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Refining the Locus for Best Vitelliform Macular Dystrophy and Mutation Analysis of the Candidate Gene ROM1

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Summary

Vitelliform macular dystrophy (Best disease) is an autosomal dominant macular dystrophy which shares important clinical features with age-related macular degeneration, the most common cause of legal blindness in the elderly. Unfortunately, our understanding and treatment for this common age-related disorder is limited. Discovery of the gene which causes Best disease has the potential to increase our understanding of the pathogenesis of all types of macular degeneration, including the common age-related form. Best disease has recently been mapped to chromosome 11q13. The photoreceptor-specific protein ROM1 has also been recently mapped to this location, and the ROM1 gene is a candidate gene for Best disease. Using highly polymorphic markers, we have narrowed the genetic region which contains the Best disease gene to the 10-cM region between markers D11S871 and PYGM. Marker D11S956 demonstrated no recombinants with Best disease in three large families and resulted in a lod score of 18.2. In addition, a polymorphism within the ROM1 gene also demonstrated no recombinants and resulted in a lod score of 10.0 in these same three families. We used a combination of SSCP analysis, denaturing gradient gel electrophoresis, and DNA sequencing to screen the entire coding region of the ROM1 gene in 11 different unrelated patients affected with Best disease. No nucleotide changes were found in the coding sequence of any affected patient, indicating that mutations within the coding sequence are unlikely to cause Best disease.

Introduction

Vitelliform macular dystrophy (Best disease) is an autosomal dominant juvenile-onset macular degeneration characterized by an accumulation of lipofuscin-like material within and beneath the retinal pigment epithelium, which results in an egg-yolk-like appearance of the macula (Best 1905). This abnormal material is associated with progressive macular degeneration which eventually leads to a severe loss of central vision in some patients. The age at clinical onset varies from early childhood to the sixth decade of life. The diagnosis can be made in many affected individuals on the basis of the classic appearance of the retinal lesions. In patients without classic lesions, the diagnosis can be made by electro-oculography (EOG) (Arden et al. 1962). Best disease shares some clinical features with age-related macular degeneration (AMD), which is the most common cause of legal blindness in the elderly. Some degree of AMD is present in as many as 20% of Caucasian individuals >70 years of age (Leibowitz et al. 1980). However, our current understanding of the pathogenesis of this common disorder is poor, and the treatment is limited. Identification of the gene which causes Best disease could lead to a better understanding of the pathogenesis of all forms of macular degeneration and could in turn lead to more effective treatments for these disorders.

Best disease has been recently linked to markers located at 11q13 (Forsman et al. 1992; Stone et al. 1992*b*). Our initial study of a single large pedigree suggested the

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most likely location of the Best disease gene to be between markers D11S871 and INT2. The gene for the retinal outer-segment-membrane protein ROM1 has recently been characterized (Bascom et al. 1993) and mapped to this location (Bascom et al. 1992*a*). ROM1 is expressed specifically in the retina and has been localized to the disk region of the rod outer segment (Bascom et al. 1992*b*). The ROM1 protein is similar in size and amino acid sequence to peripherin, the protein encoded by the RDS gene (Bascom et al. 1992*b*). Mutations in the RDS gene are associated with a subset of cases of autosomal dominant retinitis pigmentosa (Farrar et al. 1991; Kajiwara et al. 1991, 1992). We and others have recently demonstrated that the RDS gene can also cause some forms of macular degeneration, including butterfly-shaped pigment dystrophy of the fovea (Nichols et al. 1993*a*, 1993*b*), macular dystrophy (Wells et al. 1993), and retinitis punctata albescens (Kajiwara et al. 1993). On the basis of both its chromosome location and its similarity to the peripherin gene, ROM1 is a candidate gene for Best disease. Several polymorphisms in this gene have recently been identified (R. Bascom and R. McInnes, unpublished data).

A number of polymorphic markers have been reported which lie in the region of the Best disease gene. Three of these markers are highly informative simple tandem repeat polymorphisms (STRPs). Marker D11S871 (J. L. Weber, personal communication) has been previously linked to Best disease (Stone et al. 1992*b*). A polymorphism within the muscle glycogen phosphorylase gene (PYGM) (Iwasaki et al. 1992) has been recently reported. We developed an SSCP assay for a gene-duplication polymorphism within the pepsinogen A gene (PGA) reported by Evers et al. (1989). An ROM1 SSCP polymorphism is located in intron 1 of the ROM1 gene and is the result of a T insertion before the beginning of exon 2 (R. Bascom and R. McInnes, unpublished data). In the present study, we used each of these markers to refine the locus for Best disease in multiple families and examined the candidate gene ROM1, using mutation analysis.

