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Mutation analysis of the *ROM1* gene in retinitis pigmentosa

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To examine the role of *ROM1*, a homologue of peripherin/*RDS*, in autosomal dominant retinitis pigmentosa (adRP), we screened 224 adRP and 29 simplex RP probands for *ROM1* mutations. Four *ROM1* alleles were designated as potentially pathogenic because they were found only in RP patients but not in 50–100 controls nor in 249 other RP probands. The substitutions P60T and T108M were present in a single allele in a subject with typical adRP, and this allele cosegregated with the disease in the small family. The putative null allele L114 [∇1 bp] was present in an individual with atypical RP but not in three unaffected siblings. This insertion has been previously reported to cause RP only when accompanied by a peripherin/*RDS* mutation, but no peripherin/*RDS* mutations were found in any of the four probands reported here. Two substitutions (G75D, R242Q) were present in two other probands with simplex RP. These data suggest that potentially pathogenic *ROM1* mutations occur in 1% or less of patients with adRP or simplex RP. The absence of detectable peripherin/*RDS* mutations in these families suggests either that: (i) mutations in other digenic partners are required for pathogenic *ROM1* alleles to cause retinal degeneration; (ii) these *ROM1* mutations do not cause RP; or (iii) peripherin/*RDS* mutations are present but were not identified in these patients.

INTRODUCTION

The major sites of light absorption in the human retina are the membranous disks stacked in each photoreceptor outer segment. The morphogenesis and maintenance of these disks are thought to require two related disk rim integral membrane proteins, peripherin/rds and rom-1 (1,2). Mutations in the human peripherin/*RDS* gene have been associated with several different inherited retinopathies, including autosomal dominant retinitis pigmentosa (adRP) (3–5), autosomal dominant retinitis punctata albescens (6), macular dystrophy (7), and autosomal dominant butterfly-shaped macular dystrophy (8). Recently, a peripherin/*RDS* mutation has been reported to cause digenic RP when associated with *ROM1* null alleles (9). The digenic pattern of inheritance of some patients with pathogenic *ROM1* and peripherin/*RDS* alleles may reflect the non-covalent association of rom-1 and peripherin at the photoreceptor disk rim (1).

We previously identified polymorphisms and rare sequence variants at the *ROM1* locus (10). We now report four *ROM1* alleles in adRP or simplex RP probands that were not present in 50–100 control individuals nor in a large sample of other patients with RP; the absence of these rare variants from a control population suggests the possibility that they are disease-causing. Three of these alleles co-segregate with RP in the affected families, but the small size of these kindreds prevents us from concluding that these alleles cause the retinal disease. However, the absence of these mutations from controls and the predicted effects of some of these alleles on the rom-1 protein are consistent with their having a pathogenic role. Moreover, one of the alleles we identified, the putative null L114(∇1 bp), is one of the two *ROM1* mutations found by Kajiwara *et al.* (9) to be associated with digenic RP. These data provide a strong rationale for a continued search for *ROM1* mutations in RP patients with mutations in other genes known to cause RP. Our findings also suggest that the identification of other proteins that associate physically with rom-1 in the photoreceptor outer segment, as does peripherin, may contribute additional candidate digenic partners for mutant *ROM1* alleles besides peripherin/*RDS*.

RESULTS

Putative disease-causing *ROM1* sequence variants in patients with adRP or simplex RP

Proband HS770 (typical RP): two ROM1 missense mutations (P60T, T108M) in one allele. Clinical phenotype: Proband HS770 showed typical features of RP (Fig. 1A). Fundus examination at age 37 years revealed retinal vascular attenuation, with bone spicule-like pigmentary deposits scattered throughout an atrophic retina. Examination at age 48 years showed generalized depigmentation of the macular retinal pigment epithelium with more pigmentary deposits. A standard single flash electroretinogram was nonrecordable under photopic and rod-isolated conditions, and was only barely recordable with flicker and a bright flash under dark adapted conditions.

