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Linkage Analysis of X-linked Cone-Rod Dystrophy: Localization to Xp11.4 and Definition of a Locus Distinct from RP2 and RP3

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Summary

Progressive X-linked cone-rod dystrophy (COD1) is a retinal disease affecting primarily the cone photoreceptors. The COD1 locus originally was localized, by the study of three independent families, to a region between Xp11.3 and Xp21.1, encompassing the retinitis pigmentosa (RP) 3 locus. We have refined the COD1 locus to a limited region of Xp11.4, using two families reported elsewhere and a new extended family. Genotype analysis was performed by use of eight microsatellite markers (tel-M6CA, DXS1068, DXS1058, DXS993, DXS228, DXS1201, DXS1003, and DXS1055-cent), spanning a distance of 20 cM. Nine-point linkage analysis, by use of the VITESSE program for X-linked disorders, established a maximum LOD score (17.5) between markers DXS1058 and DXS993, spanning 4.0 cM. Two additional markers, DXS977 and DXS556, which map between DXS1058 and DXS993, were used to further narrow the critical region. The RP3 gene, RPGR, was excluded on the basis of two obligate recombinants, observed in two independent families. In a third family, linkage analysis did not exclude the RPGR locus. The entire coding region of the RPGR gene from two affected males from family 2 was sequenced and was found to be normal. Haplotype analysis of two family branches, containing three obligate recombinants, two affected and one unaffected, defined the COD1 locus as distal to DXS993 and proximal to DXS556, a distance of ~1.0 Mb. This study excludes COD1 as an allelic variant of RP3 and establishes a novel locus that is sufficiently defined for positional cloning.

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Introduction

X-linked cone-rod dystrophy (COD1; OMIM 34020 [http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?34020]) is a rare, progressive visual disease affecting primarily the cone photoreceptors. Affected males present with decreased visual acuity, myopia, photophobia, abnormal color vision, full peripheral visual fields, and granularity of the macular retinal pigment epithelium (RPE). Complete atrophy of the macular RPE may develop late in life, accompanied by a progressive decline in visual acuity (Pinckers and Timmerman 1981; Pinckers et al. 1981; Jacobson et al. 1989). The degree of rod-photoreceptor involvement can be variable, with increasing degeneration as the disease progresses. Photopic cone-mediated responses range from significantly decreased to absent (Meire et al. 1994). Female carriers have variable symptoms, ranging from asymptomatic to photophobic with abnormal electroretinogram (ERG), abnormal color vision, and abnormal visual evoked response (Pinckers et al. 1981; Jacobson et al. 1989). Although penetrance appears to be nearly 100%, there is variable expressivity, with respect to age at onset and severity of symptoms and findings (Hong et al. 1994).

COD1 originally was mapped to Xp11.3-21.1, in 1989 (Bartley et al. 1989). Additional evidence supporting this map location was presented by Bergen et al. (1993), Meire et al. (1994), and Hong et al. (1994). These linkage studies, however, were unable to resolve COD1 as an independent locus distinct from the retinitis pigmentosa (RP) 2 and RP3 loci and supported the hypothesis that COD1 could be an allelic variant of either RP2 or RP3, on the basis of the chromosomal location as well as clinical phenotypes. Allelic heterogeneity has been well documented for other retinal dystrophies, such as RP and Norrie disease (Chen et al. 1993; Shastry 1994; Gorin et al. 1995).

We have genotyped three extended families segregating COD1, using 11 polymorphic markers, spanning 20 cM, including both the RP2 and RP3 loci. Linkage analysis was performed with 8 markers, by use of the pro-

gram VITESSE modified to handle X-linked pedigrees (O'Connell and Weeks 1995). Recombinant mapping of informative individuals was used to further define the COD1 locus, with respect to RP2 and RP3.

