CORD9 a New Locus for arCRD: Mapping to 8p11, Estimation of Frequency, Evaluation of a Candidate Gene

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Purpose. To determine the locus of the mutant gene causing autosomal recessive cone–rod dystrophy (arCRD) in a consanguineous pedigree, to evaluate a candidate gene expressed in retina that maps to this locus, and to estimate the percentage of arCRD cases caused by mutations in this gene.

Methods. DNAs from family members were genotyped for markers covering the entire genome at an average spacing of approximately 9 centimorgans (cM). The data were input into a pedigree computer program to produce output files used to calculate lod scores. Significant linkage was revealed at 8cen, prompting the genotyping of a number of additional markers. Exons of a candidate gene were sequenced directly by standard fluorescent dideoxy methods. Haplotype analysis was performed with markers in this locus in 13 multiplex and 2 simplex CRD families in which neither parent had disease.

Results. Four-point linkage analysis gave a maximum lod score of approximately 7.6 at both D8S1769 and GATA101H09 in the large consanguineous family. Recombination events defined an interval of 8.7 cM between D8S1820 and D8S532 within which the gene must lie. This 8p11 locus (*CORD9*) is immediately distal to but distinct from the *RP1* autosomal dominant RP (adRP) locus. Two islands of homozygosity were found in this locus: The alleles of 6 of 10 markers in one of the islands and 2 of 4 in the other were homozygous. The UniGene cluster Hs.8719 (UniGene System, provided by the National Center for Biotechnology Information and available at http://www.ncbi.nlm.nih.gov/UniGene), which tags a gene with significant homology to *Dual Specificity Phosphatase 3,* maps within the *CORD9* interval and is highly expressed in the retina. To evaluate this gene as a potential disease candidate, intron–exon structure was determined, and exons were screened in the consanguineous family. No variants were found that could be related to disease. Haplotype analysis of 15 other families with CRD, using markers at *CORD9*, excluded this locus in 9 of 15.

Conclusions. A new arCRD locus (*CORD9*) has been identified corresponding to a yet unidentified gene in the 8.7-cM interval D8S1820-D8S532. No mutations were found in one candidate gene in affected members of the primary study family. Haplotype analysis of a cohort of 13 multiplex and 2 simplex families with CRD ruled out the *CORD9* gene in 9 of 15 of the families. To date, a total of 126 loci carrying gene mutations causing various forms of retinal degeneration have been mapped, and the mutant gene has been identified in 64 of them. However, only 2 loci for arCRD have been documented. This is the report of a third. (*Invest Ophthalmol Vis Sci.* 2001;42:2458–2465)

Cone–rod dystrophy (CRD) is the diagnosis encompassing a group of retinal degenerative diseases that most often initially manifest clinically as maculopathy but also show diffuse photoreceptor impairment on electrophysiological testing. There can be greater cone than rod photoreceptor dysfunction or a relatively equal loss of both cone and rod photoreceptor function.[1–5] CRD represents just one of many different clinical presentations of inherited degenerations of the retina, the largest category being retinitis pigmentosa (RP). All of these diseases are progressive, often lead to legal blindness, and can be inherited in an autosomal recessive, autosomal dominant, or X-linked manner. Many are now known to be caused by specific gene defects.[6]

To date, RP has been associated with mutations in 24 different genes and 16 chromosomal loci for which the defective gene has not yet been found.[6] The causes of CRD appear to be less numerous, but still quite diverse: four genes and six loci.[6] Two of these are of autosomal recessive inheritance: *CORD8* on chromosome 1q12-q24[7] and the locus corresponding to the *ABCA4* gene on chromosome 1p.[8] Therefore, when we found an informative, consanguineous family with an autosomal recessive cone–rod dystrophy phenotype, we first demonstrated that the disease was not linked to either of the known recessive CRD loci. We then performed a whole genome linkage search, leading to the identification of a new locus, *CORD9*, on chromosome 8p11.

Materials and Methods

Primary Family

We studied a consanguineous family from Brazil that included nine affected members inheriting autosomal recessive CRD (arCRD; Fig. 1). Two of the affected individuals in the fifth decade of life were evaluated clinically and with visual function tests, including electroretinograms (ERGs), kinetic visual fields, and static threshold perimetry.[3,9] Medical records and historical data were obtained for the other seven

