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ARTICLE

Mutation analysis of the *RPGR* gene reveals novel mutations in south European patients with X-linked retinitis pigmentosa

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The *RPGR* (retinitis pigmentosa GTPase regulator) gene has been shown to be mutated in 10–20% of patients with X-linked retinitis pigmentosa (XLRP), a severe form of inherited progressive retinal degeneration. A total of 29 different RPGR mutations have been identified in northern European and United States patients. We have performed mutation analysis of the *RPGR* gene in a cohort of 49 southern European males affected with XLRP. By multiplex SSCA and automatic direct sequencing of all 19 RPGR exons, seven different and novel mutations were identified in eight of the 49 families; these include three splice site mutations, two microdeletions, and two missense mutations. RNA analysis showed that the three splice site defects resulted in the generation of aberrant RPGR transcripts. Six of these mutations were detected in the conserved amino-terminal region of RPGR protein, containing tandem repeats homologous to the RCC1 protein, a guanine nucleotide-exchange factor for Ran-GTPase. Several exonic and intronic sequence variations were also detected. None of the RPGR mutations reported in other populations were identified in our series. Our results are consistent with the notions of heterogeneity and minority causation of XLRP by mutations in *RPGR* in Caucasian populations.

Keywords: eye diseases; retinal dystrophies; X-linked gene; retinitis pigmentosa 3 (RP3); retinitis pigmentosa GTPase regulator gene (*RPGR*); mutation analysis

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Introduction

X-linked retinitis pigmentosa (XLRP) is a severe retinal degeneration that typically causes night blindness, loss of peripheral vision within the first or second decades of life, legal blindness by 30 years of age, and often complete functional blindness by 40 or 50.[1–3] At least two loci on the X chromosome can mutate to cause retinitis pigmentosa (RP). The first, RP2 (type 2), was recently identified by positional cloning in Xp11.3.[4] A second locus, RP3 (type 3), is distal to RP2 between the DXS1110 and OTC loci in Xp21.1.[5,6] RP3 is the disease locus in the majority of XLRP families.[7–9] Deletion analysis and a positional sequencing approach led to the identification of the *RPGR* (retinitis pigmentosa GTPase regulator) gene at the RP3 locus.[10] The predicted protein contains in its amino-terminal half a tandem repeat structure highly similar to RCC1 (regulator of chromosome condensation) suggesting an interaction with a small GTPase.[10] Mutations within conserved domains provide evidence that *RPGR* is the *RP3* gene, but its function and the etiology of retinal degeneration are unknown.

The *RPGR* gene was shown to be mutated in only 10–15% of northern European *RP3* families[10,11] and in 20% of United States XLRP families.[12] Most mutations fall in exons encoding RCC1 repeats, the characteristic domain of the amino-terminal region of RPGR putative protein. It is conceivable that *RPGR* is not responsible for most cases of XLRP linked to this region and that another *RP* novel gene(s) remains unidentified. Another possibility is that identification of RPGR mutations escape detection because they are located in regulatory regions (eg promoter regions) or in additional coding sequences.[10,13] To date 29 different RPGR mutations have been identified.[10–15] Most mutations identified in European families have been different from those identified in the United States. To extend the information about the range, type, and frequency of RPGR mutations in different populations, the goal of the present study was to investigate southern European patients with XLRP. We report here the examination of all 19 exons of the *RPGR* gene in 34 XLRP families from Spain and 15 XLRP from Italy.

Materials and Methods

XLRP Patients

Patients were considered as XLRP on the basis of ocular examination, electroretinography (ERG) and assessment of visual field.[16,17] Pedigrees showed no male-to-male transmission of the disease, and females were only mildly affected, consistent with X-linked inheritance. Clinical phenotypes of families XLRP-24 and XLRP-534 have been reported elsewhere.[13] In six families, the disease state was linked to the RP3 locus; in the others, linkage analysis was not possible.