Families and Methods

Families

Sixteen members of a five-generation family (family 1; fig. 1), 36 members of a six-generation family (family 2; fig. 2), and 35 members of a six-generation family (family 3; fig. 3) were genotyped. The clinical descriptions and diagnostic criteria for families 1 and 3 have been described elsewhere (Jacobson et al. 1989; Hong et al. 1994). Ophthalmic records of males (from family 3) who had not been evaluated by Jacobson et al. (1989) were reviewed. Members of family 2 were characterized by use of medical-chart review, as well as by detailed interviews with family members, concerning visual problems associated with cone-rod dystrophy. The severity of the phenotypes as well as the age at onset were variable in all three families. The majority of the affected males had lost central vision during their late teens or early 20s. The proband (individual VI:3) and individuals V:27 and VI:11 from family 2 were examined by M.B.G. Members of family 3 were examined by J.B. and A.E.K. Individual IV:16 from family 3 was examined at age 38 years. ERG findings revealed a normal scotopic cone response; the B wave was 178/173 μV and 30/29.5 ms. Diagnostic criteria for family 2 were as described by Hong et al. (1994). For this study, the participation of family members was approved by the University of Pittsburgh Biomedical Institutional Review Board, in accordance with the guidelines of the Office for the Protection from Research Risks, and informed patient consent was obtained prior to participation.

PCR Analysis

Genomic DNA was extracted from 10–20 ml of whole blood. Mononuclear cells were isolated by use of a density gradient, in accordance with the manufacturer's conditions (Hypaque; Pharmacia). Isolated cells were lysed overnight in a 1.0% SDS, 0.5 mg proteinase K/ml solution, at 50°C. DNA was precipitated after phenol-chloroform purification by use of 0.33 vol 10 M ammonium acetate and 2.5 vol 100% ethanol. MapPairs primers (DXS993, DXS1003, DXS1068, DXS1058, DXS1201, DXS228, and DXS1055) were purchased from Research Genetics. M6CA primer sequences were 5′-TTC CTG TGC ATG GGA ACC AC-3′ and 5′-GGA TGA ACA ACC ACC CAA ATT-3′. DXS8025, DXS556, and DXS977 are described in GDB (accession numbers GDB: 643168, GDB:197006, and GDB:377123, respectively), and their map positions were estimated by use of SEG-

