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Mutations in the *RP2* Gene Cause Disease in 10% of Families with Familial X-Linked Retinitis Pigmentosa Assessed in This Study

To the Editor:

X-linked retinitis pigmentosa (XLRP; MIM 312600) is a severe form of retinal degeneration that typically presents as loss of the peripheral visual field and night blindness in affected males within the 1st or 2d decade of life, owing to primary degeneration of rod photoreceptor cells and bone spicule pigmentary deposits (Bird 1975). The disease is progressive: loss of central vision occurs later in life, and complete functional blindness often occurs by 40–50 years of age. *RP2* was the first genetically mapped retinitis pigmentosa (RP) locus (Bhattacharya et al. 1984) and showed disease segregation with the RFLP detected by the probe L1.28 (DXS7). Subsequent genetic-mapping studies have shown exceptional genetic heterogeneity for autosomal RP and XLRP, which is illustrated on the short arm of the X chromosome and on *RP2, RP3, RP15,* and *RP23* (Ott et al. 1990; McGuire et al. 1995; Thiselton et al. 1996; A. J. Hardcastle, D. L. Thiselton, T. S. Mah, M. B. Gorin, and S. S. Bhattacharya, unpublished data). Extensive homogeneity and heterogeneity analyses of worldwide XLRP family collections suggested that *RP3* (frequency 70%–75%) and *RP2* (frequency 20%–25%) are the predominant forms (Ott et al. 1990; Teague et al. 1994). The *RP3* gene was positionally cloned, facilitated by the identification of submicroscopic deletions in affected patients (Meindl et al. 1996; Roepman et al. 1996). The causative gene, a putative guanine-nucleotide-exchange factor (*RPGR*), appears to be mutated in only 15%–20% of patients with XLRP (Meindl et al. 1996; Roepman et al. 1996; Buraczynska et al. 1997), not the 70%–75% expected from the results of genetic-mapping studies (Teague et al. 1994). Investigation of splice variants, promoter/enhancer mutations, and as yet unidentified exons may account for the low mutation rate detected in this gene; however, a more likely explanation for these findings is microheterogeneity. In comparison, genetic mapping suggested that *RP2* is rare (15%–20% of patients with XLRP), and the close proximity to *RP3,* together with a lack of disease-associated deletions, has hampered the search for the *RP2* gene for many years. We reported haplotype analysis for two pedigrees, which defined both proximal and distal boundaries of the *RP2* critical interval to a region of 5 cM between *DXS8083* (Xp11.3) and *DXS6616* (Xp11.23) (Thiselton et al. 1996) and which excluded the candidate gene *TIMP1* (Hardcastle et al. 1997*b*). The *RP2* gene recently has been positionally cloned by targeting this interval by use of the YAC representation hybridization technique (Schwahn et al. 1998). *RP2* consists of five exons, encodes a polypeptide of 350 amino acids, and is ubiquitously expressed. The predicted amino acid sequence has homology to cofactor C, over 151 amino acids (30.4%), which is involved in β-tubulin folding (Schwahn et al. 1998). Currently, this provides the only functional clue for the *RP2* gene; hence, the overall function and specific role in the retina are unknown at present. We have screened our panel of patients with XLRP for mutations in the *RP2* gene, to define the spectrum of mutations causing disease. Of particular interest were the two definitive genetically defined families with *RP2* that we had described previously (families F72 and NRP; Thiselton et al. 1996).

Appropriate informed consent was obtained from patients and relatives. Of a total of 59 families with XLRP assessed in this study, 26 families were excluded from *RP2* gene mutation screening, because haplotype and linkage analyses excluded the *RP2* interval or because *RPGR* mutations had already been identified. The remaining 33 families with XLRP were assessed for *RP2* mutations.

DNA from affected male patients from each of the 33 pedigrees (and two normal male controls) was amplified with primer pairs described by Schwahn et al. (1998), by use of 150 ng of initial template DNA and with minor modifications to the suggested annealing temperatures (details available on request from the authors). Purified PCR products (8 μl of product incubated with 1 U shrimp alkaline phosphatase [SAP; Amersham Life Science] and 1 U Exonuclease I [United States Biochemical] in SAP buffer, at 37°C for 30 min followed by 80°C for 15 min) were aliquoted (5 μl) for cycle sequencing in the forward and reverse directions, by use of the ABI Prism Ready Reaction Dye Terminator cycle sequencing kit (fluorescent sequencing kit, Perkin-Elmer), in accordance with the manufacturer's instructions. Reactions then were electrophoresed on an ABI 373A automated sequencer.

