern; we also identified the founder mutation causing the disease in the Iraqi Jewish isolate that we studied, developed a screening assay for the mutation, and estimated its population frequency.

Subjects and Methods

Subjects

DNA was available from 10 patients, 7 obligatory heterozygotes, and 4 healthy siblings of eight Iraqi Jew-

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Address for correspondence and reprints: Dr. Orly Elpeleg, Metabolic Disease Unit, Shaare-Zedek Medical Center, Jerusalem 91031, Israel. E-mail: elpeleg@cc.huji.ac.il; or elpeleg@szmc.org.il

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ish families. All the patients exhibited optic atrophy, a movement disorder, and increased urinary excretion of 3-methylglutaconic acid and of 3-methylglutaric acid. Informed consent was obtained from all patients in these studies.

DNA of 75 anonymous individuals of Iraqi Jewish origin was purchased from the National Laboratory for the Genetics of Israeli Populations. An additional 10 samples from anonymous Iraqi Jews were obtained from the Israeli familial-Mediterranean-fever screening program. DNA samples of North Americans were obtained from patients with other disorders, after written informed consent was obtained.

Sequencing and Sequence Analyses

The sequences of *NOVA2*, *FLJ20084*, *FLJ22187*, and *SYMPLEKIN* were determined using genomic DNA obtained from cultured fibroblasts from three patients with type III MGA who originated from different families. DNA of other family members and of anonymous controls was obtained from peripheral leukocytes.

PCR amplification of each exon of the *FLJ22187* clone was performed by amplifying 200–400 ng of genomic DNA by use of Ready-To-Go PCR Beads (Amersham Pharmacia Biotech) with 10 pmol each primer, in a total volume of 25 μ l. Cycling parameters included initial denaturation at 96°C for 4 min; followed by cycles

of denaturation at 94°C for 1 min, annealing at 58°C for 45 s, and extension at 70°C for 90 s; and concluding with elongation at 72°C for 10 min. The PCR products were electrophoresed in 1% agarose and were stained with ethidium bromide. Oligonucleotide primers were designed using the sequence of chromosome 19 and are as follows: F1 (5'-CGTACATACGTACTGACGCA-3'), R1 (5'-TAAGCAACCACCTGACAGG-3'), F2 (5'-TCC-CAGAGCGCAGCCTGAC-3'), and R2 (5'-GCCAAGT-TGCATCAAGATCCT-3').

Automated sequencing was performed on a Beckman CEQ 2000, by the CEQ Dye Terminator Cycle Sequencing kit, according to the manufacturer's protocol (Beckman Coulter). BLAST analysis was performed for sequence-homology searches, available through the National Center for Biotechnology Information. Analysis of the amino acid sequence was performed by the PSORT software.

Mutation Screening

A 540-bp fragment containing two *RsaI* restriction sites was created using primer R2 and the "mutation-detection primer" (MDP [5'-GACCCCTCTCTTCCCC-CGTA-3']). Incubation of the normal fragment at 37°C for 3 h with *RsaI* resulted in its cleavage into three fragments (of 280 bp, 180 bp, and 80 bp), which could be separated on a 2.5% agarose gel. Incubation of the frag-

