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ORIGINAL INVESTIGATION

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Mapping and genomic characterization of the gene encoding diacylglycerol kinase γ (*DAGK3*): assessment of its role in dominant optic atrophy (*OPA1*)

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Abstract The family of diacylglycerol kinases (DAGKs) is known to play an important role in signal transduction linked to phospholipid turnover. In the fruitfly *Drosophila melanogaster*, a human DAGK ortholog, *DGK2*, was shown to underlie the phenotype of the visual mutant retinal degeneration A (*rdgA*). Previously, the gene encoding a novel member of the human DAGK family, termed *DAGK3*, was cloned and demonstrated to be abundantly expressed in the human retina. Based on these findings we reasoned that *DAGK3* might be an excellent candidate gene for a human eye disease. In the present study, we report the genomic organization of the human *DAGK3* gene, which spans over 30 kb of genomic DNA interrupted by 23 introns. In addition, we have mapped the gene locus by fluorescence in situ hybridization to 3q27–28, overlapping the chromosomal region known to contain the gene underlying dominant optic atrophy (OPA1), the most common form of hereditary atrophy of the optic nerve. Mutational analysis of the entire coding region of *DAGK3* in 19 unrelated German OPA1 patients has not revealed any disease-causing mutations, therefore excluding *DAGK3* as a major cause underlying OPA1.

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Introduction

Diacylglycerol kinase (DAGK) plays an important role in the regulation of cellular signal transduction. It initiates the regeneration of phosphatidylinositols by phosphorylation of diacylglycerol (DAG) to generate phosphatidic acid (PA) (Kanoh et al. 1990) thus controlling the intracellular concentration of the second messengers DAG and PA. While DAG is the physiological activator of protein kinase C (Nishizuka 1984), PA has been shown to modulate several enzymes in vitro, including phospholipase C-γ1 (Jones and Carpenter 1993), polyphosphoinositide kinase (Moritz et al. 1992) and *ras* GTPase-activating protein (Tsai et al. 1990).

To date, eight mammalian DAGK isozymes have been identified that differ from each other by characteristic molecular masses, enzymologic properties, substrate specificities and cell-specific expression profiles (reviewed in Sakane and Kanoh 1997). All DAGKs share a conserved putative catalytic domain at the C-terminus and two cysteine-rich zinc-finger motifs, but can be further divided into subgroups according to unique structural features, including EF-hand motifs, pleckstrin homology domains and ankyrin-like repeats.

In our search for genes involved in hereditary human eye disorders we considered one member of the DAGK family, *DAGK3*, to be an attractive candidate for human retinopathies for two reasons. First, *DAGK3* has been shown to be predominantly expressed in the human retina (Kai et al. 1994). Moreover, mutations in the gene encoding an eye-specific diacylglycerol kinase *(DGK2)* are known to cause defective phospholipid turnover in the *Drosophila melanogaster* visual mutant retinal degeneration A (*rdgA*) (Masai et al. 1993). Although there is no direct evidence for an involvement of the phosphoinositide cycle in mammalian phototransduction (Stryer 1991), the abundant presence of retinal phosphatidylinositol-related enzymes such as phospholipase C and DAGK strongly supports a functional role of these molecules in the visual system (Ferreira et al. 1993).

To assess further the involvement of *DAGK3* in human retinal diseases we first mapped the gene locus to

3q27–q28, the chromosomal region that has previously been demonstrated by genetic linkage analysis to harbor the disease gene associated with dominant optic atrophy (OPA1) (Eiberg et al. 1994). OPA1 is the most frequent form within the group of hereditary optic atrophies and has an estimated incidence of 1:50.000 (Lyle 1990). It is inherited as an autosomal dominant trait with almost complete penetrance (Kivlin et al. 1983) but highly variable expressivity. The disease is typically characterized by an insidious onset of clinical symptoms in early childhood, reduction of visual acuity, central, paracentral or cecocentral scotomas, colour vision defects and bilateral atrophy of the optic nerve, which can be seen as a pallor of the optic disc (Jaeger 1966; Kline and Glaser 1979). There is usually a slow progression of visual dysfunction throughout life (Eliott et al. 1993). Histopathologic examination of donor eyes of affected OPA1 patients suggests a primary dystrophic process in the retinal ganglion cell layer followed by ascending atrophy of the optic nerves (Johnston et al. 1979; Kjer et al. 1983).

