Identification of a gene disrupted by a microdeletion in a patient with X-linked retinitis pigmentosa (XLRP)

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The gene for the most frequent form of X-linked retinitis pigmentosa (XLRP), *RP3,* has been assigned by genetic and physical mapping to a segment of less than 1000 kbp, which is flanked by the marker *DXS1110* and the ornithine transcarbamylase (*OTC*) gene. In search of microdeletions, we have screened the DNA of 30 unrelated patients with XLRP by employing a representative set of YAC-derived DNA fragments that were generated by restriction enzyme digestion and PCR amplification. In one of these patients, a 6.4 kbp microdeletion was detected which was not present in the DNA of 444 male controls. A cosmid contig spanning the deletion was constructed and used to isolate cDNAs from retina-specific libraries. Exons corresponding to these expressed sequences as well as other putative exons were identified by sequencing more than 30 kbp of the critical region. So far, no point mutations in these putative exon sequences have been identified.

INTRODUCTION

Retinitis pigmentosa (RP) is a clinically heterogeneous group of retinal degenerations characterized by night blindness, progressive constriction of the visual field due to the early loss of peripheral photoreceptor cells, and fundus abnormalities including intraretinal bone corpuscle-like clumps of pigment. In advanced stages central vision is lost and patients become severely impaired.

Genetic analysis has defined more than 10 chromosomal regions that carry genes for RP. Several genes underlying autosomally inherited forms were identified by candidate gene approaches, including the genes coding for rhodopsin (1), the α- and β-subunits of the rod-specific cGMP phosphodiesterase (2,3), peripherin (4), and the rod outer membrane protein (*ROM1*). Mutations in the genes encoding the two latter polypeptides were recently reported as the first example of a digenic mode of inheritance in a genetic disease (5). Most cases of familial RP inherit in an autosomal recessive way. Autosomal dominant and X-linked recessive inheritance are less frequent, accounting for 10 and 6% of the families, respectively. In the UK, X-linked forms have been reported to account for 30% of the cases (6), and in Denmark for 17% (7).

Clinically, X-linked RP is considered as the most severe form, with an earlier age of onset than autosomal recessive or dominant RP. Linkage studies and heterogeneity testing in families with XLRP revealed at least two different loci, one at Xp21.1-p11.4 (*RP3*) closely linked to *OTC* and distal to *DXS7*, and a second locus (*RP2*) between *DXS7* and *DXS255* at Xp11.2-p11.4 (8,9). Upon fundoscopic examination, carriers of the *RP3* type show a characteristic, metallic-sheen tapetal reflex [OMIM#312610 (10)], whereas carrier females from *RP2* families do not [OMIM#312600 (10)]. Heterogeneity testing has supported the existence of a third X-linked locus (*RP6*), distal to *RP3* (11). So far, none of the X-linked genes has been isolated.

Previously, the chromosomal interval carrying the *RP3* locus has been defined by molecular characterization of two deletions found in patients with RP and other X-linked disorders, including Duchenne muscular dystrophy (DMD), chronic granulomatous disease (CGD), and McLeod syndrome (12,13). In this way, the critical interval could be narrowed to less than 1000 kbp (14,15). Within this region, a 70 kbp deletion has been detected recently (16). In order to perform a comprehensive search for microdeletions in XLRP patients, we have developed a technique which was inspired by the representational difference analysis (RDA) protocol (17) and resembles the YACadapt method described by Sutcliffe *et al.* (18). The essence of this technique is the generation of a defined, amplifiable subset of restriction fragments which are derived from and represent the insert of a YAC spanning a genomic region of interest. After ligation to suitable linkers, these fragments are amplified by PCR and the mixture of PCR products is used as a probe for hybridization of Southern blots containing restriction enzyme digested genomic DNA. We have employed this technique to study 30 unrelated XLRP
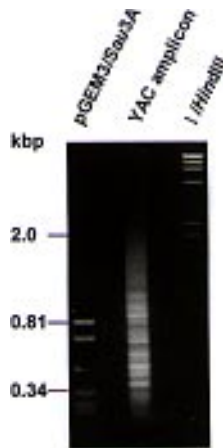
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Figure 1. PCR fragments of the *Hin*dIII amplicon from a 660 kbp YAC after separation in a 1.5% agarose gel and staining with ethidium bromide.

patients with PCR-amplified probes from a YAC spanning the *RP3* gene region. In one of those patients, a microdeletion was identified. The isolation of cosmid clones from the relevant region and their use as hybridization probes confirmed this result and enabled the construction of a cosmid contig spanning 120 kbp around this deletion. Finally, putatively transcribed sequences could be identified by screening a retinal cDNA library and by sequencing the entire cosmid clone spanning the deletion.