Material and Methods

Clinical and Electrophysiological Studies

Members of three families with Best vitelliform macular dystrophy were examined for the clinical features of Best disease (fig. 1). EOG was performed with either a UTAS-E 2000 instrument (LKC Technologies) or a custom-made portable EOG unit. A light-peak:dark-trough ratio was then calculated (Arden et al. 1962). Individuals were considered affected for linkage if they had unmistakable ophthalmoscopic evidence of the disease or an Arden ratio of <1.5. Patients were considered to be normal for linkage analysis only if their fundus examination was completely normal and they had a clearly normal Arden ratio (≥ 1.8). Blood samples from individuals in the three families affected with Best disease were collected into EDTA-containing glass tubes, and DNA was prepared using a nonorganic extraction procedure (Grimberg et al. 1989). DNA for analysis of families from the CEPH database was obtained from CEPH (Paris).

STRP Analysis

Oligonucleotide primers were synthesized with standard phosphoramidite chemistry by using an Applied Biosystems model 391 DNA synthesizer. Fifty nanograms of each patient's DNA were used as template in a 8.35-μl PCR containing each of the following: 1.25 μl of 10 × buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin); 200 μM each of dCTP, dGTP, and dTTP; 37 μM dATP; 50 pmol of each primer; 0.25 μl of α-^{35}S-dATP (>1,000 Ci/mmol; Amersham); and 0.25 U of *Taq* polymerase (Perkin Elmer Cetus). Samples were incubated in a DNA thermocycler for 35 cycles under the following conditions: 94°C for 30 s, 55°C for 30 s, and 72° for 30 s. After amplification, 5 μl of stop solution (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added to each sample. Samples were denatured at 95°C for 3 min and were immediately loaded onto prewarmed polyacrylamide gels (6% polyacrylamide and 7 M urea). Gels were electrophoresed for 3–4 h, placed on Whatman 3MM paper, and dried in a slab gel dryer. Kodak X-Omat AR film was exposed to the dried gels for 18–36 h to create autoradiograms. STRP analysis of the 40 CEPH families was performed similarly to the method outlined above.

SSCP Analysis

SSCP analysis (Orita et al. 1989) was used to assay the PGA and ROM1 polymorphisms as well as a portion of the ROM1 gene (table 1). The following primers used to assay the PGA polymorphism were chosen from published PGA sequence (Evers et al. 1989): forward 5′-CCTAGCACAAGGAATAGCCAG-3′ and reverse 5′-GAGTGCAGTGGCGCGACC-3′. The primers used to assay the ROM1 polymorphism were the same as those used for exon 2a (table 1). DNA was amplified using