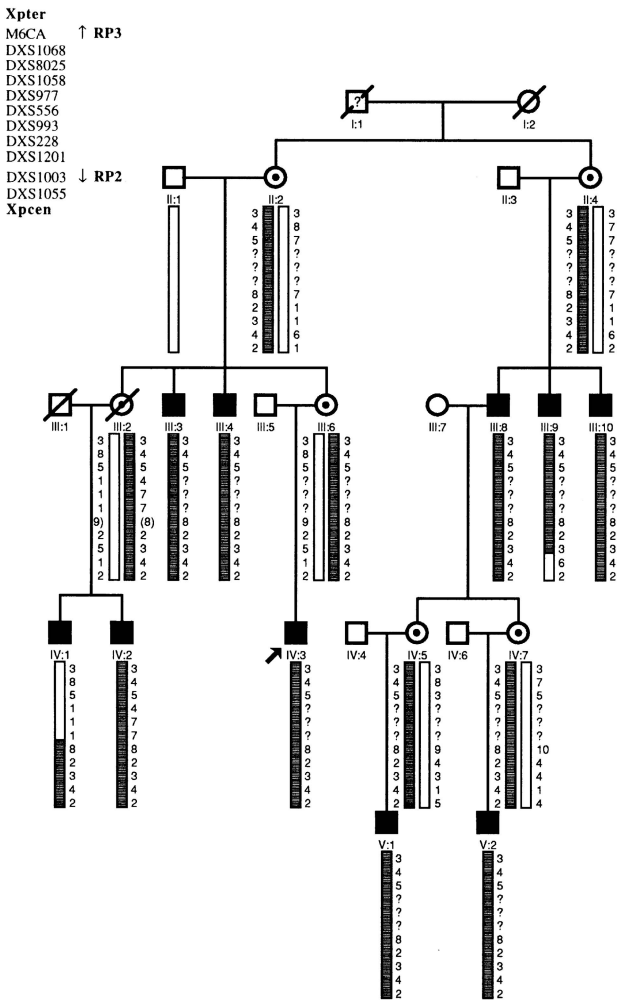

Figure 1 Haplotype analysis of family 1, segregating COD1. The at-risk haplotype is hatched, to clearly show recombinations. Carrier females are indicated by a black dot within a circle, and affected males are indicated by blackened squares. Unblackened squares and circles without a black dot denote unaffected males and noncarrier females, respectively. A question mark (?) within a square indicates that the affection status has not been established. An arrow indicates the proband. The order of the markers is shown in the key to the left of the figure and is consistent with that for families 2 and 3 (figs. 2 and 3, respectively). Alleles within parentheses were inferred, with the exception of "(99)" in figures 2 and 3, which is an arbitrary number inserted to establish phase.

MAP (Magness et al. 1993), on the basis of data derived from the Washington University Genome Center. The forward primer for each set was end-labeled with T4 kinase (Gibco) and γ-[^{32}P]-ATP (New England Nuclear/Dupont). The PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, with 1 unit *Taq* polymerase in 25 mM TAPS (N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; pH 9.3), 1.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 0.2 mM dNTP, and 0.2 μM each primer,