RESULTS

Deletion detection by YAC representation hybridization (YRH)

The principle of the method is the reduction of the complexity of the YAC-insert to a subset of small, amplifiable restriction fragments (YAC representations or 'amplicons') and their subsequent hybridization to Southern blots containing genomic DNA of patients. Prior to the generation of these amplicons, the YAC DNA was purified by preparative pulsed field gel electrophoresis and cleaved with a restriction endonuclease. Linkers were ligated to both ends, and the fragments were amplified by employing linker-specific oligonucleotides as primers. The PCR products, usually a 'smear' with some distinct bands in the size range of 0.2 to 1.0 kbp (Fig. 1), were purified, radiolabeled and used as a probe to screen Southern blots with DNA of patients.

To study the genomic region containing the *RP3* gene, we used a 660 kbp YAC (ICRFy900E0701), which harbours the cytochrome b-245 (*CYBB*) gene and part of the *OTC* gene. This YAC was also shown by PCR analysis to contain the marker *DXS1110*. Screening of *Eco*RI digested genomic DNA from 30 unrelated X-linked RP patients with the *Hin*dIII amplicon of the YAC revealed the absence of a 1.5 kbp fragment in one of them (patient 2557, Fig. 2a). To rule out a restriction site polymorphism, the DNA of this patient was cleaved with the restriction endonucleases *Bam*HI, *Bgl*II, *Eco*RV, *Hin*dIII, *Msp*I, *Pst*I, *Pvu*II, *Sst*I, *Taq*I, *and Xba*I. Aberrant restriction patterns were observed with *Bgl*II, *Pst*I, and *Pvu*II (data not shown). As expected, the proportion of the genomic sequence that can be visualized in this way depends on the restriction enzyme employed. Hybridization of the *Hin*dIII amplicon of YAC E0701 to *Eco*RI-digested
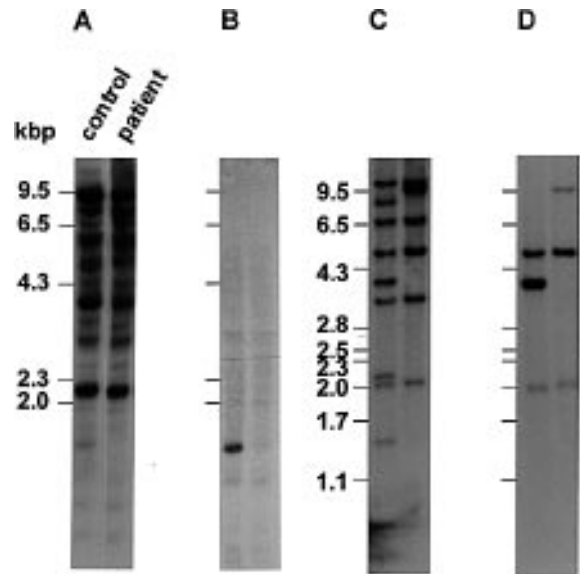

Figure 2. Autoradiographs of Southern blots containing *Eco*RI digested genomic DNA from a control and patient 2557 after hybridization with (**a**) the *Hin*dIII amplicon of YAC E0701; (**b**) clone p20; (**c**) cosmid LL242D12; and (**d**) cDNA clone R5.

genomic DNA visualized 6.5% of the sequences encompassed by the YAC, whereas the same amplicon enabled the visualization of 19% of the genomic sequences after *Sst*I digestion.

