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Refined Genetic and Physical Localization of the Wagner Disease (WGN1) Locus and the Genes CRTL1 and CSPG2 to a 2- to 2.5-cM Region of Chromosome 5q14.3

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Wagner syndrome (WGN1; MIM 143200), an autosomal dominant vitreoretinopathy characterized by chorioretinal atrophy, cataract, and retinal detachment, is linked to 5q14.3. Other vitreoretinopathies without systemic stigmata, including erosive vitreoretinopathy, are also linked to this region and are likely to be allelic. Within the critical region lie genes encoding two extracellular macromolecules, link protein (CRTL1) and versican (CSPG2), which are important in binding hyaluronan, a significant component of the mammalian vitreous gel, and which therefore represent excellent candidates for Wagner syndrome. Genetic mapping presented here in two further families reduces the critical region to approximately 2 cM. Subsequent refinement of the physical map allows ordering of known polymorphic microsatellites and excludes CRTL1 as a likely candidate for the disorder. CSPG2 is shown to lie within the critical region; however, analysis of the complete coding region of the mature peptide reveals no clear evidence that it is the gene underlying WGN1. © 1999 Academic Press

INTRODUCTION

The autosomal dominant vitreoretinopathies result from defects in ocular extracellular matrix production and help to delineate the role of the vitreous in ocular embryogenesis. Abnormal development and premature degeneration of the vitreous predispose to myopia, retinal detachment, and early onset blindness. Characteristic biomicroscopic findings include an "optically empty" vitreous or premature vitreous gel syneresis and degeneration (e.g., Snead *et al.*, 1994). Premature vitreoretinal separation leads to focal traction on the retina and predisposes to retinal hole formation and consequent ("rhegmatogenous") retinal detachment.

Among this group of conditions, Stickler syndrome (Stickler *et al.*, 1965) is the best characterized at the molecular level. The condition has major ophthalmic consequences (including congenital myopia and early retinal detachment) as well as systemic sequelae including spondyloepiphyseal dysplasia, deafness, cleft palate, and micrognathia. Mutations in the COL2A1 gene on chromosome 12q12–q13.2 are responsible for many of the cases (Richards *et al.*, 1996), while a mutation within the COL11A2 gene (Vikkula *et al.*, 1995) in a family without ocular pathology and a mutation within the COL11A1 gene on chromosome 1p21 in a family with classical Stickler syndrome (Richards *et al.*, 1996) have been described.

Those vitreoretinopathies without extraocular stigmata include Wagner syndrome (MIM 143200; Wagner, 1938). Graeminger *et al.* (1995) examined affected members of the original Wagner kindred and described the ocular findings, which include an optically empty vitreous cavity similar to that seen in Stickler syndrome, chorioretinal atrophy, cataract, and both rhegmatogenous and tractional retinal detachments (secondary to abnormal peripheral vitreoretinal adhesions). Molecular studies suggest that Wagner syndrome, like Stickler syndrome, is heterogeneous. The demonstration of a COL2A1 mutation in one family indicates that some forms represent part of the Stickler spectrum (Korkko *et al.*, 1993). Brown *et al.* (1995) demonstrated linkage to a 35-cM region of 5q13–q14 in Wagner's original kindred with a peak lod score of 5.8 with the

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marker D5S815. In addition, erosive vitreoretinopathy (Brown *et al.*, 1994), which is characterized by abnormal vitreous gel structure and retinal pigment epithelial and electroretinographic abnormalities, is also linked to 5q13–q14 and may be allelic. Genes encoding two extracellular macromolecules, link protein (CRTL1) and versican (CSPG2), which are important in binding hyaluronan, an important component of the vitreous gel, lie within this chromosomal region and represent excellent candidates for the condition. We present genetic and physical data that refine the localization of WGN1 and allow ordering of known polymorphic microsatellites. In addition we show that both CRTL1 and CSPG2 lie within or close to the critical region.

MATERIALS AND METHODS

Clinical details. Families W1 and D1 (Figs. 1 and 2, respectively) are previously published kindreds with Wagner syndrome (Fryer *et al.*, 1990; Jansen, 1962). Sixteen other families with a history suggestive of autosomal dominant vitreoretinopathy were identified in which no evidence of arthropathy, cleft palate, hearing loss, cardiovascular abnormalities, or other medical conditions was found. Blood samples were obtained from available family members, and DNA was extracted using conventional methods.