ROM1 genotype: A band shift was detected in exon 1 of proband HS770 by SSGE (Fig. 1B); this specific shift was not identified in 100 control alleles. The variant allele cosegregated

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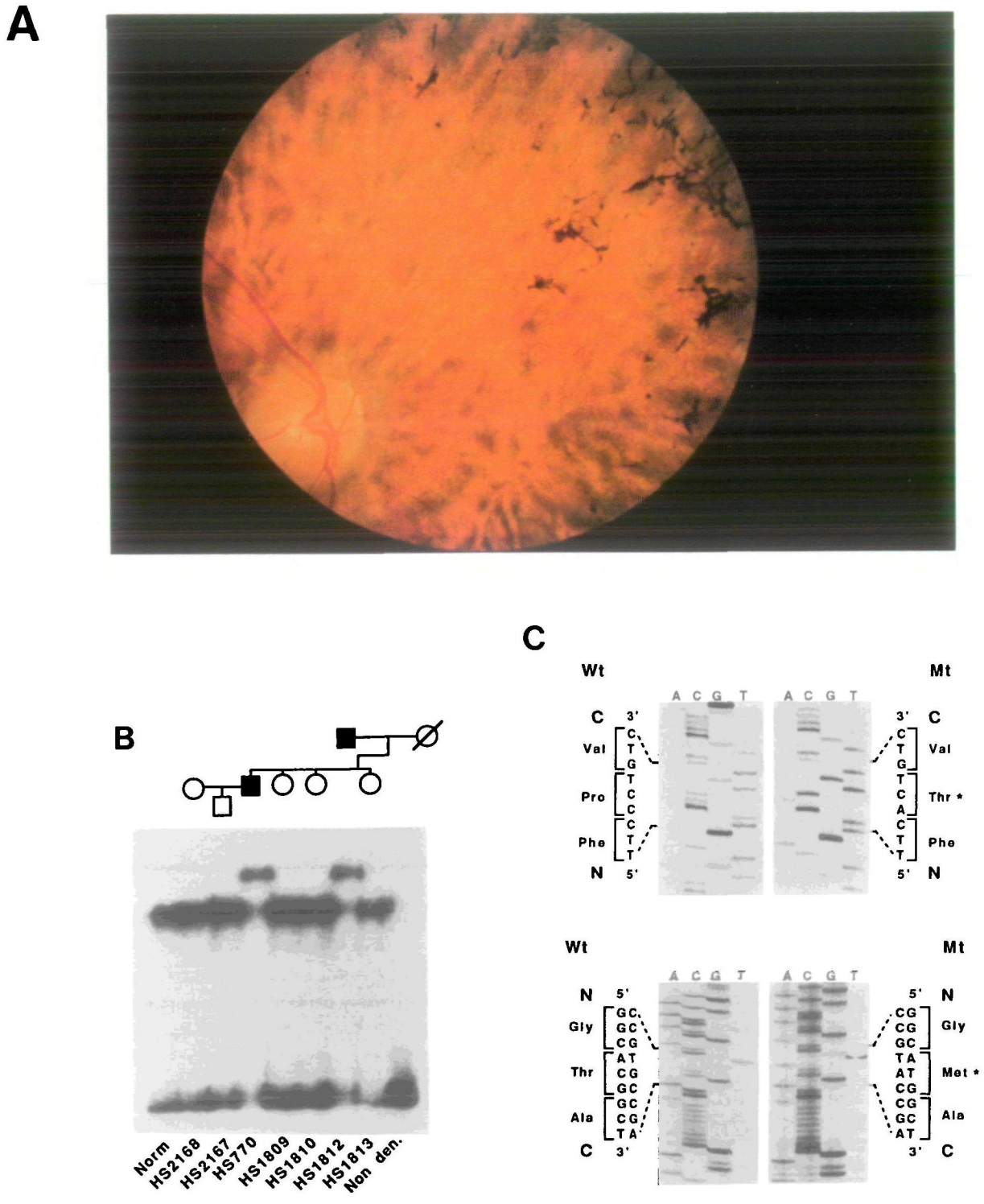

Figure 1. (**A**) Fundus of proband HS770 (typical adRP) showing retinal vascular attenuation and bone spicule-like pigmentary deposits scattered throughout an atrophic retina. (**B**) Cosegregation of a mutant *ROM1* SSGE-detectable allele (P60T, T108M) with adRP in the affected family. The proband HS770 has the mutant allele as does the affected father HS1812. The three unaffected siblings (HS1809, HS1810, and HS1813) and the unaffected son (HS2167) do not have the P60T, T108M allele. (**C**) Direct sequencing of wild type and mutant *ROM1* alleles from a control and a typical adRP proband (HS770), respectively. The mutant allele DNA fragment was cut out from a dried SSGE gel, reamplified, and sequenced. Two nucleotide changes were identified on the single mutant allele, resulting in the amino acid substitutions P60T (A) and T108M (B).