For further analysis, the amplicon was cleaved with *Hin*dIII to remove linker sequences and the resulting fragments were cloned into the *Hin*dIII digested pGEM3 vector. Subsequently, individual clones from the amplicon library were used as probes on Southern blots containing patient and control DNA. Among the first 20 clones tested, one clone (p20) was deleted in the patient's DNA (Fig. 2b) but not in controls. Cloning of the corresponding genomic region was achieved by using clone p20 (a 300 bp *Hin*dIII fragment, see Fig. 4) as a probe to screen an X-specific cosmid library. Four positive cosmids were obtained and hybridized to *Eco*RI digested DNA. An aberrant hybridization pattern was observed in the patient when these cosmids were used as probes: four fragments of 8.0, 3.8, 2.1 and 1.5 kbp, respectively, were absent, and one aberrant fragment of 9 kbp was seen (Figs 2c and 4). These data enabled us to determine the size of the deletion as 6.4 kbp.

Construction of a cosmid contig and identification of exon sequences

For the isolation of cosmid contigs from the critical region we have used the *Hin*dIII amplicon of the YAC to screen an X chromosome-specific cosmid library. Eighty-five positive cosmids were picked and spotted on a nylon membrane. Cosmid walking was performed by using the YAC end clones, generated by ligation mediated PCR, and cDNAs derived from the *CYBB* and *OTC* genes as starting points. Overlapping cosmids were identified which linked (i) the right end clone and *CYBB* and (ii) the left end clone and *OTC*. Cosmids detecting the microdeletion in patient 2557 were located between the *CYBB* and *OTC* contigs and were employed as a third anchor point for cosmid walking. This intermediate contig has been expanded to a size of at least
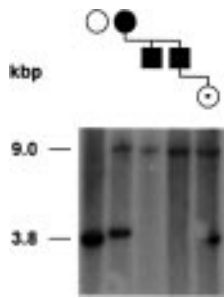
Figure 3. Autoradiogram of a Southern blot containing *Eco*RI restricted DNA from a control (left open circle), patient 2557, his mother and daughter as well as one affected brother after hybridization with a subclone of the cDNA probe R5. The 9 kbp fragment represents the deletion breakpoint whereas the 3.8 kbp fragment corresponds to the unaffected X chromosome.

300 kbp and overlaps with the contig around the *CYBB* gene. In order to identify transcribed sequences, we have screened an adult retinal cDNA library and identified six clones, which recognized three X-chromosomal *Eco*RI fragments on genomic Southern blots (Fig. 2d). Sequence analysis of the six cDNAs and the corresponding genomic fragments revealed five exons (fragments Z–V, Fig. 4). cDNA clone R5 was shown to comprise exons V–Z and detected a 7.5 kbp transcript in heart, brain, placenta, lung liver, muscle, kidney, and pancreas (data not shown). A poly(A)-tail was identified in fragment Z and a polyadenylation signal (AATAAA) was present in the corresponding genomic sequence of the 4.7 kbp *Eco*RI fragment. Therefore, fragment Z is considered to represent the 3′ end of a gene. This fragment also revealed a 94.7 % identity with a human EST (em_est: hsgs01161). No open reading frame was present in the cDNA clone R5 and database searches failed to detect significant homologies with known gene sequences. Hybridization of the cDNA to a Southern blot containing *Eco*RI digested DNA from two carriers of the patient's family and an affected brother revealed cosegregation of the deletion breakpoint fragment with the disease (Fig. 3).

Shotgun cosmid sequencing enabled us to determine 32895 bp of DNA sequence encompassing the 6.4 kbp microdeletion. Exon prediction by GRAIL identified three additional putative exons;

one proximal (fragment U) and two distal (fragments A and B) to the retinal cDNA (see Fig. 4).

By employing the SSCP technique, mutation screening was carried out in 29 XLRP patients for all putatively transcribed sequences (Table 1). Bandshifts in fragments Z, Y, V and U were found both in patients and in controls. Therefore it is most likely that these variants represent polymorphisms rather than disease-related changes.

Recently, another gene has been cloned from the *RP3* critical region, designated *SRPX* or *ETX1* (16,19). In order to locate this gene with respect to the candidate sequences isolated in this study, we have used primers from the 5′- (exon 1) and the 3′- (exon 10) end of the *SRPX* gene. Both sequences were found to be present on our cosmid contig encompassing the critical region. However, no *SRPX* sequences were deleted in patient 2557 and exon 1 of this gene was mapped approximately 50 kbp distal to the deletion breakpoint (Fig. 5).