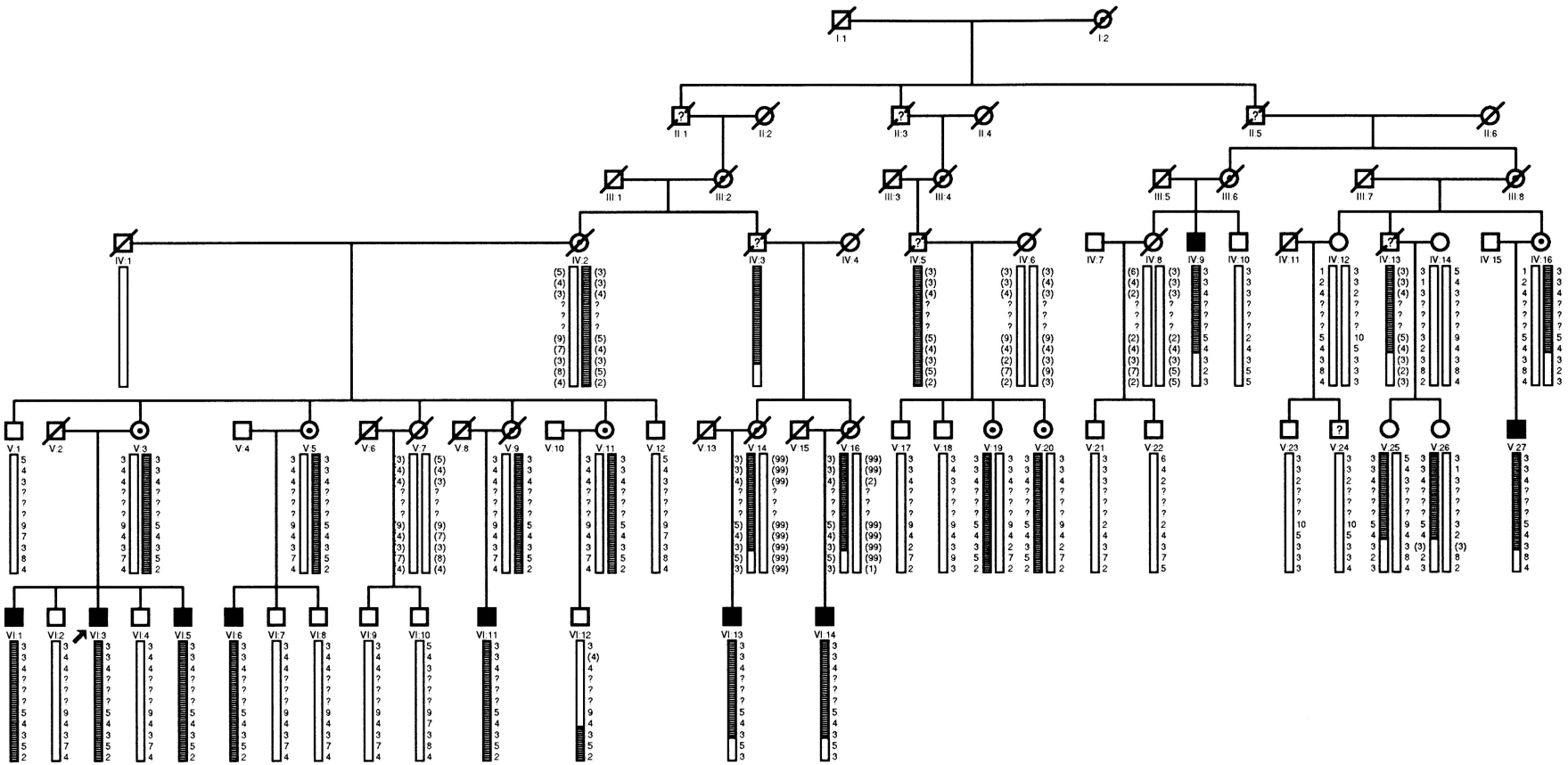

Figure 2 Haplotype analysis of family 2, segregating COD1. Symbols are as defined in the legend to figure 1.