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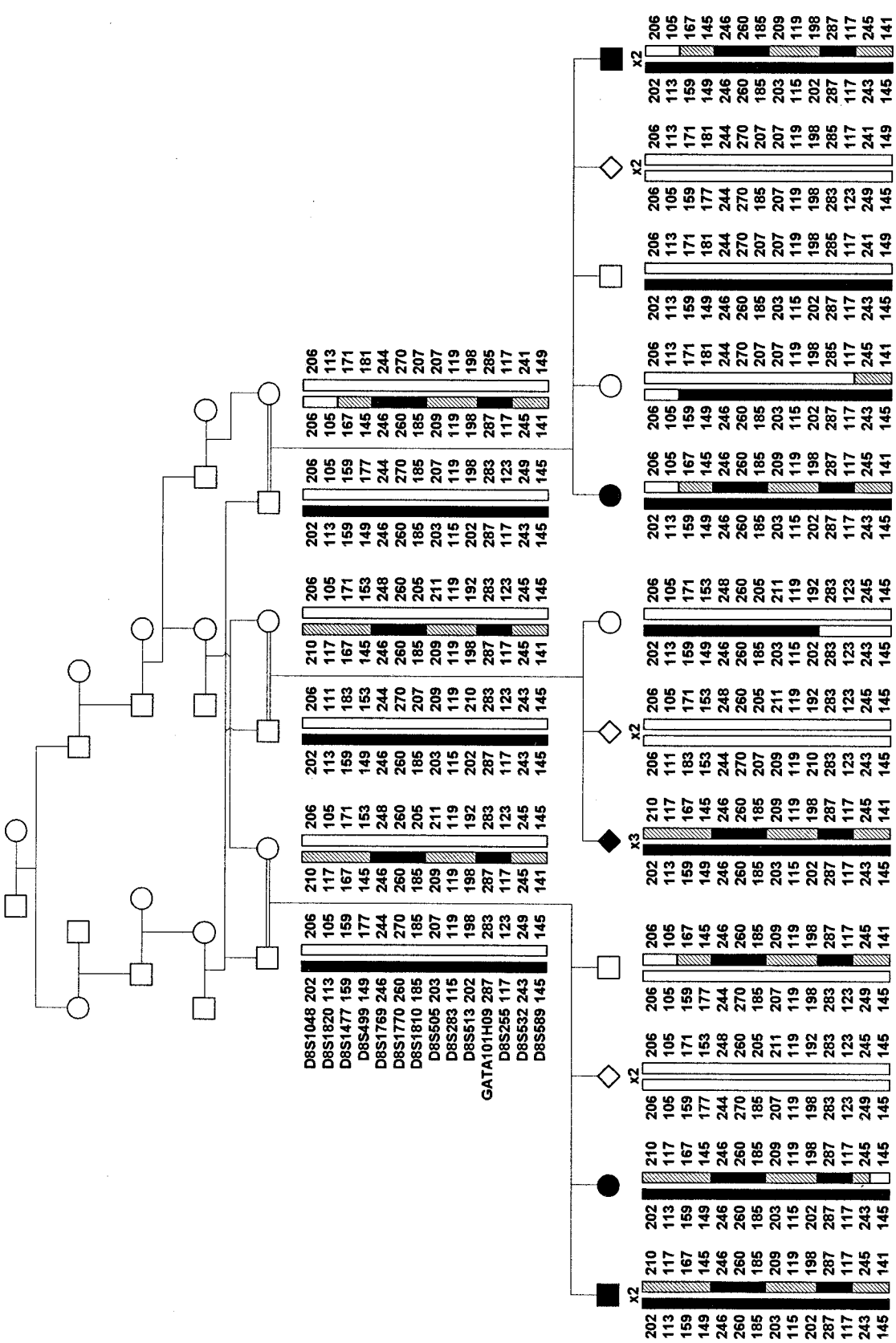

FIGURE 1. Pedigree of the Brazilian family segregating severe arCRD. *Circles*: females; *squares*: males; *filled symbols*: affected members; *open symbols*: unaffected members. Symbols with a multiplier shown below in the sixth generation represent multiple individuals with identical genotype and phenotype. *Squares* or *circles*: sibling groups of the same sex; *diamonds*: multiple individuals of both sexes. Haplotypes are shown for 14 markers spread across a 12.5-cM region spanning the *CORD9* locus on chromosome 8p11. *Black bar*: the affected haplotype in the left arm of the pedigree. Regions of nonconcordance between the affected haplotypes on the left and right sides are *shaded*, whereas normal haplotypes are *white*. Marker D8S1820, at 56.04 cM from the 8p telomere on the Marshfield Genetic map, reveals a crossover between the mother of the sibship on the *right* and her two cousins, which places the locus proximal to *(below,* on the figure) this marker. D8S532, at 64.75 cM, detects a crossover in the first unaffected sibling of the sibship on the *right*, who has inherited both affected alleles at and proximal to this marker, placing *CORD9* distal to it *(above)*. Within the 8.71 cM between D8S1820 and D8S532, there are two islands of homozygosity that potentially further refine the locus.

