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A frameshift insertion in the cone cyclic nucleotide gated cation channel causes complete achromatopsia in a consanguineous family from a rural isolate

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Complete achromatopsia is genetically heterogeneous and segregates with mutations in *CNGA3* or *CNGB3* genes, which respectively encode for α - and β -subunits of the cyclic-nucleotide-gated (CNG) cation channel expressed in cone photoreceptors. High incidence of the disease (1 in 60) was detected in a rural isolate in central Chile. We excluded previously reported mutations in a consanguineous kindred with five affected members. Genotype analysis with short tandem repeat polymorphic (STRP) markers provided evidence to search for the causative mutation in *CNGB3*. Two sequence variations, c.492_493insT and c.488A>G, flanking an adenosine (A₅) repeat in exon 4 were identified. The frameshift mutation creates two consecutive stop codons in exon 5 that would induce premature translation termination. The severely truncated β -subunit is likely to render a nonfunctional cone CNG channel and cause total colour blindness in this kindred.

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Introduction

Complete achromatopsia (total colour blindness; ACHM3; OMIM 262300) is an autosomal recessive disease with an estimated incidence of 1:30 000,¹ characterised by total inability to distinguish colours, reduced visual acuity, photophobia and nystagmus. The disease was genetically mapped to chromosome 2q11.2-q12 (*ACHM2* locus)^{2,3} and chromosome 8q21-q22 (*ACHM3* locus),^{4,5} which comprise *CNGA3* and *CNGB3* genes, respectively. Mutations in the genes for the α -subunit (*CNGA3*) and β -subunit (*CNGB3*) of the CNG channel expressed in cones are the primary cause of complete achromatopsia in families with diverse geographic or ethnic origin.^{6–9}

Mutation analysis of affected members from a consanguineous family resident in a rural isolate in central Chile with high disease incidence (1:60),¹⁰ allowed us to exclude all previously reported *CNGA3* or *CNGB3* mutations in these

subjects. Homozygosity of STRP markers for the ACHM3 locus prompted us to search for the underlying genetic defect in *CNGB3*. We identified two close nucleotide changes that segregate with the disease, a transition and a single nucleotide insertion. The frameshift insertion, that would cause premature translation termination, is likely responsible for complete achromatopsia in this kindred.

Materials and Methods

Subjects

Ten subjects of Hispanic descent from a rural isolate of 600 inhabitants were diagnosed with complete achromatopsia. These patients exhibited complete inability to discriminate colours, reduced visual acuity (<0,2), nystagmus, and photophobia. Clinical symptoms were evident in early childhood. Fundus examination revealed atrophic macular changes in two older patients. Electroretinographic examination carried in two patients showed reduced cone photopic flicker response.

Personal interviews and records from the local government were used to establish family relationships for 82 individuals and to construct the family pedigree accounted

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for in this study (Figure 1). Peripheral blood was obtained after informed consent from eight available subjects, five affected and three healthy relatives. This research project was approved by the Ethics Committee at INTA, Universidad de Chile.

Genotype analysis with STRP markers

Genomic DNA isolated from white cells by standard phenol-chloroform procedure was used for genotyping with STRP markers D2S2333, D2S113 and D2S2264 on 2q11-q12 and D8S1119, D8S1707, D8S167, D8S273 on 8q21- q22. Marker amplification was carried out by PCR and analysed by denaturing polyacrylamide electrophoresis (see Appendix).

Linkage analysis

We used a modified version of the MLINK 4.03 programme¹¹ compiled for analysis of pedigrees with up

to seven consanguinity-loops. An autosomal recessive model of inheritance with no phenocopies and complete penetrance was assumed. The frequency of the disease-causing allele was set to 0.13, reflecting the approximate frequency of the disease in this community. To reduce the computational burden of multipoint linkage analysis, the entire 8q21-q22 haplotype was recoded into a single three-allele marker system, assuming no recombination among such markers.

