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Analysis of the *RPGR* Gene in 11 Pedigrees with the Retinitis Pigmentosa Type 3 Genotype: Paucity of Mutations in the Coding Region but Splice Defects in Two Families

Ricardo Fujita,[1] Monika Buraczynska,[1,*] Linn Gieser,[1] Weiping Wu,[1] Patricia Forsythe,[1] Magnus Abrahamson,[3] Samuel G. Jacobson,[5] Paul A. Sieving,[1] Sten Andréasson,[4] and Anand Swaroop[1,2]

Departments of [1]Ophthalmology and [2]Human Genetics, W. K. Kellogg Eye Center, University of Michigan, Ann Arbor; Departments of [3]Clinical Chemistry and [4]Ophthalmology, University of Lund, Lund, Sweden; and [5]Department of Ophthalmology, Scheie Eye Institute, University of Pennsylvania, Philadelphia

Summary

X-linked retinitis pigmentosa (XLRP) is a severe form of inherited progressive retinal degeneration. The *RP3* (retinitis pigmentosa type 3) locus at Xp21.1 is believed to account for the disease in the majority of XLRP families. Linkage analysis and identification of patients with chromosomal deletion have refined the location of the *RP3* locus and recently have led to the cloning of the *RPGR* (retinitis pigmentosa GTPase regulator) gene, which has been shown to be mutated in 10%–15% of XLRP patients. In order to systematically characterize the RPGR mutations, we identified 11 retinitis pigmentosa type III (RP3) families by haplotype analysis. Sequence analysis of the PCR-amplified genomic DNA from patients representing these RP3 families did not reveal any causative mutation in *RPGR* exons 2–19, spanning >98% of the coding region. In patients from two families, we identified transition mutations in the intron region near splice sites (IVS10+3 and IVS13-8). RNA analysis showed that both splice-site mutations resulted in the generation of aberrant RPGR transcripts. Our results support the hypothesis that mutations in the reported *RPGR* gene are not a common defect in the RP3 subtype of XLRP and that a majority of causative mutations may reside either in as yet unidentified RPGR exons or in another nearby gene at Xp21.1.

Introduction

X-linked retinitis pigmentosa (XLRP) is one of the more severe forms of inherited retinal degenerations that are characterized by nightblindness, pigmentary retinopathy, and progressive loss of peripheral vision (Heckenlively 1988). XLRP is clinically and genetically heterogeneous; the major XLRP loci, *RP3* (retinitis pigmentosa type 3) and *RP2* (retinitis pigmentosa type 2), have been mapped to Xp21.1 (Musarella et al. 1991; Aldred et al. 1994) and Xp11.3-11.23 (Bhattacharya et al. 1984; Thiselton et al. 1996), respectively. In addition, the *RP6* (retinitis pigmentosa type 6) locus has been postulated at Xp21.3 by heterogeneity analysis (Ott et al. 1990), and *RP15* (retinitis pigmentosa type 15) has been localized at Xp22.13-22.11 in one kindred (McGuire et al. 1995). *RP3* is believed to be the predominant form of XLRP, representing 60%–90% of the affected families (Musarella et al. 1990; Ott et al. 1990; Aldred et al. 1994; Teague et al. 1994).

The *RP3* locus had been genetically localized within a relatively small region at Xp21.1, between the flanking markers DXS1110 and *OTC* (Musarella et al. 1991; Aldred et al. 1994). The coincidental location, at Xp21.1, of a large chromosomal deletion in a patient, BB, who had retinitis pigmentosa as well as Duchenne muscular dystrophy and chronic granulomatous disease, made the proximal region of the deletion a primary focus of *RP3*-cloning efforts (reviewed in Egan and McInnes 1996; Fujita and Swaroop 1996). However, recent genetic linkage and haplotype analysis in a large XLRP family have placed the mutation outside the BB deletion (Fujita et al. 1996). Furthermore, two independent XLRP patients have been reported to have deletions ~300 kb centromeric to the BB proximal breakpoint (Meindl et al. 1995; Roepman et al. 1996*a*). Consistent with these findings, the gene *RPGR* (retinitis pigmentosa GTPase regulator) has been identified 300–400 kb centromeric to the BB deletion, and microdeletions and missense and nonsense mutations in XLRP patients have demonstrated its role in RP3 disease (Meindl et al. 1996; Roepman et al. 1996*b*).

Although *RP3* is anticipated to account for the disease in a majority of XLRP patients, the reported mutation data showed a remarkably low frequency of causative *RPGR* mutations. Meindl et al. (1996) identified only

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Address for correspondence and reprints: Dr. Anand Swaroop, W. K. Kellogg Eye Center, University of Michigan, 1000 Wall Street, Ann Arbor, MI 48105. E-mail: swaroop@umich.edu

*On sabbatical leave from the Department of Medicine, Medical School, Lublin.

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two deletions and five single-base substitutions (two nonsense and three missense) in a study of 74 XLRP patients, whereas Roepman et al. (1996*b*) reported three missense mutations and one deletion mutation in 5 of 28 XLRP families. It is evident that the number of identified *RPGR* mutations is not close to the expected frequency for the *RP3* disease (for review, see Fujita and Swaroop 1996). To evaluate the role of *RPGR* in RP3 disease and to gain molecular insights into the pathogenesis of XLRP, we determined the Xp genotypes of 38 XLRP pedigrees, to identify those with the *RP3* subtype. DNA samples from the RP3 families were then analyzed for mutations in 18 of the 19 reported *RPGR* exons (spanning >98% of the coding region). Here we report the haplotype data for 11 RP3 families, along with the results of *RPGR*-mutation analysis. Identification of causative mutations in only 2 of 11 independent RP3 patients provides strong evidence for additional, as yet unidentified, RPGR exons and/or the existence of another *RP3* gene in the Xp21.1 chromosomal region.