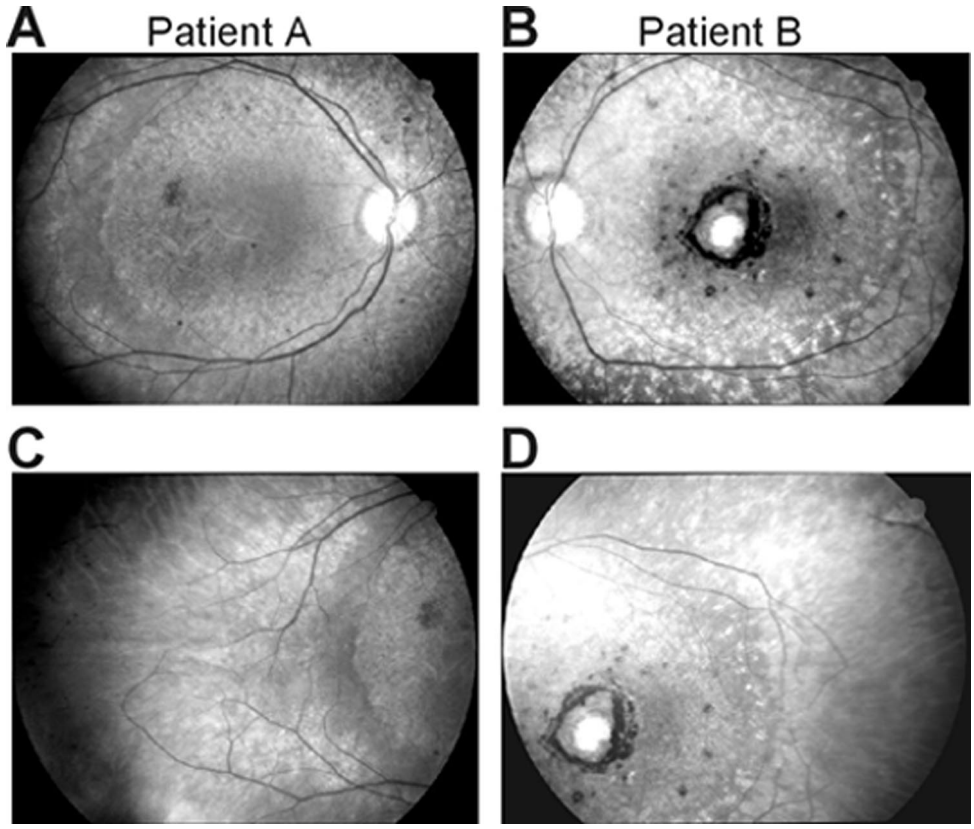

FIGURE 2. Fundus photographs of the central (**A**, **B**) and temporal retina (**C**, **D**) of two affected members of the primary family segregating arCRD. Photographs are of patient A's right eye (**A**, **C**) at age 48 years and patient B's left eye (**B**, **D**) at age 43 years.

affected members. This process was facilitated by a medically qualified unaffected member of the family.

Additional CRD-Affected Families

Fifteen other CRD-affected families, each with a small number of affected members were ascertained from two clinical centers specializing in the study of retinal degenerations (University of Illinois Eye and Ear Infirmary, Chicago, IL; Scheie Eye Institute, Philadelphia, PA). Diagnoses were based on published clinical and ERG criteria.[3,5] In all families, parents did not report or manifest disease. In 13 of the families, there were two or more affected siblings (multiplex), which suggests autosomal recessive inheritance. In the remaining two simplex families, there were three and four unaffected siblings, respectively.

The study protocol adhered to the tenets of the Declaration of Helsinki for all individuals. Informed consent for all procedures was obtained from all subjects after the nature of the studies had been fully explained.

Genotyping

DNAs were extracted from blood leukocytes by standard methods. The DNAs of the primary family were analyzed for the alleles of 400 polymorphic, high-quality, short tandem repeat markers spanning the genome, with an average spacing of approximately 9 centimorgans (cM) by the Marshfield Mammalian Genotyping Service (provided in the public domain by Marshfield Clinic, Marshfield, WI, and available at http://www.marshfieldclinic.org/research/genetics/genotyping_service/mgsver2.htm). The data were input directly to the Cyrillic pedigree information package (Exeter Software; Setauket, NY) to produce output files for the Linkage program suite (provided by the Human Genome Mapping Project Resources Center, Cambridge, UK and available at http://www.hgmp.mrc.ac.uk/genomeweb/linkage.html). PCR primers for additional markers tested in the 8p12-q12 locus (determined as the critical interval by analysis of the Marshfield data) and selected from the Marshfield genetic map of chromosome 8 (provided in the public domain by Marshfield Clinic, and available at http://www.marshfieldclinic.org/research/genetics/map_markers/maps/indexmapframes.html) were purchased from Research Genetics (Huntsville, AL). The markers were amplified by PCR from genomic DNAs in the presence of [α-^{32}P]dCTP, electrophoresed in 7% acrylamide denaturing gels, and exposed to x-ray film, as described previously.[10] For each marker, all members of the family were run side by side in the same gel.

Haplotype analysis was performed on the 15 other CRD-affected families using the markers D8S499, D8S2319, and D8S1125. Genomic DNAs were extracted, amplified with the appropriate markers and electrophoresed as described for the markers of the primary family. All members of these families were also run side by side in the same gel.

