

Mutations in the Pre-mRNA Splicing-Factor Genes *PRPF3*, *PRPF8*, and *PRPF31* in Spanish Families with Autosomal Dominant Retinitis Pigmentosa

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PURPOSE. Mutations in the systemically expressed pre-mRNA splicing-factor genes *PRPF3*, *PRPF8*, and *PRPF31* have recently been associated with autosomal dominant retinitis pigmentosa (adRP). This study was intended to identify mutations in *PRPF3*, *PRPF8*, and *PRPF31* in 150 Spanish families affected by adRP, to measure the contribution of mutations in these genes to adRP in that population, and to correlate RP phenotype expression with mutations in pre-mRNA splicing-factor genes.

METHODS. Denaturing gradient gel electrophoresis (DGGE) and direct genomic sequencing were used to evaluate the complete coding region and flanking intronic sequences of the *PRPF31* gene, exon 42 of *PRPF8*, and exon 11 of *PRPF3* for mutations in 150 unrelated index patients with adRP. Ophthalmic and electrophysiological examination of patients with RP and their relatives was performed according to preexisting protocols.

RESULTS. Three nonsense mutations caused by insertion and deletion sequences and two missense mutations (Arg2310Gly) and within the stop codon of the *PRPF8* gene (TGA→TTG), were detected in five unrelated heterozygous patients. Three patients were heterozygous carriers of different nonsense mutations in exon 8 of the *PRPF31* gene and one Thr494Met mutation was found in exon 11 of the *PRPF3* gene. Cosegregation of the mutation in *PRPF8* and *PRPF3* with adRP was observed. However, two nonsense mutations in *PRPF31* causing adRP detected in two families showed asymptomatic carriers.

CONCLUSIONS. Nine mutations, six of which are novel, in the pre-mRNA splicing-factor genes *PRPF3*, *PRPF8*, and *PRPF31*, causing adRP have been identified in the Spanish population.

Their contribution to adRP is approximately 5% after correction in relation to mutations found in other genes causing adRP. The patients carrying a mutation in the pre-mRNA splicing-factor *PRPF8* gene showed a type 1 diffuse RP. The existence of asymptomatic carriers of the nonsense mutation in the *PRPF31* gene suggests incomplete penetrance for these mutations in the families. (*Invest Ophthalmol Vis Sci.* 2003;44:2171-2177) DOI:10.1167/iov.02-0871

Retinitis pigmentosa (RP) is a group of hereditary retinal degenerations with a worldwide incidence of approximately 1 in 3500 individuals. Clinical characteristics include night blindness, loss of peripheral visual field, characteristic changes in the ocular fundus, and depression of the normal ocular electrophysiological responses.¹ The genetics of RP is complex, and the disorder may be inherited through an autosomal dominant (adRP), autosomal recessive (arRP), X-linked (XLRP),^{2,3} or digenic⁴ trait. Mutations within six genes (*RHO*, peripherin/*RDS*, *RPI1*, *NRL*, *CRX*, and *FSCN2*) that encode proteins specifically expressed in photoreceptor cells have been reported to cause adRP.⁵⁻⁹ Such proteins are involved in specific functions in the retina, such as the visual transduction cycle and structural components of the rod and cone photoreceptor cells or transcription factors. Furthermore, genetic linkage studies and mutation detection resulted in the characterization of mutation in genes with systemic expression implicated in adRP.¹⁰⁻¹²

Three of these genes, *PRPF3* (RP18), *PRPF8* (RP13), and *PRPF31* (RP11), are members of the pre-mRNA splicing-factor components of the U4/U6-U5 tri-snRNP particle¹³⁻¹⁵ that is dynamically assembled and dissociated at each round of the splicing cycle. Although little is known about the association and dissociation of the U4/U6 and U5 snRNP, protein-protein interaction and rearrangement are believed to take part in the formation and function of the tri-snRNP particle.¹⁶ It is therefore plausible that some mutation in one of the protein components of the tri-snRNP affects its structure and the splicing function. Mutations in the pre-mRNA splicing-factor genes reported so far in humans seem to produce pathologic effects in the retina only. The mutations reported to date in *PRPF8*¹¹ and *PRPF3*¹² are clustered within a 14 (exon 42) and 2 (exon 11)-codon stretch respectively, whereas mutations in *PRPF31*¹⁰ are more dispersed within the transcript sequence.