Multiplex SSCA Screening

Genomic DNA for amplification reactions was extracted from the blood of affected individuals and other family members by standard procedure.[18] All individuals were informed about the objectives of the study and volunteered to participate. DNA samples were screened for mutations within the *RPGR* gene, by multiplex PCR/SSCA analysis.[19] The screen was performed for all 19 exons of the *RPGR* gene, using oligonucleotide primers described elsewhere.[10] Genomic DNA samples (200 ng) were co-amplified for three pairs of RPGR primers in a 12 µl reaction containing 7.5 mM $MgCl_2$, 7.5 mM Tris-HCl (pH 8), 7.5m M Hepes, 50 mM KCl, 2 mM DTT, 0.01% gelatin, 2.5% formamide, 6.25 mM dNTPs, 0.5 mM each primer and 0.5 units Taq polymerase (Perkin Elmer, Foster City, CA, USA). After an initial denaturation step at 94°C for 5 min, PCR was performed for 30 cycles; each cycle consisted of 30 s of denaturation at 94°C, 45 s of annealing at an appropriate temperature, and 45 s at 72°C for extension; 4 µl of stop solution (95% formamide, 60 mM NaOH, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) were added to 3 µl of each sample. Amplification products were denatured for 4 min at 95°C, chilled in ice for 5 min and loaded on to SSCA gels. Gel formulations include 9% acrylamide/bis (60:1), 8% glycerol and 8% acrylamide/bis (50:1), 6% glycerol. Gels were run for 6–12 h at 4°C, 1200 V, and stained using a silver nitrate protocol.[20]

Reverse Transcriptase-PCR Analysis

Total RNA was isolated by the guanidine isothiocyanate-phenol method[21] from Epstein-Barr virus-immortalised lymphocytes of probands of families XLRP-401, XLRP-DA, and XLRP-192. The cDNA was synthesised using random hexamers as primers and Superscript II RNase H Reverse (Life Technologies, Milan, Italy) according to the manufacturer's instructions. Subsequent amplification was done with primers: RT2 (5'-GTT TAA AAA TGA TGT CCC TGT A-3') and RT3 (5'-TTG ACA CAT GTT GGC TTG CTG A-3') for the splice site mutation in XLRP-401 (annealing 60°C); RT7 (5'-GTT AGG TCT TCC CAA TCA GC-3') and RT9 (5'-TTA GAG CAC AAA GTA GGA ATG AAG-3') for the splice site mutation in XLRP-DA (annealing 60°C); RT13 (5'-TTA AAA TTA TCA CCA GTT CA-3') and RT15 (5'-TTG CTC CTC TAT TCC ATT TC-3') for the splice site mutation in XLRP-192 (annealing 54°C). The PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at an appropriate temperature for min and extension at 72°C for 2 min, then a final extension at 72°C for 10 min with the same reagents described above.

Sequencing of PCR Products

In order to define mutations/sequence changes, DNA fragments that exhibited mobility shifts on SSCP analysis, as well as normal fragments and RT-PCR products for splice site mutations, were reamplified, gel purified using a Geneclean (Bio 101 Inc., Vista, CA, USA) gel extraction kit, and used as

templates in a direct dideoxynucleotide chain termination reaction using dye terminator chemistry (Perkin Elmer) according to the manufacture's protocol. The primers employed for PCR reactions were used for sequencing and the products were analysed on an ABI PRISM 377 DNA sequencer (Perkin Elmer). All mutations were confirmed by sequence analysis in both directions in the affected individuals of each pedigree.

Results

In the cohort of 49 apparently unrelated XLRP families (34 Spanish and 15 Italian), seven novel mutations were identified in hemizygous affected males and heterozygous carrier females from eight XLRP families by multiplex-SSCP and direct automated sequencing. All seven mutations segregated with the disease in appropriate family members that were available for the study. Table 1 summarises the mutations observed in this study.

Missense Mutations

Two missense mutations (see Table 1) in the RCC1 domain were found in two families, one Spanish (XLRP-G9; Figure 1c) and one Italian (XLRP-RC; Figure 1d). The first (T99N) is Thr- > Asn at nucleotide 355 in exon 4 (Figure 2a); the second (I289V) is Ile- > Val at nucleotide 924 in exon 8 (Figure 2b). Both amino acidic substitutions are within residues which are conserved across evolution.[10] These mutations were present in carrier females, affected males, absent in healthy males of each family and also in 120 control chromosomes suggesting that these are causative of the disease.