RT-PCR Analysis

Total RNA was extracted from retinal tissue using extraction reagent (TRIzol; Gibco BRL, Grand Island, NY), according to the manufacturer's instructions. The tissue had been dissected from human eyes and snap frozen in liquid nitrogen. One microgram of this RNA was incubated with 100 ng of random primer at 70°C for 10 minutes. The samples were placed on ice and Moloney murine leukemia virus (MMLV) reverse transcriptase buffer, 10 mM dithiothreitol (DTT), 1 mM each of the dNTPs (all from Gibco BRL), and 0.5 U RNAsin (Promega, Madison, WI) were added. After equilibration at 37°C for 2 minutes, 200 U MMLV reverse transcriptase was added, and the reactions were incubated at 37°C for 1 hour. The samples were heated to 95°C and 2 μl of the cDNA was used in PCR reactions.

Mutation Screening

The coding exons of *DUS3*-like and the flanking splice junctions were screened for mutations by direct sequencing. The following primers were used: exon 1, 5′-CGCTTTGTAATTGGTGCAGCTC-3′ and 5′-CCAGCGTGGAGAGCC-AGCTG-3′ (PCR product, 416 bp); exon 2, 5′- GCTGCTCTGGACTCACTCAAG-3′ and 5′-CCTGTCTGCCACCCTTCT-CC-3′ (PCR product, 425 bp); exon 3, 5′-GTCTTCCCCAACAAC-