Subjects, Material, and Methods

XLRP Families

The diagnosis of XLRP in the families in this study was made by ocular examination, complemented by visual field testing and/or electroretinograms (ERGs) (Jacobson et al. 1989; Andréasson et al. 1993; Sieving 1995). In all pedigrees, there was RP in male family members and no male-to-male transmission. Female family members reported no visual symptoms or any degree of visual impairment; many obligate carriers and those indicated in figure 1 were documented, by ocular examination and visual function tests, to have features typical of the heterozygous state of XLRP. Clinical phenotypes of families XLRP-122 and XLRP-127 have been reported elsewhere (Andréasson et al. 1997). Blood samples were collected from >150 independent XLRP families. DNA and/or RNA was extracted from lymphocytes by use of standard procedures. Informed consent was obtained from patients and normal subjects after the nature of the procedures had been fully explained. The research procedures were in accordance with institutional guidelines and the Declaration of Helsinki.

Haplotype Analysis

Twenty polymorphic markers at the Xp22.13–Xp11.22 chromosomal region (spanning XLRP loci *RP15, RP6, RP3,* and *RP2*) were used for haplotype analysis. Polymorphic markers and conditions for PCR amplification have been described elsewhere (Fujita et al. 1996; Genome Database).

Mutation Analysis

Eighteen primer sets that amplify *RPGR* exons 2–19 (for primer sequences, see Meindl et al. 1996) were synthesized at the Biomedical Research Core Facility of the University of Michigan. Amplified products were directly sequenced with the corresponding forward and/or reverse PCR primers, by the Sequenase™ PCR Product Sequencing kit (USB Amersham). Ten microliters of PCR-amplified DNA (5–50 ng) was treated with 10 units of exonuclease I and 2 units of shrimp alkaline phosphatase for 30 min at 37°C. The enzymes were inactivated by incubation at 80°C for 15 min. An aliquot of treated template DNA (6 μl) was mixed with the sequencing primer (1–5 pmol) (in 11 μl of total volume), and the mixture was denatured at 100°C for 3 min. After the template and primer were annealed at 4°C for 10–15 min, the following was added: 2 μl of 5 × Sequenase™ reaction buffer, 1 μl of 0.1 M DTT, 0.4 μl of labeling buffer, 1.6 of μl water, 0.5 μl of ^{35}S[dATP], 1.9 μl of glycerol diluent, 0.125 μl of inorganic pyrophosphatase (5 units/μl), and 0.125 μl of Sequenase™ (13 units/μl). After the sample had been kept on ice for 5 min, dideoxynucleotide termination reactions were performed at 45°C for 5 min. Samples were denatured at 90°C for 3 min and were loaded onto a 6% glycerol-tolerant sequencing gel prepared with Sequagel™ concentrated solution (National Diagnostics).

Restriction Analysis

An aliquot (10–15 μl) of the PCR-amplified product was digested with the indicated restriction enzyme (obtained from New England Biolabs), as suggested by the manufacturer. The restriction digests were electrophoresed in a 1.5%–2% agarose gel.

Reverse Transcriptase–PCR (RT-PCR) and Sequence Analysis

Total RNA was extracted from lymphocytes of probands of families XLRP-122 and XLRP-127 and from normal human retina by the guanidine isothiocyanate–phenol method (Chomczynski and Sacchi 1987). cDNA was synthesized by reverse transcription using reagents from the 3′-RACE kit (GIBCO-BRL) and was used for amplification of the RPGR sequence (Meindl et al. 1996) by use of primers F8 (from exon 10; forward) (5′ TGG TAG TTT TTG CTG CTC CTC ATC 3′) and B6 (from exon 15; reverse) (5′ CCA TGC ACC TTC ACA TTT TCC T 3′). For the analysis of RNA from family XLRP-122, 1 μl of the PCR product was reamplified by use of F8 and a nested primer B5 (from exon 14; reverse) (5′ TTC AGT AAG AGC TGT ATC C 3′). Approximately 30 ng of the reamplified product was sequenced with the F8 primer. For the analysis of RNA from family XLRP-127, 1 μl of the F8-B6 PCR product was reamplified by use of B6 and a nested primer F9 (from exon 11; forward) (5′ CAC GAT GTT CTG AGA GAA ACC TCC 3′), and the reamplified product was sequenced with the B5 primer.

Results

Identification of RP3 Families by Haplotype Analysis

From 38 XLRP families (28 North American, 1 South African, and 9 Swedish) characterized with polymorphic Xp markers, we identified 11 pedigrees (6 North American and 5 Swedish) in which the disease locus cosegregated with markers in the *RP3* region and recombined with *RP2*. (The disease locus in 8 of the 11 families also recombined with *RP15,* and in 7 of the families it recombined with *RP6*.) Haplotypes of these 11 RP3 pedigrees are shown in figure 1.