Microdeletions

Two different microdeletions were identified among the affected members of three unrelated families. One deletion (ΔT545), which is a single thymine deletion at base 545 in exon 6, was found in affected males of Spanish family XLRP-G4 (Figure 1f), causing a frameshift and premature termination signal in codon 174. This mutation has also been reported in another Spanish family XLRP-24,[13] but the two families are unrelated – at least in available pedigrees – and came from different parts of Spain. The second (ΔAT296–297) was a deletion of two bases pairs at 296–297 in exon 3, causing a frameshift and premature termination signal in codon 86. This mutation has been identified in an Italian family XLRP-534 described previously.[13]

Splice-site Mutations

In three patients, splice site mutations were detected that cosegregated with the disease. The first (IVS2-2) was observed in an Italian family (XLRP-401; Figure 1b). It is an A- > G transition (Figure 3a) at position –2, at the first intronic nucleotide of the splice-acceptor site of exon 3 (sequence designation is according to Meindl *et al*[10]). To determine the effect of this mutation, we sequenced the RT-PCR products, spanning exons 2 and 3, from the proband lymphocyte RNA. Our results show that the mutant transcript of 606 bp includes intron 2, resulting in a failure to recognise the affected intron (Figure 3a). Intron 2 is therefore retained in the final mRNA product. As a result, a new stop codon is encountered within the first three nucleotides of the intron, resulting in a severely truncated polypeptide of

Table 1 RPGR mutations in patients with XLRP

Mutation	*Exon*	*Mutation at DNA level*	*Mutation at RNA and protein level*	*Family*	*Origin*
Missense:					
T99N	4	C->A at 355 nt	codon 99 (Thr->Asn)	XLRP-G9	Spain
I289V	8	A->G at 924 nt	codon 289 (Ile->Val)	XLRP-RC	Italy
Deletion:					
ΔAT296-297	3	del AT at 296-297 nt	frameshift and stop codon at 86	XLRP-534[a]	Italy
ΔT545	6	del T at 545 nt	frameshift and stop codon at 174	XLRP-G4	Spain
				XLRP-24[a,b]	Spain
Splicing:					
IVS2-2	3	A->G at 216-2 nt	change in splice donor	XLRP-401	Spain
IVS8+1	8	G->A at 839+1 nt	change in splice acceptor	XLRP-DA	Italy
IVS13-2	14	A->G at 1632-2 nt	change in splice donor	XLRP-192	Spain

Codon numbering starts with the first in-frame methionine of the *RPGR* gene (Meindl *et al*;[10]). All mutations were tested in 120 control chromosomes; [a]described in Miano *et al*;[13] [b]Family linked to RP3 locus.