Segregation of the mutation, with the disease, was

Figure 1 cDNA and predicted amino acid sequence of the *RP2* gene

shown by SSCP analysis. PCR products were labeled by incorporation of $\alpha[^{32}P]$-dCTP during amplification. Denatured PCR products were resolved by SSCP analysis (Orita et al. 1989), in nondenaturing 0.5 × mutation-detection–electrophoresis gels (FMC Bioproducts) in 0.6 × Tris-borate EDTA buffer run at 8–10 W for 14–18 h. Autoradiographs were exposed at −80°C for 1–12 h. In addition, DNA from 50 female controls (100 chromosomes) was amplified, together with DNA from a patient harboring each mutation found, and was separated under the same conditions.

To complete an effective mutation screen at the sequence level, the wild-type *RP2* gene sequence was compiled by complete sequencing of DNA from two unaffected males (see above) and by cloning of the cDNA, because no DNA sequence information was publicly available. Human brain cDNA (~150 ng, from Dr. M. Cheetham) was amplified by use of the 1F/5R primers described by Schwahn et al. (1998). PCR was performed by use of proofreading *Taq* polymerase (KlenTaq, Clontech), with an initial denaturation at 94°C for 3 min followed by 40 cycles of 94°C for 20 s, 57°C for 20 s, and 68°C for 1 min 30 s. The resultant product was cloned directly into pGEM-T Easy (Promega) in accor-