Mutation screening

Coding exons and intron-exon boundaries were PCR-amplified using primers within flanking introns. At least three independent reactions per amplicon were pooled for sequence analysis using an ABI model 377 DNA sequencer. Mutations were verified on both DNA strands (see Appendix).

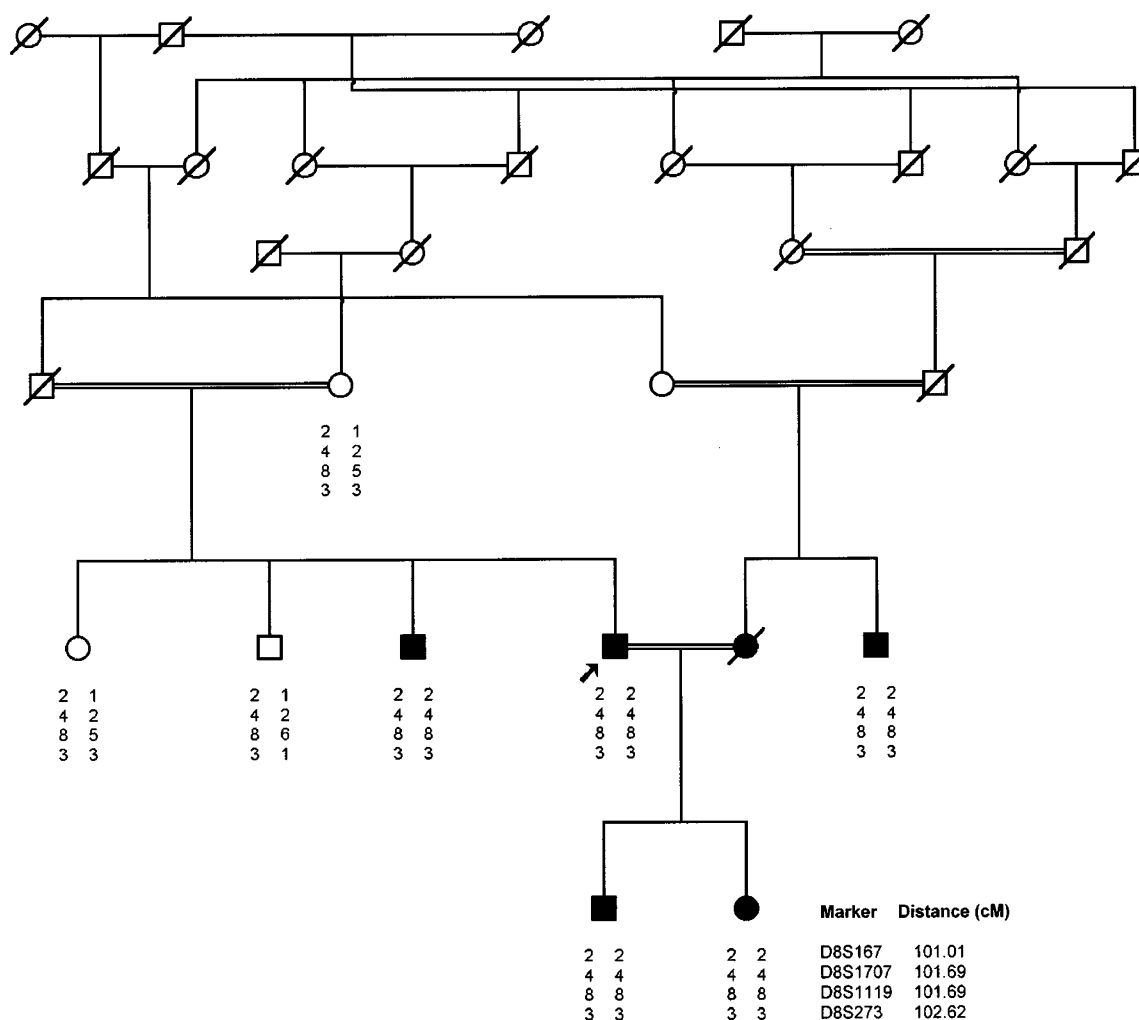


Figure 1 Pedigree of the family with complete achromatopsia. Genotyping data for 8q21-q22 STRP markers are given below individuals available for analysis. The markers are ordered from centromere to telomere. The arrow indicates the proband.