Here, we report the complete genomic organization of *DAGK3* and the mutational analysis of the entire coding region of the gene in the genomic DNA of 19 individuals affected with dominant optic atrophy. No disease-associated mutations were identified, suggesting that *DAGK3* is not a major factor underlying OPA1 aetiology.

Materials and methods

Isolation of genomic clones containing *DAGK3*

The RPCI-1 human P1 bacterial artificial chromosome (PAC) library (Ioannou et al. 1994) was kindly provided by P. deJong (Roswell Park Cancer Institute, Buffalo, N.Y.) and was screened with a full-length *DAGK3* cDNA probe obtained by reverse transcription-polymerase chain reaction (RT-PCR) amplification of total human retinal RNA using oligonucleotide primers 5′-DAGK3 (CCAAGTAAGGGAATAAACCG) and 3′-DAGK3 (TGCTTTCTCTCTTGGTTTAG), designed according to published cDNA sequences (GenBank Accession No. D26135; Kai et al. 1994). Hybridization was performed at 65°C as described by Church and Gilbert (1984). Final washing conditions were at a stringency of 0.2×SSPE, 0.1% SDS.

Positive clones were isolated and DNAs were prepared from overnight cultures by standard alkaline lysis techniques. Purified DNA was *Eco*RI digested, electrophoresed on 0.8% agarose, blotted onto Hybond N+ nylon membranes (Amersham) and hybridized to seven overlapping RT-PCR products together covering the entire coding sequence of the *DAGK3* gene. The nucleotide positions of the seven fragments (1A, 1B, 2A–C, 3A, 3B) with respect to the published cDNA and the sequences of the primers used for RT-PCR are as follows: 1A (–103 to +508, 611 bp): 5′-DAGK3b (ATCGCAAACCTGCAATGAGC) and DAGK-6 (AGGGACAGGTAGCACACAACA); 1B (+464 to +796, 333 bp): DAGK-5 (ATCCCCAGTGGTGTACCTG) and DAGK-2 (GAGCCAGAGTCATCCATCC); 2A (+756 to +1265, 509 bp): DAGK-1 (TCCCATTGCTGGTGCTCC) and DAGK-8 (CATGACAAGTTCGCCCTTG); 2B (+1212 to +1472, 260 bp): DAGK-7 (GGCCAGGTGAGAAGTCTGA) and DAGK-10 (ACCACAGGCCAAAACACGG); 2C (+1229 to +1667, 239 bp): DAGK-9 (ACTTTTTCCGTGATACTCCAG) and DAGK-4 (GTCCAGCATCACCAAGGGG); 3A (+1626 to +1926, 301 bp): DAGK-3 (ATCCTGAAAGACATTGAGCA) and DAGK-12 (CCCCATCACACTCCAACTCA); 3B (+1886 to +2414, 529 bp): DAGK-11 (CAAGAAACTCCACGACCAC) and 3′-DAGK3.

Characterization of exon/intron junctions and structure of *DAGK3*

*Hinc*II, *Hind*III and *Pst*I restriction fragments of overlapping PAC clones dJ207E13 and dJ131A16, containing the entire coding region of *DAGK3*, were separately subcloned into the plasmid vector pBluescript SK(–) (Stratagene). Exon-containing fragments were identified by colony hybridization of the seven cDNA fragments described above. Positive clones were sequenced using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham) with vector- and exon-specific primers. The exon/intron boundaries were identified by alignment of the genomic sequence with the published *DAGK3* cDNA (Kai et al. 1994) using the MacVector sequence analysis software (release 4.0).

Chromosomal localization

Radiation hybrid mapping was performed by PCR using a commercially available panel of 25 human×hamster hybrid cell line DNAs (BIOS Corporation, New Haven, Conn.) and oligonucleotide primers DAGK-F (AAACCAAGAGAGAAAGCAAG) and DAGK-R (AATTCCCAGTTTCTCCGCTC) designed from the 3′ untranslated region (UTR) of the *DAGK3* cDNA (Kai et al. 1994). Fluorescence in situ hybridization (FISH) mapping was performed using PAC clone dJ131A16 as previously described (Köhler and Vogt 1994).