Mutations in the rhodopsin (*RHO*) and peripherin/*RDS* genes are the cause of 20% of the adRP in the Spanish population.¹⁷ Mutation in other genes associated with adRP such as *NRL*¹⁸ and *RPI1* (unpublished results) have also been detected in the Spanish population, contributing to 5% of total cases of adRP in Spain. To determine the contribution of mutation in the pre-mRNA splicing-factor genes *PRPF3*, *PRPF8*, and *PRPF31* in adRP-carrying Spanish families, screening for mutation in the complete coding region of the *PRPF31* gene was performed. Because mutations in the *PRPF3* and *PRPF8* gene causing adRP reported so far are clustered in exons 11 and 42

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Supported in part by grants from Fondo de Investigación Sanitaria (FIS 99/0780, 01/0081-01), ONCE, and Fundación ONCE.

Submitted for publication August 26, 2002; revised December 3, 2002; accepted December 14, 2002.

Disclosure: **M. Martínez-Gimeno**, None; **M.J. Gamundi**, None; **I. Hernan**, None; **M. Maseras**, None; **E. Millá**, None; **C. Ayuso**, None; **B. García-Sandoval**, None; **M. Beneyto**, None; **C. Vilela**, None; **M. Baiget**, None; **G. Antiñolo**, None; **M. Carballo**, None

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TABLE 1. Primers and DGGE Screening Conditions

Primers*	Gene	Fragment Size (bp)	Gradient (%)†	Running Temp. (°C)
Exon 8 Forward: 5'-GC-ccccacctctctctttcttct-3' Reverse: 5'-ggaggggcatgacgcagtg-3'	<i>PRPF31</i>	282	55-90	60
Exon 42 Forward: 5'-GC-gatagcagtagggataaggtgag-3' Reverse: 5'-gctgaagcaggagggcaggaac-3'	<i>PRPF8</i>	344	40-70	60
Exon 11 Forward: 5'-GC-tccagctggagaagtgact-3' Reverse: 5'-ccacaagtgagaaaggcatt-3'	<i>PRPF3</i>	344	50-75	50

* GC, 5'-CGCCCGCCGCGCCCGCGCCCGCCCGCCCGCCCGCCCG-3'

† 100% denaturant, 7 M urea and 40% (vol/vol) formamide in TAE buffer.

respectively, screening for mutation in these exons was performed. Four novel nonsense mutations and one missense mutation were detected in exon 42 of the *PRPF8* gene, three truncating protein mutations in the *PRPF31* gene and one previously reported missense mutation Thr494Met in *PRPF3*¹² were found.

MATERIAL AND METHODS

Informed consent was obtained from all subjects who participated in the study and the research adhered to the tenets of Declaration of Helsinki.

Ophthalmologic and Electrophysiological Studies

A complete ophthalmic examination of patients was performed. The examination consisted of best correct visual acuity with Snellen optotypes, color vision with the Farnsworth 32-hue test, computerized perimetry (recorded on the Octopus 500; Interzeag, Schlieren, Switzerland), and biomicroscopy and fundus examination after pupillary dilatation.

Cone, rod, mixed, and photopic flicker (30 Hz) electroretinograms (ERGs) and electroculograms (EOGs) were performed and recorded according to the standard testing protocols proposed by the International Society for Clinical Electrophysiology of Vision (ISCEV).¹⁹