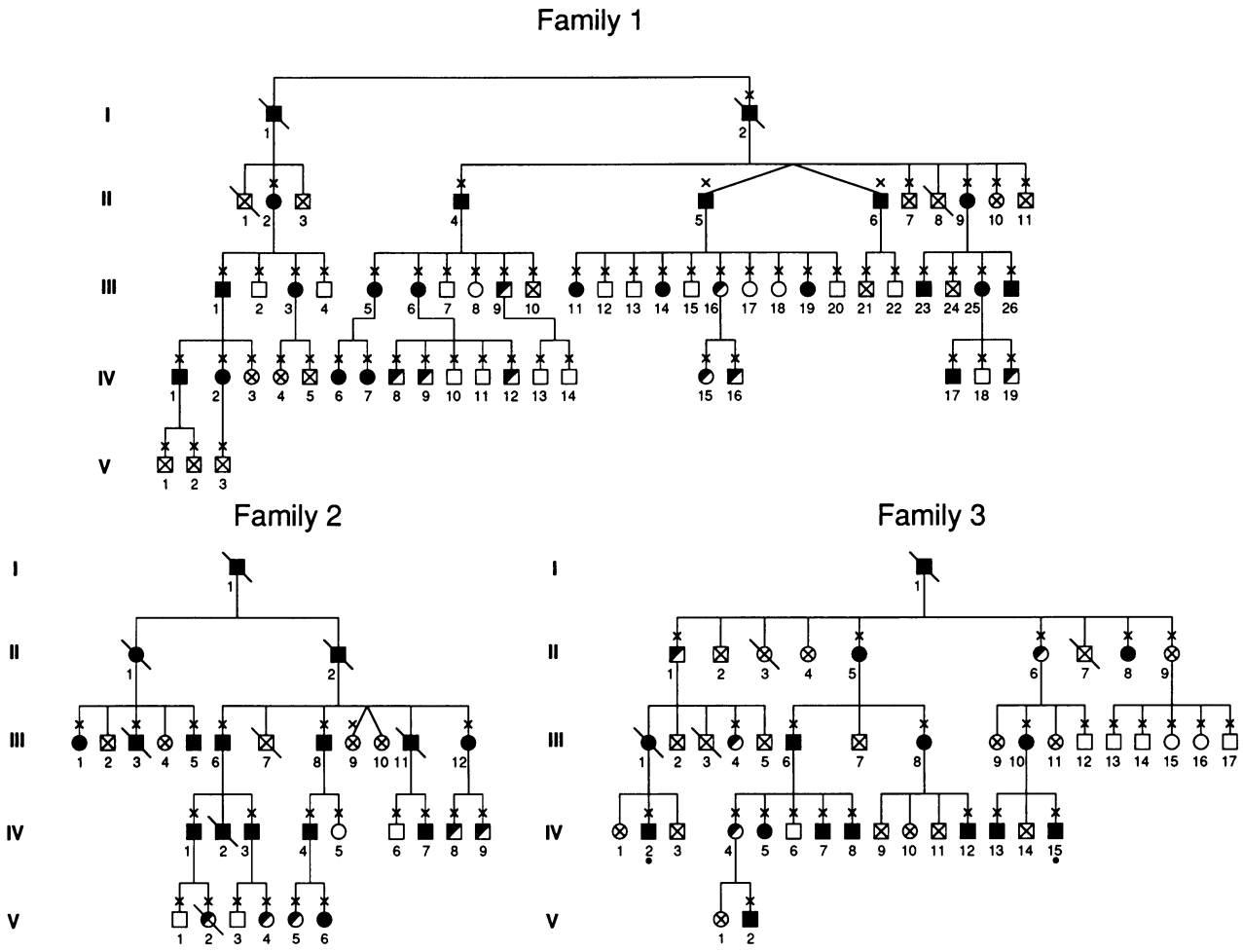

Figure 1 Pedigrees of the three families affected with Best disease. Family 1 was previously linked to 11q13 (Stone 1992*b*). Patients who were examined are indicated with a small "x" above their symbol. Blackened symbols indicate either ophthalmoscopically affected patients or patients who are obligate carriers of the disease because they have affected children. Half-blackened symbols indicate patients with equivocal retinal findings but a depressed EOG ratio. Unblackened symbols indicate patients who had normal appearing maculas and a normal EOG ratio. Patients who either were not seen or for whom a definite diagnosis could not be made are indicated by an "x" inside their symbol. Patients who were clinically examined but from whom a blood sample was not available are indicated by a black dot beneath their symbols.

either radioactive incorporation or a silver-staining procedure. For the former, DNA was amplified and internally labeled with α-^{35}S-dATP as described above, with the exception that the annealing temperature used was 60°C for 30 s. For detection with silver staining, DNA was amplified as described above, with the exception that 200 μM of each dNTP was used and α-^{35}S-dATP was omitted from the reaction. After amplification, stop solution was added as indicated above. Samples were denatured and electrophoresed on fan-cooled nondenaturing 5% (radioactive incorporation) or 8% (silver staining) polyacrylamide gels (5% glycerol and 0.5 TBE) at 20 W for approximately 4 h at room temperature. Autoradiograms for radioactive incorporation were created by exposing Kodak X-Omat AR film to the dried gels for 24–36 h. Gels for silver staining were prefixed to the shorter of the two glass plates by coating it with silane (Sigma) before pouring the gel. After electrophoresis, gels were silver stained according to a method reported elsewhere (Bassam et al. 1991). In brief, gels were fixed by soaking in 10% acetic acid for 20 min. After three successive 2-min rinses with dH_2O, gels were stained for 30 min in a solution containing $AgNO_3$ (1 g/liter) and formaldehyde (1.5 ml/liter). The gels were then rinsed briefly (<20 s) in dH_2O and developed for 4–7 min in a 4°C solution containing Na_2CO_3