Patients

Twenty affected subjects of German origin who are unrelated on the basis of genealogy were ascertained from the University Eye Hospitals in Berlin, Essen and Tübingen. Diagnosis of dominant optic atrophy was based on the presence of typical clinical features, including reduced visual acuity, colour vision deficits, abnormal visual-evoked potentials, optic disc pallor and family history of the disease (Table 1). In three cases (patients 1–3), blood samples from additional affected and unaffected family members were available for DNA and haplotype analysis.

Haplotyping and single-strand conformation analysis (SSCA)

Genomic DNAs were extracted from blood lymphocytes and were used as templates for PCR amplification. For haplotype analysis, microsatellite markers at *D3S1601* and *D3S1265*, closely flanking the *OPA1* locus, were used (Bonneau et al. 1995; Johnston et al. 1997). Primer sequences and PCR conditions were taken from the literature. For SSCA, PCR primer pairs flanking the 24 exons of the *DAGK3* gene are listed in Table 2. PCR conditions were an initial denaturation of 1 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at the exon-specific annealing temperature (Table 2) and 30 s at 72°C followed by a final extension of 5 min at 72°C. For SSCA, DNA was amplified in the presence of [^{32}P]dCTP (3000 Ci/mmol) and electrophoretically separated in a 6% non-denaturing polyacrylamide gel with or without 5% glycerol at 4°C, 25 W constant power for 2–11 h. PCR products with an aberrant mobility shift were cycle-sequenced as described above.

Results and discussion

Characterization of the genomic organization of *DAGK3*

Screening of the human RPCI-1 PAC library with the full-length *DAGK3* cDNA probe resulted in the isolation of six PAC clones: dJ91H22, dJ91L22, dJ115B1, dJ115H3, dJ131A16 and dJ207E13. Southern blots of *Eco*RI-digested PAC clones were probed with seven overlapping cDNA frag-

Table 1 Clinical data of OPA1 patients. (*VEP* visual evoked potential, *n.k.* not known)

Patient No.	ID	Age onset/ age[a] (years)	Progression	Visual acuity (OD/OS)	Colour vision defect	VEP (Amplitude/latency)	Disc appearance	Family history
1	3884-OAK	7/31	Slow	0.4/0.5	Non-specific	Diminished/ normal	Temporal pallor	Yes
2	250568996-DION	23/29	No	1.0/1.0	Non-specific	Normal	Normal	Yes
3	31XDION-12261	n.k.	n.k.	0.9/0.9	n.k.	n.k.	Temporal pallor	Yes
4	4917-OAK	20–30/51	Slow	0.2/0.4	Tritan defect	Diminished/ normal	Temporal pallor	Yes
5	5675-OAK	n.k.	n.k.	n.k.	n.k.	n.k.	Pallor diffuse	Yes
6	5579-OAK	6/13	No	0.3/0.2	Non-specific	Diminished/ prolonged	Temporal pallor	n.k.
7	4410-OAK	43/54	Rapid	0.1/0.1	Tritan defect	Diminished/ prolonged	Diffuse pallor	Yes
9	4195-OAK	13/16	No	1.0/0.8	Non-specific	Diminished/ normal	Temporal pallor	No
10	4999-OAK	12/24	No	0.4/0.4	Non-specific	n.k.	Temporal pallor	Yes
11	1331-OAK	35/38	Slow	0.4/0.5	Non-specific	n.k.	Diffuse pallor	No
12	4518-OAK	3/7	No	0.3/0.4	Non-specific	n.k.	Temporal pallor	Yes
13	555-OAK	6/19	No	0.3/0.3	Deutan defect	n.k.	Temporal pallor	Yes
14	542-OAK	8/23	No	0.4/0.4	Protan defect	n.k.	Temporal pallor	Yes
15	4049-OAK	12/22	No	0.4/0.3	Tritan defect	n.k.	Temporal pallor	Yes
16	1338-OAK	49/57	Slow	0.4/0.2	Deutan defect	n.k.	Temporal pallor	n.k.
18	3050-OAK	54/56	Slow	0.2/0.2	Tritan defect	n.k.	Temporal pallor	Yes
19	3288-OAK	Childhood/43	No	0.1/0.1	Deutan defect	n.k.	Temporal pallor	n.k.
20	53/931296 DHON	6/10	Slow	0.3/0.3	Tritan defect	Diminished/ normal	Temporal pallor	Yes
21	57/926096 DHON	Childhood/71	Slow	0.03/0.04	Tritan defect	Diminished/ prolonged	Diffuse pallor	Yes
22	63/X-DION	Childhood/56	Slow	0.05/0.05	n.k.	n.k.	Temporal pallor	Yes