Microsatellite analysis. Microsatellite markers D5S1962, D5S626, CRTL1, D5S2094, D5S644, and D5S2103 were PCR amplified using primers described elsewhere (Dib *et al.*, 1996; Hecht, 1991). Standard PCRs were performed using 100 ng genomic DNA in a 20-μl reaction volume containing 3.7 mM $MgCl_2$, 67 mM Tris–Cl (pH 8), 166 mM $(NH_4)_2SO_4$, 0.17 mg/ml BSA, 3 mM each dNTP, 10 pmol of each primer, and 0.1 IU of *Taq* polymerase (Gibco BRL). Samples were overlain with mineral oil and processed through 30 cycles of amplification consisting of 1 min at 94°C (denaturation), 1 min at 57°C (annealing), and 1 min at 72°C (extension). The final extension step was 10 min. All products were run on an 8% polyacrylamide gels and silver stained according to standard methods.

Linkage analysis. Linkage analysis was performed using the MLINK program (Lathrop *et al.*, 1984) using the genetic distances given by Dib *et al.* (1996).

RNA extraction. Total RNA was extracted from approximately 10^7 fibroblast cells using the Triazol reagent (Gibco BRL) in accordance with the manufacturer's instructions. Once isolated, RNA was treated with RQ1 RNase-free DNase.

RT-PCR. Typically 1 μg of RNA was transcribed in a 20-μl reaction volume using a reverse transcription kit (Promega) according to the manufacturer's guidelines. cDNA at a dilution of 1/20 was used as a template for subsequent PCR amplification (see above). PCR products were then resolved on a 2% agarose gel, isolated from the gel, and purified using the GeneClean II kit (Stratech Scientific). The products were used for direct sequencing (ABI Prism Dye terminator cycle sequencing; Perkin–Elmer, Applied Biosciences Division).

Mutation analysis of CSPG2 and CRTL1 using genomic DNA. The genomic structure of CSPG2 had previously been defined. However, flanking intron sequence was unavailable (Naso *et al.*, 1994). A comprehensive screen of the gene therefore required redefinition of the intron/exon boundaries prior to effective analysis. Intron/exon boundaries were elucidated using a modification of the vectorette system (Riley *et al.*, 1990). BAC DNA was prepared using a modified miniprep technique (Qiagen). The DNA was digested with a panel of restriction enzymes (*Alu*I, *Eco*RV, *Pvu*II, *Rsa*I) to produce blunt-ended fragments. These were ligated to a blunt-ended synthetic vectorette oligonucleotide (Cambridge Research Biochemicals). The resultant products were used as template for PCR amplification. PCR was performed using a linker-specific primer (5′–3′ TCTCCCTTCTCGAATCGTAACCGTTCGTAC) and an exon-specific primer. Second-round PCR was then performed using a nested linker-specific primer (5′-3′ CGAATCGTAACCGTTCGTACGAGAATCGCT) and an exon-specific primer. PCR products were sequenced using methods described above. The primers thus designed for mutation screening are listed in Table 3. Primers used for a limited screen of the CRTL1 gene are described by Brown *et al.* (1995).

For SSCP/heteroduplex analysis, 1 vol of PCR product was mixed with 1 vol of formamide loading dye and denatured at 96°C for 5 min prior to loading on a 6% acrylamide gel. Gels were run at 350 V overnight at 4°C and silver stained according to standard protocols.

RESULTS

Genetic Mapping of Families with Wagner Disease

Linkage was independently confirmed to chromosome 5q14.3 in 4/18 families. The remaining families, for whom pedigree structures have not been shown, were of insufficient size to confirm linkage. Haplotype data are included for the two families, W1 and D1 (Figs. 1 and 2), containing recombinants that enable refinement of the Wagner disease critical region.

For family W1 using the regional microsatellite polymorphisms D5S626 and D5S1719, 2-point lod scores of 2.98 (θ = 0) were obtained (Fig. 1). Penetrance was assumed to be 100% and disease allele frequency 0.0001. For family D1, 2-point lod scores of 3.68 and 3.67 (θ = 0.0) were obtained with D5S428 and CRTL1, respectively (Fig. 2). Lod scores for the two families together for markers D5S626, D5S2094, and CRTL1 are given in Table 1; a maximum combined lod score of 4.3 was obtained with the marker D5S2094.

Haplotype analysis in family W1 (Fig. 1) revealed a multiply informative recombination event in individuals III-5/IV-8. This defines a distal boundary for the critical region of interest. Individuals III-1 and III-5 are affected first cousins whose affected mothers are both deceased. Thus refinement of the critical region relies upon the identification of entirely separate allele sets from the identified disease haplotype. By this means the markers D5S617, D5S1719, and D5S107 and the intragenic microsatellite of the CRTL1 gene (found within the CRTL1 promoter) are excluded from the critical region while the markers extending proximally from this point cosegregate with the disease in all affected individuals. Haplotype analysis on the large Dutch family, D1 (Fig. 2), reveals a recombination event in affected individual V-2, which places D5S626 as the flanking marker at the proximal boundary of the critical region.