Polymerase Chain Reaction

Genomic DNA was prepared from peripheral blood lymphocytes (QI-Amp DNA Blood Mini Kit; Qiagen, Valencia, CA). Gene coding exons were amplified with primers located in the flanking intronic region (Table 1). One PCR primer in each pair included a 40-base GC-rich segment (GC-clamp) attached to its 5' end to facilitate the detection of mutations by denaturing gradient gel electrophoresis (DGGE). PCR reactions were performed in a 50- μ L volume of buffer (20 mM Tris-HCl [pH 8.55], 16 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 150 μ g/mL BSA, and 10% DMSO) containing 500 to 200 ng human genomic DNA, 25 picomoles of each primer, 10 nanomoles of each deoxyribonucleoside triphos-

phate, and 1.5 units of *Taq* polymerase. Incubation was performed for 40 cycles consisting of 30 seconds at 94°C, 30 seconds at 59°C, and 30 seconds at 72°C, followed by 5 minutes at 94°C and 5 minutes at 72°C. Electrophoresis of 8 μ L of final PCR reaction volume was performed on 1.5% agarose gel, to test the amplification reaction.

Mutation Detection

Mutation analysis of the coding region of *PRPF31* (exon 8), *PRPF8* (exon 42), and *PRPF3* (exon 11) was performed by DGGE.^{20,21} Electrophoretic conditions—that is, the running temperature and the concentration range of the denaturing gradient of formamide-urea for each different PCR product are shown in Table 1. For DNA sequencing, PCR products were purified using chromatography columns (Qiaquick; Qiagen). DNA sequencing was then performed (OpenGene automated DNA sequencing system, Visible Genetics (Toronto, Ontario), and Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit; Amersham Pharmacia Biotech, Piscataway, NJ).

Subcloning of *PRPF8* and *PRPF31* Alleles

The sequence variation detected by DGGE was characterized directly from the chromatograms of heterozygous samples. However, to demonstrate the exact sequence of samples showing insertion and/or deletion, sequences were cloned into a vector (pCR2.1-TOPO) with a cloning system from Invitrogen (San Diego, CA), used according to the manufacturer's instructions. Minipreparations of 25 clones were PCR amplified with the primers used in the mutation screening and analyzed by DGGE. Three clones corresponding to mutant and wild-type allele were selected and sequenced as described earlier.

RESULTS

One hundred fifty index patients from adRP-affected Spanish families, previously excluded for mutations in the rhodopsin, peripherin/*RDS*, *NRL*, *CRX*, and *RPI* genes, were screened for mutations at the pre-mRNA splicing factor *PRPF31* gene by

TABLE 2. Mutation Detected in Pre-mRNA Splicing-Factor Genes Causing adRP in Spanish Families

Family	Gene	Location	Mutation	Protein Alteration/Predicted Change
S240	<i>PRPF31</i>	Exon 8	732-737delins 20 bp	M244fsX248
M637	<i>PRPF31</i>	Exon 8	769-770insA	K257fsX277
M368	<i>PRPF31</i>	Exon 8	828-829delCA	H276fsX277
M323	<i>PRPF8</i>	Exon 42	6928A→G	R2310G
V17	<i>PRPF8</i>	Exon 42	6943-6944delC	L2315fsX2358
SJD1	<i>PRPF8</i>	Exon 42	6974-6994del 21 bp	V2325fsX2329
M618	<i>PRPF8</i>	Exon 42	6893-6896delins 7 bp	L2298fsX2337
V541	<i>PRPF8</i>	Exon 42	7006T→C	Stop2336fsX2377
S269	<i>PRPF3</i>	Exon 11	1482C→T	T494M