[a]Age at final examination

ments (1A, 1B, 2A–C, 3A, 3B) covering the entire coding sequence of the *DAGK3* gene. A single PAC clone, dJ207E13, contains the most 5′ end of *DAGK3*, including fragments 1A, 1B, 2A–C and 3A, whereas the remaining PAC clones all hybridized to cDNA probes 2A–C, 3A and 3B and thus contain the 3′ end of the gene (data not shown). Overlapping PAC clones dJ207E13 and dJ131A16 were selected for further characterization of the *DAGK3* locus (Fig. 1).

Based on sequential hybridizations of overlapping cDNA fragments to high-density colony filters of subcloned PAC restriction fragments, exon-containing plasmids were identified and sequenced. This allowed us to deduce the genomic organization of the *DAGK3* gene, which consists of 24 exons spanning more than 30 kb of genomic DNA (Fig. 1). As shown in Table 3, the sizes of the exons range from 50 to 191 bp, while those of the introns vary from 100 bp to over 5 kb. Intron sizes were determined by restriction mapping or PCR analysis with primers flanking the respective introns. All acceptor and donor splice site junctions contain the invariant dinucleotides AG and GT, respectively, and boundary sequences that are in excellent agreement with 5′ and 3′ consensus splice sequences (Shapio and Senapathy 1987; Table 3).

Several functional domains have been defined in *DAGK3*, including two Ca^{2+}-coordinating EF-hand sequences, two zinc finger-like sequences and a putative ATP-binding motif (Kai et al. 1994). A comparison with the genomic exon/intron structure shows that the various protein domains are not strictly correlated with single exon boundaries, e.g. the second zinc finger-like motif is encoded in part by exon 11 (36 amino acids) and exon 12 (11 amino acids).

Several human tissues, including liver, kidney, spleen and testis, as well as hepatoma cell line HepG2 were found to express a catalytically inactive DAGK3 carrying an internal deletion of 25 amino acid residues (Kai et al. 1994). Our data demonstrate that the 25 amino acid residues are entirely encoded by exon 15, suggesting that the truncated DAGK3 isoform is the result of a skipping of exon 15. Both, the 3′ acceptor and the 5′ donor sequences of exon 15 have excellent splice site discrimination scores, 2.5 and 5.3, respectively, and therefore should represent strong splicing

Table 2 Oligonucleotide primers used for single-strand conformation analysis of *DAGK3*