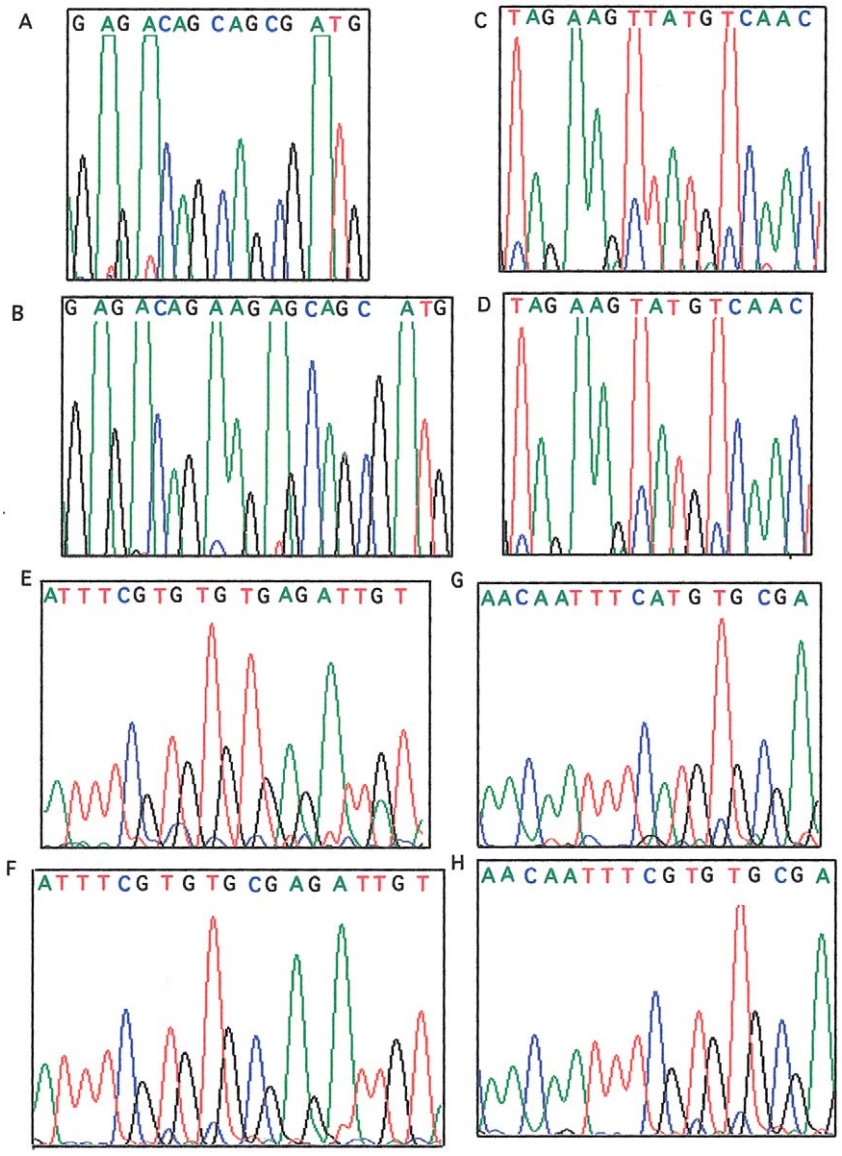

Figure 2 Electropherograms depicting mutations in patients with XLRP and normal sequences in the *RP2* gene. *A,* 5-bp deletion in exon 2 (del688–692) in affected male from family NRP. *B,* Normal sequence of corresponding region in exon 2. *C,* 1-bp insertion (T) in exon 4 (929insT) in affected male from family RP3877. *D,* Normal sequence of corresponding region in exon 4. *E,* Nonsense mutation in exon 2 (C358T) in affected male from family F15. *F,* Normal sequence of corresponding region in exon 2. *G,* Missense mutation in exon 2 (G353A) in affected male from family F72. *F,* Normal sequence of corresponding region in exon 2.

dance with the manufacturer's instructions. DNA was prepared from 1F/5R PCR-positive colonies (QIAprep Spin Miniprep kit, Quiagen) and was sequenced bidirectionally on the ABI sequencer, by use of the vector and internal primers described by Schwahn et al. (1998). The cDNA sequence for the *RP2* gene is shown in figure 1, along with the predicted amino acid sequence. The amino acid sequence deviates from the published sequence by one residue (residue 169; Asp[D]→Asn[N]). Sequence information from all patients, covering the entire coding region and the exon/intron boundaries, was examined for mutations by use of Sequence Navigator software (Perkin Elmer), to compile normal and patient sequences and to compare them directly to the cloned *RP2* cDNA.

The electropherograms in figure 2 show mutations found in our patient pool, with normal sequences depicted below each mutation sequence. A 5-bp deletion