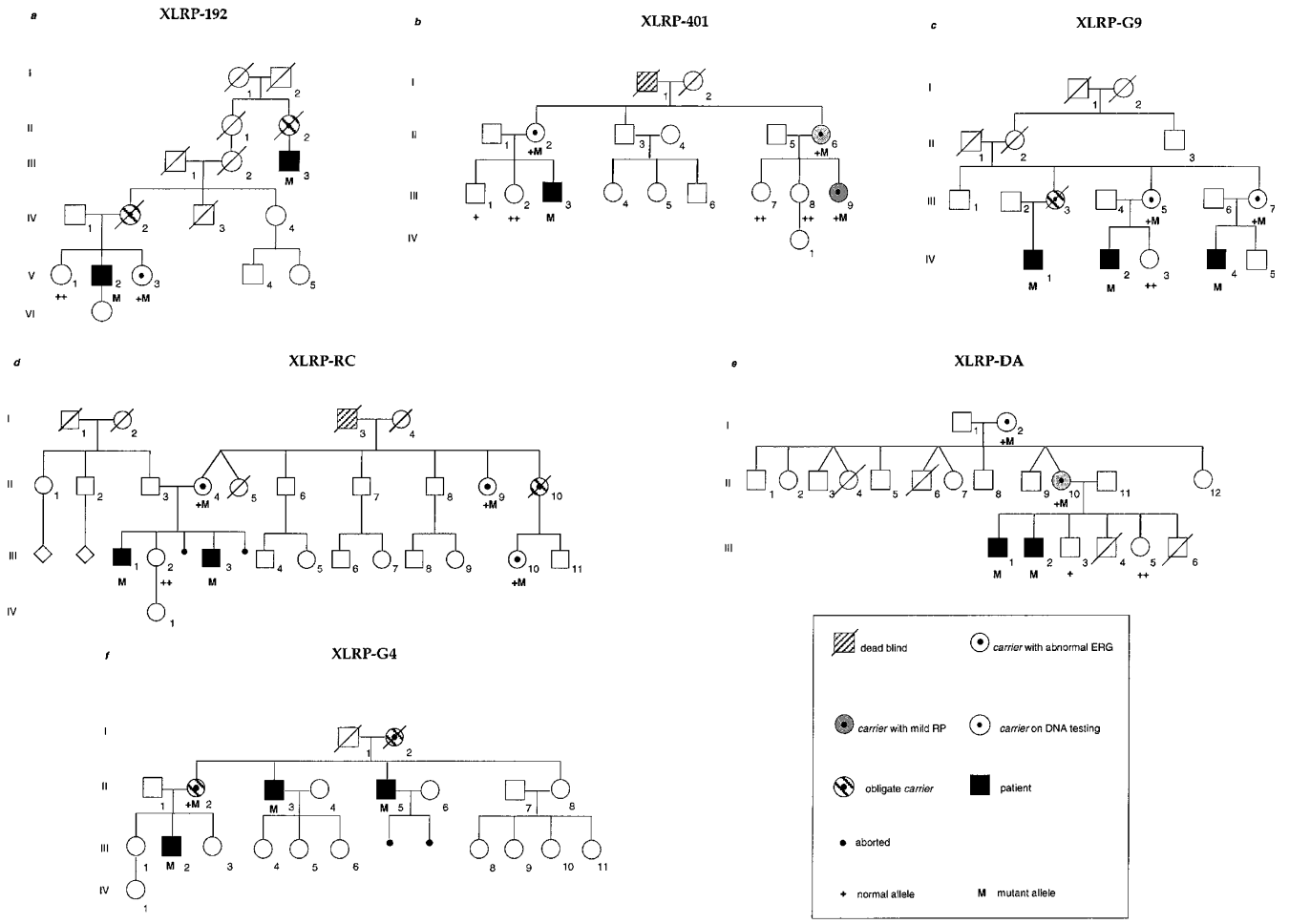

Figure 1 *Segregation of RPGR mutations in six XLRP families. a) family XLRP-192 is segregating for the splice site mutation IVS13-2; b) family XLRP-401 for the IVS2-2; c) family XLRP-G9 for the T99N; d) family XLRP-RC for I289V; e) family XLRP-DA for the IVS8 + 1; and f) family XLRP-G4 for ΔT545 (See Table 1). Symbols used are described at the bottom of the figure*

193 residues lacking the RCC1 domains. The sequence alteration generated a new HpaII restriction site and the loss of a BstNI restriction site, which segregate with the disease in the family (data not shown).

The second splice site mutation (IVS8 + 1) is a G- > A transition (Figure 3b) at position + 1 in the splice-donor region of exon 8, observed in another Italian family, XLRP-DA (Figure 1e). Sequence analysis of RT-PCR products from lymphocyte RNA from the proband was performed with primers that span exons 7 to 9. The sequence change results in the skipping of exon 8 (156 nt), and leads to an in-frame deletion of an RCC1 repeat (Figure 3b).

The third splice site mutation (IVS13-2) was observed in a Spanish family, XLRP-192 (Figure 1a). It is also an A- > G transition (Figure 3c) at position –2 in the splice-acceptor site of exon 14. Sequence analysis of RT-PCR products from lymphocyte RNA from the proband was carried out with primers spanning exons 13 to 15. Similar to mutation IVS8 + 1, this sequence alteration results the skipping of an exon (exon 14, 185 bp), and leads to an in-frame deletion in the carboxy-terminal region of the RPGR protein (Figure 3c). This consequence is a protein with a small 'charged domain'.[12] These three mutations were absent in 70 XLRP and 120 control chromosomes, indicating that they are not simply frequent variant alleles in these populations.