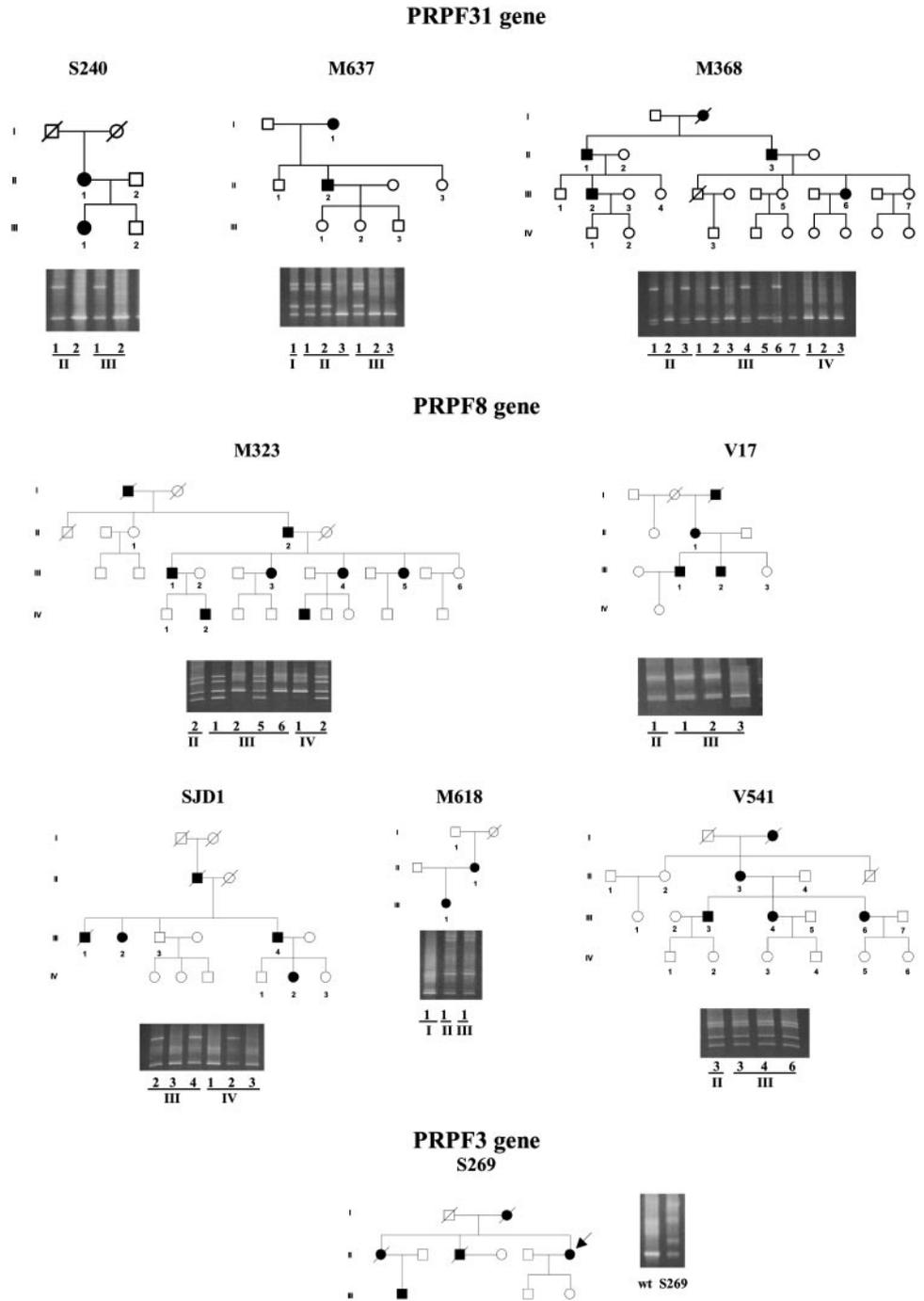


FIGURE 1. Pedigrees and DGGE analysis of adRP-affected families. *Filled symbols:* affected individuals. Segregation of RP and mutation in each family was performed by DGGE of a DNA PCR fragment containing exon 8 of *PRPF31*; exon 42 of *PRPF8*, and exon 11 of *PRPF3*. In member III-4 of family M368 and member II-1 of family M637, cosegregation between RP and mutation was not found.

DGGE. The sequence variation detected by DGGE was characterized by direct sequencing. Because insertion or deletion nucleotides were detected in sequencing of heterozygous samples, direct cloning of PCR products was performed. Sequencing of the mutant and wild-type allele was performed, as described in the Material and Methods section. Three insertion-deletion alterations were identified in the coding sequence of the *PRPF31* gene. These sequence alterations predict a truncated protein (Table 2).