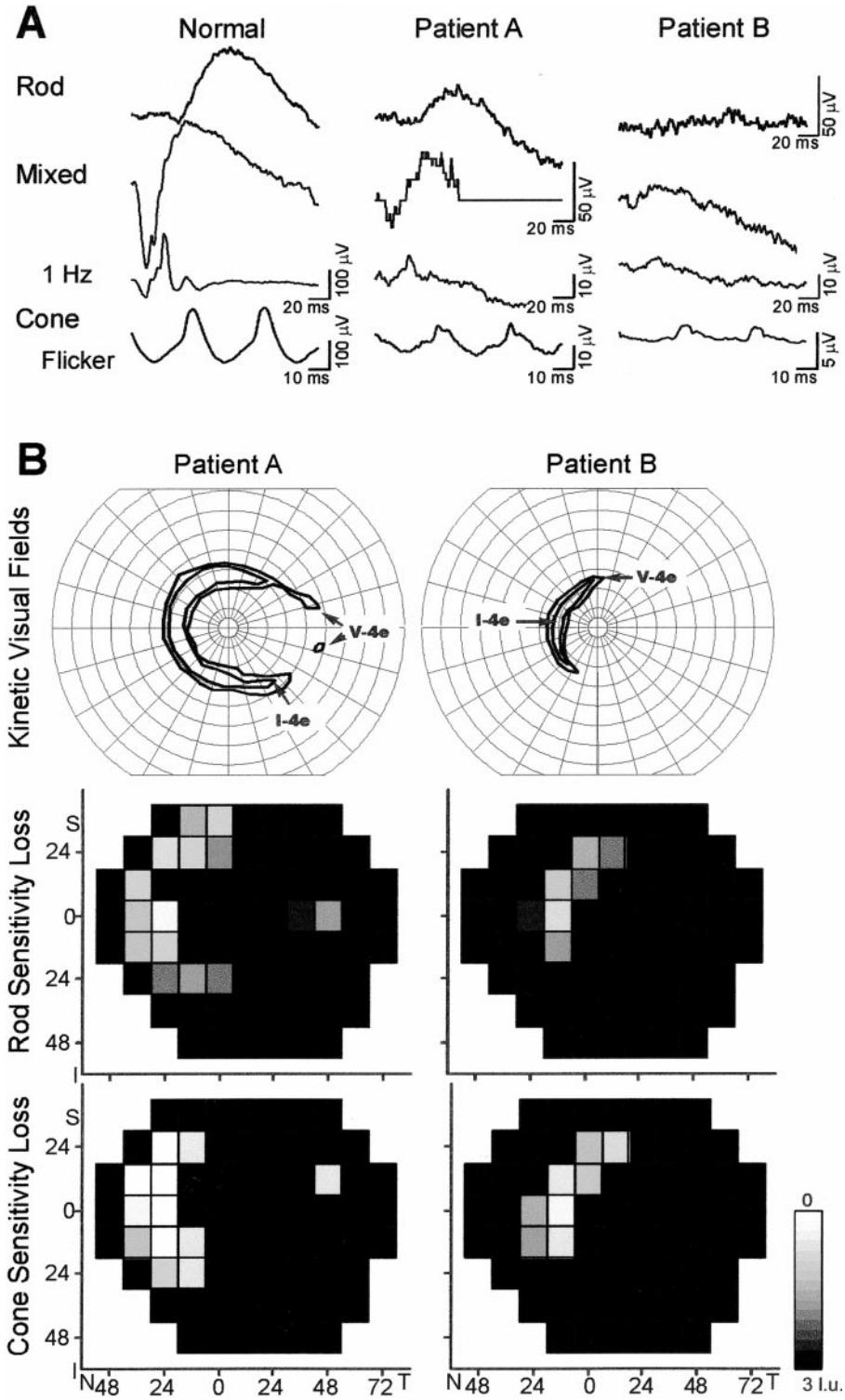

FIGURE 3. Visual function test results in two affected members of the primary family segregating arCRD. (**A**) Rod, mixed cone–rod, and cone ERGs from a representative normal subject (*left panels*) are compared with those from patients A (age 41) and B (age 43). ERGs are from the right eye of the patients. Calibrations are *below* and to the *right* of the responses. (**B**) Kinetic visual fields using V-4e and I-4e test targets (*top panels*) and gray-scale maps of sensitivity loss from rod (*middle panels*) and L/M-wavelength cone (*bottom panels*) static threshold perimetry in the same two patients. Perimetric results in patient A are from the right eye; results in patient B are from the left eye, but are displayed as right eye data for ready comparison. Scales have 16 levels of gray, representing 0 (*white*) to 3 (*black*) log units. N, nasal; T, temporal; S, superior; I, inferior fields.

CTGGC-3′ and 5′-CAAATTCCAGGCACCCTGTTC-3′ (PCR product, 319 bp); and exon 4, 5′-GCCCCATGTCACCCTTTCCC-3′ and 5′-CCTATCTCCAGCTGGGAGCC-3′ (PCR product, 343 bp). Reactions were performed in a 50-μl volume with 50 ng of genomic DNA; 20 pmol of each primer; 200 μM each dATP, dCTP, dGTP, dTTP; 10 mM Tris-HCl (pH 8.3); 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 1 U *Taq* DNA polymerase (Gibco BRL). After the initial denaturation step at 96°C for 3 minutes, the samples were processed through 35 cycles of 92°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. A final extension step was performed at 72°C for 10 minutes. After amplification, PCR products were processed using a PCR purification kit (Qiaquick; Qiagen, Valencia, CA) according to the manufacturer's instructions. The PCR product was eluted into 30 μl of 10 mM Tris (pH 8.5), and 5 μl of this purified product was used in a sequencing reaction. Both the forward and reverse strands of the PCR products were directly sequenced (ABI 377 sequencer, using the ABI Prism BigDye Terminator cycle sequencing ready-reaction kit; PE Applied Biosystems, Foster City, CA). Sequencing reactions were set up according to the manufacturer's instructions, using either the forward or reverse primers detailed earlier.

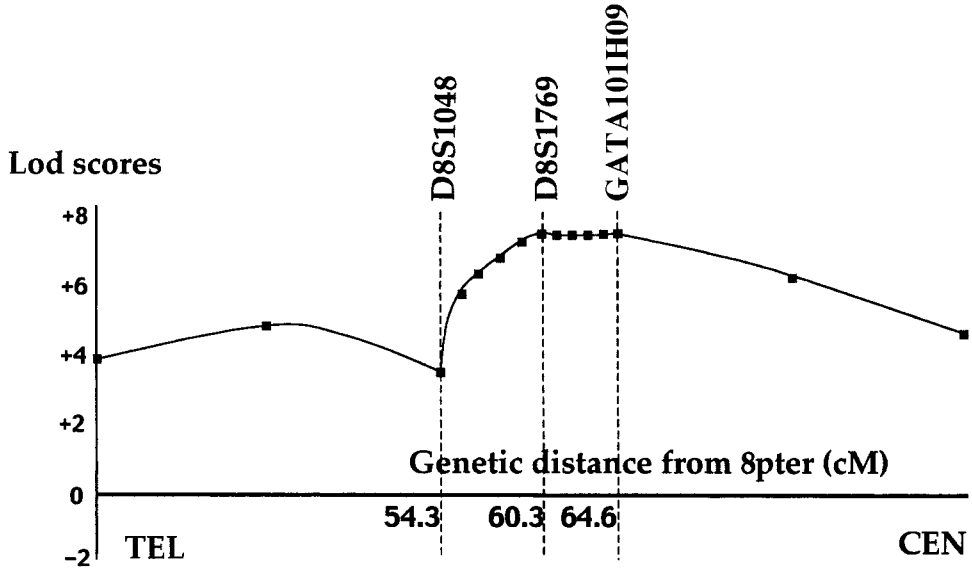

FIGURE 4. Four-point linkage analysis of chromosome 8p11-q11 markers D8S1048, D8S1769, and GATA101H09 against cone-rod dystrophy in the consanguineous Brazilian pedigree. Genetic distances are taken from the Marshfield genetic map, measured from the 8p telomere. Lod scores were calculated using the program Linkmap from the Fastlink version of the Linkage package available through the Human Genome Mapping Project Resources Center (Cambridge, UK). Allele frequencies were estimated from alleles sampled within the family, as for the two-point analysis. The maximum lod score, both at D8S1769 and GATA101H09, was 7.6.

RESULTS

Disease Expression of the Primary CRD-Affected Family

Affected members of this family had childhood-onset visual acuity impairment. The disease progressed over decades to major loss of central and then peripheral visual function. At late stages of disease, patients retained only islands of vision in the midperiphery. Funduscopic appearance (Fig. 2) and visual function test results (Fig. 3) in two affected family members in the fifth decade of life are shown. Visual acuities in both patients were 20/200 or worse, and the refractive error was modest myopia with astigmatism. There were small posterior subcapsular cataracts.