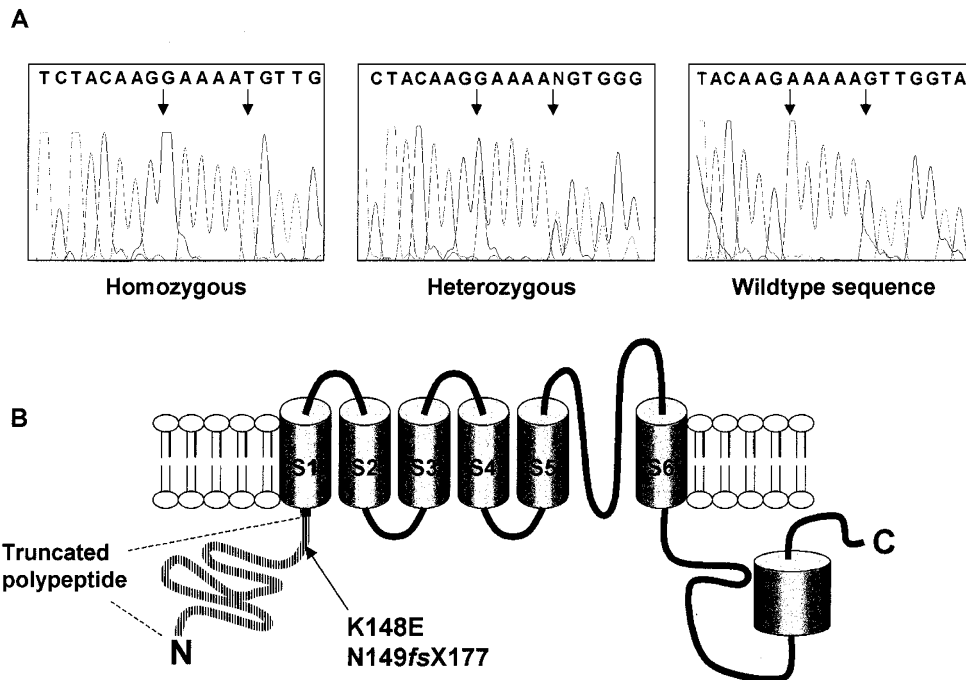


Figure 2 (A) Electropherogram sections of exon 4 sequence around the A5 repeat for homozygous, heterozygous and wildtype subjects. (B) Folding model for cone CNG β -subunit. The arrow indicates the approximate position of the changes in protein sequence.

Results

Five affected subjects and three unaffected siblings (Figure 1) were genotyped to define whether the disease segregates with genetic markers for the ACHM2 or ACHM3 loci in this family. ACHM2 markers failed to display any haplotype-phenotype association. Conversely, all patients showed homozygosity for D8S167, D8S1707, D8S1119 and D8S273 markers spanning a 1.6 cM interval in 8q21-q22. Whereas, their unaffected siblings were heterozygous. Constructed haplotypes for chromosome 8q21 markers are shown in Figure 1.

Given that allele frequencies for these STRP markers are unknown in this population and that LOD score analysis may be influenced by incorrect specification of such frequencies,¹² either equal allele frequency for each marker or allele frequencies drawn from public databases were assumed to compute LOD scores. The frequency of the linked haplotype (2-4-8-3) was set equal to the frequency of the least frequent allele in the haplotype. Under these conditions, LOD scores of 2.15 (equal allele frequency) and 2.74 (allele frequency from databases) were calculated.

Sequence analysis of the eighteen exons from the proband (Figure 1) revealed two nucleotide changes in *CNGB3*, a transition (c.488A>G) that results in a K to E substitution, and a single frameshift insertion (c.492_493insT) flanking an adenosine (A₅) repeat in exon 4. All affected individuals were homozygous for both nucleotide changes; whereas heterozygotes displayed the mixed sequence

pattern illustrated in Figure 2A. Two unaffected relatives, whose family relationships were not clearly established (consequently, not included in the pedigree), were also heterozygous for the described sequence modifications. The frameshift insertion, not detected in 100 control chromosomes, creates downstream consecutive nonsense codons at positions 178 and 179.