with the disease in the small family (Fig. 1B). In addition to the proband (HS770), the affected father (HS1812) had the mutant allele while the three unaffected sisters (HS1809, HS1810, HS1813) and the unaffected son (HS2167) did not. The son was of an age (19 years) at which he would be expected to have manifestations of RP on ophthalmologic examination,

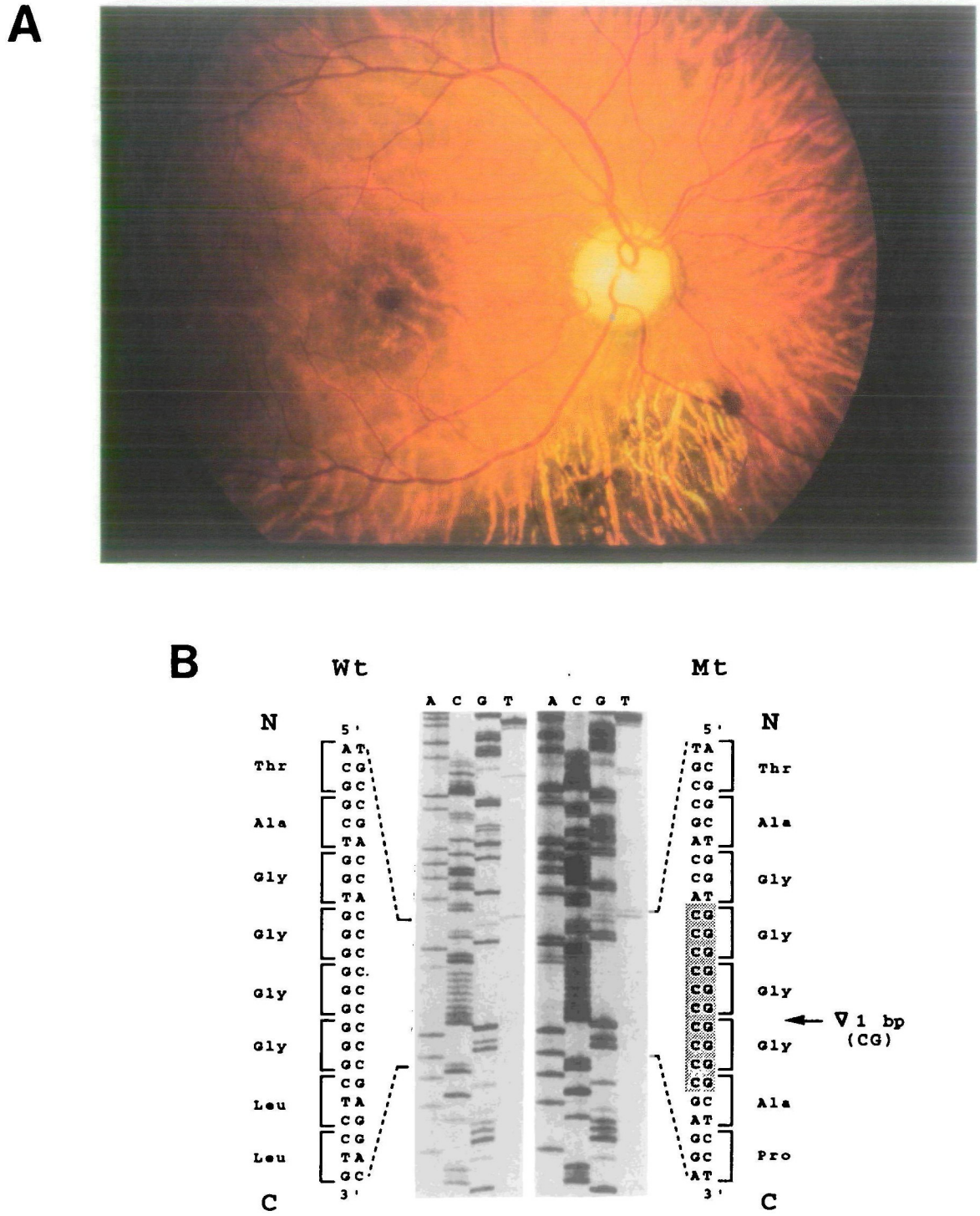

Figure 2. (A) Fundus of proband 56–1 (atypical RP). Beaten metal perifoveal lesions with no discrete flecks are present. The right eye (shown) contains a patch of retinal pigment epithelial atrophy along the inferotemporal arcade. (B) Direct sequencing of wild type and heterozygous mutant *ROM1* alleles from a control and atypical RP patient (56–1) respectively. The mutant allele is seen against a wild type background and results from a single bp insertion in the sequence encoding the third transmembrane domain of rom-1.