In the central retina of the right eye of patient A (Fig. 2A), there was a large area of discoloration, suggesting retinal pigmented epithelial disturbances. Patient B showed a macular coloboma-like lesion with a central atrophic scar surrounded by a ring of pigmentary change (Fig. 2B). The central retinal area was discolored as in patient A; there were, in addition, small, discrete, white-yellow patches. In both patients, the fundus temporal to the macula was less pathologic appearing (Figs. 2C, 2D). Both patients had attenuated retinal vessels. Patient B showed temporal pallor of the optic nerve head, whereas this was not as evident in patient A.

Visual function test results are shown in Figure 3. Compared with ERGs of a representative normal subject, all waveforms of the two patients were severely abnormal (Fig. 3A). Patient A showed reduced amplitudes but normal timing of both rod and cone ERGs. Patient B had greater reductions in amplitude. Figure 3B shows kinetic perimetry (top panels) and static threshold perimetry in the dark-adapted (middle panels) and light-adapted (bottom panels) states. Sensitivity losses for rods (500-nm stimulus, dark-adapted) and long- (L) and middle (M)-wavelength cones (600-nm stimulus, light-adapted) were calculated from locus-specific normal values and are shown as gray-scale maps (0–3 log units). The psychophysical tests were conducted to position the anatomic fovea at the central fixation target (central scotoma thus truly "central"). Patients otherwise used eccentric fixation loci of approximately 20° from the fovea. By kinetic perimetry, both patients retained midperipheral islands of vision and could see both a large bright target (V-4e) and a smaller bright target (I-4e). Static perimetry also indicated detectable rod and cone sensitivity mainly in the nasal midperiphery. Some loci had no loss of rod or cone sensitivity, whereas other loci showed losses of 1 to 3 log units.

Additional CRD-Affected Families

All 15 probands reported symptoms of decreasing central vision. In addition to reduced visual acuity, these individuals and their affected family members showed color vision disturbances, relative or absolute central scotomas with minimal-to-moderate peripheral field restriction by kinetic perimetry, and ERG abnormalities. ERG b-wave results in patients with detectable signals conformed to the two patterns we have described in CRD[3,5]: cone responses substantially worse than those of rods or cone and rod responses equally reduced. A spectrum of macular changes were observed that varied from a blunted foveal reflex or nonspecific mottling of the foveal retinal pigment epithelium, to bull's eye–appearing macular lesions, to geographic atrophy. Most but not all patients had peripheral retinopathy, which ranged from regions of hypopigmentation to pigment clumping (anterior to the vascular arcades) to large patches of atrophy. Despite a spectrum of visual function and clinical findings in the different families, there was intrafamilial consistency of phenotype with those members at advanced stages showing a more severe version of the disease pattern found in their younger less-affected relatives.

Genotyping of the Primary Family

Analysis of the initial genome-wide scan suggested a locus in a large region around the centromere of chromosome 8 from 8p12 to 8q12. This locus has been named *CORD9* (Hugo Gene Nomenclature Committee, http://www.gene.ucl.ac.uk/nomenclature/). Two-point lod scores were calculated for markers in this region from the original genotyping and from additional markers tested by us. There were two peaks: one at D8S1769 (60.3 cM from the p telomere in the Marshfield genetic map) and the other at GATA101H09 (64.6 cM). Multipoint analysis showed a flat approximately 4-cM peak from D8S1769 to GATA101H09 with a lod score of approximately 7.6 for both markers (Fig. 4). The pedigree (Fig. 1) reveals a consanguineous family with three informative sibships. The three fathers of the sibships are brothers, whereas two of the mothers are sisters and the third is their first cousin. Each of the fathers is third cousin to each of the mothers. Crossovers at D8S1820 and D8S532 place the mutant gene proximal to (be-