DISCUSSION

The identification of submicroscopic deletions or other small chromosomal rearrangements has been instrumental for the positional cloning of numerous disease genes (20). The YRH method used in this study allows the screening for microdeletions of chromosomal regions as large as YAC inserts by employing a representative set of small, amplified restriction fragments as probe in Southern blot hybridization. Although not all of the sequences which are covered by the YAC are visualized with this method, a representation of up to 19% can be achieved with a single hybridization. The resolution of this technique can be enhanced by the use of several restriction endonucleases either for the generation of amplicons or for the digestion of the genomic DNA. In this way, amplicons can be generated that will cover nearly the entire region of interest (17). In contrast, the generation of probes by *Alu* PCR (21,23) requires the presence of *Alu* repeat motifs in an amplifiable distance; thus, these probes are much less representative and the resolution of this approach is limited. As shown here, the YRH method allows the generation of a dense array of clones from a genomic region of 660 kbp, and enabled us to detect a 6.4 kbp microdeletion in a patient with XLRP. Eight putative exons could be identified in the vicinity of this deletion, six of which seem to belong to the gene. Two of these were deleted
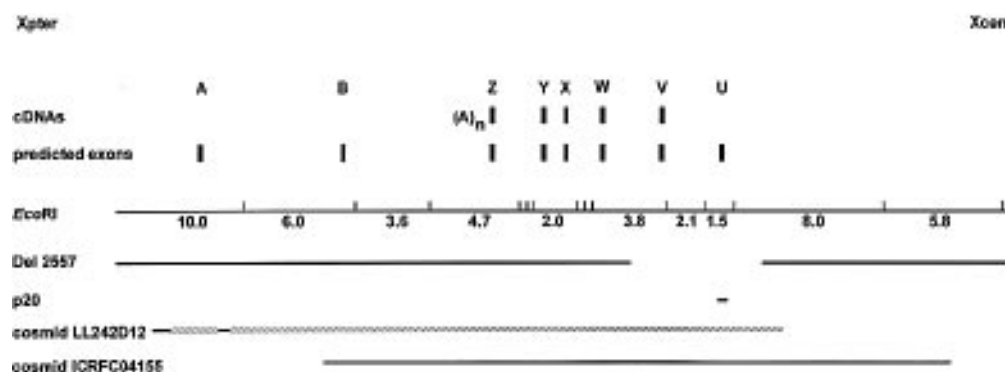

Figure 4. *Eco*RI restriction map of a 40 kbp region surrounding the 6.4 kbp microdeletion in patient 2557 and location of putative exons as identified by cDNA screening (fragments V, W, X, Y and Z) and exon prediction from genomic sequences (fragments U, W, X, A and B). A polyA-tail was present at fragment Z. The amplicon clone p20 hybridizes to the 1.5 kbp *Eco*RI fragment and was shown to be deleted in patient 2557. The same clone was used to isolate cosmid LL242D12, which encompasses the microdeletion 2557. The sequenced parts of cosmid LL242D12 are given as shaded boxes.
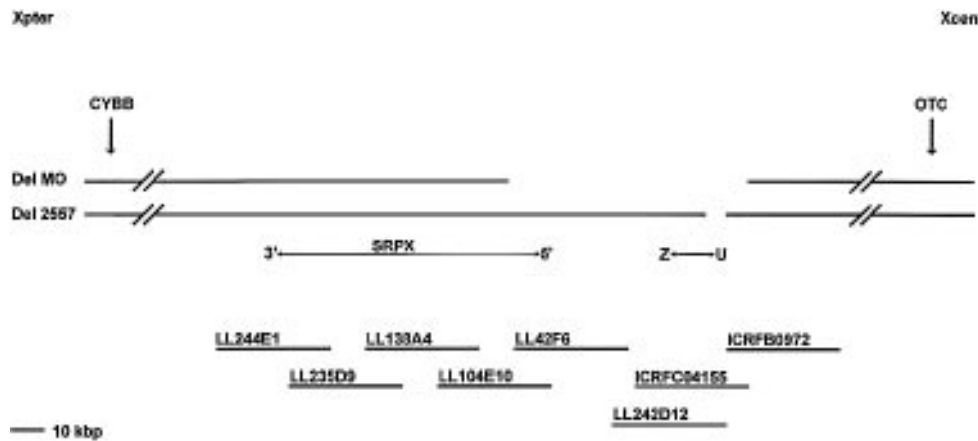
Figure 5. Physical map of the *RP3* critical region as defined by deletion mapping in patient 2557 and the recently identified deletion MO, which was reported to be 70 kbp in size (16). The two flanking markers *CYBB* (cytochrome b-245) and *OTC* (ornithine transcarbamylase) were present on a 660 kbp YAC. Exon 1 of the previously described *SRPX* gene is located on cosmids LL42F6 and LL104E10, and exon 10 was mapped to cosmid LL244E1. The ICRF cosmid clone C04155 and exon 1 of the *SRPX* gene were shown to be deleted in patient MO (16). The deletion 2557 is 6.4 kbp in size and approximately 30 kbp proximal to exon 1 of the *SRPX* gene.