Family XLRP 101.—Patient A26 carries allele 2 of the marker *MAOA,* inherited from the noncarrier maternal grandmother A13, which indicates a crossover with the *RP2* region. The carrier female A16 is homozygous for allele 1 of DXS84, whereas allele 2 is present in other affected chromosomes, indicating a recombination with *RP6* and *RP15* loci.

Family XLRP 102.—This pedigree has been described elsewhere as *RP3* (Fujita et al. 1996).

Family XLRP 112.—Recombination with the *RP2* markers is evident from alleles of *MAOB* and DXS1003 loci in patient A287. A crossover event with *RP15* is suggested by discordance of DXS989 alleles between patients A287 and A284.

Family XLRP 115.—Evaluation of haplotypes of patients A675, A457, and A455 indicates that alleles flanking the *RP3* locus are in phase (markers M6–DXS7) and that those in the *RP2, RP6,* and *RP15* region recombine with the disease locus.

Family XLRP117.—An adult male A459, ascertained as unaffected on the basis of clinical examination, carries alleles identical to those of his affected brother (A458), from the markers proximal to DXS7, excluding *RP2* as the disease locus in this family.

Family XLRP 121.—Haplotype comparison of patient A361 and heterozygote carriers A808 and A809 indicates that only markers *CYBB* and M6 (which are in the *RP3* region) are in phase with the disease. A recombination event is observed with DXS6679, a marker ≈4 kb proximal to the *RPGR* gene. Lack of informative polymorphic variations between M6 and DXS6679 does not exclude *RPGR* as the disease locus. Obligatory recombinations with markers in the region of *RP2, RP6,* and *RP15* exclude these loci.

Family XLRP 122.—The two patients, A364 and A365, share a common haplotype between markers DXS1214 and DXS6679, which includes the *RP3* locus. Obligatory recombinations are observed with markers in the *RP2* region.

Family XLRP 125.—Clinical examination of the female individual A803 indicates a carrier status for RP. However, haplotype analysis shows two crossover events that occurred in either heterozygote carrier A375 or her deceased mother, leaving DXS6679 (at the *RP3* locus) as the only marker in phase with the disease and excluding *RP2, RP6,* and *RP15* as the disease loci.

Family XLRP 127.—Haplotype analysis of A384 and A383 shows that the disease segregates in phase with all tested markers between *CYBB* and DXS7, spanning the *RP3* locus. Proximal and distal recombinations rule out *RP2, RP6* and *RP15* as the disease loci in this family.

Family XLRP 129.—Two affected patients, A395 and A393, show a common haplotype with markers DXS1214, *CYBB* and DXS6679, demonstrating linkage to the *RP3* locus. Obligatory recombination with DXS7 and proximal markers excludes *RP2*.

Family XLRP 151.—Two obligatory recombinations, revealed by the analysis of affected brothers A656 and A658 and affected distant uncle A174, restrict the disease locus to a region between *CYBB* and DXS1068, demonstrating *RP3* as the XLRP locus.

Paucity of RPGR Coding Region Mutations in RP3 Patients

Genomic DNA from one affected member of each of the 11 RP3 families (shown in fig. 1) was subjected to PCR amplification of *RPGR* exons 2–19, which include >98% of the coding region. (The reported primer set for the *RPGR* exon 1 [Meindl et al. 1996] did not amplify specific products from the genomic DNA and, therefore exon 1 has not been analyzed in this study.) Comparison of the *RPGR* sequences from the 11 patients with the reported cDNA sequence did not reveal any variations in the coding region. The genomic sequence of 3–25 nucleotides (depending on the distance between the primer and the splice site) at the intron-exon junctions was also examined for mutations. In two patients, sequence variations that result in possible splice defects were detected in the intronic region (described below).

Aberrant Splicing of the RPGR Transcript in Family XLRP-122

Analysis of the *RPGR* sequence in patient A364 from family XLRP-122 (see fig. 1) revealed an A→G transition at the third base pair downstream of the exon 10 nucleotide 1304 (sequence designation is according to Meindl et al. 1996) in the splice-donor region of intron 10 (the mutation is designated as "IVS10+3"). This sequence alteration generated an *Mwo*I restriction site (5′ GCNNNNNNNGC 3′, where the underlined nucleotide represents the mutation), permitting an easy identification of the mutation. Sequencing and *Mwo*I restriction analysis of exon 10 amplified products from other family members revealed that the IVS10+3 mutation cosegregated with RP in hemizygotes or heterozygotes (data not shown). This sequence change was not detected in 100 unaffected and 51 affected X chromosomes, indicating that it is not a frequent variant allele