Table 1

ROM1 Primers for Mutation Analysis

Exon	Primer Sequences	Assay
1a	Forward 5′-TGCACTCCCTTGGGCAGAGA-3′ Reverse 5′-AGCCAGGAAGGTGCCAAGGT-3′	SSCP
1b	Forward 5′-CGTCATCCTCCTCTGTAGTG-3′ Reverse 5′-GTGAGCCAAGGCAGTCACCA-3′	SSCP
1c	Forward 5′-TGGCTTTGCCTGGGAGTCTG-3′ Reverse 5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCGGCCGGATTCAAGGCAGCAGGAGG-3′	DGGE (50%–80% for 11 h)
2a	Forward 5′-CCTTCTGAACACCTGTGCCCT-3′ Reverse 5′-ACCCTTGGGCCCAGAGGTT-3′	SSCP
2b	Forward 5′-CTTTCAGACTCCTACGCCCA-3′ Reverse 5′-GGAGGAGGTGTCAGATGCTT-3′	SSCP
3	Forward 5′-TCTTGGAACCGCTGACTCTC-3′ Reverse 5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCGGCCCCTTGGTAAGGAGTTGTGAGGT-3′	DGGE (50%–80% for 9 h)

(30 g/liter), formaldehyde (1.5 ml/liter), and $Na_2S_2O_3$ (2 mg/liter). Finally, 10% acetic acid was added to stop development. Gels were then rinsed two times for 2 min each with dH_2O. After overnight drying at room temperature, permanent copies were created by exposing EDF film (Kodak) to the gel by using a light box for 7–11 s.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

One of each of the primer pairs used for DGGE analysis contained a 40-bp GC-clamp at the 5′ end (Sheffield et al. 1989). Each patient's DNA (500 ng) was used as template in a 100-μl PCR containing each of the following: 10 μl of 10 × buffer (670 mM Tris-HCl pH8.8, 67 mM $MgCl_2$, 160 mM ammonium sulfate, 100 mM β-mercaptoethanol, and 50% dimethylsulfoxide), 37.5 nmol of each dNTP, 50 pmol of each primer, and 1.5 U of *Taq* polymerase (Perkin Elmer Cetus). Samples were incubated at 94°C, 60°C, and 72°C for 30 s at each temperature in a DNA thermocycler (Perkin Elmer Cetus), for a total of 40 cycles. The optimum gradient of DNA denaturant for parallel DGGE was determined by analyzing each DNA fragment with perpendicular DGGE as described elsewhere (Meyers et al. 1985). Ten to 20 μl of the amplified DNA product were analyzed on an 8% polyacrylamide (37.5:1 acrylamide:bis) denaturing gradient gel containing a 50%–80% linear gradient of DNA denaturants (100% denaturant is defined as 40% formamide and 7 mol urea/liter). Samples were electrophoresed at 150 V at 60°C for 9 or 11 h (table 1). Gels were stained with ethidium bromide and were photographed under UV transillumination with Kodak Tri-X film.

DNA Sequencing

Primers used in the screening assay (table 1) were also used for DNA sequencing. In addition, primer 5′-GGCCAAGGCATCTTGTATTGG-3′ was used to sequence the promoter region upstream of the start codon. The sequenced regions include the entire coding region, the 5′ and 3′ splice sites of the two introns, a 168-bp segment immediately upstream of the start codon, and a 49-bp segment immediately downstream of the stop codon. PCR-amplified DNA samples were prepared for sequencing by electrophoresis on low-melting-temperature agarose gels. Gels were stained briefly with ethidium bromide, and the desired band was cut from the gel. DNA was isolated using a commercially available kit (Promega). Purified DNA was sequenced in a DNA thermocycler using a dideoxy-termination kit (Promega). After 30 cycles of extension at 95°C for 30 s and at 70°C for 30 s, 3 μl of stop solution was added to each sample. After denaturation, 3 μl of each sample was loaded onto 6% polyacrylamide gels (19:1 acrylamide:bis and 7 M urea). After electrophoresis for approximately 3 h, gels were dried and exposed to Kodak X-Omat AR film for 24–48 h.