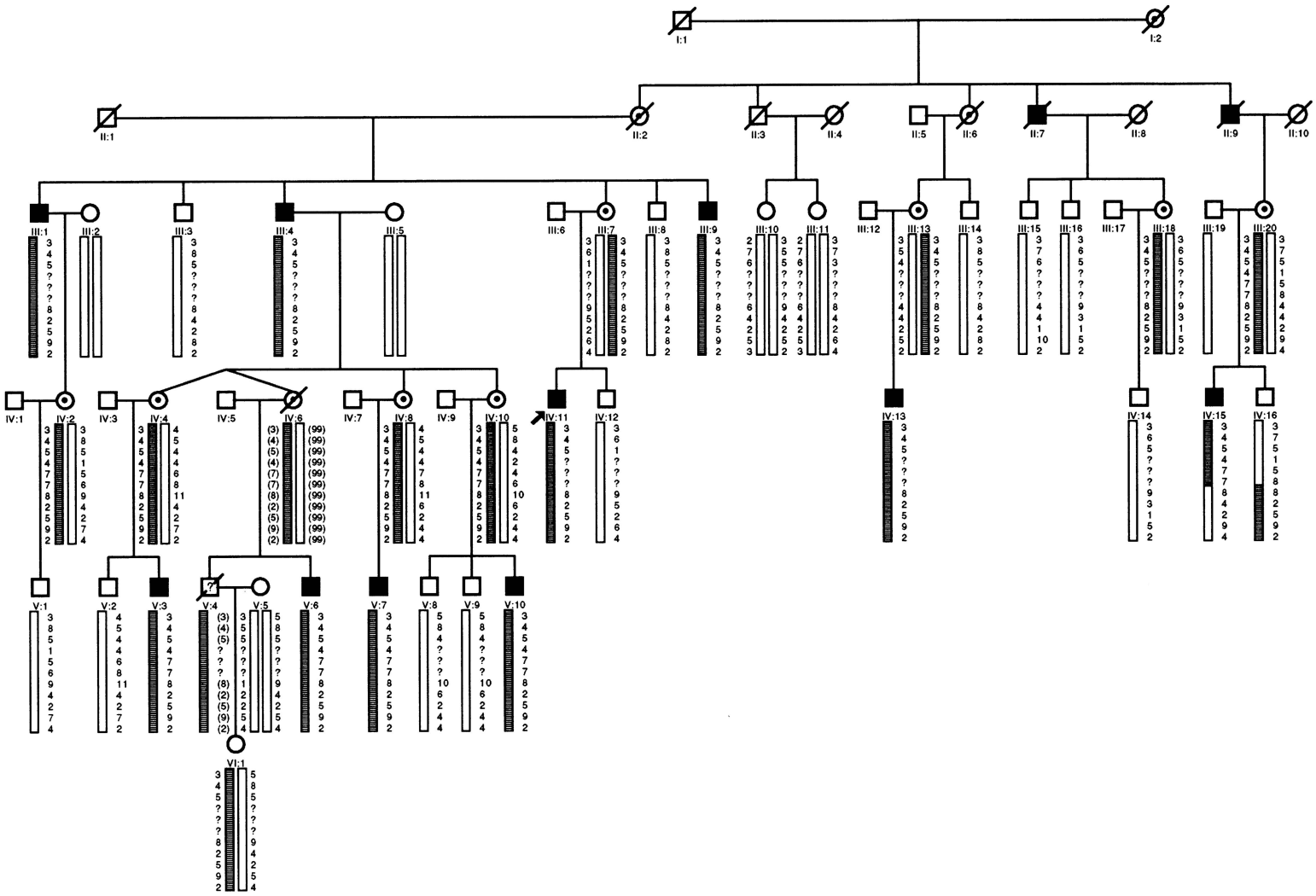

Figure 3 Haplotype analysis of family 3, segregating COD1. Symbols are as defined in the legend to figure 1.

Table 1

Two-Point LOD Scores between the COD1 Locus and Markers Spanning Xp11.3-p11.4

	LOD Score at θ =					
Marker	.00	.05	.10	.20	.30	.40
M6CA	4.0458	3.76442	3.45707	2.76369	1.95841	1.03532
DXS1068	5.52884	12.08237	11.17213	8.8279	6.02898	2.87917
DXS1058	9.4829	8.70317	7.88757	6.13495	4.19265	2.05966
DXS993	12.58392	12.15392	11.16337	8.76569	5.97544	2.8354
DXS228	9.49005	9.59043	8.88967	7.05514	4.84172	2.31421
DXS1201	3.81624	8.33671	7.72942	6.07873	4.09985	1.89182
DXS1003	−1.75344	11.28836	11.10132	9.33397	6.72528	3.52923
DXS1055	−7.46604	3.26174	3.89578	3.54291	2.34304	.84782

in a final volume of 20 μl. PCR products were electrophoresed on a 6.0% polyacrylamide, 7 M urea denaturing sequencing gel and were dried and analyzed by autoradiography. Product sizes were determined by comparison with an M13 sequencing ladder.

Linkage Analysis

Linkage analysis was performed by use of the linkage program VITESSE, modified to handle X-linked data. VITESSE, which uses a novel algorithm to perform rapid multipoint likelihoods, was able to handle the complete nine-point analysis without having to break the eight-marker framework map into smaller sets. We thus were able to extract maximum information on the position of the trait locus. The frequency of the COD1 allele in the general population was ~.0001, with a penetrance of .99. The order and distances of the markers used were determined by use of the most recent genetic map of the X chromosome (Nelson et al. 1995). The number of alleles for each marker was as described in GDB. Because allele frequencies were not available, alleles were assumed to be at equal frequencies for all of the linkage analyses.

Sequencing

Ten milliliters of whole blood was collected from individuals VI:3 and VI:6 from family 2. Peripheral lymphocytes were collected as described above. Whole RNA was isolated by use of the RNAgents kit (Promega), in accordance with the manufacturer's protocol. First-strand cDNA synthesis was performed by use of an oligo(dT) primer, under standard reverse-transcriptase–PCR conditions with 1 unit of reverse transcriptase (Perkin-Elmer). The RP3 gene, RPGR (*r*etinitis *p*igmentosa *G*TPase *r*egulator) (Meindl et al. 1996), was amplified in two overlapping segments, of 1.8 kb (5′) and 0.8 kb (3′), by use of the following primers: 5′ end—forward, 5′-ACC GTC CTC TAC AGC CTCC-3′, and reverse, 5′-CTA CTT CCT CAT CTG AAA ATGC-3′; and 3′ end—forward, 5′-CGC AGC CAG CTA CGA CTA TC-3′, and reverse, 5′-TTA CAA TAC ACT TGG TGA CTG TG-3′.