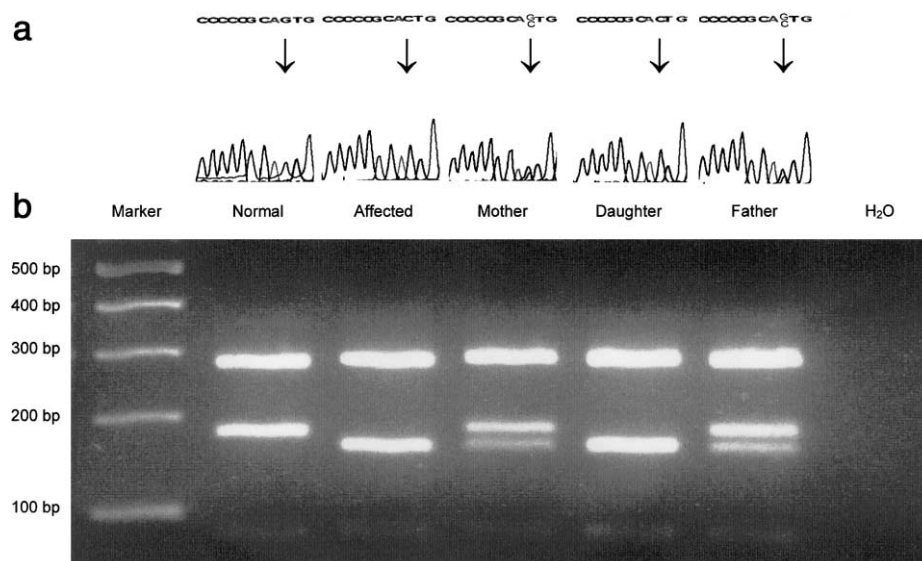


Figure 1 Demonstration of founder mutation in *OPA3* in Iraqi Jewish family. *a*, Sequence of intron 1/exon 2 boundary of *OPA3*, in normal control, affected patient, and three family members. The G→C mutation was present in the homozygous state in the daughter and in the heterozygous state in the mother and the father. *b*, Gel electrophoresis of PCR-amplification products cut with *RsaI*. A 540-bp fragment in the region of interest was amplified by primers R2 and MDP, the latter of which was specifically designed to introduce an additional *RsaI* cleavage site into the product formed using the mutant sequence as template. Treatment with *RsaI* produced a 280-bp band in all patients; in addition, a 180-bp fragment was visible in normal individuals, a 161-bp fragment was apparent in affected patients, and the 180-bp and 161-bp bands were both present for obligate heterozygotes.

ment created by amplification of the mutant DNA yielded four fragments (of 280 bp, 161 bp, 80 bp, and 19 bp). The mutation could be detected by the appearance of a 161-bp band instead of the 180-bp band.

Northern Blot Analysis

Multiple-tissue northern filters, loaded with poly(A+) RNA (2 μ g/lane) derived from a number of human tissues, were purchased from Clontech. In addition, we isolated total RNA (20 μ g) from cultured fibroblasts by use of Trizol reagent (Life Technologies). The RNA was separated on a 1.2% agarose/3% formaldehyde gel and was blotted onto a Nytran nylon membrane (Schleicher and Schuell) in the presence of 20 \times SSC. Blots were prehybridized and then were hybridized with Express Hyb solution (Clontech) at 68°C. The probe was composed of human *OPA3* cDNA, was 619-bp long, was random-primer labeled with α -[³²P]-dCTP (DuPont/New England Nuclear), and was prepared by PCR amplification of normal cDNA, by primers CF1 (5'-GGTTGC-GCGTGCCCTGTGA-3') and CB1 (5'-ACGTTAGGTACATAGGCCATG-3'). The same set of filters was also probed with β -actin.

Results

The gene for type III MGA, *OPA3*, was previously shown, through linkage studies, to reside between polymorphic markers *D19S918* and *D19S412*. Of the many genes located within this region, we chose four for intensive investigation. Two genes—*NOVA2* (neuro-oncologic ventral antigen 2) and *SPK* (or *SYMPLEKIN*, encoding hun-

tingtin interacting protein I)—were chosen owing to their expression in brain and to their putative connections with the clinical manifestations of the disease. Exhaustive sequencing, however, revealed no mutations in either gene. Two other clones—*FLJ20084* and *FLJ22187*—were studied, specifically because their function was totally unknown. Sequencing of *FLJ20084* revealed no mutations; however, the *FLJ22187* clone could not be amplified from the cDNA of patients with type III MGA, although control cDNA yielded the expected 619-bp band (data not shown). We next elucidated the genomic structure of the *FLJ22187* clone, by BLAST sequence analysis. The complete gene has two exons: exon 1 is located in clone CTB-124I16 (GenBank accession number GI:10799401), and exon 2 is located in cosmid R32889 (GenBank accession number GI:4079613). This information enabled us to design primers for the coding region of *OPA3* in genomic DNA.