but the exam was normal. Two missense mutations P60T (CCT→AXT), T108M (AX→ATG) were identified in the variant allele by direct sequence analysis. The two mutations were subsequently shown to be on the same allele by cutting out the variant band from a dried SSGE gel, eluting the DNA and amplifying it for direct sequencing (Fig. 1C). Neither of these two substitutions was present in 200 control alleles examined by allele-specific oligonucleotide hybridization. P60T is situated in the intradiskal loop between transmembrane domains one and two, while T108M occurs in the third

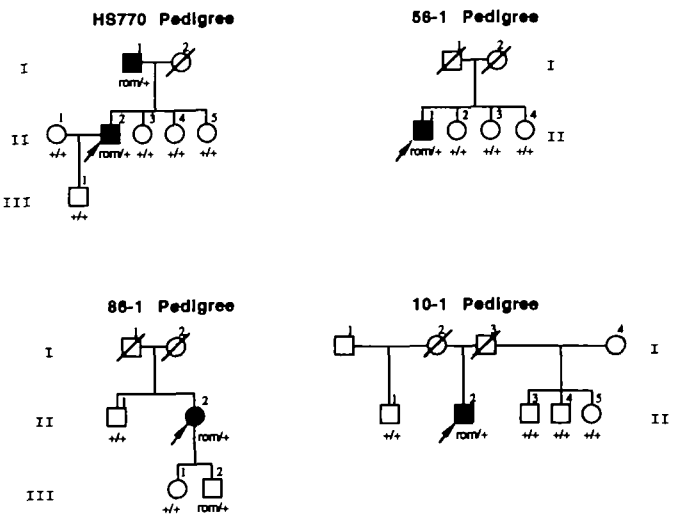

Figure 3. Pedigrees of probands HS770, 56–1, 86–1 and 10–1. Individuals with RP are represented by shaded symbols, and unaffected individuals by open symbols. The genotypes are shown, with + denoting a wild type *ROM1* allele and 'rom' a mutant allele. The mutant *ROM1* allele is P60T and T108M in pedigree HS770, L114 [∇1 bp] in pedigree 56–1, G75D in pedigree 86–1, and R242Q in pedigree 10–1.

transmembrane domain (Fig. 4). The two substitutions involve amino acids which are not conserved with peripherin/rds, but which are conserved with mouse rom-1.

Proband 56–1 (atypical RP): ROM1 putative null allele (L114 [∇1bp]). Clinical phenotype: At age 40 the visual acuity of the proband was 20/20 in the right eye and 20/40 in the left eye. The optic disks were pale and there was a one disc area zone of retinal pigment epithelial atrophy centered on fixation in each eye. Tiny drusen-like deposits were most notable at the edges of the atrophic areas. In the right eye (Fig. 2A) there was a 1.5×4 disc diameter zone of RPE atrophy with some clumps of pigment within the atrophic zone. No classic bone spicules were seen in either eye. Fluorescein angiography revealed normal choroidal filling with relative hyperfluorescence in the area of RPE atrophy. Electroretinography of the proband was essentially normal except for the light adapted responses, which were diminished.

ROM1 genotype: In the atypical RP proband (56–1) a single G insertion was identified in exon 1 of *ROM1*, in a run of nine Gs starting at nucleotide 346 (Fig. 2B). The insertion occurs in the region encoding the third transmembrane domain, and results in a frameshift that would produce a truncated protein of only 130 amino acids (13.1 kDa) compared to the normal 37 kDa (351 amino acids) rom-1 polypeptide. Seventeen new amino acid residues are introduced by the frameshift, starting from codon 114.

Three unaffected siblings of the proband (Fig. 3) did not have this *ROM1* mutation. One of the unaffected siblings later developed multifocal choroiditis, a condition not thought to be genetic.

Proband 86–1 (typical RP): ROM1 missense mutation (G75D). Clinical phenotype: At age 54 years this female proband noted that she had decreased visual acuity and was found on fundus examination to have a diffuse retinal pigment epithelial atrophy.