in the patient and his affected brother but not in 444 unrelated controls. Thus, this gene may have a causal role in *RP3*, the most common X-linked form of retinitis pigmentosa. SSCP screening has revealed several other sequence alterations within these putative exons but none of these were confined to patients with XLRP. The apparent absence of point mutations in the cDNA that spans the 6.4 kbp deletion is not too surprising, since none of the exons which have been identified so far seem to code for protein. On the other hand, mutation detection by SSCP might be hampered by the size of the PCR fragments, which is >200 bp and therefore not optimal for the detection of all sequence alterations (24). As judged from the presence of a poly(A) tail in one of the cDNA clones, the exons identified so far may be derived from the 3′ untranslated part of the transcript. The 3′ untranslated regions of mRNAs are known to contribute to its stability and are responsible for the intracellular transport (25). Therefore, the observed deletion of two exons from this region in patient 2557 may result in an unstable or erroneously located mRNA molecule. Alternatively, the disease phenotype may be due to the deletion of sequences important in chromatin structure (e.g., matrix attachment sites) or replication (26). Deletion of 5′ regulatory sequences of a not yet identified adjacent gene is another possibility. Recently, a novel gene has been identified from the *RP3* critical region called *SRPX* (16) or *ETX1* (19). Exon 1 of *SRPX* was reported to be deleted in a patient with XLRP (patient MO, ref. 16), but the search for clinically relevant point mutations in the coding region of this gene, performed in 79 unrelated patients with XLRP, was unsuccessful (16,19). This strongly argues against a role of this gene in the etiology of *RP3*. The MO deletion is much larger than the one described here and includes the entire cosmid C04155 (see Fig. 5). Therefore it encompasses all putative exons described in this report including the cDNA that spans the 6.4 kbp deletion in patient 2557. In contrast, this 6.4 kbp deletion is at least 50 kbp proximal to the 5′ end of the *SRPX* gene. In conclusion, we have isolated the 3′ end of a gene that is deleted in two patients with XLRP but not in healthy controls. Therefore, we consider it as a strong candidate for the long-sought *RP3* gene. Elongation of this cDNA and mutation screening in coding regions of the gene should soon reveal if it is really involved in the etiology of *RP3*.

MATERIALS AND METHODS

Purification and restriction enzyme digestion of YAC DNA

DNA from the YAC (ICRFy900E0701) was isolated in 1% LMT (w/v) agarose plugs (40 μl each plug) using standard procedures (27). The YAC DNA of 40 plugs was separated from the yeast chromosomes on a preparative 1% low melting agarose gel using pulsed field gel electrophoresis (PFGE) with 0.5× TBE (45 mM Tris-borate–EDTA, pH 8.3) as electrode buffer. The 660 kbp YAC was cut out of the gel and the DNA was purified using gelase (Epicentre Technologies). The DNA (400 ng) was digested using 4 U of either the restriction enzyme *Hin*dIII (New England Biolabs), *Bam*HI (BRL) or *Bgl*II (BRL) according to the manufacturers instructions, phenol-chloroform extracted, precipitated (NaCl/ethanol) and dissolved in water (10 ng/μl).