Sequence Variations

Several base substitutions (exonic and intronic) were detected by multiplex-SCCA and specified precisely by sequence analysis, in both patients and control samples

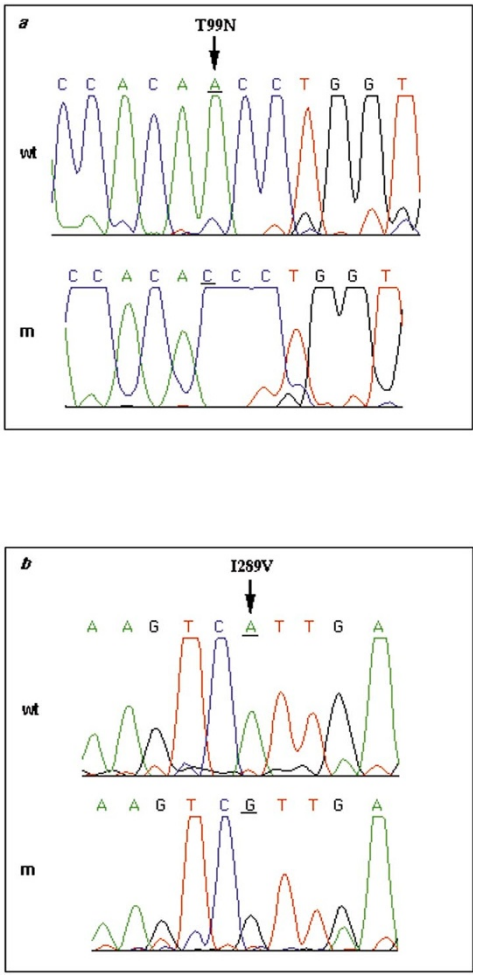

Figure 2 *Sequence analysis from PCR products of patients (m) and controls (wt). The PCR amplification was performed with primer pairs as described in Meindl* et al[10] *Sequencing reactions were carried out with appropriate exon primers for mutations T99N (a) and I289V (b)*

(see Table 2). We identified seven new sequence variants in some of 120 control chromosomes and 22 XLRP patients. They include two silent mutations in exon 2 (S47S), exon 7 (K244K) and one conservative substitution in exon 14 (T533M) and four intronic variants in intron 10 (IVS10 + 16), intron 12 (IVS12-101; IVS12-130), intron 17 (IVS17 + 46). Additional sequence variants detected during our studies have been described elsewhere. They include two silent mutations in exon 11 (R425K; I431V), in unaffected individuals, producing conservative amino acid substitutions.[11,12] We also identified one silent mutation in exon 10 (A388A), one conservative substitution in exon 14 (G566E), and three intronic variants in intron 1 (IVS1-15), intron 13 (IVS13 + 11), intron 18 (IVS18 + 11), all previously described by Buraczynska.[12] Any affect of these changes on splicing has not been determined.

Discussion

Long-range sequencing in X-linked retinitis pigmentosa led to the discovery of mutations in the *RPGR* gene.[10] Expression of RPGR has been demonstrated in all neuronal layers of the retina and in RPE. The protein is seen both in cytoplasm and nucleoplasm, particularly in the vicinity of nuclear pores, in membranous organelles surrounding the nucleus and the inner and outer segments of both rod and cone photoreceptors.[22,23] But all studies indicate that RPGR is expressed at surprisingly low levels in the retina, and it is unclear how mutations in this gene cause retinal degeneration.

Mutations in *RPGR* (see Figure 4) have been identified in only 10–15% of northern European RP3 families[10,11] and in 20% of United States XLRP families.[12] The low percentage of XLRP families with RPGR mutations is consistent with recent reports indicating that RPGR mutations were found in XLRP families less frequently than the 60–90% level originally predicted by linkage analysis of XLRP pedigrees.[7–9,12] Thus, other genes might be quantitatively more important as causes of XLRP. On the other hand, it remains a possible alternative that because the sensitivity of mutation–detection methods is not adequate, mutations may have escaped detection, possibly because they occur in regulatory regions (eg promoter region) or in additional coding sequences.[10,13]

Studies of different populations might reveal a range of mutations that would help to distinguish between these alternatives. Here, among 49 southern European XLRP families, eight carried seven different RPGR mutations in coding regions and splice sites. None of the 29 RPGR mutations already reported in other populations were seen. This result demonstrates the diversity of RPGR mutations responsible for XLRP in different populations. It is consistent with the inference that most RP3 mutations are of independent origin.[24]

Different clinical presentations have been recognised in XLRP,[3] and some genotype–phenotype correlations have been attempted in hemizygous males and heterozygous carrier female in XLRP families.[25–27,15] The location and nature of RPGR mutations might help to understand the molecular basis of XLRP and the role of the RPGR protein in photoreceptor function. Six of