These primers were employed to determine the *OPA3* mutation causing type III MGA in our cohort of patients. We sequenced *OPA3* coding exons, as well as their adjacent splice-site regions, using genomic DNA of three affected individuals. Each patient exhibited a homozygous G→C change at the -1 position of intron 1 in the 3' (acceptor) splice site. This information permitted the development, by use of genomic DNA, of a restriction-enzyme-based assay of the mutation. We designed a PCR primer, called "MDP," containing a specific nucleotide mismatch 2 bases upstream of the G→C mutation, so that the amplified fragment would contain an additional *RsaI* restriction site in the mutated allele but not in the normal allele. Amplification and cleavage

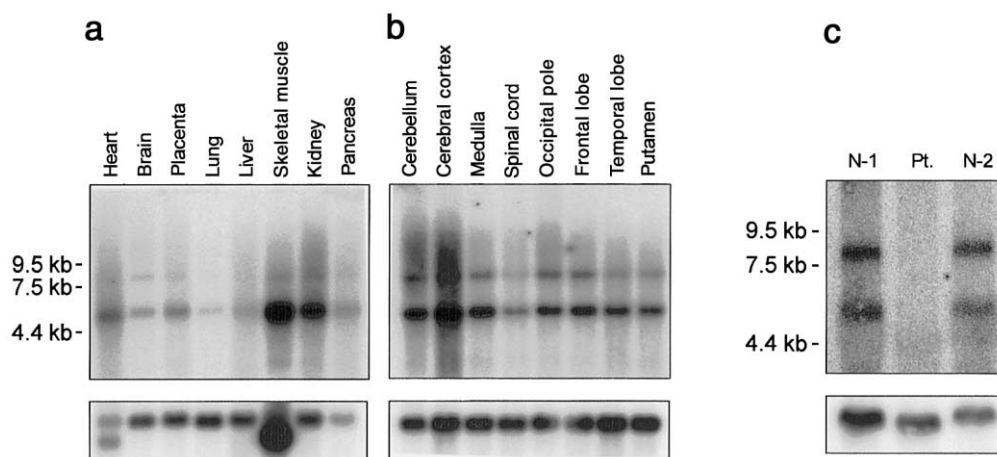


Figure 2 Northern blot analyses by 619-bp probe to human *OPA3* cDNA. *a*, Probe hybridized to 5.0-kb band and secondary 8.0-kb band in all tissues tested. Expression appeared greatest in skeletal muscle and kidney. The lower panel shows the band for β -actin, which served as a control for RNA loading. *b*, Same bands on human multiple-tissue northern blot, demonstrating expression in all parts of brain. *c*, 5.0-kb and 8.0-kb bands of RNA from normal fibroblasts (N-1 and N-2), which hybridized with *OPA3*-cDNA probe, whereas no bands appeared on use of RNA from fibroblasts from one Iraqi Jewish patient (Pt.).

with *RsaI* revealed that the G→C mutation segregated with type III MGA in 10 affected individuals, 7 obligate heterozygotes, and 4 healthy siblings. An example is presented in figure 1. The mutation was also found in 8 of 85 anonymous Israelis of Iraqi Jewish origin but in 0 of 55 North Americans.

Northern blot analysis by a 619-bp *OPA3*-cDNA probe revealed 5.0-kb and 8.0-kb messages that were ubiquitously expressed, most prominently in skeletal muscle and kidney (fig. 2a). Within the brain, the cerebral cortex, the medulla, the cerebellum, and the frontal lobe, compared with other parts of the brain, had slightly increased expression (fig. 2b). The message was present in normal cultured fibroblasts but was not present in fibroblasts obtained from an affected individual (fig. 2c).