The samples were amplified for 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min, followed by extension at 72°C for 10 min. PCR products were subcloned into the PCR II vector, in accordance with the manufacturer's protocol (Invitrogen TA cloning kit). Sequencing was performed by use of M13 forward and reverse primers and the following two internal primers for the 1.8-kb fragment: 5′-CTG CCC TAA CTG AGG ATG GAAG-3′ and 5′-GAG TCC CTT CTA TTG GAGG-3′ (ALFexpress CY5 cycle sequencing kit; Pharmacia). Sequence reactions were analyzed by use of the ALFexpress automated sequencer.

Results

Linkage analysis was performed by use of the following markers: tel-M6CA–1.5 cM–DXS1068–DXS1058–4.0 cM–DXS993–6.0 cM–DXS228–2.25 cM–DXS1201–2.25 cM–DXS1003–DXS1055. The analysis was conducted by use of a model of X-linked inheritance with a penetrance of .99 and a disease-allele frequency of .0001. Nine-point linkage analysis calculated a maximum combined LOD score of 17.5 between markers DXS1058 and DXS993, a distance estimated to be <4 cM. Two-point linkage analysis, using identical parameters, placed the disease locus closest to marker DXS993, with a combined LOD score of 12.58 (recombination fraction of θ = .00) (table 1).

Three recombinants, two affected and one unaffected, were further genotyped by use of two markers between DXS1058 and DXS993 (DXS977 and DXS556) to further narrow the critical region of COD1 (fig. 2). Individual IV:1 in family 1 defined the COD1 region proximally, through a recombination between markers DXS993 and DXS556, thus excluding RP3, which is distal to marker DXS556 (Meindl et al. 1996). Individual IV:15 in family 3 defined the critical region distally, through a recombination between DXS993 and

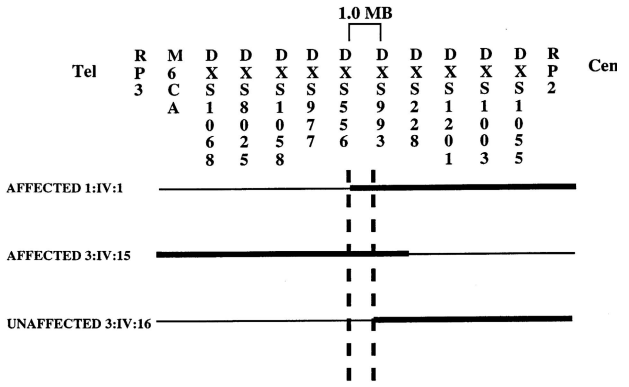

Figure 4 Map of the recombinations defining the critical COD1 region. The affected individuals define the critical region between DXS556 and DXS228, on the basis of recombinations. The unaffected individual narrows the critical region, on the basis of a recombination between DXS993 and DXS556. The dashed lines depict the smallest interval shared by affected individuals. The boldface lines represent the affected haplotypes. The marker order for DXS977 and DXS556 was determined by use of Segmap (Magness et al. 1993). This map is not drawn to scale.

DXS228, excluding RP2, which is proximal to DXS228 (Thiselton et al. 1996). Data on individual IV:16 in family 3, an unaffected male (established by examination and confirmed by ERG testing at age 38 years), were used to define the critical region flanked by DXS993 and DXS556, on the basis of the inclusion of the affected haplotype at DXS993 and a recombination between DXS993 and DXS556. These recombinants refined the COD1 locus to a region that is flanked distally by DXS556 and proximally by DXS993, an estimated physical distance of <1 Mb. Although markers DXS1068 and DXS1058 (distal) and DXS1055 and DXS1003 (proximal) were <1.0 cM from each other, allelic association was not determined, on the basis of the exclusion of these regions by recombinations.

In all three families, RP2 was excluded by recombination events at markers distal to the RP2 locus. RP3 was excluded in families 1 and 3 on the basis of recombinations at markers proximal to the RP3 locus. In family 2, RP3 could not be excluded by recombinations; therefore, RPGR, the transcript of the RP3 gene, was sequenced to screen for mutations. The entire coding region was isolated from leukocyte mRNA of affected individuals VI:3 and VI:6 from family 2 and was cloned and sequenced as two overlapping DNA fragments of 1.8 kb (5′) and 0.8 kb (3′). No mutations were identified when the coding region was compared with the published sequence and a normal, control sequence. Sequencing was not performed on 5′ or 3′ UTRs or on RPGR promoter sequences. On the basis of these data, we have tentatively excluded the presence of the RP3 gene in this family. Therefore, COD1 is defined as a locus distinct from RP2 and RP3.