A Physical Map of the Critical Region for WGN1

YACs from both the CEPH "mega" YAC library (Chumakov *et al.*, 1992) and the ICI library (Anand *et al.*, 1990) have been screened with STSs that span the critical region. For this purpose the proximal boundary was defined by the marker D5S626, and the distal boundary was defined by the marker D5S107. The critical region is contained within the CEPH YAC

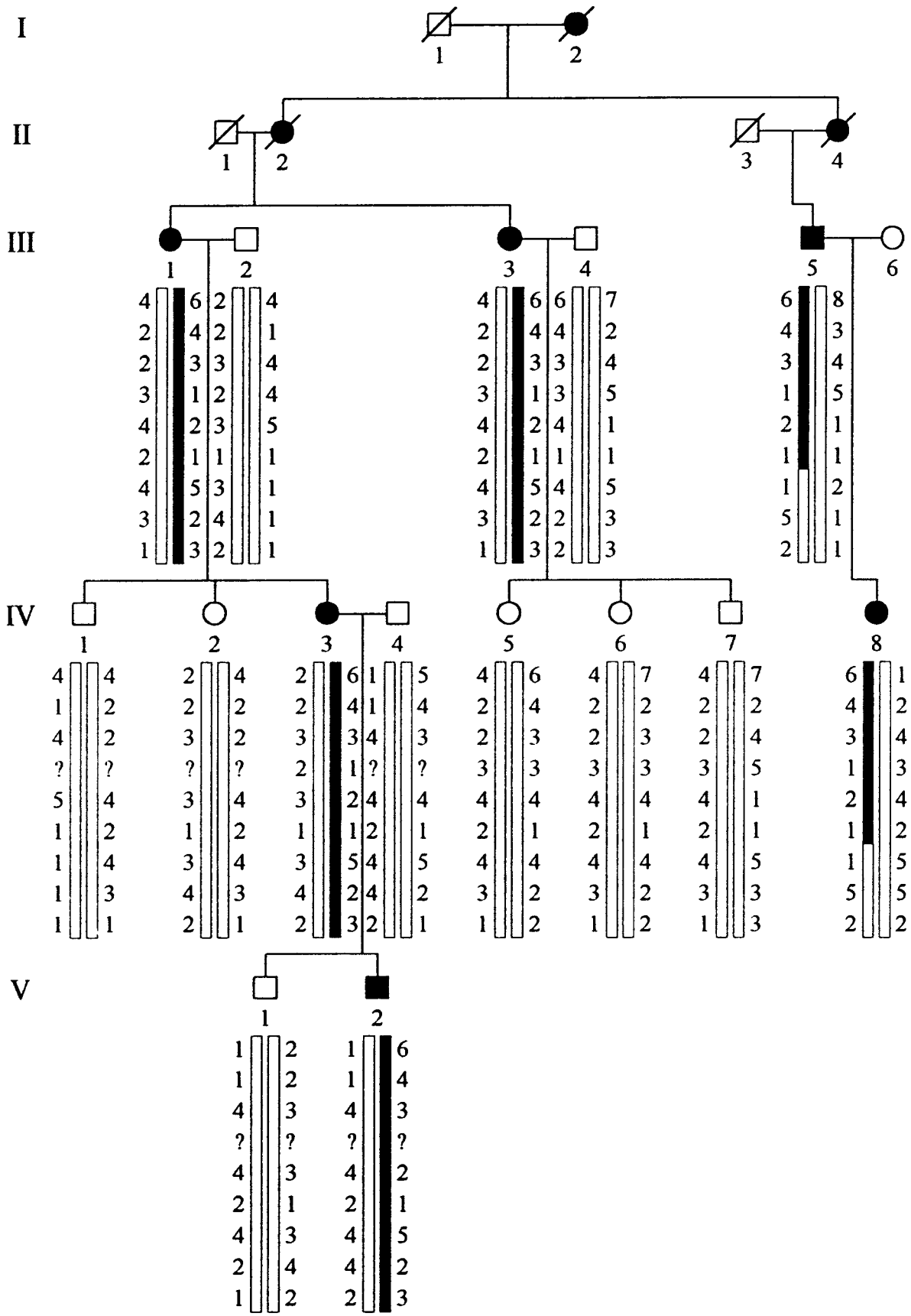

FIG. 1. Haplotype analysis of family W1. Haplotypes correspond, from top to bottom, to markers D5S2029, D5S626, D5S2094, D5S1726, D5S1719, AFMb298zg1, CRTL1, D5S107, and D5S617. A recombination event is demonstrated in individuals III-5/IV-8 proximal to CRTL1.