Exon	Primer	Sequence (5′→3′)	Fragment size (bp)	T_A (°C)
1	5′ DAGK3	CCAAGTAAGGGAATAAACCG	210	62
	DAGK-13R	AGAAGAAATCCCGTCCCAG		
2	DAGK-53F	ATTTACTTGGCCCAGCTTC	194	60
	DAGK-22R	AAAGGCTAAAAAACACAGGG		
3	DAGK-67F	CCCTTTGCTTCTCTCCACTG	245	61
	DAGK-54R	GCGCAAGGTCTTCCCATC		
4	DAGK-24F	CAAGAAGCTAGAGGGCCT	155	60
	DAGK-55R	ATTCCCTACTTTTCCCACAA		
5	DAGK-40F	CTAAACAGAAAATGTCCTTGG	287	58
	DAGK-41R	GGAATAATGGATAGAACCCC		
6	DAGK-56F	CATCAAAACTTCAGCCATCC	189	60
	DAGK-35R	ATTCTTCCTCTGCTTGCC		
7	DAGK-42F	CTCTGGTTGTCTCCTTTTGAT	151	60
	DAGK-15R	ACTCCAAAGCCACCCCATC		
8	DAGK-57F	GAGGCAGAAGGCAGGTAGAC	234	60
	DAGK-66R	TTCCTGAATGGCAAGAGATC		
9	DAGK-68F	ATCTCAGGGCAGCCAGCT	197	61
	DAGK-26R	TGGGGGAAGAGGTTTGAAG		
10	DAGK-58F	TACGTGGCTATTCACAAGC	165	58
	DAGK-36R	CAGTAGAACAAGGCAGTAGATG		
11	DAGK-69F	GGTTCTGCCATTTTCCCTG	189	58
	DAGK-59R	GTTGAAAAGGAAGCAGGGC		
12	DAGK-28F	TTGCTCACTTTCACTCACTC	183	58
	DAGK-60R	AACCCGGAGAAGCTACAG		
13	DAGK-43F	TTGTGTTAGGCTTCTTTTTCC	145	58
	DAGK-44R	GTGCAATCGGATTTAGAGC		
14	DAGK-61F	CCTATTGGATGAAGCAGAGC	177	57
	DAGK-38R	CTGAGTCGTGTCGCCCTA		
15	DAGK-30F	GAACCCTCATCCCTGCCT	208	55
	DAGK-62R	AAGGGAGGGGGAGAAATAAG		
16	DAGK-45F	TAACAAGCCCATTTTGGCC	158	53
	DAGK-46R	TAAATAAGCAGAAAACGCCC		
17	DAGK-63F	CTCAAACATTCTACAGTCCCAT	174	59
	DAGK-39R	CCCAGGGTACCACCATTAG		
18	DAGK-47F	TGCTGTGGTGGCAAATGCA	273	64
	DAGK-48R	CCAGAGGGACAACAGAAGGC		
19	DAGK-70F	GGACCCTGAGATAGATAGCG	225	57
	DAGK-64R	AGAGAGCAGGTGAAAGGC		
20	DAGK-49F	CATGAGCATTTCAAGTATGGA	194	59
	DAGK-33R	CCAGGAGCAGAACCAAGATC		
21	DAGK-34F	TGAGGACACTAACAGATGGCC	274	59
	DAGK-65R	CTACTGGGCATTGACCTAAAAC		
22	DAGK-50F	CTGAGACCAAATGTGTATGCA	207	56
	DAGK-51R	TGGGTGACAAAGAGGCATAC		
23	DAGK-71FN	AAAAAGCTCACTGTGTTCTC	135	52
	DAGK-73R	GCTTCTCATCCCCATCTC		
24	DAGK-52F	GCGACTCACTCTCCCTTAG	205	56
	DAGK-R	AATCCCAGTTTCTCCGCTC		

signals (Penotti 1991). Thus far, the functional significance underlying the aberrant splicing event in some human tissues is unclear and needs further elucidation.

Mapping of the human *DAGK3* gene to chromosome 3q27–q28

To determine the chromosomal location of *DAGK3*, we applied a combination of two independent methods. We initially sublocalized *DAGK3* by PCR hybrid mapping using oligonucleotide primers that were designed from the locus-specific 3′-UTR of the gene. On the basis of the presence of the expected 778-bp PCR product in human genomic DNA and hybrid cell lines containing human chromosome 3 (SM-423, SM-507, SM-860, SM-861, SM-1079), and the absence of this fragment in the remaining 20 hybrids lacking chromosome 3 as well as the parental hamster ovary cell line CHO-104, we assigned the *DAGK3* locus to chromosome 3.

In addition, biotin-labelled PAC clone dJ131A16, containing exons 9 to 24 of *DAGK3* (Fig. 1), was used as a probe in FISH experiments on human metaphase spreads. Hybridization signals were repeatedly produced on both chromatids of chromosome 3q27–q28, whereas background signals were distributed randomly (Fig. 2). These results allowed us to refine further the subchromosomal position of the *DAGK3* locus to the 3q27–q28 region, confirming previous reports of *DAGK3* mapping performed by Fitzgibbon et al. (1995).

Mutational analysis of *DAGK3* in OPA1 patients

In several studies the autosomal dominant *OPA1* gene has been mapped to 3q28–q29 (Eiberg et al. 1994; Bonneau et al. 1995; Lunkes et al. 1995; Jonasdottir et al. 1997; Seller et al. 1997; Votruba et al. 1997, 1998), a chromosomal region overlapping the *DAGK3* location. We therefore decided to perform a mutational screen of the 24 exons of *DAGK3* in 19 OPA1 patients by SSCA (patient 3 was excluded from this analysis; see below).