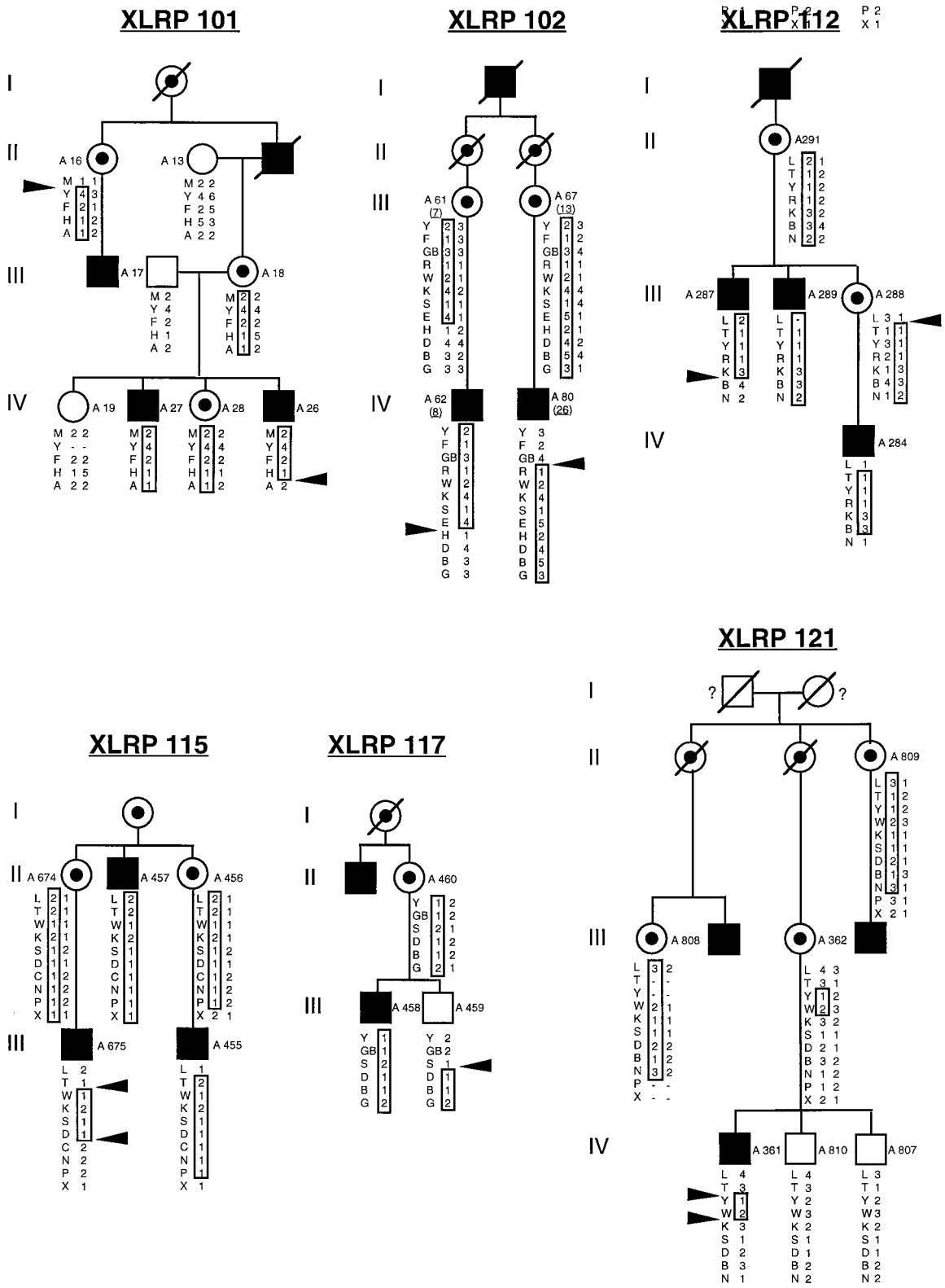

Figure 1 Determination of the RP3 status in 11 XLRP families. *RP3* was identified as the disease locus by haplotypes shown here. For each pedigree, only selected individuals with critical recombinations and relevant markers are shown. Blackened squares denote affected individuals; circles containing black dots denote carrier females; and unblackened squares and circles denote unaffected individuals. Markers spanning the *RP3* locus that are in phase with the disease are shown as boxed, and obligatory recombinations with the *RP2, RP6,* and *RP15* loci are indicated by arrowheads. The markers are, in correlative order from DXS989 at Xp22.13 (*top*) to DXS6849 at Xp11.23 (*bottom*), are pter–DXS989 (L) -DXS1214 (T), DXS84 (M), CYBB (Y), DXS1110 (F), DXS8349 (GB) -DXS8170 (R), M6 (W), DXS6679 (K), DXS1068 (S), DXS997 (E), DXS556 (H), DXS7 (D), MAOA (A), MAOB (B), DXS8080 (C), DXS1003 (N), DXS426 (G), DXS1367 (P), DXS6849 (X), and cen. *RP3* is genetically mapped between DXS8349 and DXS6679 (Fujita et al. 1996; present study, family XLRP-121). The *RPGR* gene maps between the M6 and DXS6679 loci (Meindl et al. 1996). *RP2* is genetically localized centromeric to DXS8080 and is linked to the indicated proximal markers (Thiselton et al. 1996). the markers DXS989 and DXS1214 are in the region of *RP15* and *RP6,* respectively (Ott et al. 1990; McGuire et al. 1995).