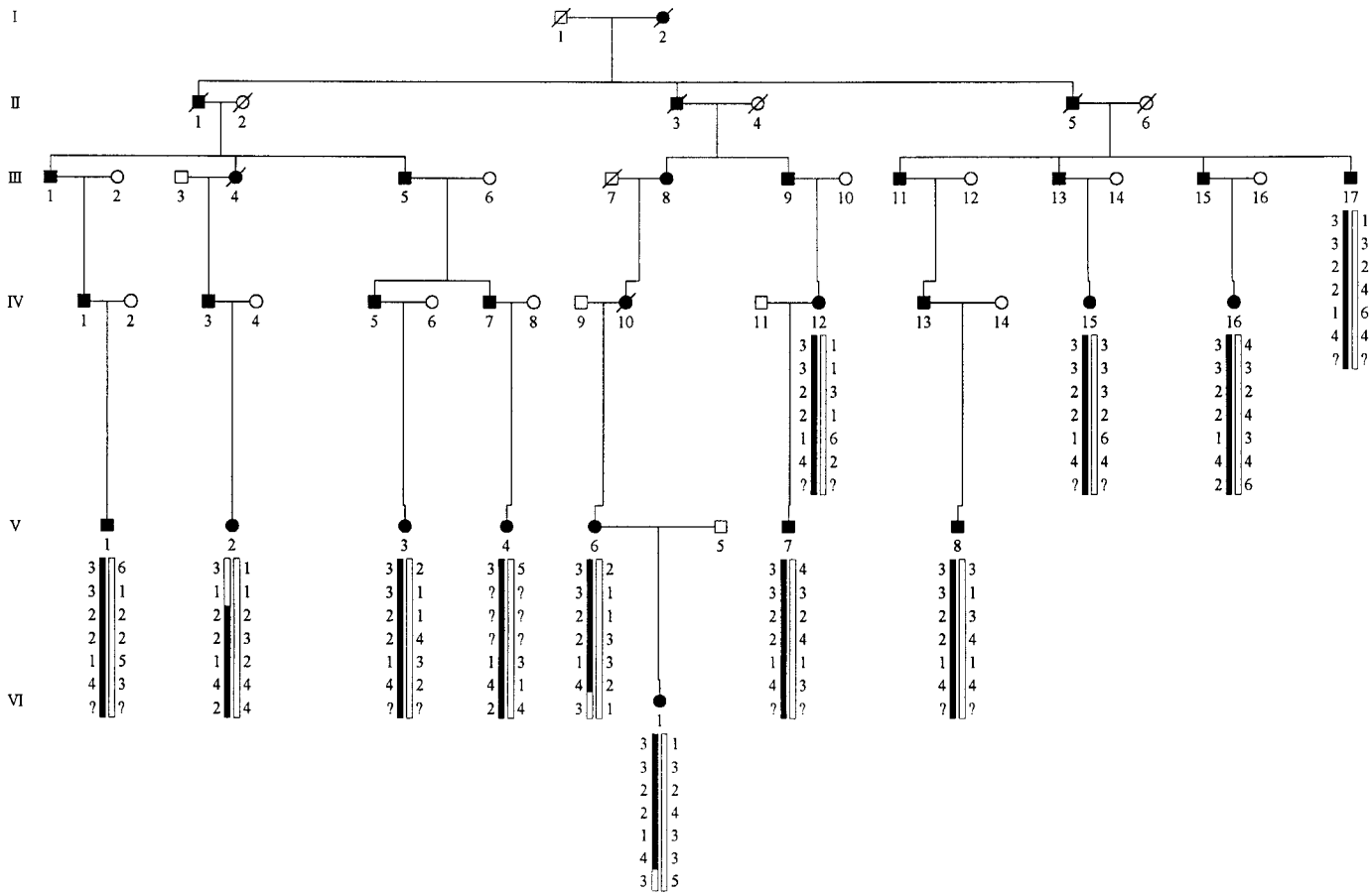

FIG. 2. Haplotype analysis of family D1. Haplotypes correspond, from top to bottom, to markers D5S641, D5S626, D5S1959, D5S2094, D5S428, CRTL1, and D5S644. A recombination event is demonstrated in individual V-2, which is distal to D5S626. In individual V-6, a recombinant distal to CRTL1 has been identified.

934c2, a 1780-kb coligated clone. A contig spanning the critical region was assembled and allowed the physical order of markers from that region to be deduced (Fig. 3). This suggests a marker order of D5S626–(D5S1959–D5S2094)–(WI-20868–D5S1726–D5S1948)–(D5S1719–CRTL1)–AFMb298zg1–D5S2641–D5S107 and places CRTL1 as the distal flanking marker of WGN1. The order of polymorphic markers is consistent with Généthon physical and linkage data (Cohen *et al.*, 1993; Dib *et al.*, 1996). The gene that encodes versican (CSPG2) is also placed within the critical region between the markers D5S2094 and D5S1948 (Fig. 3).