Analysis of exon 4 revealed a complex pattern of band shifts as the result of a combination of three different variations in introns 3 and 4. Sequence changes IVS3–31T→C and IVS3–11T→C appear to be common polymorphisms with observed frequencies of the T alleles in control chromosomes of 37.0% and 53.2%, respectively (Table 4). The third alteration, IVS4+11G→A, was found once in patient 12 as well as in 4 out of 94 control alleles. An additional heterozygous sequence change, IVS5+12C→A, was observed in patient 11 but not in any other of the remaining 37 *OPA1* alleles nor in 188 unaffected control chromosomes (Table 4). This sequence alteration as well as the rare intronic base change IVS4+11G→A do not appear to influence the strength of the respective 5′ donor splice site signals. However, a possible functional effect on the correct splicing mechanism still needs to be assessed. In addition, the lack of available DNA samples from crucial family members of patients 11 and 12 has not allowed us to test for segregation of the rare variations with the disease. Finally, in exon 10 an A to G transition at nucleotide 947 results in a change of a lysine to an arginine residue at codon 316. This alteration was found in patients 4, 5 and 21 but also in

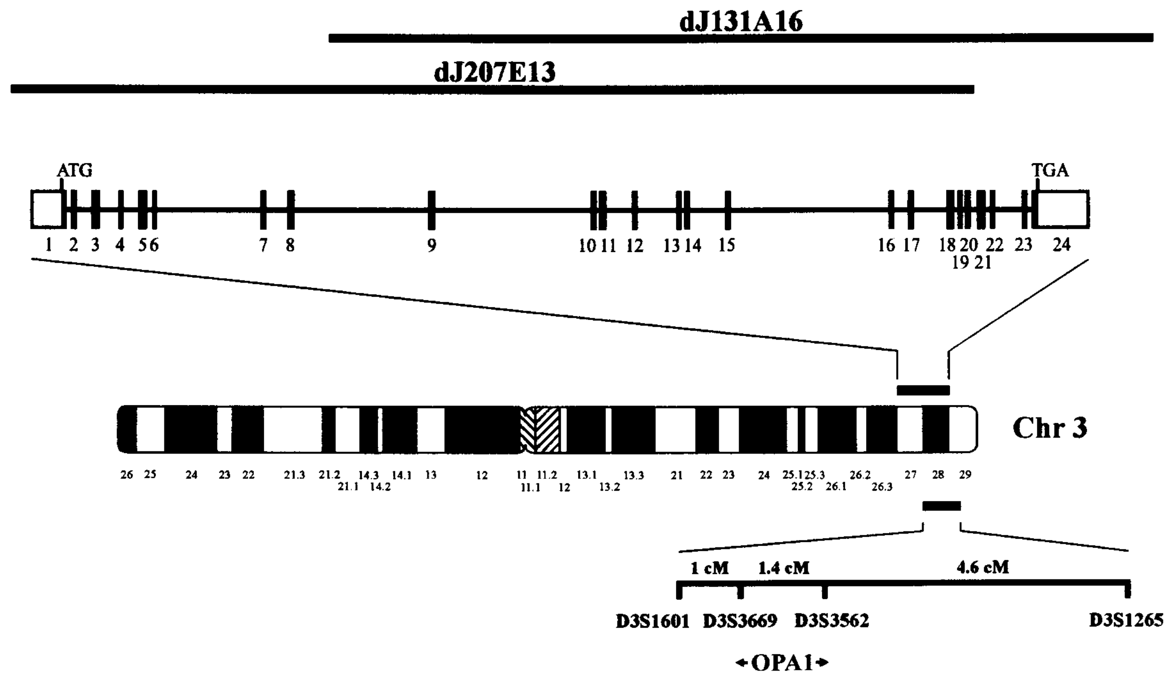

Fig. 1 Location and genomic organization of human *DAGK3*. *Top* PAC clones dJ207E113 and dJ131A16 containing the entire *DAGK3* gene are indicated by *horizontal bars*. The 24 exons of the gene are represented by *filled boxes*, the 3′ and 5′ untranslated regions by *open boxes*. *DAGK3* (Fitzgibbon et al. 1995; present study) and *OPA1* (Jonasdottir et al. 1997) have been mapped to overlapping genomic regions on 3q indicated by *solid bars* on each side of the idiogram of chromosome 3

Table 3 Exon/intron boundaries of the human *DAGK3* gene. Intronic and exonic sequences are given in lower and upper case letters, respectively