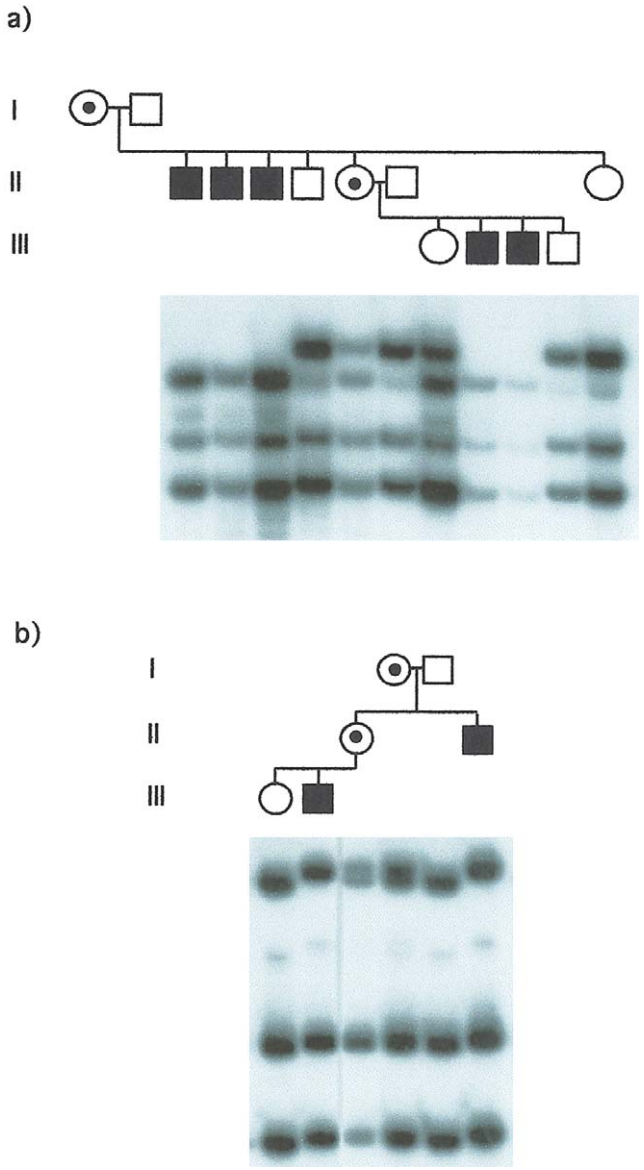

Figure 3 SSCP-based cosegregation analysis of two novel *RP2* mutations. Pedigree symbols are aligned to correspond to the appropriate gel lane. *A,* Segregation of the exon 2 C358T mutation in family F71. *B,* Segregation of the 929insT mutation in family RP3877.

in exon 2 (del688–692) was detected in family NRP, and the sequence of an affected male from this pedigree is shown in figure 2*A,* above the normal sequence of the gene (fig. 2*B*). Figure 2*C* shows the sequence of exon 4 from an affected male in family RP3877. When compared with the normal sequence of the gene in this region (figure 2*D*), a 1-bp insertion (T) is apparent (929insT; see fig. 1), thus causing a frameshift. The most common mutation observed in our sample set was a nonsense mutation converting CGA (Arg; fig. 2*F*) to TGA (termination codon; fig. 2*E*) in exon 2 (C358T; see fig. 1), at a CpG dinucleotide. This mutation was found in three apparently unrelated pedigrees, namely, F15, F71, and RP227. One missense mutation was observed in exon 2 in family F72, namely, a CGT (Arg; fig. 2*H*) to CAT (His; fig. 2*G*) change at nucleotide position 353 (G353A; see fig. 1), which is identical to the missense mutation reported by Schwahn et al. (1998). In summary, only 6 of the subset of 33 families appeared to harbor mutations in the *RP2* gene.

Segregation of mutations, with the disease, was confirmed by SSCP analysis, examples of which are seen in figure 3*a* (segregation of C358T mutation in family F71) and in figure 3*b* (929insT in family RP3877). To rule out the presence of nonpathogenic sequence variants, all described mutations were analyzed by SSCP analysis and were excluded in 50 independent female controls.

Table 1 summarizes the results of our mutation screen of the subset of 33 pedigrees with XLRP and the mutations described in the original publication (Schwahn et al. 1998). Of particular interest to us were the two families we previously had reported as defining the genetic interval for *RP2* (families F72 and NRP; Thiselton et al. 1996). A mutation was identified in exon 2 in an affected male from family F72; this missense mutation (G353A, Arg118His) had been reported previously (Schwahn et al. 1998) and is the only missense mutation found to date. We also found an *RP2* gene mutation in the second pedigree (NRP). This 5-bp deletion (del688–692) in exon 2 leads to a premature termination of translation two amino acids downstream, thus leading to a severely truncated polypeptide. Further phenotypic studies of these two families may show slightly differing disease progression or severity, reflecting the significance of a subtle point mutation (basic-to-basic amino acid substitution) and of severe truncation in a protein of unknown function. One of the original families (F15) described as showing linkage to the RFLP L1.28 (Bhattacharya et al. 1984) also was included in our sample subset of 33 pedigrees. In this family, disease segregates with the nonsense mutation in exon 2 (C358T). The same mutation was observed in two other families, F71 and RP227, which apparently are unrelated, making this the most common *RP2* mutation to date. A 1-bp insertion in exon 4 (929insT) was identified in pedigree RP3877. This mutation leads to a frameshift coding for 18 novel amino acids, followed by premature termination. In summary, from the total set of 59 families with XLRP assessed in this study, ~10% (6/59) of cases of familial disease were caused by mutations in the *RP2* gene. When considered together with the original screen, no mutations in exons 3 and 5 have been detected to date. The significance of the distribution of mutations is not known, because the gene is novel; therefore, without an interspecies/gene family sequence with which to