TABLE 1

Results of Two-Point Linkage Analysis between the Disease Gene and Markers from 5q14.3

	Lod score for families W1 and D1 combined at θ								
Marker	0	0.01	0.05	0.1	0.2	0.3	0.4	Z_{max}	θ_{max}
D5S626		2.56	**2.90**	2.71	2.06	1.31	0.59	2.90	0.05
D5S2094	**4.30**	4.21	3.81	3.30	2.30	1.36	0.55	4.30	0
CRTL1		3.40	**3.63**	3.35	2.47	1.49	0.60	3.65	0.04

Exclusion of CSPG2 as a Candidate Gene for WGN1

Analysis of the coding sequence via RNA. Primers were designed to amplify segments of the CSPG2 transcript (Table 2). Identification of CSPG2 mRNA from lymphoblasts proved unreliable because of low expression levels. As expression in fibroblasts was higher, this allowed reliable RT-PCR analysis in one family from which RNA was available (Family W2, Black *et al.*, submitted for publication). Sequencing of the products revealed no mutations. There was no evidence of altered fragment size during RT-PCR amplification.

Amplification of exons 13–15 from RNA in both patients and normal controls revealed two products that differed by approximately 150 bp. Sequencing of each fragment revealed that the shorter fragment corresponded to that expected and contained the published gene structure, with exon 15 linked to exon 14 (Fig. 4a). The longer fragment corresponded to an alternatively spliced version of CSPG2, in which the short 144-bp intron 14 remains unspliced and is a part of the mature RNA molecule. Sequencing demonstrated that this would introduce a termination codon 17 residues into