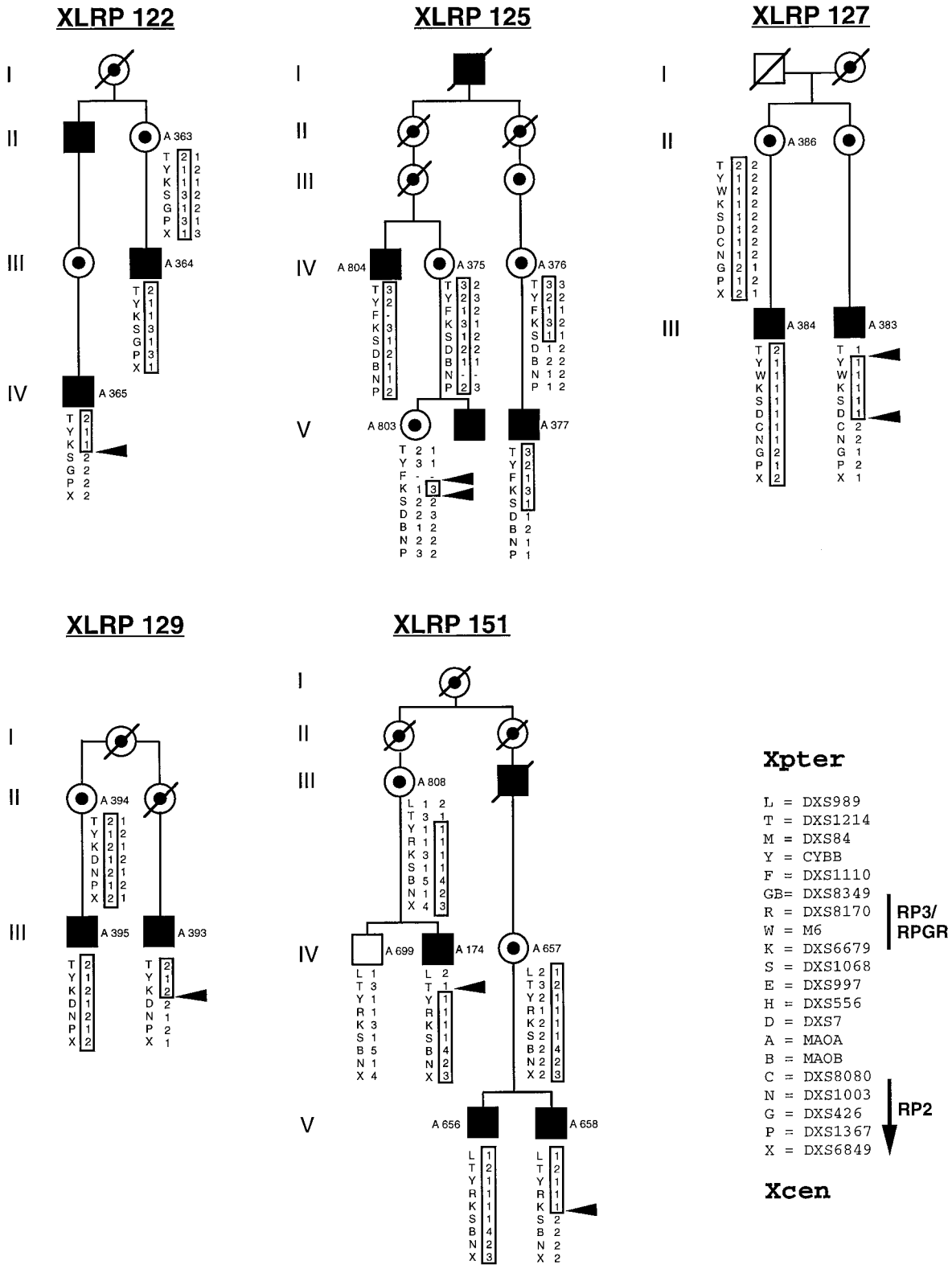

Figure 1 (continued)