We detected three deletion and/or alterations into the coding sequence (exon 42) of the *PRPF8* gene that predict a change in the open reading frame of the protein and a shift of the stop codon (Table 2). Two additional missense mutations were detected in the *PRPF8* gene. One A→G change at position 6928 was found that creates a new restriction site for

ApaI. This mutation causes an Arg2310Gly substitution at the *PRPF8* protein. A second substitution T→C in the TGA stop codon of the *PRPF8* gene that predicts a protein with 41 additional amino acid residues was detected in one adRP family (Table 2). Analysis for mutations of the exon 11 performed in the *PRPF3* gene detected the previously reported Thr494Met mutation in one patient with RP.

Segregation by DGGE or restriction analysis (not shown) and direct genomic sequencing in each family of the detected mutation in the index case were performed (Figs. 1, 2). The mutations in *PRPF8* and *PRPF3* were carried by all patients with RP but were absent in nonaffected members of the family. However, two different nonsense mutations in the *PRPF31* gene, detected in families M368 and M637, were carried by two asymptomatic individuals, suggesting the possibility of

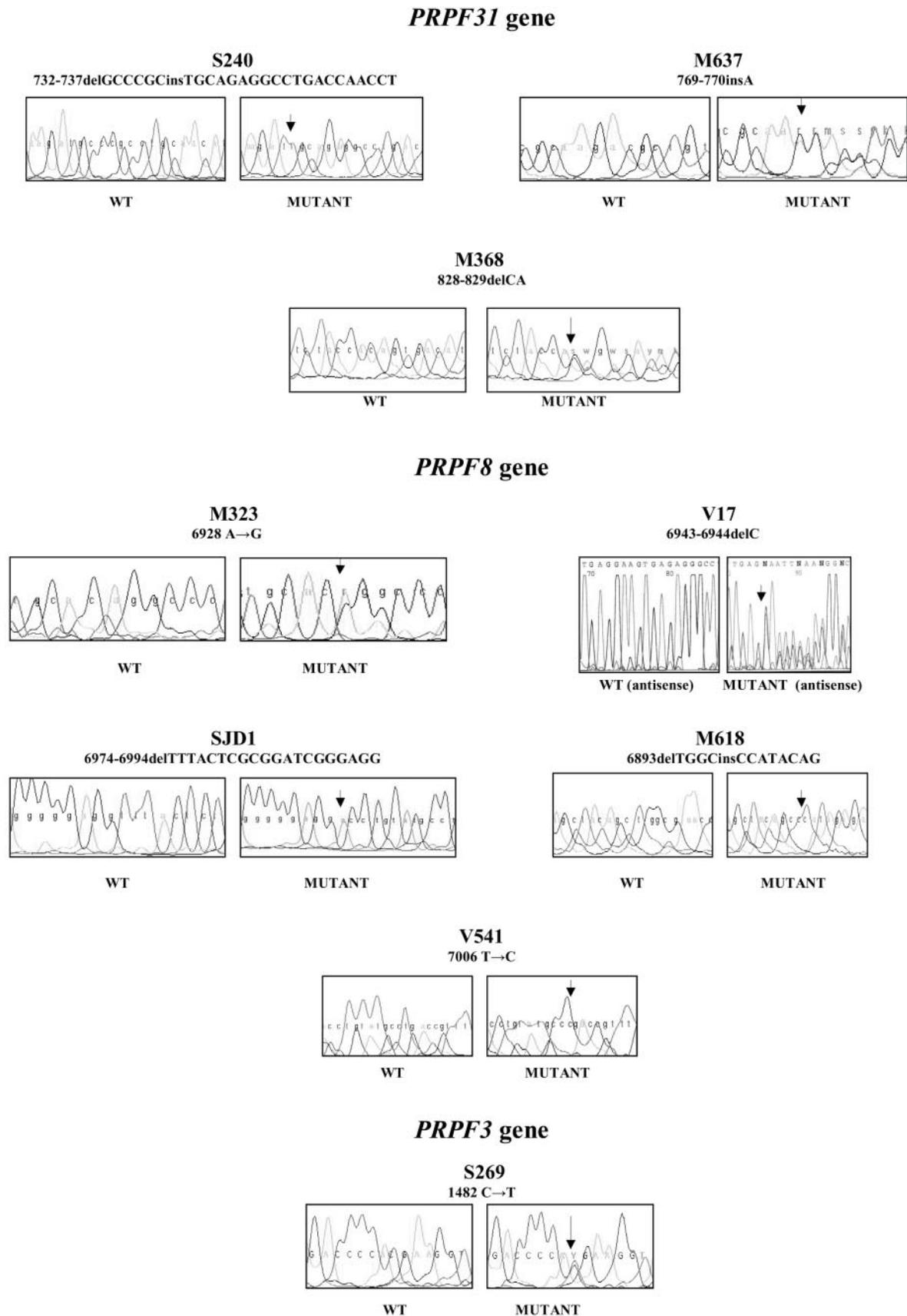


FIGURE 2. DNA sequence of the index patients carrying mutation in pre-mRNA splicing-factor genes. Direct genomic sequencing (M637, M323, V17, S269) and sequencing from DNA PCR fragments cloned in the pCR2.1-TOPO vector (S240, M368, SJD1, M618, V541) were performed. Deleted and/or inserted sequences are indicated at the *top* of each picture.

TABLE 3. Clinical Features of adRP Families Carrying the *PRPF8* Mutation

Family	Subject	Age (y)	Onset of NB	Onset of ↓ VF	Onset of ↓ VA	Present VF	Present VA		ERG	Fundus	Cataract Age (y)
							R	L			