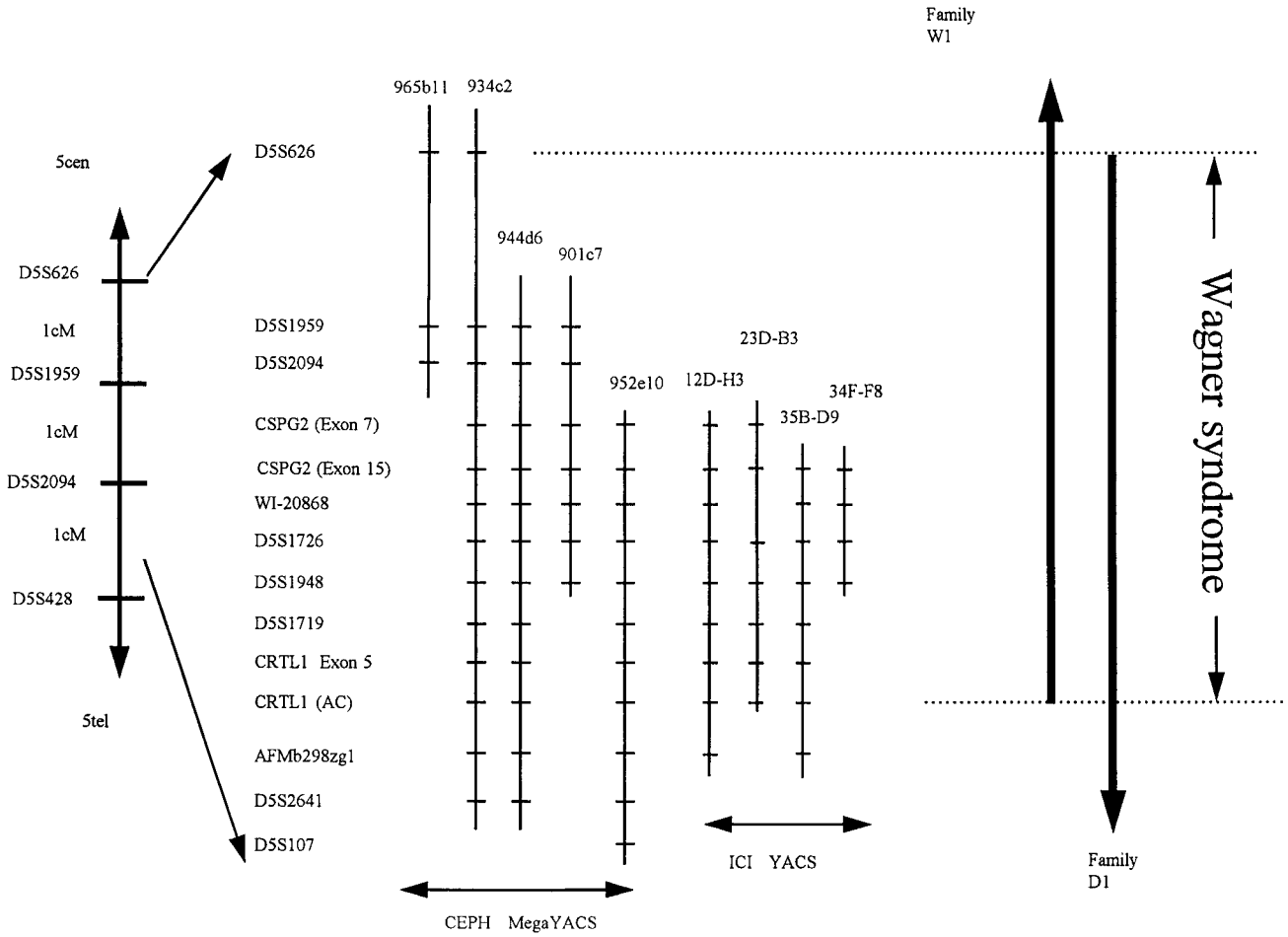

FIG. 3. Physical and genetic localization of WGN1 locus. Recombination events between the putative vitreoretinopathy gene and the microsatellite markers from chromosome 5q14.3 define a proximal boundary in familiy D1 between the marker D5S626 and the distal boundary by a recombination event with CRTL1. The WGN1 critical region of 2–2.5 cM is shown with ordering of the known polymorphic markers and positions of candidate genes CRTL1 and CSPG2 on a YAC contig of the region. The disease gene lies in a region of less than 2–2.5 cM between the markers D5S626 and CRTL1.

the intron that would theoretically result in the production of a protein lacking the terminal complement regulatory protein-like domain. The biological significance and the consequences of these findings on versican function remain uncertain.

Analyses of CSPG2 on genomic DNA. A comprehensive screen of the gene required sequencing of intron/exon boundaries prior to analysis. Primers were then designed to amplify each exon with its flanking intron sequence (see Table 3). The exon sequences of the glycosaminoglycan binding regions encoded by exons 7 and 8 (2961 and 5262 bp, respectively) were analyzed in fragments of between 200 and 300 bp by PCR and SSCP/heteroduplex analysis. In addition, 13 and 24 pairs of primers were designed, respectively, from the published sequences (primer sequences available on request). Each amplicon was then analyzed in genomic DNA from affected individuals in all 18 families. PCR products were tested by SSCP/heteroduplex analysis. Of the 38 PCR products from exons 7 and 8, 13 showed

TABLE 2

CSPG2 Primer Sequences for RT-PCR Amplification

Exon	Primer 1	Primer 2	Approximate size (bp)	Annealing temperature (°C)
1–3	gccccgagcctttctggggaag	caaggtaggctgacttttcc	394	55
3–6	cgtcatgtacgggattgaag	cacactcttttgcagcctc	419	55
5–7	gcaggagtcaggacttatg	ggataaactgggtgatgcag	432	55
7–8	ctgtaactgagcacaaagtg	gaggggaaaacagcagg	1186	60
8–13	gtggccacactctacttctg	caacacagtcttctccagc	880	55
9–13	cacctgttatcctactgaaac	caacacagtcttctccagc	493	55
13–15	ccatctcacctatacgtgc	cgaggtgataggaaaggcac	418/~550	60