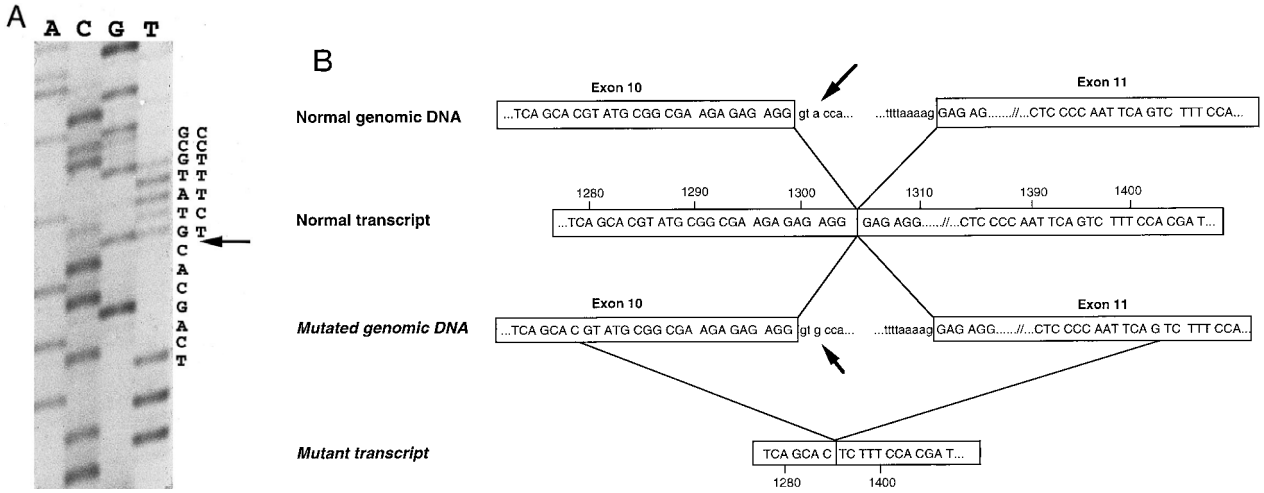

Figure 2 *A,* Sequence of total *RPGR* RT-PCR products (sense strand) derived from lymphocyte RNA of the proband in family XLRP-122. The arrow indicates the position of a new splice site produced by the IVS10+3 mutation. Beyond this, two sequences can be read; one corresponds to the normal RPGR transcript, and the other corresponds to an aberrant transcript (shown to the right of the normal sequence). Both transcripts appear to be produced at equal efficiency. *B,* Schematic representation of *RPGR* genomic DNA and transcripts in normal (unaffected) chromosomes and in mutant chromosomes of family XLRP-122. The A→G mutation (position IVS10+3) in the affected chromosome (indicated by the arrow) results in inefficient splicing, and, in addition to the normal RPGR transcript, an aberrant transcript is generated by activation of cryptic donor and acceptor sites within exons 10 and 11, respectively. The aberrant transcript retains the reading frame and would result in a truncated RPGR protein with an internal deletion of 37 amino acids. Numbers correspond to the sequence of RPGR cDNA reported by Meindl et al. (1996).

in the population (data not shown). No other *RPGR* sequence variation was detected in family XLRP-122.

To determine the effect of IVS10+3 mutation on splicing, RT-PCR analysis was performed, using the lymphocyte RNA of the proband. Sequencing of the total RT-PCR products spanning exons 10 and 11 demonstrated two types of RPGR transcripts, which appear to be generated with equal efficiency: one was the normal transcript generated by the joining of exon 10 with exon 11, whereas the other transcript was produced by activation of both a cryptic splice-donor site in exon 10 (GT dinucleotide at 1285–1286) and a cryptic splice-acceptor site in exon 11 (AG dinucleotide at position 1394–1395) (fig. 2*A* and *B*). The latter contained a deletion of 111 nucleotides. This aberrant transcript would result in an RPGR protein with an internal deletion of 37 amino acids. We therefore hypothesize that the RPGR splice defect resulting from the IVS10+3 mutation leads to RP in family XLRP-122.

Aberrant Splicing of the RPGR Transcript in Family XLRP-127

DNA from patient A384 (see fig. 1) from family XLRP-127 presented an A→G sequence change in the splice-acceptor region of intron 13 (fig. 3*A*), 8 bp upstream of nucleotide 1632 in exon 14 (the mutation is designated as "IVS13-8"). This sequence alteration destroys a recognition site for the *Apo*I restriction enzyme (5′ PuA*A*TTPy 3′, where the underlined nucleotide is changed to G). *Apo*I digestion of exon 14 PCR products from other members of this family (see fig. 1) showed that the IVS13-8 mutation cosegregated with the disease (data not shown). No other sequence variation was detected in this family. The A→G mutation was not detected in 167 independent X chromosomes (116 normal and 51 XLRP chromosomes; of these, 22 normal and 11 XLRP chromosomes were of Swedish origin), suggesting that it is not a common allelic variant in the unaffected or XLRP population.

To determine the effect of this mutation, we sequenced the total RT-PCR products, spanning exons 13 and 14, obtained by use of lymphocyte RNA of the proband. Our results show that the mutant transcript includes the last seven nucleotides (TTTACAG) of intron 13, resulting in a frameshift with multiple stop codons in exon 14 (fig. 3*B* and *C*). It also appears that the use of a newly created splice-acceptor site is virtually exclusive, at least in the lymphocytes. The truncated RPGR protein produced by the mutant transcript is predicted to be the cause of RP in family XLRP-127.

Polymorphisms in the RPGR Gene

During the mutation analysis, we identified two single-base substitutions that appear to be polymorphic variants in the *RPGR* sequence (data not shown). The first one was in exon 10 at nucleotide 1223 (the designation is based on the *RPGR* sequence reported by Meindl et al. 1996), where a G→A change was detected in ~15% of chromosomes. This sequence variation changes codon 388, GCG changes to GCA, both coding