Although families 1 and 3 were from New York and Iowa, respectively, a similar haplotype was observed among the affecteds from each family. Affected males in these two pedigrees, with the exception of the two recombinants, have identical allele sizes for markers DXS1068, DXS1058, DXS977, DXS556, and DXS993.

Discussion

COD1 has been hypothesized to be an allelic variant of either RP2 or RP3. Using three large families segregating COD1, we have defined the critical region to be a locus distinct from those for RP2 and RP3. The RP2 locus has been mapped to a region that lies proximal to marker DXS1201, in a 4–5-cM interval flanked by markers DXS8083 and DXS6616 (Thiselton et al. 1996). Obligate recombinants proximal to this marker were observed in all three families, thus excluding RP2 as the COD1 locus. The RP3 gene, RPGR, which was identified recently, maps distal to marker DXS1068 (Meindl et al. 1996). In families 1 and 3, obligate recombinants proximal to DXS1068 excluded RP3 as the COD1 locus. The coding region of RPGR was sequenced in family 2, and no mutations were identified, thus tentatively excluding RP3. These data support our hypothesis that COD1 is a unique locus flanked by RP2 and RP3.

Linkage analyses of X-linked RP families have mapped three loci (RP2, RP3, and RP15) to the short arm of the X chromosome, with RP3 accounting for the majority of cases (Chen et al. 1989; Musarella et al. 1990; Ott et al. 1990; Teague et al. 1994; Bergen et al. 1995; McGuire et al. 1995). Mutation analysis of RPGR from affected males of X-linked RP families has determined that only 9.5% (7 of 74 families) are due to mutations in this gene, suggesting that a novel locus could be responsible for a subset of X-linked RP cases (Meindl et al. 1996). The low frequency of RPGR mutations identified in affected males and the mapping of COD1 as a distinct locus in proximity to RP2 and RP3 suggest that some cases of X-linked RP could be allelic with COD1. For family 1, we have previously reported clinical heterogeneity, suggesting that identical mutations within the same gene could lead to varying phenotypes (Hong et al. 1994). Although most individuals with late-stage cone-rod disease show considerable rod-photoreceptor involvement, this is possibly a secondary result of the progressive degeneration of the cone photoreceptors. However, in family 1 there are individuals with a disproportionate amount of rod-photoreceptor dysfunction at an early stage of the disease. None of the individuals in the families studied presented with phenotypes that are distinctive for RP, such as peripheral pigmen-

tation, night blindness, or peripheral vision loss. Phenotypic heterogeneity has been well documented for other progressive retinal diseases with differing clinical presentations. For example, mutations in the peripherin gene have been described in families segregating either autosomal dominant RP or macular dystrophy (Shastry 1994; Gorin et al. 1995).

Analysis of the most recent physical map of the X chromosome suggests that the COD1 critical region lies within a 1.0-Mb interval. This small region facilitates both the construction of a physical contig spanning the COD1 region and the use of positional cloning techniques to identify and characterize the COD1 gene.

Haplotype analysis of the three families has provided evidence supporting a possible founder effect. Families 1 and 3 are unrelated for at least six generations; however, the affected males share alleles at five markers covering a distance of 4.0 cM. Identification of the COD1 gene will resolve whether or not there is a possible founder effect explaining the disease origin in these two families.

We have defined COD1 as a locus distinct from both RP2 and RP3 and have narrowed the critical region to a distance suitable for the initiation of positional cloning techniques. The identification and analysis of the COD1 gene may resolve the issues of allelic heterogeneity. In addition, the characterization of the COD1 gene will further our understanding of the biology pertaining to degenerative eye diseases.

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