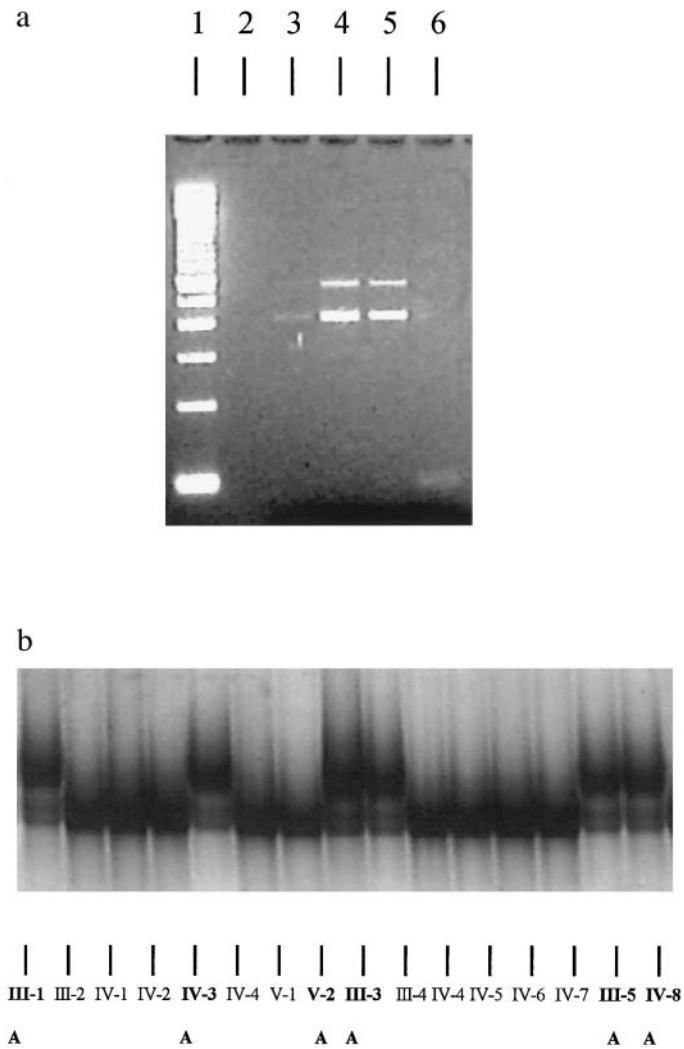

FIG. 4. Analysis of versican (CSPG2) gene. (**a**) Analysis of 3′ end of CSPG2 by RT-PCR. RT-PCR between exons 13 and 15 reveals two bands, of approximately 400 and 550 bp. Sequencing revealed that the larger band represents a fragment including intron 14 (bp), suggesting alternative splicing between exons 14 and 15. Lane 1, 100-bp marker. Lane 2, negative control (Water). Lane 3, normal control: lymphoblastoid cell line. Lane 4, Wagner syndrome patient: fibroblast cell line. Lane 5, normal control: fibroblast cell line. Lane 6, genomic DNA. (**b**) SSCP/heteroduplex analysis of CSPG exon 7 fragment on vitreoretinopathy family W2. The amplicon (Size 274 bp, situated at nucleotide positions 2124–2395 within cDNA) demonstrates a mobility shift that cosegregated with the disease phenotype (marked "A" beneath affected individuals).

sequence variants in patient samples; however, with one exception these either did not cosegregate with the disease phenotype in that family or were found in unaffected normal controls.

A mobility shift on SSCP/heteroduplex analysis was detected within exon 7 in family W2, which was shown to segregate with the disease phenotype (Fig. 4b). This was not present in 100 normal chromosomes of similar racial origin. Sequencing analysis revealed this to be an A-G missense substitution at position 2331, resulting in an alanine to threonine substitution.

DISCUSSION

Wagner syndrome, an inherited vitreoretinopathy without extraocular manifestations, has been shown, by molecular analysis, to be heterogeneous. Mutations in COL2A1 suggest that a small number of families have a mild form of Stickler syndrome in which extraocular manifestations are mild or absent. The remaining families are linked to chromosome 5q14; none of the families linked to this region have any extraocular sequelae. There is as yet no evidence for further loci implicated in autosomal dominant isolated vitreoretinopathy.

There is a variety of ocular manifestations, involving both anterior and posterior segments of the eye, among families linked to 5q14. Anterior segment abnormalities include microphthalmia, anterior segment dysgenesis, congenital glaucoma, cataract, and lens subluxation (Black *et al.*, submitted for publication). Indeed family W1, originally described by Fryer, includes two members who have congenital glaucoma. This suggests that defects in this gene have wider effects on ocular development than those confined to the posterior segment and vitreous (e.g., Black *et al.*, submitted for publication; Fransden, 1966).

The posterior segment changes are also heterogeneous: Wagner syndrome and erosive vitreoretinopathy demonstrate classical features of a vitreoretinopathy including abnormal vitreous gel development, premature vitreoretinal degeneration, and a predisposition to retinal detachment. The family D1 was originally described by Jansen and is recognized to have a higher risk of retinal detachment than that originally described by Wagner. This strengthens the suggestion that there is interfamilial variation in the effects and prognosis of mutations at this locus. Additional findings, in particular in erosive vitreoretinopathy, suggest a primary retinal or retinal pigment epithelial defect leading to symptoms and signs of a receptor dystrophy.

Initially Brown *et al.* (1995) demonstrated linkage to a 35-cM region of 5q13–q14 flanked by the markers D5S650 and D5S409. We have previously refined the region to a 5-cM region between D5S626 and D5S2094 (Black *et al.*, submitted for publication). Genetic mapping presented here has refined this further to a 2- to 2.5-cM region flanked by the markers D5S626 and CRTL1. On genetic grounds the distance between D5S626 and D5S428 is approximately 3 cM, although the exact position of CRTL1 is undefined.