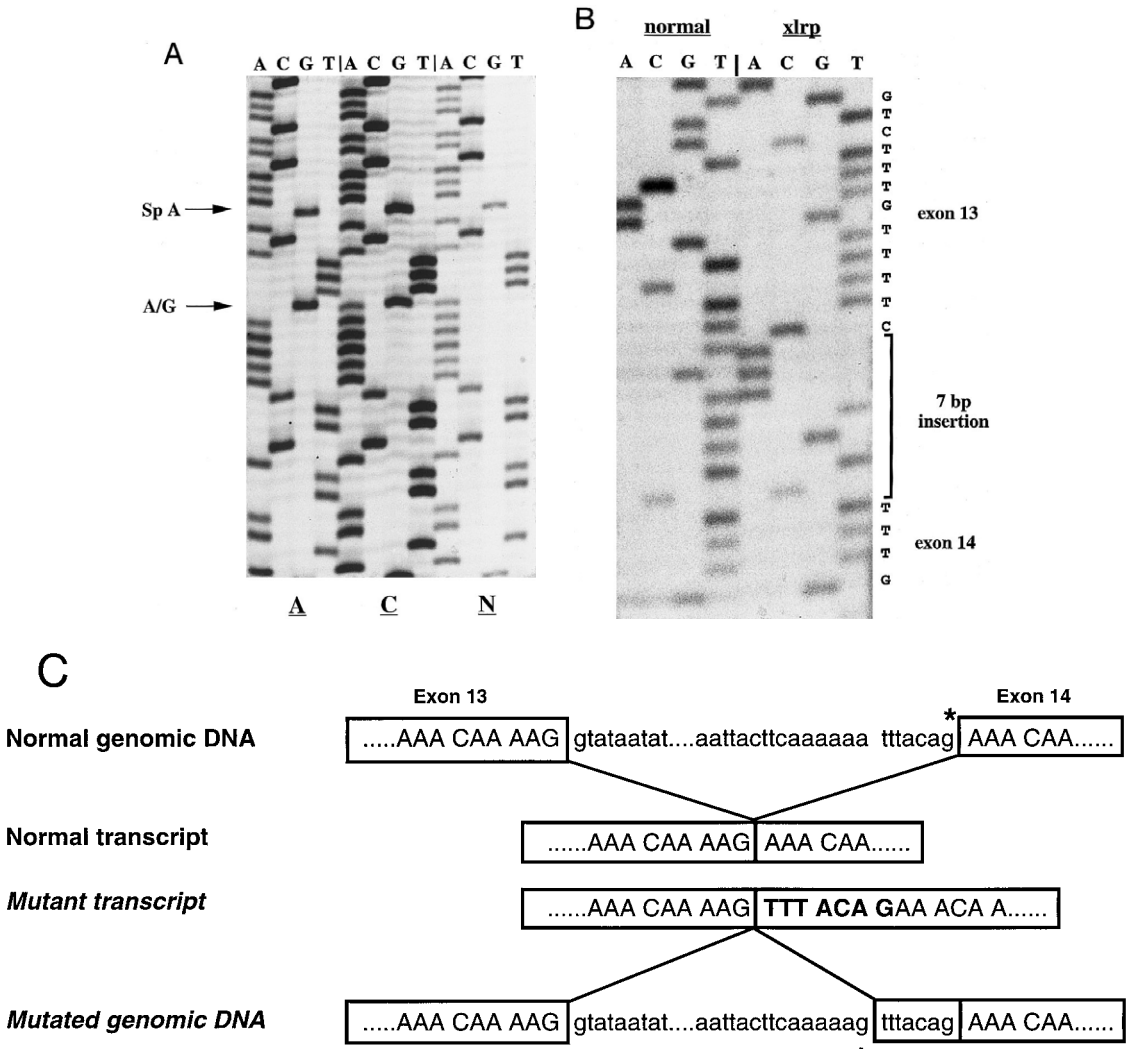

Figure 3 *A,* Genomic sequence at intron 13–exon 14 boundary of family XLRP-127. Lane A, A384 patient. Lane C, A386-heterozygote carrier. Lane N, Unaffected male. Sequencing of the amplified product that includes exon 14 was performed by use of the forward primer. The sequence change (A/G) and splice-acceptor site (SpA) are indicated. The A386-heterozygote carrier shows both A and G at the −8 position. *B,* Sequence of total *RPGR* RT-PCR products from retinal RNA from an unaffected individual and from lymphocyte RNA from the proband in family XLRP-127. The sequence of the antisense strand is shown here. The normal sequence presents the correct splice event between exon 13 and 14, whereas the mutated, xlrp product shows an addition of seven nucleotides as a consequence of the newly created splice-acceptor site. The human retinal RNA was processed as a control in parallel with the patient's sample. *C,* Schematic representation of *RPGR* genomic DNA and transcripts in normal (unaffected) chromosomes and in mutant chromosomes of family XLRP-127. Asterisks (*) indicate the 3′ splice site.

for an alanine residue. The other variant was observed in the splice-donor region of intron 18. This change represented a T→C transition, 11 bp downstream of nucleotide 2300 of exon 18, where C is observed with 20% frequency in XLRP chromosomes. Sequencing of the total RT-PCR products derived from the lymphocyte RNA of a patient with this sequence variation did not reveal any effect on splicing of the RPGR transcript (data not shown).

Discussion

After an intense search spanning more than a decade, the first RP gene was identified by the positional cloning strategy (reviewed in Egan and McInnes 1996; Fujita and Swaroop 1996). This gene, *RPGR,* was isolated from the Xp21.1 chromosomal region (Meindl et al. 1996; Roepman et al. 1996*b*) that includes the *RP3* locus. The *RPGR* gene appears to be constitutively expressed; however, low levels of transcripts are detected in the retina and retinal pigment epithelium (RPE), the sites of disease manifestation in XLRP. Nevertheless, nonsense and missense mutations and intragenic deletions were demonstrated in *RPGR,* confirming its involvement in causing RP3 disease (Meindl et al. 1996; Roepman et al. 1996*b*). A low frequency of reported RPGR mutations (7 of 74 examined by Meindl et al. [1996] and 5 of 28 analyzed

by Roepman et al. 1996*b*) presented a dilemma, since the RP3 subtype is expected to account for 60%–90% of XLRP (Ott et al. 1990; Musarella et al. 1990; Aldred et al. 1994; Teague et al. 1994). Several explanations were proposed to account for the low frequency of RPGR mutations in XLRP: (i) a low sensitivity of the SSCP method used for mutation screening, (ii) the use of all XLRP families (and not genetically characterized RP3 families), (iii) alternative splicing with as yet unidentified novel exon(s) in the retinal/RPE RPGR transcript, and (iv) another gene in the *RP3* region. To distinguish among these possibilities and to gain further insights into the pathogenesis of the disease, we identified RP3 pedigrees from our cohort of XLRP families and searched for specific causative RPGR mutations in these pedigrees.