To confirm the open reading frame of *OPA3*, we performed an expressed-sequence-tag search, sequenced the region 5' to *FLJ22187*, and located a stop codon (tga) at position -150 with respect to the ATG translation start site (fig. 3). *OPA3* consists of this 5' UTR, an open reading frame encoding 179 amino acids, and >970 nucleotides of 3' untranslated sequence (fig. 3). The protein product is predicted to be a 20-kD peptide and to consist of 61% α helix, 12% extended strand, 4% β turn, and 22% random coil. The sequence contains a mitochondrial targeting peptide, NRIKE, at amino acid residues 25–29 and is predicted, with a probability of .87, to be exported to the mitochondrion. The gene product's first 114 amino acids are 54% identical to those of *Drosophila melanogaster* cytochrome B heme and 28% identical to those of a hypothetical coiled-coil protein of *Schizosaccharomyces pombe*. A conserved motif of 22 amino acids exists among the human, the *D. melanogaster*, and the yeast homologues of *OPA3* (fig. 4).

Discussion

Metabolic diseases characterized by MGA have confused students for decades. Of the four categories of MGA, the most frustrating to study remains type IV, a category in which patients present with phenotypes that have not yet been characterized by a primary enzymatic or molecular defect; some instances of this type may eventually be assimilated into other existing disease categories, including the other three types of MGA. The first step toward revealing the pathophysiology of these three types has been made—that is, they have all had their causative genes isolated. Type I MGA results from absence of 3-methylglutaconyl-CoA hydratase activity, type II MGA results from mutations in tafazzin, and we now report the sequence, genomic organization, and mRNA product of *OPA3*, the gene responsible for type III MGA. The prominent expression of *OPA3* mRNA in the cerebral cortex,

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                                     tga
gacctggaagtttcctcagagcgtacgtacatcacgtacgacgcac
gcatggccacgagagggcggggaggagtgaagagggtgagggcgc
cccgccagtcagcaaggttgccgtgcccctgtgagaccgccaag
1 atgggtggtggcgcttcctatggcgaagctgtatacttgggc
  M V V G A F P M A K L L Y L G
46 atccggcaggtcagcaagccctgtgccaacctattaaggaggcc
  I R Q V S K P L A N R I K E A
91 gcccgccaagcagattcttcaagacctatctgcctcccgcgcg
  A R R S E F F K T Y I C L P P
136 gctcaagctgatcactgggtggagatgcggaaccaagatgcgcatc
  A Q H Y H W V E M R T K M R I
181 atgggtctccggggcacggtcatcaagccgtgaacgaggaggcg
  M G F R G T V I K P L N E E A
226 cgagctgagctgggagcagagctgctgggagcagccaccatcttc
  A A E L G A E L L G E A T I F
271 atcgtggcgccgctgccttagctgagtagctggcaccaccag
  I V G G G C L V L E Y W R H Q
316 gcgcagcagccacaaggaggaggagcagcgtgctgctggaac
  A Q Q R H K E E E Q R A A W N
361 gcgctgggagcaggtgggccaacctggcctggcctggaagcg
  A L R D E V G H L A L A L E A
406 ctgcagcgcaggtgcagcggcgccaccagggcgccctggag
  L Q A Q V Q A A P P Q G A L E
451 gaactgcgcacagagctgcaagaggtgcgcccagctctgcaat
  E L R T E L Q E V R A Q L C N
496 cccggcggctccgcttcccagcagctgctgctccaagaatag
  P G R S A S H A V P A S K K *
gagcttgctgagtggaacctgaattggacatggcctatgtacct
aacgtggccttctcccgcaccaccttgctgctgctggcccagt
ggaaaccaccaggatcttgatgcaactggcattgggtaccctc
gctgataagagcagccgttacctgccaactgggaccagcaggtgaa
gcgttgcaacatagccccctccatcatcctaccctcctatcccc
cactccaaccaggacgacctgcaaggtcccagccagcagggacac
cgtgggcaactctggcaaatgaaaaaatggaacctgggtctgagct
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gtgttaaccttactgggacactcactgttacactggaacacct
tcttctttttgtcaactcggaacagaccactgtaaggaatgcaagt
gtgtgcaagtggccttttctcccctcacccttaaggtcagctc
tagctgagcatcagtgctctcttaaggaggaaaaaacggtgagg
ctgggagcgggtggctcagcctgtaactctagcacttgggaggc
cgaggcgggggagtcacttgaggtcagagttccagaccagcctg
gccaacatggtgaaactcgtctctactaaaaatataaaaattag
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tgaggcaggagaattgcttgaaacctagaggtggaggtgctgctg
agccaagatggcaccattgcaacctagcctgggcaacagagcaag
acaccgtcttaaaacaaaagtaaccggggcgtggtggtgggtgct
ctgtaactcagctacttgggaggtgagcagaggagaattgcttg
aacctgggaggtggaggccaagattgtaccactgtattccagccc
gggtgacagagcaagactgtctc