The physical and genetic data presented here suggest a marker order of D5S626–D5S1959–D5S2094–D5S1948–D5S1726–D5S1719–CRTL1–D5S107, which is consistent with the Généthon physical map of the region (Cohen *et al.*, 1993). Brown *et al.* (1995) identified two possible candidate loci for the condition, link protein (CRTL1) and versican (CSPG2), both of which map cytogenetically to 5q13–q14. Both are extracellular matrix components that bind hyaluronan and that have been identified in the vitreous (Reardon *et al.*,

TABLE 3

CSPG2 Intronic Primer Sequences Flanking Exons for Mutation Screening on Genomic DNA

Exon (No. of fragments)	Primer 1	Primer 2	Approximate size (bp)	Result	Annealing temperature (°C)
3 (2)	gaaagagactactgtccttg	ctcaatgcttctgaaccagg	290	—	60
	ctgcttatccattcacatattg	cctttgtagtcctgaccaatc	280	Polymorphism	60
4 (1)	cagtgggagatttgaacaggc	caagttaataatgaagcctgtggc	290	—	62
5 (1)	gccacaggcttcattattaacttg	gaacttctggtcttccctcag	260	—	60
6 (2)	cttccccaccatatttatcc	ctcaccccaagtagaccac	280	—	60
	catggaggaacggctttgac	caagacatggaatccactcc	266	—	60
7 (15)	gagcacttaacaaacactggg	ggttggactgtggctttctg	270		62
	caggcgctttctacgccac	gcagcatttggtatgttgac	220	—	62
8 (26)	gtggcagcgagaattcttg	gttcagccaacaagcgatgc	300	—	60
	gttaaagaagacagtatgtgacgg	catgacgtgtctaccccacca	200	—	60
9	gtaaagcctataatatggtagc	cgaccaacataaattctccc	280	Polymorphism	60
10	gcaacctcacactaaatgg	gcaggaaagattatgcattg	220	—	60
11	gacaaaactaagagagtcatgag	ccaagtttgaatgacgtatgtgc	300	—	64
12	ctcctacgtactaagtacgac	cattacgtgttagtcacgtgt	240	—	60
13	gaatttgagacacaaattcctg	ccatcgttgctcttacgttac	290	—	64
14 (2)	gattagataccactgcaaagatg	gctgatgaggaatttttaaag	290	—	62
	gcttgcggccagcccccctg	gttcatgcaggtaattttagg	250	—	60
15	ctgcataccaaaggacttattc	cgaggtgataggaaaggcac	250	—	64

1998). Since our data place CSPG2 between D5S2094 and D5S1948, within the critical region, both remain excellent potential candidates for WGN1.

In family W1 genetic data demonstrate a recombination between the disease gene and the microsatellite polymorphism within the CRTL1 promoter, suggesting that this does not lie within the candidate region. However, the gene is not absolutely excluded on genetic grounds since physical evidence suggests that the coding sequence lies proximal to the polymorphism. We, like Brown *et al*. (1994), have not found mutations after PCR/SSCP/heteroduplex screening of the majority coding sequence in our families. In combination, these data suggest that CRTL1 is excluded as a candidate gene for WGN1.

We therefore screened the entire coding sequence of CSPG2 via RNA amplification in 1 family and analysis of genomic DNA in 18 families that fit the clinical criteria of Wagner disease/erosive vitreoretinopathy. No definitive mutations have been found within the families tested, although a threonine–alanine substitution was demonstrated within exon 7 in one family (W2) of Anglo-Saxon origin, a large kindred with an autosomal dominant vitreoretinopathy that has been shown to be tightly linked to this region (Black *et al.*, submitted for publication). This variant was not present in the control chromosomes analyzed. The substitution is within one of the two large proteoglycan binding domains of CSPG2, and its effect is unclear. The lack of confirmatory evidence of other mutations in the remaining families suggests that this may not be a pathogenic mutation.

The evidence does not strongly support CSPG2 as a candidate for Wagner syndrome. Although it is expressed in vitreous and is situated within the critical region, the lack of mutations in our families argues against CSPG2 being the gene underlying WGN1 and related conditions. The clinical resource of 18 families is likely to be sufficient to provide a reliable basis for screening. Furthermore, since the families exhibit interfamilial phenotypic variability, it is unlikely that a single mutation is responsible for the disorder in all families. It should be noted, however, that the degree of heterogeneity of the condition is unknown. Therefore in some families, the phenotype may be caused by mutations at different loci. Mutation analysis of further families proven to be linked to 5q14 would be valuable to confirm these results and formally exclude CSPG2 as a candidate.

Nevertheless, our data suggest that neither CSPG2 nor CRTL1 is likely to be the gene responsible for WGN1. This leaves no obvious candidate genes within the critical region and suggests that a positional cloning approach to the identification of the gene is likely to be necessary. For the families described, there is little scope for the refinement of the distal boundary of the critical region by genetic means. However, the identification of microsatellites from the proximal portion of the region between D5S626 and D5S2094 may potentially refine the position of the proximal boundary. The development of a genomic contig and a detailed transcript map of this region will then be required for identification of a gene that is important in early ocular development and vitreous gel construction and underlies vitreoretinopathies that map to 5q14.3.

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