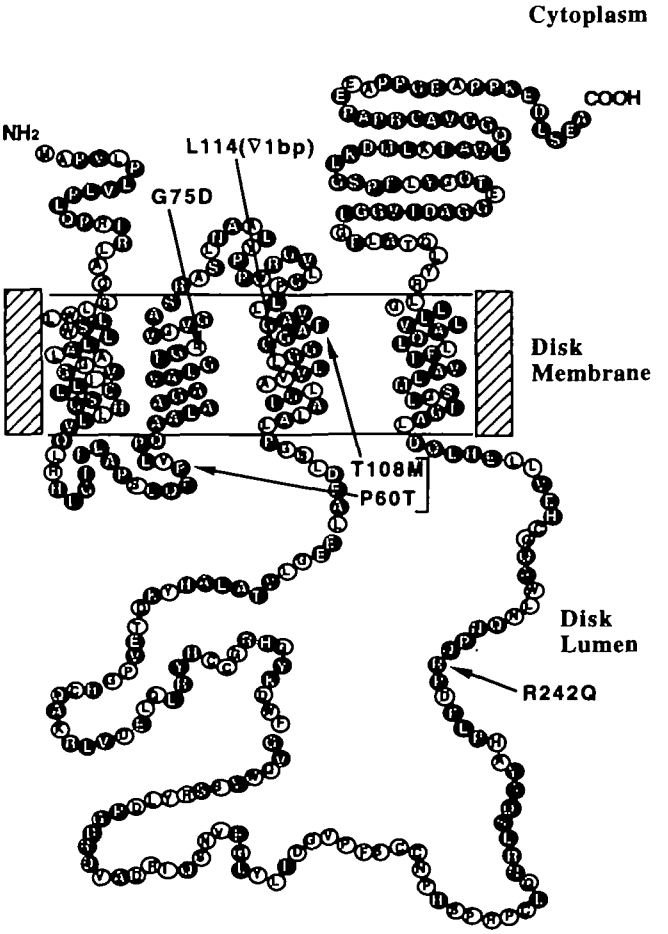

Figure 4. Distribution of the *ROM1* alleles described in this report. The model illustrates the topology of the rom-1 protein in the disk membrane and compares the sequence of rom-1 and peripherin/rds by showing identical amino acids as open circles and substitutions as closed circles.

At age 58 years the diagnosis of RP was suggested by the presence in the peripheral retina of some atrophic areas and a few bone spicule-like clumps of pigment. Macular degeneration was thought to be 'old cystoid macular edema'. The electroretinogram was non-recordable. Her visual acuity was 20/40 in the right eye and 20/100 in the left eye.

ROM1 genotype: A non-conservative substitution G75D (GG→GAC) was identified in the second transmembrane domain of rom-1 (Fig. 4). This mutation was detected in the 26 year old son (Fig. 3) of the proband, but not in her brother or daughter. None of the family members other than the proband were available for examination, although the son is presently free of clincal symptoms. The deceased parents of the proband were reported to have normal vision up to the time of their deaths (in their late 70s to early 80s). The G75D substitution was not observed in 100 control alleles (data not shown). G75 is not conserved in peripherin/rds but is conserved in mouse rom.1.

Proband 10-1 (typical RP): ROM1 missense mutation (R242Q). Clinical phenotype: At age 38 years this male proband experienced poor night vision and constricted visual

Table 1. The primer sets and restriction enzymes used to PCR amplify and digest, respectively, the three *ROM1* exons

Exon amplified	Primer sets	Location of primers*	Restriction enzyme	Size of digested fragments (bp)
1	5′-CTGACTCAGCATCCTGCC-3′	−56	*Ava*II	200, 165
	5′-GGATTCAAGGCAGCAGGAGG-3′	+676		243, 124
2	5′-CCTCTATCTCCAGACATCCT-3′	+947	*Taq*I	172, 152
	5′-GGAGGAGGTGTCAGATGCTT-3′	+1270		
3	5′-TCTTGGAACCGCTGACTCTC-3′	+1306	*Pst*I	204, 135
	5′-CTTGGTAAGGAGTTGTGAGG-3′	+1644		

* The nucleotide numbers refer to numbers from Bascom *et al.* (38).

fields; an electroretinogram at the time was nonrecordable. Fundus examination at age 50 years revealed waxy pallor of both optic disks with very attenuated vessels. In addition, bone spicule-like pigmentation and RPE atrophy were observed extending from the main temporal arcades to the periphery, and only a small area of retinal epithelium remained unaffected. Visual acuity was 20/100 in the right eye and 20/200 in the left eye.