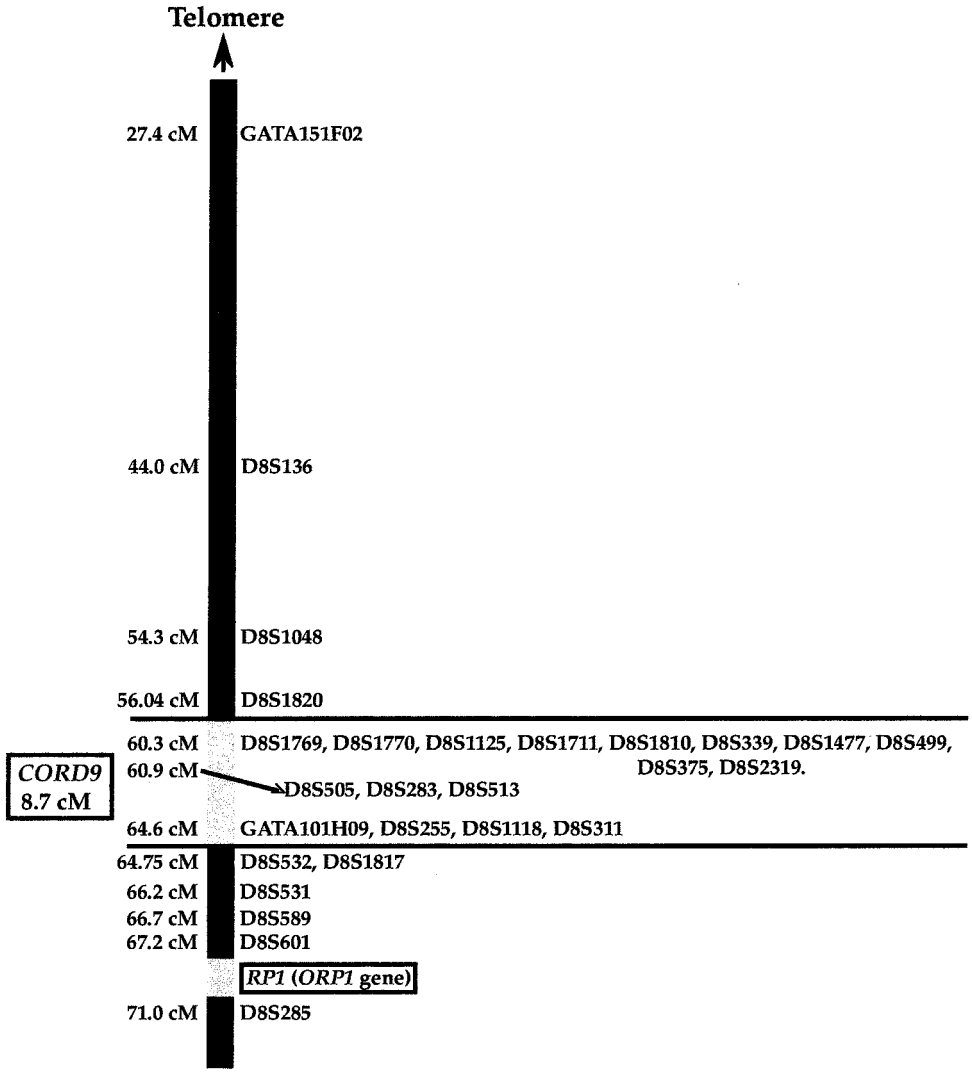

FIGURE 5. Chromosome 8 locus of *CORD9*. Markers and genetic distances were taken from the Marshfield Genetic map. Ten markers are listed at 60.3 cM in the Marshfield map. The nine affected individuals inherited the same homozygous alleles for six of these markers, and the same heterozygous alleles for the other four. At 64.6 cM there are four markers listed in the map. The nine affected individuals inherited the same homozygous allele for two of the markers and the same heterozygous alleles for two of the markers. A fifth marker was uninformative and a sixth segregated proximal to D8S532. The same heterozygous alleles were inherited for D8S505, D8S283, and D8S513 (at 60.9 cM) which lie between these two possible regions of homozygosity (*batched areas*).

low) the former and distal to (above) the latter (legend to Fig. 1). According to the Marshfield genetic map, the distance between D8S1820 and D8S532 is 8.7 cM (Fig. 5).

Because this is a consanguineous family, we searched for a region of homozygosity within the 8.7-cM locus. Figures 1 and 5 show two possible homozygous regions within the locus separated by a region of heterozygosity. Figure 5 also shows that D8S532 (cM 64.8), at the proximal flank of *CORD9*, is clearly distal to the distal flank of the *ORP1* gene, D8S601 at cM 67.2.[11,12] *ORP1* is the site of mutations responsible for one of the genetic forms of adRP known as RP1.[13–15].

Gene Candidate

At the time the *CORD9* locus was first discovered, examination of Genemap 99 (provided by NCBI and available at http://www.ncbi.nlm.nih.gov/genemap/) identified the expressed sequence tag (EST) stSG4713 within the candidate region. This EST was part of the UniGene cluster Hs.8719 (ESTs, weakly similar to dual-specificity protein phosphatase 3; UniGene System provided by NCBI at http://www.ncbi.nlm.nih.gov/UniGene). Of the ESTs within this cluster, 48% (43/89) originated from eye or brain cDNA, making this EST, hereafter referred to as dual-specificity protein phosphatase 3-like (*DUS3*-like), a good candidate for the *CORD9* gene. The complete coding sequence of *DUS3*-like was obtained on the basis of overlapping EST sequences available within GenBank (provided by NCBI and available at http://www.ncbi.nlm.nih.gov/GenBank) and RT-PCR analysis. The mRNA obtained was 1689 bp, contained an open reading frame (ORF) between nucleotide 135 and nucleotide 1008, and encoded a predicted protein of 211 amino acids. The genomic organization of *DUS3*-like was determined by comparing the sequence of the cDNA with the genomic sequence of clone RP11-2I13 available in GenBank (accession number AC015779). *DUS3*-like contains four exons and spans 7.5 kb. RT-PCR analysis confirmed a high level of expression of this gene in retina and brain but also showed it was widely expressed in many other tissues (Fig. 6). Therefore, because the complete genome structure for this relatively short retinally expressed gene was available, we directly sequenced it in family members, but no DNA changes were observed.