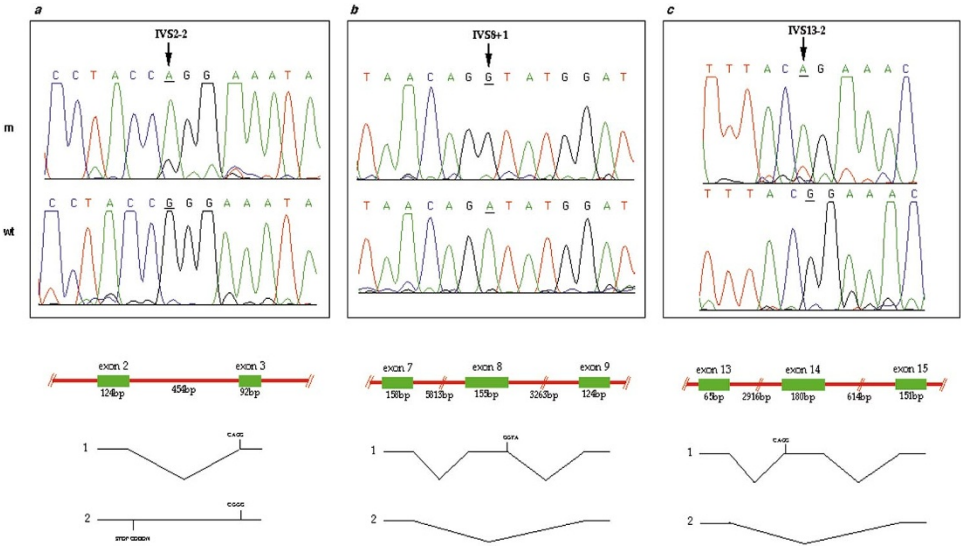

Figure 3 *Sequence analysis from PCR products of patients (m) and controls (wt). At the bottom: schematic representation of the effects in the three splice mutations*

Table 2 RPGR sequence variations in XRLP patients

Sequence variation	*Nucleotide change*	*Exon/intron*	*Effect on coding sequence*	*Frequency observed (%)*
S47S[a]	T or G at 200 nt	exon 2	Silent mutation	4.3
K244[a]	G or A at 791 nt	exon 7	Silent mutation	3.3
A388A[b]	G or A at 1223 nt	exon 10	Silent mutation	11.2
R425K[c]	G or A at 1333 nt	exon 11	Conservative substitution (codon 425; Arg->Lys)	3.4
I431Vc	A or G at 1350 nt	exon 11	Conservative substitution (codon 431; Ile>Val)	3.4
T533M[a]	C or T at 1657 nt	exon 14	Conservative substitution (codon 533; Thr->Met)	0.1
G566E[d]	G or A at 1756 nt	exon 14	Conservative substitution (codon 566; Gly->Glu)	5.6
IVS1-15[d]	A or G at 87-15 nt	intron 1	Not determined	1.7
IVS10+16[a]	A or G at 1304+16 nt	intron 10	Not determined	5.0
IVS12-101[a]	T or A at 1565-101 nt	intron 12	Not determined	34.0
IVS12-130[a]	T or C at 1565-130 nt	intron 12	Not determined	3.0
IVS13+11[d]	A or G at 1631+11 nt	intron 13	Not determined	3.0
IVS17+46[a]	C or T at 2208+46 nt	intron 17	Not determined	2.0
IVS18+11[b]	T or C at 2300+11 nt	intron 18	Not determined	27.0

[a]Variations described here; [b]in Fujita *et al*[14] and Buraczynska *et al*[12]; [c]in Roepman *et al*[11] and Buraczynska *et al*[12]; [d] in Buraczynska *et al*[12].

the newly observed mutations, as others reported earlier, fall in RCC1 repeats and one falls in the 'charged' domain.[12] The amino-terminal half of the predicted RPGR protein contains 6–7 repeats with similarity to repeats within RCC1, a protein which is essential for nucleocytoplasmic transport, though it might also be involved in protein trafficking through the Golgi apparatus.[28] Most RPGR mutations fall in the RCC1 repeats, providing evidence that it is an important functional domain. Recently, studies to identify proteins that interact with *RPGR*, showed that the RCC1-like domain of *RPGR* interacts with phosphodiesterase delta subunit.[29] This interaction links *RPGR* to the visual transduction cascade that regulates the rod phosphodiesterase holoenzyme,[30] though no specific related activity (eg of guanine nucleotide exchange) has been identified. The large group of XLRP families in which no mutations have been detected in *RPGR* provide one starting point for the study of interacting proteins or additional mutations that regulate *RPGR*.