ROM1 genotype: A substitution R242Q (cGA→CAA) was identified in the sequence encoding the conserved central hydrophilic loop of rom-1. Analysis of the family DNA revealed that three unaffected half brothers and an unaffected half sister of the proband did not have the *ROM1* mutation (Fig. 3). The R242Q mutation has not been identified in 100 control alleles (data not shown). Although R242 is conserved in mouse rom-1, this residue is glutamine in human and mouse peripherin/rds.

Mutation analysis of the peripherin/*RDS* gene

In view of the possibility that the four probands with variant *ROM1* alleles may have digenic RP involving both the *ROM1* and peripherin/*RDS* genes, we examined the peripherin/*RDS* gene of these patients for mutations. No abnormalities were identified in the entire coding region of the peripherin/*RDS* gene by SSCP analysis or by direct sequencing of the genomic DNA products obtained from amplification of each exon and its flanking sequences. In addition, a southern blot of *Msp*I digested patient genomic DNA hybridized with a ^{32}P labelled human peripherin/rds cDNA failed to reveal any major alterations of the gene structure in any of the probands (data not shown).

DISCUSSION

Although a definite relationship between retinal degeneration and the four alleles reported here cannot be established on the basis of cosegregation of the allele and the disease because of the small size of the affected families, there are several reasons for considering that at least some of these *ROM1* mutations may be disease-causing. First, none of these mutations were detected in 100 alleles from unaffected controls (and in the case of P60T and T108M, in 200 control alleles), nor in either the disease or control alleles of the other 249 other RP probands examined, indicating that if any of these alleles are benign variants, they are extremely rare ones. Secondly, one of the mutations we found, a single G insertion in codon L114 [L114(∇1bp)] in a subject with atypical RP, is the same putative null allele that was associated with RP only when it was coinherited with a peripherin/*RDS* mutation in the digenic RP families reported by Kajiwara *et al.* (9). Thus, there is a strong precedent for suspecting that at least this allele may participate in the pathogenesis of RP in the family reported here. Thirdly, the changes in the rom-1 amino acid sequence introduced by these alleles are potentially disruptive to normal protein structure or processing. Although the R242Q substitution would result in the loss of a positive charge from the rom-1 protein, this residue is a glutamine in the peripherin/rds polypeptide, and consequently, this substitution may have no significant effect on rom-1.

If the mutant *ROM1* allele in proband HS770 with two substitutions (P60T, T108M) is disease-causing, one or both of the two mutations may be pathogenic. The P60T substitution may alter the structure of the loop joining the first and second transmembrane domains (Fig. 4), since proline residues often introduce kinks into peptide chains (11). The replacement by methionine of T108—which is located in the third transmembrane domain—may interfere with the oligomerization of that domain with other rom-1 transmembrane domain(s), since subtle changes in the nature of certain side chains can disrupt oligomerization. For example, substitution of a valine by leucine in the transmembrane domain of the human erythrocyte sialoglycoprotein glycophorin (GpA) impairs its dimerization (12,13).

The non-conservative substitution G75D of proband 86–1 replaces a neutral with a negatively charged residue in the middle of the second transmembrane domain of rom-1 (Fig. 4). The introduction of a charged amino acid into a transmembrane domain can lead to retention and degradation of the polypeptide in the ER. This effect was observed, for example, by Bonifacino *et al.* (14), when they replaced single amino acids in the central region of the transmembrane domain of the Tac antigen with aspartic acid.

The mutant *ROM1* allele of the atypical proband 56–1 [L114(∇1bp)], is likely to be a disease-causing null allele for several reasons. First, a premature termination codon in the shifted reading frame may result in a severe reduction in the level of mRNA from the mutant *ROM1* allele, a phenomenon often observed for other nonsense mutations (15). Secondly, if this allele is translated, the resulting truncated protein would lack several potentially critical domains. The truncated rom-1 protein made from the L114(∇1bp) allele would lack the highly conserved central hydrophilic domain, the fourth transmembrane domain, and the carboxyl tail. In addition, the protein sequence introduced by the shifted reading frame contains charged arginine and helix-breaking proline residues which would be expected to disrupt the spanning potential of

the third transmembrane domain. Indeed, a loss of membrane spanning potential in the truncated protein predicted from the L114(∇1bp) allele is evident from the hydropathy profile of this truncated mutant (not shown). Thirdly, mutations in the peripherin/*RDS* gene that are located 3′ to L114 are pathogenic in both mouse and humans. In the *rds* mouse, the retroviral type insertion which produces a true null (16) occurs in codon 230 (17), a position considerably C-terminal to codon 114. In the human peripherin/*RDS* gene a nonsense mutation C-terminal to the third transmembrane domain, in codon 258, causes a type of macular dystrophy (7).