Discussion

Evidence for association of complete achromatopsia to the *CNGB3* gene in this kindred was provided by homozygosity of all four markers spanning a 1.6 cM interval that encompasses the *CNGB3* gene in all affected subjects. Unaffected siblings exhibited heterozygous haplotypes (Figure 1).

Sequencing analysis of *CNGB3* exons in the proband lead to the identification of two close nucleotide changes in *CNGB3*. These sequence modifications were confirmed in all available individuals in this kindred. The c.488A>G transition causes a missense substitution (K148E) of an amino acid residue, conserved among human and mouse cone *CNGB3* subunits, that is located in the protein amino terminus. However, *CNGB1* subunits from human, mouse and bovine rods bear glutamic acid in this position. The frameshift insertion (c.492_493insT) creates two consecutive nonsense codons in exon 5 that would cause premature termination of protein translation. The truncated polypeptide is only 177-amino acids long and lacks all membrane spanning segments, according to the folding model (Figure

2B). Therefore, it is highly likely that complete achromatopsia in this kindred arises from loss of *CNGB3* function attributable to the single base insertion (c.492_493insT).

This family, with four consanguineous marriages, belongs to a community with a high inbreeding coefficient.¹⁰ Conceivably, the disease mutation was spread among family members and other inhabitants of the rural isolate due to consanguinity.

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Appendix

Methods supplement

Genotype analysis with short-tandem-repeat polymorphic (STRP) markers Genomic DNA was isolated from blood white cells according to a standard procedure.¹ STRP markers spanning 10.3 cM on human 2q11-q12 and 1.6 cM on human 8q21-q22 were used for genotyping of individuals. Amplification of STRP markers by standard PCR was carried out with 50 ng of genomic DNA in 25 μ l of PCR buffer (20 mM Tris HCl pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 0.2 mM each dNTP, 1 μ M of the forward and reverse primers and 0.6 U Taq DNA polymerase (Life Technologies, Rockville, MD, USA). PCR primer sequences for D8S1119, D8S1707, D8S167, D8S273, D2S2333, D2S113 and D2S2264 amplicons were obtained from The Genome Database. The PCR program for STRP marker D8S1707, D8S273, D2S2264 amplification consisted of 34 cycles of 94°C for 40 s, 60°C for 60 s and 72°C for 60 s, followed by a single cycle of 94°C for 40 s, 60°C for 60 s and 72°C for 10 min. The cycling parameters for STRP D8S167 and D2S2333 markers were 34 cycles of 94°C for 40 s, 58°C for 60 s and 72°C for 60 s, followed by a single cycle of 94°C for 40 s, 58°C for 60 s and 72°C for 10 min. Amplification of STRP D8S1119 and D2S113 markers consisted of 34 cycles of 94°C for 40 s, 63°C for 60 s and 72°C for 60 s, followed by a single cycle of 94°C for 40 s,

63°C for 60 s and 72°C for 10 min. Amplicons were separated by electrophoresis on 6 or 8% polyacrylamide-8 M urea gels, according to Bassam.²

Screening for previously reported mutations in *CNGA3* and *CNGB3* Coding exons and intron-exon boundaries were PCR-amplified from genomic DNA isolated from affected individuals with primers located within flanking introns. Primer sequences for amplification exon 4 through 18 on *CNGB3* were from Sundin *et al*.³ Primers designed for amplification of *CNGB3* exons 1, 2 and 3 and *CNGA3* exons 5 and 7 are indicated in Table 1. Amplification reactions were carried out as described above. PCR programmes consisted of 32 cycles (94°C for 60 s; 53°C for 150 s; 72°C for 60 s) followed by a single cycle (94°C for 60 s; 53°C for 60 s; 72°C for 10 min) for *CNGA3* exons 5 and 7, and 35 cycles (94°C for 40 s; 63°C for 60 s; 72°C for 40 s), followed by an extension cycle (94°C for 40 s; 63°C for 60 s; 72°C for 10 min), for *CNGB3* exons 5, 6, 9, 10, 11 and 13. At least three independent reactions per amplicon were pooled and fractionated by electrophoresis on 2% agarose gels in TAE buffer. DNA fragments were recovered from agarose using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) and quantitate spectrophotometrically. Sequen-