Nested PCR of ROM1

The possibility that an intragenic deletion in ROM1 causes Best disease was investigated using a PCR-based strategy. The entire gene was amplified in one affected individual from each family who was affected with Best disease and also shown to be heterozygous for the ROM1 polymorphism within intron 1. The resulting PCR products were analyzed on a 4% polyacrylamide gel in order to demonstrate a single band of the expected size. These same PCR products were then used

Table 2

Pairwise Linkage Data: *Z* Value and Recombination Distance (θ)

	Z AT θ =								
MARKER AND FAMILY	.05	.10	.15	.20	.25	.30	.40	Z_{max}	MAXIMUM θ
D11S871:									
1	6.4	6.2	5.7	5.1	4.5	3.7	1.9	6.4	5.4
2	−.1	.3	.4	.4	.4	.3	.1	.4	18.7
3	2.3	2.1	1.9	1.7	1.4	1.1	.6	2.5	.0
Total	8.6	8.6	8.0	7.2	6.2	5.1	2.7	8.7	7.1
PGA:									
1	5.8	5.3	4.7	4.2	3.5	2.9	1.5	6.3	.0
2	.9	.8	.6	.5	.3	.2	.1	1.0	.0
3	1.7	1.5	1.3	1.0	.8	.6	.2	1.9	.0
Total	8.5	7.6	6.6	5.6	4.6	3.6	1.7	9.3	.0
D11S956:									
1	10.1	9.2	8.2	7.2	6.2	5.1	2.6	10.9	.0
2	3.0	2.8	2.5	2.2	1.8	1.5	.7	3.2	.0
3	3.8	3.4	3.0	2.6	2.2	1.7	.8	4.1	.0
Total	16.9	15.4	13.8	12.0	10.2	8.2	4.1	18.2	.0
ROM1:									
1	7.3	6.6	5.9	5.1	4.3	3.4	1.7	8.0	.0
2	.8	.7	.6	.5	.4	.2	.1	.9	.0
3	1.0	.9	.7	.5	.4	.3	.1	1.2	.0
Total	9.1	8.1	7.1	6.1	5.0	3.9	1.8	10.0	.0
PYGM:									
1	9.7	9.0	8.2	7.3	6.2	5.1	2.7	10.0	1.4
2	3.4	3.0	2.6	2.2	1.8	1.4	.6	3.8	.0
3	1.7	1.7	1.5	1.4	1.1	.9	.4	1.7	7.5
Total	14.8	13.7	12.4	10.8	9.2	7.4	3.7	15.0	2.4

as template in a nested PCR which amplified the region containing the polymorphism. These PCR products were then analyzed on an SSCP gel.

Linkage Analysis

Genotypic data from the autoradiograms were entered into the Linkage Interface software on a Macintosh computer (Nichols et al. 1992). Marker data were transferred to a DOS-compatible computer for linkage analysis with the computer program LINKAGE (version 5.1) (Lathrop and Lalouel 1984). For the analysis, the disease was assumed to be autosomal dominant with a 99% penetrance (Stone et al. 1992*b*). Allele frequencies were assumed to be equal for each marker. Since families 2 and 3 had alleles of D11S871 which were not present in the originally reported family, this assumption increased the lod score (*Z*) for family 1 slightly, from the previously reported value for this marker. The MLINK program was used for pairwise analysis. Estimates of sex-averaged recombination between polymorphic markers and odds of marker order with and without inclusion of the disease locus were calculated using ILINK.

Results

Clinical Characterization

Three families were studied, including the one reported in our earlier paper (Stone et al. 1992*b*). The pedigrees are shown in figure 1. A total of 103 patients had complete retinal examinations. Ninety-eight were at 50% risk for Best disease because of a known affected sibling or parent. The remaining five were children of a family member (patient II-9 from pedigree 3 in fig. 1) whose diagnosis could not be definitely determined. Thirty-eight patients were found to have macular lesions consistent with the diagnosis of Best disease. Forty-three patients with normal or near-normal retinal examinations underwent EOG. Seventeen of these patients had an abnormal Arden ratio and were consid-