Intron	Exon		Intron	
3′ Splice acceptor	No.	Size (bp)	5′ Splice donor	Size (kb)
	1	67[a]	...AAT gtgagt	0.23
aattgtgtctcttcag A...	2	77	...GAG gtaaga	0.55
ttttgccccttctcag C...	3	166	...CAG gtacac	0.68
tcttttttttcctcag A...	4	63	...CTG gtaagt	0.56
cttttgttcatcgcag A...	5	171	...AGT gtaagt	0.27
ctgtgttttcttacag T...	6	50	...GCG gtaagt	3.30
ctttgctattttctag G...	7	75	...CCT gtgagt	0.75
tctttggattccacag A...	8	123	...TCT gtaagt	0.43
cctattacccctgcag G...	9	118	...CTC gtgagt	>5.0
ctttttttcttttcag A...	10	89	...GAG gttcgt	0.10
cgcttccctcccgcag G...	11	117	...ACG gtgggt	0.90
tcatttctccccccag T...	12	93	...CGG gtaagt	1.30
tcctttcttgtcacag G...	13	60	...CAG gtacct	0.20
tcttttacctttcaag T...	14	80	...AAG gtacgt	1.20
cttttttttttccatag A...	15	75	...AGG gtatgg	>5.0
tcttcttcttccccag G...	16	86	...TTG gtaagc	0.51
tctgtgtgtcttgcag A...	17	90	...GAG gtgagt	1.10
gtgctgtcctccacag G...	18	162	...GTG gtgagt	0.20
cacgttaccttcgcag G...	19	65	...CAG gtaagc	0.15
ctgctttgtggtccag G...	20	91	...GAG gtaagt	0.30
tctctccgtcctccag T...	21	191	...AAG gtaagc	0.25
gctgtctcctctgcag A...	22	121	...CCA gtgcgc	3.60
gtgttctgggttccag G...	23	61	...ACG gtgagt	0.10
gcctctttgcttttag A...	24	99[a]		

[a]Sizes correspond to the coding regions of these exons

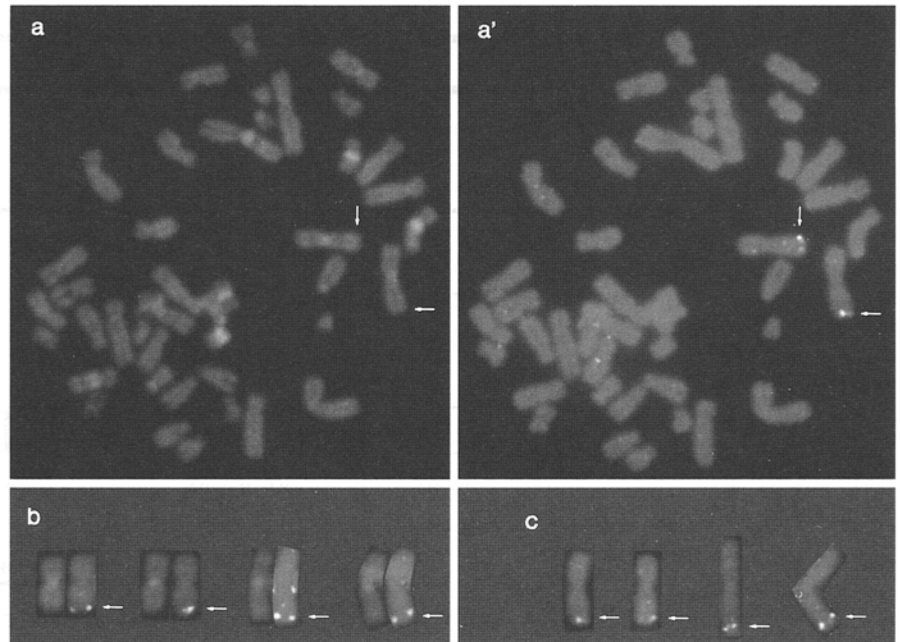

Fig. 2a–c Fluorescence in situ hybridization and 4′-6′-diamidino-2-phenylindole counterstaining (*blue*) on a human metaphase spread (**a**, **a′**) and on chromosomes 3 (**b**, **c**). Hybridization with PAC clone dJ131A16 as probe results in specific signals on 3q27–q28