Table 1 Primer sequence used for *CNGA3* and *CNGB3* mutation screening

Target gene	Amplicon	Primer name	Primer sequence
<i>CNGB3</i>	exon 1	B-E1F	GGCACAGTCATAAATACAGAGG
		B-E1R	CATGTACCAGATGGTGCCAGG
<i>CNGB3</i>	exon 2	B-E2F	GATCTGATTGACAATTAAGTGG
		B-E2R	CTAGATAATTCACCTAGTCAAGG
<i>CNGB3</i>	exon 3	B-E3F	CATCTTTGTAGCTCATGTTGCC
		B-E3R	ATTCAGCTAAAGGGGAGAGTGG
<i>CNGA3</i>	exon 5	A-E5F	GAAGCGCCTGGCCCTGGCC
		A-E5R	CCTGCAAATAAGCAGATACC
<i>CNGA3</i>	exon 7	A-E7F	AAAGGTGGGCACAACTACC
		A-E7R1	CAGCCTTCAGCTTGCTGCGG
		A-E7R2	CCTTTGAGAGGCAGAACAGG
		A-E7R3	GCGCCCATGCTCTGGGATTG
		A-E7R4	AAGCAGGAGACAGATGCTGC

cing of amplicons was carried out using an ABI model 377 DNA sequencer and compared to the wild type mRNA sequence. Mutations were verified by sequencing with both forward and reverse primers.

Screening a control population for the nucleotide changes in exon 4 Genomic DNA from 50 control individuals with similar ethnic background was screened by standard PCR for the presence of the two sequence changes in exon. Four independent amplification reactions per DNA sample were carried out with primers designed to discriminate between bases A or G at nucleotide 488 and A or T bases at position 492. Primer pairs and their sequences are listed in Table 2. The amplification products were separated by agarose gel electrophoresis, stained with ethidium bromide and their size compared to appropriate standards.

Table 2 Primer sequences used for *CNGB3* SNP screening

Primer name	Primer sequence	Amplicon size (bp)
488A-F	GCGTCAAAGAACAGCCCTCTACAAGA	125
E4II-R	CCCCTCAGCACTTCTTTCTTCCAG	
488G-F	GCGTCAAAGAACAGCCCTCTACAAGG	179
E4-R	TCTCAGGGTCTGCTTTTGGGAGATCCAA	
492A-R	GGTGAGGAGAGATCTCCCTCTACCAACT	178
E4-F	TCTTCATGGGCCCCTCTCTATTGCCA	
E4II-F	GCTCCTGTTATAAATGAGTATGCCG	110
492T-R	GAGGAGAGATCTCCCTCTACCAACA	

Electronic database information

The human *CNGB3* gene Locus ID 54714, Accession No. NT023717; complete sequence, clone RP11-298P6, Accession No. AC013751; human *CNGB3* mRNA, Accession No. AF272900; human *CNGB1*, Accession No. AI004485; bovine *CNGB1*, Accession No. AI150392; mouse *CNGB3* mRNA, Accession No. NM013927 and mouse *CNGB3* mRNA, Accession No. AJ243572, were from GenBank: <http://www.ncbi.nlm.gov> Allele frequency was obtained from: <http://www2.mc.duke.edu/depts/medicine/medgen/allelefreqs/frequency.html>, <http://www.gdb.org/gdb-bin/genera/genera/hgd/Allele>, and <http://lpg.nci.nih.gov/cgi-bin/markerSearch>

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