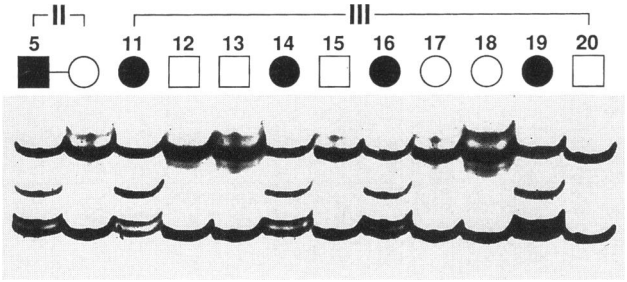

Figure 2 Segregation of the ROM1 SSCP polymorphism with the Best disease phenotype. A section of family 1 (fig. 1) is shown above the silver-stained SSCP gel. Shaded symbols indicate affected patients, and unshaded symbols indicate unaffected patients. A spouse is shown connected with a horizontal line to patient II-5. The upper pair of bands segregate with the disease in all three families studied.

ered to be affected. The remaining 26 patients had a normal EOG ratio ($\geq$1.8) and were considered to be unaffected for this study. All patients who did not satisfy these criteria were considered "unknown" for the linkage analysis. Blood samples were obtained from 104 family members and 20 spouses of affected patients with children. In addition, blood samples were also obtained from eight unrelated patients with a clinical diagnosis of Best disease.

Genetic Mapping

Five markers demonstrated $Z > 8$ for the three families studied (table 2). In addition, each of the three families demonstrated $Z > 3$ for at least one of the markers tested. Markers D11S956 and PGA demonstrated no recombinations in any of the families and resulted in Z values of 18.2 and 9.3, respectively. The SSCP polymorphism within ROM1 also segregated with the disease in all three families and resulted in $Z = 10.0$ (fig. 2). Markers D11S871 and PYGM both demonstrated multiple recombinations with Best disease.

Markers D11S956 and PYGM were typed in 40 CEPH families. Linkage analysis indicated that the most likely marker order (with sex-averaged recombination distances) is centromere D11S956 (5 cM) PYGM telomere. The odds that this order is correct are >1,000 times that of the next most likely order. The three families in this report were used to determine the approximate location of marker D11S871 on this genetic map (table 3).

Mutation Analysis of ROM1

Since there were no recombinations between Best disease and the polymorphism in the ROM1 gene, we began a search for ROM1 mutations in a single affected individual from each of the three families affected with Best disease, as well as in eight additional unrelated individuals with the clinical diagnosis of Best disease. Primers were selected which flank each of the ROM1 exons (table 1). Both SSCP and DGGE techniques were used to screen the ROM1 exons for mutations. No evidence of a base substitution was observed in any affected individual. Direct sequencing of the ROM1 gene (see Material and Methods) in a single affected individual from the largest family with Best disease also failed to reveal any sequence changes compared with a normal control.

A deletion containing the entire ROM1 gene was excluded as a cause of Best disease in these families, since at least one affected individual in each family was heterozygous for the ROM1 intragenic polymorphism. Furthermore, nested PCR of affected individuals was used to exclude the possibility of a partial deletion within the gene (data not shown; see Material and Methods).

Discussion

This report suggests that Best vitelliform macular dystrophy is not genetically heterogeneous. In addition, these data indicate that Best disease can be further localized to the 10-cM region between markers D11S871 and PYGM (odds ratio >1,000:1). ROM1 has been shown by somatic hybrid analysis to lie within this region, between PGA and PYGM (Bascom et al. 1992*a*). The location of markers PGA and ROM1 with respect to D11S956 could not be definitely determined (data not shown). In addition, a polymorphism within the

Table 3

θ Between Markers

	D11S956	PGA	ROM1	PYGM
D11S871	θ = 4.7 (Z = 12.7)	θ = .0 (Z = 7.0)	θ = 11.1 (Z = 4.9)	θ = 6.8 (Z = 15.0)