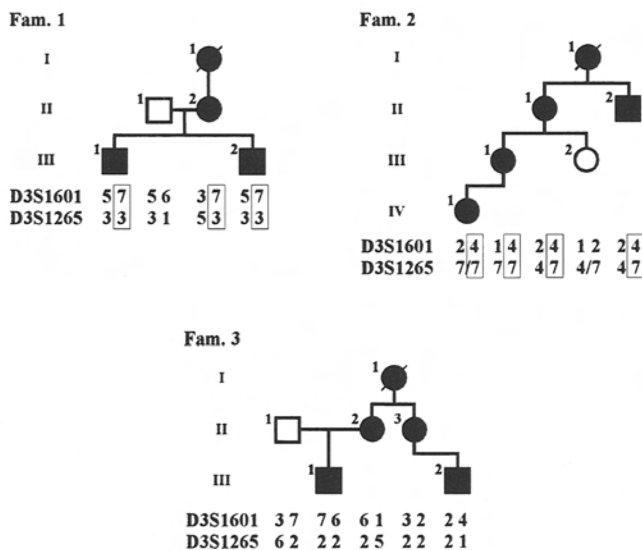

Fig. 3 Pedigrees and genotyping data of three multigeneration OPA1 families. Note that affected individuals in family 3 do not share a common allele at *D3S1601*, suggesting locus heterogeneity of OPA1 in this pedigree

Table 4 Sequence alterations identified in the *DAGK3* gene and their frequencies

Sequence alteration (functional consequences)	Frequencies (no. of alleles)	
	OPA1 patients	Controls
IVS3–31T→C (polymorphism)	T=16; C=22	T=35; C=59
IVS3–11T→C (polymorphism)	T=17; C=21	T=50; C=44
IVS4+11G→A (unknown)	G=37; A=1	G=90; A=4
IVS5+12C→A (unknown)	C=37; A=1	C=188; A=0
A947G (K316R)	A=35; G=3	A=74; G=20

20 out of 47 control individuals, suggesting that this amino acid change represents a neutral polymorphism. Taken together, of the five sequence variations identified in the OPA1 patients, three appear to be common polymorphisms while the biochemical significance of two nucleotide alterations is presently unknown.

While our mutational analyses were in progress, the Human Transcript Map reported a refined localization of the *DAGK3* gene approximately 20–25 cM centromeric to the *OPA1* minimal candidate region (http://www.ncbi.nlm.nih.gov/cgi-bin/SCIENCE96) thus physically excluding this gene from the *OPA1* locus. This is in full agreement with the results of our mutational analysis, which does not support a role of *DAGK3* in OPA1.

Genetic heterogeneity in OPA1

In more than 50 families from different ethnic populations, genetic linkage studies have mapped the dominant optic atrophy locus to the distal long arm of chromosome 3 (e.g. Eiberg et al. 1994; Bonneau et al. 1995; Lunkes et al. 1995; Jonasdottir et al. 1997; Seller et al. 1997; Votruba et al. 1997, 1998). However, there is recent evidence of genetic heterogeneity in this condition (Seller et al. 1997) with at least two additional OPA loci on Xp11.4–p11.2 and on 18q12.2–q12.3 (Assink et al. 1997; Kerrison et al. 1998).

Three of our patients (nos. 1, 2 and 3) are part of multigeneration pedigrees of whom blood samples of several affected and unaffected members were available for genetic linkage analysis. We genotyped all individuals at polymorphic DNA markers at *D3S1601* and *D3S1265* known to flank the *OPA1* candidate region (Bonneau et al. 1995; Johnston et al. 1997; Fig. 1, Fig. 3). In families 1 and 2 seg-

regation of marker alleles at both loci are consistent with linkage of *OPA1* in these families to distal 3q (Fig. 3). Moreover, haplotyping revealed two distinct haplotypes, suggesting that independent mutations underlie the OPA1 condition in the two families. Genotyping of members of family 3 demonstrates that the four affected individuals do not share a common disease allele at the highly informative locus *D3S1601*, located approximately 1–2 cM distal to the *OPA1* region (Votruba et al. 1998). These findings suggest locus heterogeneity in our OPA1 pedigree. Currently, additional affected and unaffected members are being recruited to pursue further the genetic localization of the disease locus in this family.

The present study represents the first characterization of the genomic organization of a member of the DAGK family. This work will greatly contribute to the future study of the evolution of the DAGK family in higher animals. Moreover, the knowledge of the detailed gene structure of *DAGK3* will facilitate the assessment of the gene in other human eye diseases and finally will allow further analysis of the biological properties and functional relevance of *DAGK3* in ocular tissues.

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