More recently, it has become possible to produce a comprehensive list of known and predicted genes in the *CORD9* region using the Genome Web Browser (provided by the University of California Santa Cruz and available at http://genome.ucsc.edu/index.html). A recent version of this revealed 28 known genes and a slightly larger number of predicted genes in the approximately 11-mb centromeric interval between markers D8S1820 and D8S532. It may therefore require further genetic studies to make identification of the gene involved a more feasible undertaking.

FIGURE 6. RT-PCR analysis of DUS3-like. (**A**) Intra-exonic primers from *DUS3*-like (5′-CATGGCTAACAACCGCCGGG-3′ and 5′-CCTCCAC-GAGGGTAAGGTGG-3′) were used to amplify cDNA generated from retina and a number of other tissues that were available commercially (Clontech). *Lane 1*, retina; *lane 2*, brain; *lane 3*, fetal brain; *lane 4*, cerebellum; *lane 5*, spinal cord; *lane 6*, kidney; *lane 7*, liver; *lane 8*, fetal liver; *lane 9*, trachea; *lane 10*, lung. (**B**) GAPDH control.

Genotyping of the 15 Additional CRD-Affected Families

To estimate the frequency of the *CORD9* mutation in arCRD, we performed genotype analysis on 13 multiplex and 2 simplex families in which neither parent had disease, using markers from the locus. We first tested D8S499 because it is a tetranucleotide repeat and was among the markers in the region of homozygosity at 60.3 cM (Fig. 5). If D8S499 was uninformative, we also tested D8S2319 and D8S1125, tetranucleotide and trinucleotide repeats, respectively, in the same region of homozygosity. In eight of the families, D8S499 did not segregate with disease and in one additional family that was uninformative for D8S499 and D8S2319, D8S1125 did not segregate with disease. This locus could not be ruled out from segregating with disease in the other six families. We use the phrase "could not be ruled out from segregating with disease" because even though the affected individuals in these six families inherited the same unique pair of alleles for the markers tested, the probability of this occurring by chance is high. For example, in a sibship of one unaffected and two affected siblings, the chance that the two affected siblings will uniquely inherit the same pair of alleles of a marker is almost 5 in 100. Therefore, based on our very small sample of 15 families, the frequency of *CORD9* mutations can be no more than 6 (40%) of 15 and is almost certainly much lower.

DISCUSSION

We have performed an entire genome linkage search in a large consanguineous pedigree of Brazilian origin, segregating a severe form of recessive CRD, and have found a new locus *CORD9* at 8p11. We identified the candidate gene *DUS3*-like by uncovering a UniGene cluster of ESTs that maps to the *CORD9* locus and has a high frequency of expression in the retina. After determining the mRNA sequence from overlapping EST sequences and RT-PCR analysis, the genomic organization of *DUS3*-like was determined from a genomic clone available in GenBank. The four exons of the gene were directly sequenced in members of the Brazilian family, but no DNA changes were observed.

Haplotype analysis of 13 multiplex and 2 simplex North American CRD-affected families using markers from the *CORD9* locus, ruled out 9 of them, leaving a maximum of 6 (40%) that could have disease-causing mutations in the gene at this locus. It is likely that the actual percentage of mutations is substantially lower. In studies of the *PDE6B* gene in 30 families with autosomal recessive retinal degeneration, the gene could be ruled out from segregating with disease in 21 families. Exon screening by single-strand conformation polymorphism (SSCP) of the probands from the nine remaining families revealed a homozygous mutation in one and no mutations in either allele of the other eight probands.[16]

There are at least 40 genes that can cause nonsyndromic RP when mutated (24 genes and 16 additional loci for which the mutant gene has not yet been discovered). Of the 17 arRP genes that have been identified, not one has been shown to have mutations causing more than a small percentage of all arRP.[17–24] There are four genes and six additional loci for nonsyndromic CRD.[6] Only one gene and one locus (with the gene not yet identified) have been associated with arCRD. Mutations in this one gene (*ABCA4*) have been estimated to account for as much as 70% of cases of arCRD in one study[25] but a lesser percentage in another.[26] Further molecular studies of patients diagnosed with CRD using published classification schemes[3,5] should clarify the frequency of the different genotypes and their relation to phenotype.

In summary, we have identified a new locus for arCRD (*CORD9*) at 8p11. We have shown that disease can be caused by mutations in the gene at this locus in no more than 6 (40%) of a cohort of 13 multiplex and 2 simplex CRD-affected families, and suggest that this maximum frequency will turn out to be considerably lower for the gene. We have also identified the *DUS3*-like candidate gene in the *CORD9* locus, determined its exon–intron structure, screened it, and found no mutations associated with disease in the primary arCRD family. Although CRD is relatively rare, the pathogenesis of this disease resembles many aspects of the more common age-related macular degeneration. Identification of the gene mutated in *CORD9* will, therefore, highlight a new candidate for possible involvement in susceptibility to this increasingly significant disease of older adults.

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