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Figure 3 *OPA3* gene and protein sequence. Nucleotide 1 corresponds to nucleotide 22 of *FLJ22187*. The first three nucleotides, shown in boldface, comprise the nearest stop codon 5' to the open reading frame. The arrow after nucleotide 142 denotes the border between exons 1 and 2.

the medulla, the cerebellum, and the frontal lobe is consistent with the spasticity and the ataxia observed in patients with type III MGA, who lack a functional gene product.

The product's function remains unknown, and homologues to proteins of other species have not been illustrative; however, several findings suggest that the *OPA3* protein plays some role in mitochondrial processes:

1. *OPA3* contains a mitochondrial targeting signal. Al-

Human	10	K	L	L	Y	L	G	I	R	Q	V	S	K	P	L	A	N	R	I	K	E	A	A
<i>D. melanogaster</i>	10	K	L	A	L	L	A	I	K	H	I	S	K	P	I	G	N	L	I	K	Q	T	A
Yeast	7	K	I	G	S	L	L	V	R	T	L	S	K	P	I	A	N	T	I	K	A	Q	A

Figure 4 Conserved motif of 22 amino acids within human, *D. melanogaster*, and yeast homologues of *OPA3*. Numbers indicate the position of the first amino acid in the conserved region; letters in boldface indicate amino acids conserved among all three species.

though supporting evidence from cell-biological studies would be required, the putative mitochondrial localization of *OPA3* would be consistent with the optic atrophy observed in patients with type III MGA. Individuals with mitochondrial disorders also exhibit optic atrophy; specifically, autosomal dominant optic atrophy results from mutations in *OPA1* (which encodes a dynamin-related mitochondrial protein), and Leber hereditary optic atrophy (variably associated with a movement disorder) results from mtDNA mutations (Riordan-Eva and Harding 1995; Delettre et al. 2000). Notably, digitonin-permeabilized fibroblasts from two patients with type III MGA exhibited a 50% reduction in ATP production and a fourfold increase in lactate:pyruvate ratio (Wanders et al. 1992; A. Saada and O. Elpeleg, unpublished data), both of which are indications of impaired mitochondrial function.

2. The presence of increased MGA itself suggests mitochondrial involvement. Fibroblasts from patients with type II MGA contain a markedly reduced amount of cardiolipin, a phospholipid found exclusively in the inner mitochondrial membrane and required for optimal function of respiratory-chain enzymes (Vreken et al. 2000). Mitochondrial respiratory-chain defects have been reported in several patients with type IV MGA (Ibel et al. 1993; Besley et al. 1995), and MGA has been reported in patients with other mitochondrial respiratory-chain defects, including mtDNA-depletion syndromes, mtDNA deletions, and ATP-synthase deficiency (Gibson et al. 1992; Holme et al. 1992; Scaglia et al. 2001). The link between MGA and mitochondrial respiratory-chain defects likely involves the mevalonate shunt; this pathway produces 3-methylglutaconyl-CoA from mevalonate via dimethylallyl pyrophosphate (Edmond and Popjak 1974), an intermediate in the synthesis of cholesterol and polyisoprenoids, such as ubiquinone. It has been speculated that defective ubiquinone biosynthesis could lead to increased levels of precursors (e.g., mevalonate) in the pathway, thereby increasing synthesis of 3-methylglutaconic acid (Kelley and Kratz 1995). We have seen no beneficial effect, on administration of an active form of ubiquinone, coenzyme Q₁₀, to three patients with type III MGA (Cos-teff et al. 1998).