The close proximity of XLRP loci at Xp makes it difficult to distinguish among different genetic subtypes; the two major loci, *RP3* and *RP2,* are separated by 10–15 cM. Our haplotype analysis of 38 XLRP pedigrees identified 17 families in which we were able to ascertain the genetic status. In 11 of these families, the disease locus cosegregated with markers spanning the *RP3* locus at the Xp21.1 chromosomal region and recombined with other XLRP loci (*RP2* in all 11 families, *RP6* in 7 families, and *RP15* in 8 families). Since, thus far, in no family has the condition been linked to the *RP6* locus, and since only one kindred exists in which the condition is linked to the *RP15* locus, we concluded that all 11 families (shown in fig. 1) belong to the RP3 subtype. In a majority (21 of 38) of studied families, the genetic subtype has not been assigned, because of the lack of key recombinants between the *RP3* and *RP2* loci. The criterion of obligate-crossover events to define the XLRP subtype, used in our studies, is more stringent than the heterogeneity testing and multipoint analysis that elsewhere have been utilized to determine the estimated frequency of RP3 (Ott et al. 1990; Musarella et al. 1990; Teague et al. 1994). However, the relative proportion (11/17 [65%]) of RP3 in our cohort of genetically subtyped XLRP pedigrees is consistent with earlier reports.

Mutation analysis in the 11 RP3 families showed a paucity of causative mutations in the RPGR-coding region. However, we were able to identify splice-site mutations in two of the families. Mutations in the invariant splice-site nucleotides often result in splicing defects that include exon skipping, intron retention, or utilization of a cryptic splice signal (Maquat 1996). The consensus splice-donor sequence is RG↓GTRAGT (R = purine; and ↓ = splice site), where the GT dinucleotide is nearly invariant. At first glance, the IVS10+3 transition mutation (A→G at position +3) in family XLRP-122 would not seem to have any effect on RPGR splicing; nonetheless, RT-PCR results show that the efficiency of correct exon 10–exon 11 splicing is compromised by this sequence change (see fig. 2*A*). Although a normal RPGR transcript is also generated in the lymphocytes of the proband, we suggest that either the splice defect is more pronounced in the retina/RPE or the aberrant RPGR protein has a dominant-negative effect. In any scenario, the splice defect due to the IVS10+3 mutation appears to be the cause of RP in family XLRP-122.

The consensus sequence at the 3′ splice site is $(Y)_{11}$NYAG↓R (Y = pyrimidine; R = purine; and ↓ = splice site), where the AG dinucleotide is nearly invariant. The A→G mutation in family XLRP-127 is apparently at a variant nucleotide position (see fig. 3*C*). Although this change produces an AG dinucleotide, the neighboring sequence (AATTACTTCAAAAAG↓(T) in intron 13 still does not conform to the consensus at the splice-acceptor region. RNA analysis showed that this mutation created a new splice signal, which was utilized almost exclusively in the lymphocytes. The utilization of this splice-acceptor site, which is closer to the branch point in the intron, provides in vivo support for the 5′→3′ scanning model of splice-site selection (Lang and Spritz 1983; Smith et al. 1993), although other possible mechanisms (Chiara and Reed 1995; Maquat 1996; Pasman and Garcia-Blanco 1996) cannot be ruled out.

The results presented here confirm that mutations in the *RPGR* gene represent only a small proportion of the genetic defects in XLRP. Our studies support the notion that the majority of disease-causing mutations in *RP3* families are localized in sequences other than the 18 *RPGR* exons and their corresponding splice-site regions that have been analyzed in this investigation. We propose the following three possibilities to explain the low frequency of RPGR mutations:

1. It is possible that a majority of mutations in RP3 patients are in exon 1 or in an as yet uncloned exon(s) that may be present in an alternately spliced transcript expressed in the retina or RPE. Nevertheless, the haplotype analysis of XLRP patients with polymorphic markers between *CYBB* and DXS1058 has not revealed any linkage disequilibrium, suggesting an independent origin of many, if not all, causative RP3 mutations (R. Fujita, P. Forsythe, and A. Swaroop, unpublished data). In addition, although no full-length human retinal/RPE RPGR cDNA has been isolated yet, RNA analysis using northern or rapid amplification of cDNA ends (RACE) experiments consistently yielded a similar-size transcript, of 2.9 kb (Meindl et al. 1996; Roepman et al. 1996*a,* 1996*b*).
2. The disease-causing mutations may occur in the regulatory sequence (e.g., promoter) or intronic regions of the *RPGR* gene. Such mutations are much less common, compared with the coding region or splice-site mutations, and probably could not account for the disease in 80% of RP3 patients.

3. Genetic heterogeneity exists at the *RP3* locus, and *RPGR* may not be the only *RP3* gene at Xp21.1. Haplotype data in family XLRP-121 (reported in fig. 1), along with our previous studies in family XLRP-102 (Fujita et al. 1996), suggest that the location of the *RP3* gene is between the DXS8349 and DXS6679 loci. Since mutations in the *RPGR* gene cannot account for the disease in the majority of RP3 families, we are tempted to consider the possibility of another *RP3* gene in the Xp21.1 region. In addition, the RP disease in patient BB remains an enigma (for discussion, see Fujita et al. 1996).

Further studies are in progress to resolve the molecular and genetic complexity at the *RP3* locus and to understand the ontogeny of severe retinal degeneration observed in patients with XLRP.

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