Table 1

Mutation Spectrum of the *RP2* Gene in XLRP

Family/Patient	Exon	Intron	Nucleotide Position[a]	Mutation
10004[b]	1		Del16–18	Del Ser6
2553[b]	1		76	Nonsense mutation CAG→TAG, Gln26Stop, 325 aa missing
2967[b]		1		LINE1 (long interspersed repeated sequence 1) retrotransposition
2613,[b] F72	2		353	Missense mutation, CGT→CAT, Arg118His
F15, F71, RP227	2		358	Nonsense mutation, CGA→TGA, Arg120Stop, 231 aa missing
1168[b]	2		453	Nonsense mutation, TAC→TAG, Tyr151Stop, 200 aa missing
3102[b]	2		Del453	Frameshift, 199 aa missing
NRP	2		Del688–692	Frameshift, 2 aa novel and 117 aa missing
RP3877	4		929	Insertion (T)/frameshift mutation, GTA→GTT, 18 aa novel and 23 aa missing
2448[b]	4		Del	Frameshift, 55 aa missing

NOTE.—Del = deletion, and aa = amino acids.

[a] See figure 1.

[b] Results from the study by Schwahn et al. (1998).

identify conserved residues and domains, functional assessment of pathogenic sequence alterations can only be speculative.

Interestingly, however, the spectrum of mutations has a trend toward severe protein truncation, with only one missense mutation identified in two families (reported by Schwahn et al. [1998] and F72 in this study). This suggests a critical role for this residue in protein folding/function. Because mutations in the *RP2* gene that cause XLRP are skewed toward protein truncation, more subtle missense mutations may cause a variant phenotype such as X-linked congenital stationary night blindness (CSNBX), which also shows genetic heterogeneity (Hardcastle et al. 1997*a*). In this study we did not search for upstream or intronic mutations; however, we do not anticipate a large increase in the percentage of cases of XLRP attributable to *RP2* gene mutations, since genetic-mapping studies suggest that a maximum of 25% of XLRP cases are linked to this interval (Teague et al. 1994).

The only indication of the functional role of this novel protein in the retina or, indeed, in any tissue is its homology to cofactor C, which in itself is unique. Cofactor C was identified as part of the β-tubulin folding pathway (Tian et al. 1996) and cooperates with the cytosolic chaperonin (CCT) and other cofactors to produce properly folded β-tubulin subunits that can form heterodimers with α-tubulin. The precise role of cofactor C is unknown, but it appears to function as a chaperone late in this folding pathway, binding near native β-tubulin (Tian et al. 1996, 1997). Cofactor C also copurifies with microtubules and also may be a microtubule-associated protein (MAP). Therefore, *RP2* possibly is also a molecular chaperone involved in tubulin biogenesis or a specialized MAP modulating some aspect of microtubule function. For example, photoreceptors have a specialized microtubule cilium connecting inner and outer segments, and *RP2* may be involved in either cilium biogenesis or maintenance. Alternatively, *RP2* may act as a chaperone in cooperation with CCT, by acting to fold proteins other than β-tubulin, possibly including retina-specific proteins. Studies of the cellular and subcellular localization of *RP2* will provide valuable insights into its physiological function and its relationship to other essential retina proteins (these experiments are currently under way).

The identification of *RPGR* as the causative gene for *RP3* (Meindl et al. 1996) has not accounted for all the cases of disease mapped to this genetic interval. Approximately 20% of families with XLRP have mutations in this gene (Meindl et al. 1996; Roepman et al. 1996; Buraczynska et al. 1997), compared with the 75% estimated from genetic-mapping studies (Teague et al. 1994), suggesting the potential for microheterogeneity around this locus. Currently, new XLRP loci are being identified (*RP23;* A. J. Hardcastle, D. L. Thiselton, T. S. Mah, M. B. Gorin, and S. S. Bhattacharya, unpublished data), suggesting that the level of heterogeneity for this disease is far greater than originally hypothesized. Because mutations in the *RP2* gene can cause only a maximum of 25% of XLRP cases, as determined from genetic-mapping studies (Teague et al. 1994) and more-recent mutation studies (Schwahn et al. 1998; this study), $\leqslant$60% of XLRP cases are still unaccounted for. The cloning of the *RP2* and *RP3* genes should facilitate

disease definition in a significant proportion of XLRP pedigrees; however, the genetics of XLRP is still complex and ill defined. The majority of families with XLRP may have mutations in one or more genes that lie in the interval between *RP2* and *RP3,* which potentially is allelic with the newly identified location for CSNBX (*CSNB4;* Hardcastle et al. 1997*a*).

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