Generation and cloning of amplicons

PCR linkers were ligated to 100 ng of restriction fragments in 30 μl of T4 ligase buffer using 400 U of T4 DNA ligase (BRL). Depending on the restriction enzyme, linkers as described by Lisitsyn *et al*. (17) were used: RBgl24 (1.5 μg) and RBgl12 (0.75 μg) to the *Bgl*II fragments; RBgl24 (1.5 μg) and RHind12 (0.75 μg) to the *Hin*dIII fragments and RBam24 (1.5 μg) and RBam12 (0.75 μg) to the *Bam*HI fragments. PCR amplification of the YAC fragments was performed in a total reaction volume of 100 μl, containing 10 μl of the ligation mixture, and 20 μl 5× PCR buffer (67 mM Tris–HCl, pH 8.8, 4 mM MgCl$_2$, 16 mM (NH$_4$)$_2$SO$_2$, and 320 μM (each) dATP, dGTP, dCTP, dTTP). The tubes were heated to 72°C using a thermocycler (Perkin-Elmer), 5 U Taq polymerase (AmpliTaq, Perkin-Elmer Cetus) were added, the reactions were covered with mineral oil and incubated for 5 min at 72°C to fill in 5′ protruding ends of the ligated linkers. Subsequently, 2 μg of the PCR primers were added, and the amplicons were generated during 20 cycles of 1 min 95°C and 3 min at 72°C with a final extension for 10 min at 72°C. The amplified fragments were purified using a Centricon 100 microconcentrator (Amicon), diluted to 100–200 ng/μl and used as a probe on Southern blots.

Table 1. Summary of putatively transcribed sequences from cosmid C2 as identified by cDNA isolation or exon prediction from genomic DNA sequences and database comparison

Fragment	Mode of identification	Size (bp)	Genomic *Eco*RI fragment	SSCP fragments and primers Size (bp)	Sequence (5′→ 3′)	Polymorphic
U	GRAIL	680	1.5 kbp	312	atctgctggttgttcaggct	+
					agcttggcctttccttcttc	
				212	cacttccctcaataatgatcag	–
					ggctttcatcgacagaagtgat	
				379	agtggtctcattcttaagcttc	–
					gttcattaccagctagagctc	
				232	gagtgcaactgaaatgtcttct	–
					tcctgaacatctcctacaatct	
V	cDNA/GRAIL	>1000	3.8 kbp	350	ctacttgaagtcacagaaagc	+
					cagaaacctcagtaggaacc	
				322	aggtgcagactctggtctg	+
					ccacggacaaaagtgcctat	
				410	cttgatttggatgctgatcag	+
					agattaaggcttggaaagcag	
W	cDNA/GRAIL	185	3.8 kbp	272	tcttactcttctctgatggtc	–
					ggatctcaggatttaagcatc	
X	cDNA/GRAIL	61	2.0 kbp	235	tagaacctgcttaaagattcag	–
					agcttataattacccaattatgg	
Y	cDNA/GRAIL	96	2.0 kbp	248	tcactgagagcatcaggtctt	+
					gagttaaattatgtggattgcca	
Z	cDNA/EST/ GRAIL	514	4.7 kbp	258	gttaagtgaatgtttcacttatg	+
					acaatacacttggtgactgtga	
				382	gagtcaggaatcatcagaatatc	–
					gtaagattgcaaatattgcctta	
A	GRAIL	97	10.0 kbp	203	ttctcataccagaagcaggg	–
					ggcagatccgtgcggcc	
B	GRAIL	113	6.0 kbp	288	ctagtttcttattcttacaaggt	–
					tggccaacatggcaaaactc	

The location of the different fragments is shown in Figure 4.

Prior to cloning, linkers were removed from the amplified fragments by digestion with 10 U of the restriction enzyme per 1 µg DNA and subsequently separated from primers by a preparative gel electrophoresis in 1% low melting agarose. DNA was purified using the QiaQuick gel purification kit (Qiagen) and subsequently ligated into phosphatase treated pGEM3 using standard procedures (28). The inserts of individual clones were obtained by direct colony PCR with the vector-primers T7 and SP6 (17).