There are three possible explanations for the presence of the L114(∇1bp) allele in proband 56–1 with atypical RP, one being that its presence is entirely coincidental to the occurrence of RP in this patient and that the mutation is pathogenic only when paired with a defect in peripherin/*RDS* to cause digenically inherited RP (9). Alternatively, the L114(∇1bp) allele may have reduced penetrance in certain genetic backgrounds such as those of the unaffected carriers of L114(∇1bp) in the kindreds reported by Kajiwara *et al.* (9), but this mutation may lead to pathology in other genetic contexts, such as that of proband 56–1, even in the absence of a mutation in peripherin/*RDS*. A third possibility is that this *ROM1* mutation never causes retinal degeneration in the absence of a mutation in peripherin/*RDS* or in some other retinal gene, and that proband 56–1 actually has digenic RP, but we have failed to identify the digenic partner of the *ROM1* mutation. Since the digenic inheritance described by Kajiwara *et al.* (9) is likely to reflect the fact that rom-1 and peripherin non-covalently associate in order to execute a joint function at the disk rim (1), the most probable digenic partners of rom-1 besides peripherin are other proteins that also physically associate with rom-1 in the rod outer segment. For example, structural proteins identified by electron microscopy at the disk rim (18) are potential associates of rom-1 that could participate with it in digenic inheritance.

Although the above discussion has focused on the L114(∇1bp) allele, the same arguments may equally well apply to the other potentially pathogenic alleles presented in this report. Pedigree 86–1 might well illustrate digenic inheritance, in that individual III-2 carries the same *ROM1* mutation as his affected mother but is said not to have any visual impairment at age 26 years. However, he has not been available for clinical assessment, and an absence of symptoms at his age is not unusual in dominant RP. Consequently, no conclusions can yet be made about his clinical phenotype or the possibility of digenic RP in this family.

If the *ROM1* mutations presented here are indeed disease-causing, then the presence of only five such mutations in 253 RP probands suggests *ROM1* mutations are a relatively uncommon cause of RP. That RP may result from mutations in any one of many different genes is now well established, with locus heterogeneity having been demonstrated for dominant, recessive and X-linked RP (19). For dominant RP alone, at least six genes other than *ROM1* have been implicated: rhodopsin on 3q (20), peripherin on 6p (21), and unidentified genes on 7p (22), 7q (23), 19q (24), and in the pericentric region of chromosome 8 (25).

Alternatively, *ROM1* mutations by themselves may not cause adRP, unlike many peripherin/*RDS* mutations (26,27). The apparent lack of an ocular phenotype in individuals heterozygous for null *ROM1* alleles (9) distinguishes them from individuals heterozygous for null peripherin/*RDS* alleles who have photoreceptor degeneration; this discrepancy in phenotypes must reflect some fundamental difference in the biology of the proteins. Unlike peripherin/rds, rom-1 may not be essential for disk morphogenesis but may modify the rim formation, for example by ensuring disk rim closure. In this case the functions of rom-1 and peripherin/rds could partially overlap such that normal quantities of rom-1 may allow normal disk morphogenesis in a heterozygous peripherin/*RDS* L185P background. Targeted mutagenesis of the murine *Rom-1* gene will be necessary to determine whether rom-1 is critical for disk morphogenesis in rod photoreceptors, as is peripherin/*RDS* (17). The availabililty of such mutant mice will also make it possible to examine directly the pathogenic potential of individual rom-1 alleles such as those described here.

Sakuma *et al.* (28) have recently reported a small adRP pedigree in which an affected mother and daughter both carry the L114(∇1bp) *ROM1* allele. Notably, no mutations in peripherin/RDS were found in that family, a finding consistent with the data and conclusions we make here.

MATERIALS AND METHODS

Patient population

The adRP and simplex RP patient populations were not further subdivided on the basis of phenotypic differences. The probands used in this study had been previously screened for potentially pathogenic rhodopsin mutations but none was identified.

Polymerase chain reaction (PCR)

The full coding region of *ROM1* and its intervening introns were amplified by PCR (29) as a single fragment from human genomic DNA (~300 ng/100 µl reaction) using the primer pair 5′-GGCCAAGGCATCTTGTATTG-3′ and 5′-CTTGGTAAGGAGTTGTGAGG-3′ (10). Each exon was subsequently amplified from the full length PCR product using nested primers (Table 1). PCR reactions were done in the standard Cetus PCR buffer. 35 step-cycles (98°C for 30 s, 68°C for 2 min) were performed in a Perkin Elmer Cetus DNA thermal cycler. The cycle conditions for second round PCR were 98°C for 30 s, 65°C for 2 min.