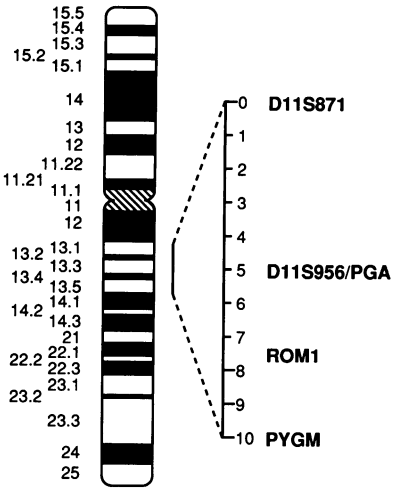

Figure 3 Locus for Best disease, on 11q13. A depiction of chromosome 11 is shown on the left. The five polymorphic markers which include the region for Best disease are shown on the genetic map expanded to the right of the chromosome (for explanation, see text). The scale is arbitrarily set at 0 cM at marker D11S871. Since the exact location of PGA with regard to D11S956 could not be definitely determined, it is shown coincident with marker D11S956. ROM1 is shown at an arbitrary location between PGA and PYGM, on the basis of somatic-cell hybrid data (Bascom et al. 1992*a*). Markers D11S871 and PYGM demonstrate multiple recombinants with the Best disease phenotype, whereas the three internal markers do not demonstrate recombinants. These data place Best disease within the 10-cM region between markers D11S871 and PYGM.

ROM1 gene demonstrates no recombinations with the disease phenotype (maximum Z [Z_{max}] = 10.0). As figure 3 demonstrates, ROM1 is included in the 10-cM region which harbors the Best disease gene.

Several pieces of data implicate ROM1 as a strong candidate gene for Best disease. First, ROM1 encodes an integral membrane protein which is expressed in the rod outer segment (Bascom et al. 1992*b*). Although Best disease affects the macula more severely than it affects the periphery, abnormal lipofuscin material is known to accumulate in the retinal pigment epithelium of the entire fundus. Furthermore, the EOG findings of Best disease reflect an abnormal response of the entire retina, not just the macula. The ROM1 protein has significant similarity to peripherin, another photoreceptor-specific protein which is the product of the RDS gene. We and others have recently identified different mutations in the RDS gene in families with different retinal degenerations, including macular dystrophies (Kajiwara et al. 1993; Nichols et al. 1993*a*, 1993*b*; Wells et al. 1993). Thus, a gene closely related to ROM1 has already been shown to be involved in a macular disease. These observations suggest that the photoreceptor-specific protein ROM1 could be involved in Best disease. The failure to identify recombinations between an ROM1 intragenic polymorphism and the disease phenotype in three families demonstrates that ROM1 and Best disease are tightly linked.

Several possibilities can explain the failure to detect a mutation in ROM1 in patients with Best disease. First, it may be that a different retina-specific gene near ROM1 is actually the Best disease gene. Three other diseases which affect the retina have been previously linked to this area: Usher syndrome type 1 (Kimberling et al. 1992; Smith et al. 1992), autosomal dominant neovascular inflammatory vitreoretinopathy (Stone et al. 1992*a*), and familial exudative vitreoretinopathy (Li et al. 1992). These studies suggest that 11q13 may be the site for a cluster of retina-specific genes. Alternatively, it is possible that an ROM1 coding-sequence mutation does cause the disease in these three families but was not detected by the methods used in the present study. Although the same ROM1 allele segregates with the disease in these three families, two of these families have a different PGA D11S956 ROM1 haplotype, suggesting that at least two of the families are not recently related to one another. In addition to the single affected individual from each of the three families, eight additional unrelated patients with the clinical diagnosis of Best disease were also used in the screening assay. It is very unlikely that our screening assay would miss multiple different point mutations within the ROM1 gene. Furthermore, direct sequencing of the ROM1 gene would be expected to detect a mutation, even if it were not detected by using SSCP or DGGE. Thus, unless all Best patients descended from a single progenitor, it is unlikely that this disease is caused by point mutations or deletions within the coding sequence of the ROM1 gene.

We would not have detected a distal promoter mutation or a branch-splicing mutation within the introns, as these regions were not included in the PCR products which we assayed with SSCP, DGGE, and direct sequencing. Therefore, the results of this study do not completely exclude ROM1 as a candidate gene for Best disease. However, if ROM1 is the disease-causing gene, it is unlikely that the disease-causing mutation (at least in the individuals studied) is a single base substitution resulting in an amino acid change. Definitive determination of the relationship between ROM1 and Best disease will require the sequencing of the promoter and intron branch-site sequences from affected individuals with Best disease, the demonstration of a recombina-

tion between the Best disease phenotype and the ROM1 gene, or the identification of another gene near ROM1 as the disease-causing gene.

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