3. The carboxy terminal motif, SKK, of the *OPA3* gene product somewhat resembles a peroxisomal targeting signal (Gould et al. 1989), and there is precedent

for the existence of mixed mitochondrial/peroxisomal proteins. In particular, 3-hydroxy-3-methylglutaryl-CoA lyase has both a mitochondrial targeting signal and a peroxisomal targeting motif and resides both in mitochondria and in peroxisomes (Ashmarina et al. 1994). The SKK motif found in the *OPA3*-gene product, however, has been specifically shown to target proteins to the cytoplasm (Gould et al. 1989), mitigating against a dual localization for *OPA3*.

Whatever the role played by the *OPA3*-gene product may be, a specific G→C intron 1 acceptor splice-site mutation causes the type III MGA phenotype in the patients whom we studied. This base substitution violates the GT-AG rule, changing the splice-junction score from 86.9% to 70.9% (Shapiro and Senepathy 1987). The resulting lack of mRNA expression manifests as the absence of an *OPA3* band on a northern blotting of fibroblasts from affected patients and as an inability to amplify the *FLJ22187* clone by use of patients' cDNA. The complete absence of an *OPA3* transcript is associated with early-onset optic atrophy and a movement disorder of variable severity, which begins in early adolescence. Milder mutations in *OPA3*, however, may cause optic atrophy with onset later in life and perhaps without additional neurological abnormalities.

The G→C splice-site mutation in *OPA3* segregated with type III MGA in the Iraqi Jewish families that we studied and was also detected in 8 of 85 anonymous individuals of Iraqi Jewish origin. This suggests that the mutation has a long history within this genetic isolate—which represents the original Middle-Eastern Jewish gene pool, derived from ~120,000 Jews who were exiled to Babylon in 586 B.C., after the destruction of the First Temple and of Jerusalem. The relative isolation of this population, for 2,500 years, resulted in a high (3.3%) carrier rate for the common mutation in the factor XI gene (Shpilberg et al. 1995), in a remarkably high (39%) carrier rate for the common mutation in the *MEFV* gene (which causes familial Mediterranean fever [Stoffman et al. 2000]), and in a frequency of 5% for the autosomal dominant disorder pseudocholinesterase deficiency (Zlotogora et al. 2000). According to the Israeli Central Bureau of Statistics, the number of Iraqi Jews living in Israel in 1998 was ~253,200. With a carrier frequency of ~1/10 for the founder mutation in *OPA3*,

the predicted number of patients affected with type III MGA would be 633. The actual number is lower, probably because the gene pool has been diluted. Since 1948, 127,000 Iraqi Jews have immigrated to Israel, and large communities exist in India, in the United Kingdom, in Montreal, and in Australia. It would be of great interest to determine the frequency of the OPA3 splice-site mutation and its associated disorder in these genetic isolates as well. It will also be of critical importance to identify (1) other patients with type III MGA and (2) other OPA3 mutations, to determine the phenotypic spectrum of this disorder.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

BLAST, <http://www.ncbi.nlm.nih.gov/BLAST/>
GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for human OPA3 [FLJ22187] [accession number GI:14761409], clone CTB-124I16 [accession number GI:10799401], cosmid R32889 [accession number GI:4079613], *S. pombe* hypothetical coiled-coil protein [accession number GI:6723925], and *D. melanogaster* homologue [accession number GI:7301068])

National Laboratory for the Genetics of Israeli Populations, <http://www.tau.ac.il/medicine/NLGIP/nlgip.htm>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for type III MGA [MIM 258501])

PSORT WWW Server, <http://psort.nibb.ac.jp/>

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