For SSCP analysis, 0.5–1.0 µl of a ^{32}P dCTP (~3000 Ci/mmol) was added per 50 µl PCR reaction. Each ^{32}P labelled, amplified exon was digested with an appropriate restriction enzyme for 3 h (Table 1) to produce optimum size fragments for SSGE (30,31). Digestion involved adding 1 µl (10 U) of restriction enzyme to 10 µl aliquots from the PCR reactions.

Single stranded gel electrophoresis (SSGE)

SSGE gels were run under three sets of conditions, to increase the probability of detecting single base pair substitutions. SSGE was performed using both the conditions of Hayashi (32) and of S.Reeders (personal communication). Using the method of Hayashi, a 5% acrylamide gel (99 acrylamide : 1 bis-acrylamide) containing 5% glycerol was run overnight (12–16 h) at room temperature at 180 V in 0.5×TBE running buffer. The two other conditions involved the use of 5% acrylamide gels (59 acrylamide: 1 bis-acrylamide) with 0% or 10% glycerol. The 0% and 10% glycerol gels were run at 40 W at 4°C in 0.5×TBE running buffer for 2.0 h and 4.5 h, respectively.

Once a variant allele was detected by SSGE, the restriction enzyme digested exon fragment containing the mutation (see Table 1 for restriction enzymes) was identified by separating the digested exon fragments on a 2% low melt agarose gel. Each fragment was then cut out, denatured, and run in a separate lane on an SSGE gel thereby allowing the fragment with the mutation to be identified. If a putative disease-causing mutation was identified, the complete *ROM1* gene (coding and intron splice site regions) of the patient was then sequenced to establish that no other mutations were present in the gene.

Direct sequencing

PCR amplified fragments were separated on and then cut from 5% polyacrylamide gels or 1% agarose gels. The DNA fragments were removed from polyacrylamide gel slices by elution in TE pH 7.4 buffer (10 mM Tris–HCl, 1 mM EDTA) at 37°C overnight or from agarose gel slices by spinning

through sterile glasswool. The DNA was then purified by extraction with phenol and chloroform and precipitated. The amplified fragments were sequenced directly using a CircumVent DNA sequencing kit (New England Biolabs). To confirm all nucleotide changes, both strands of each mutant fragment were sequenced.

The amino acid substitutions P60T and T108M were shown to be present on the same allele by cutting out the polymorphic band from a dried SSCP gel, resuspending it in water, and reamplifying the *ROM1* fragment. The dried SSCP gel was re-exposed to Kodak X-O-mat AR film to confirm that the band had indeed been cut out. The amplified band was then sequenced (i.e. in the absence of the wild type allele).

Allele-specific oligonucleotide hybridization

Approximately 200 ng of each amplified fragment was separated on a 0.8% agarose gel and then transferred to Hybond-N^+ or Hybond-N (Amersham) membranes. The blots were prehybridized and hybridized with 4× SSC, 100 µg/ml sheared salmon sperm DNA, 5× Denhardt's solution and 0.05% SDS. Prehybridization was performed for at least 4 h at 10°C below the predicted T_m of the primers, after which the labelled oligonucleotides were added for hybridization (a period of ~16 h). The oligonucleotides were ^{32}P end-labelled (33). The blots were washed in 0.4×SSC and 0.1% SDS for 1.5–2.0 h at temperatures ranging from 4°C to 10°C below the predicted T_m of the oligonucleotide. The blots were then exposed to Kodak X-O-mat AR film at –80°C for 1 to 6 h.

The allele-specific oligonucleotides used in this experiment were:

P60T Normal 5′-GGACAGGGAACTG-3′
Mutant 5′-GGACAGTGAACTG-3′
T108M Normal 5′-CTGGCACGGCTGG-3′
Mutant 5′-CCAGCCATGCCAG-3′

Peripherin/*RDS* mutation screening

The entire coding region of the peripherin/*RDS* gene was amplified from genomic DNA and sequenced using three primer sets as described previously (8,34–36). Hybridization of patient genomic DNA with the human peripherin/rds cDNA was done using conditions identical to that previously reported for the rom-1 cDNA (37).

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