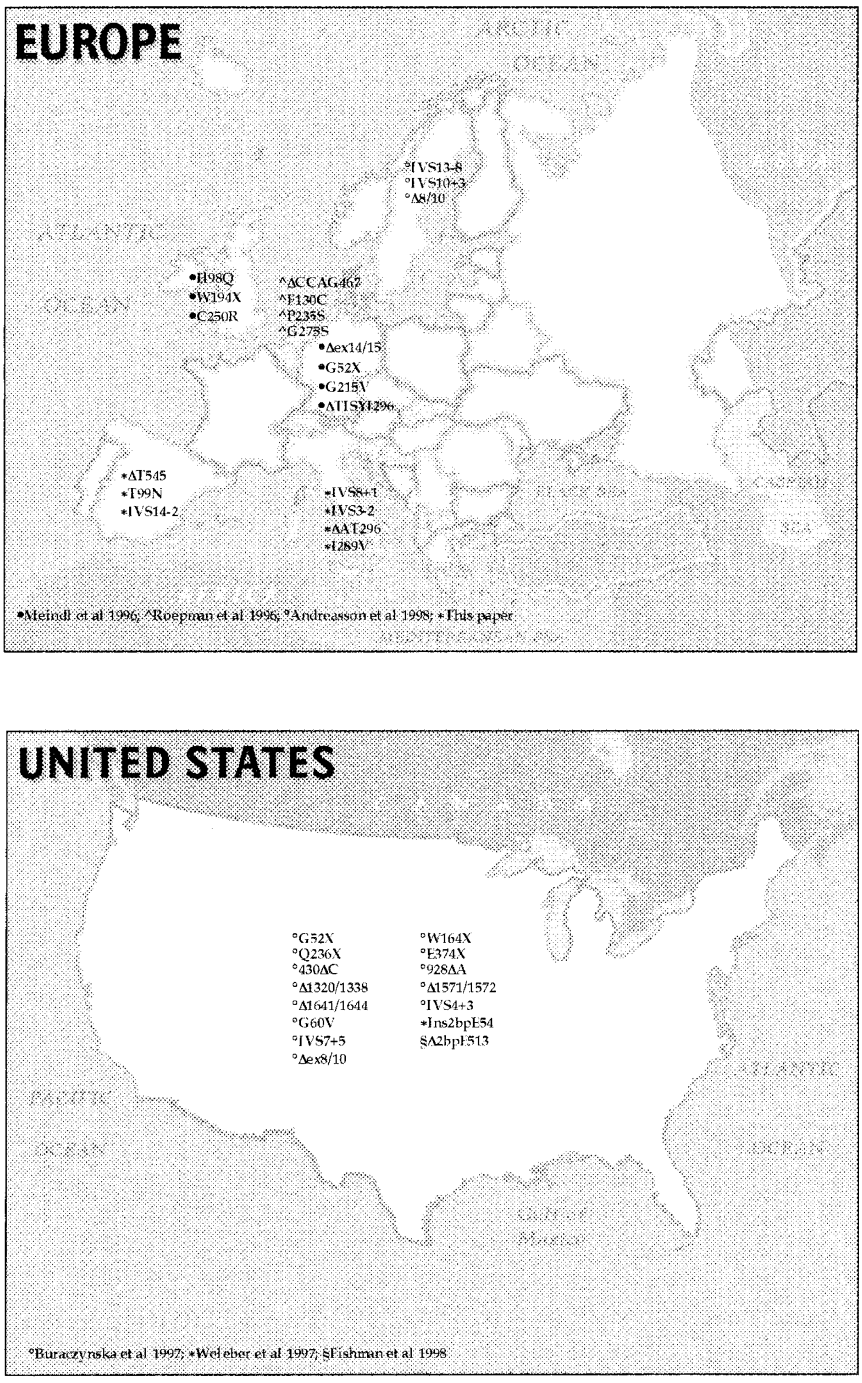

Figure 4 *Distribution of RPGR mutations found in the United States and Europe*

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