Southern blot analysis

Lymphoblastoid cell line GM07947B (BB) was obtained through the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). DNA from RP patients was isolated from EBV transformed cell lines using standard procedures. Restriction enzyme digestions were performed according to the manufacturers instructions and fragments were separated on a 0.8% (w/v) agarose gel in 40 mM Tris–acetate, 1 mM Na$_2$EDTA, pH 7.5. Alkaline blotting onto GeneScreen Plus membrane (DuPont) was performed for 2–5 h as recommended. When the amplicon was used as a hybridization probe, 150 ng of DNA was labelled and when plasmid or cosmid inserts were used, 5–10 ng of DNA was labelled by random primer extension (29,30). Hybridization was performed after competing repetitive sequences as described (31), except for amplicon probes, which were preassociated in the presence of 1 mg of sheared and denatured total human DNA (Hybridime).

Cosmid library screening

Gridded filters from an X chromosome-specific cosmid library (LL0XNC01, Lawrence Livermore National Library) were screened according to the accompanying instructions by using the clone p20 or the entire *Hin*dIII amplicon as a probe. Positive clones were identified and the Lawrist 16 vector arms were removed by cleavage with *Sfi*I (New England Biolabs) and subsequent preparative gel electrophoresis [0.8% (w/v) LMT agarose].

Screening of cDNA libraries

Cosmid LL242D12 was used to screen two cDNA libraries, established from adult retina: an oligo-dT primed library in λgt10, (courtesy of J. Nathans) and an oligo-dT and randomly primed (5′ stretch) library in λgt10, (Clontech HL 1132a, Lot # 17951). Screening of 500 000 p.f.u. of each library, plated on *E.coli* LE392, was performed mainly as described previously (32).

Inserts from purified cDNAs were subcloned into the pGEM3 vector and sequenced by primer walking starting from the T7 and SP6 sites, and using an automatic ABI 370A DNA sequencer and a Taq DyeDeoxy terminator cycle sequencing kit (ABI).

Shotgun cosmid sequencing

The cosmid DNA was prepared and sequenced as previously described (33). In brief, after sonication of the DNA, a 0.8–1.4 kbp fraction was subcloned into the *Sma*I site of M13mp18. Templates were prepared through magnetic bead technology and sequenced using dye-primer chemistry. The raw data were collected using ABI373A automated sequencers and assembled with the XBAP program (34). Gaps were closed using custom made primers on M13 templates, PCR products or cosmid DNA in combination with Taq dye terminator chemistry (Perkin Elmer) or internal labeling (Pharmacia). Homology searches against the EMBL database were performed using BLAST (Version 1.4) (35) and FASTA (Version 2.0) (36). Gene prediction programs GRAIL (37) and XPOUND (38) were used. Sequence alignments were done by 'Global Alignment Program' (39).

SSCP analysis

Putatively transcribed sequences were amplified by PCR using the primers (Isogen Bioscience, The Netherlands) indicated in Table 1. The reactions were carried out in a total volume of 50 µl and the presence of 10 mM Tris–HCl pH 8, 50 mM KCl, 3 mM MgCl$_2$, 0.5 mM each dNTP, 10 ng bovine serum albumine (Biolabs), 125 ng of each primer, 100 ng template DNA and 1.25 U Taq DNA polymerase (Boehringer). If the sample was used for SSCP, the reaction was carried out in 20 µl, containing 0.006 mM dCTP instead of 0.5 mM and 0.2 µl α-[^{32}P]dCTP (ICN, 3000 Ci/mmol). Amplification was performed for 35 cycles, each 1 min 92°C, 1 min 60°C and 2 min 72°C. SSCP analysis was carried out in non denaturing polyacrylamide gels as described (40).

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NOTE ADDED IN PROOF

In a directly flanking cosmid (ICRFB0972), we have recently identified novel exons by shotgun cosmid sequencing and screening of the retina cDNA library. These exons correspond to the 5′ and middle part of the gene described here. Sequence comparison revealed homology to the human *RCC1* gene [Ohtsubo *et al.* (1987) *Genes Dev.* **1**, 585–593] and SSCP analysis in our patients detected several bandshifts and at least one non-conservative (Pro→Ser) amino acid substitution which were not observed in 80 control males.