M-323	II-2	77	20	35	55	<10°	<0.1	<0.1	NR	RP	NA
	III-1	50	21	28	NA	10°	0.9	0.8	NR	RP	NA
	IV-2	18	7	NA	NA	AS	0.7	0.7	NR	RP	NA
M-618	II-1	45	5	5	22	10°	0.3	0.3	—	RP	15
	III-1	19	5	5	NA	30°	0.5	0.5	—	RP	18
V-17	II-1	64	20	30	45	10°	0.1	0.1	NR	RP	NA
	III-1	40	10	15	28	10°	0.8	0.9	NR	RP	NA
	III-2	34	57	10	30	10°	0.9	1.0	NR	RP	NA
SJD-1	III-3	31	NA	NA	NA	N	1.0	1.0	N	—	NA
	III-2	70	10	15	15	—	—	—	—	RP	NA
	III-3	68	NA	NA	NA	N	—	—	—	N	NA
	III-4	65	10	15	15	>10°	0.2	0.1	NR	RP	NA
	IV-1	30	NA	NA	NA	N	1.0	1.0	N	N	NA
	IV-2	29	10	15	15	10°	0.3	0.4	NR	RP	NA
	IV-3	28	NA	NA	NA	N	1.0	1.0	N	N	NA

AS, annular scotoma; N, normal; NA, not available; NB, night blindness; NR, nonrecordable; RP, typical retinitis pigmentosa; VA, visual acuity; VF, visual fields.

incomplete penetrance. None of these sequence variations was found in 70 analyzed control subjects.

The clinical characteristics of the adRP-affected families are depicted in Tables 3 and 4. Patients carrying a mutation in *PRPF8* or *PRPF31* showed night blindness as the first symptom of RP, with age at onset between 5 and 20 years, followed by onset of visual field loss in the second half of the third decade of life. Fundoscopy of patients with RP who were carriers of a mutation in *PRPF8* or *PRPF31* revealed attenuated arterioles, waxy pallor of the optic disc, bone spicule pigmentation in the midperiphery, and atrophy of the retinal pigment epithelium (RPE; Fig. 3). The patient carrying a mutation in *PRPF3* showed a mild RP phenotype, with the first symptoms (night blindness) of RP by the age of 40 years. Only this patient was clinically examined, because the rest of family refused to participate in this study.

DISCUSSION

Recently, mutations in genes with systemic expression, such as the pre-mRNA splicing-factor genes that form part of the spliceosome complex, have been associated with adRP.¹⁰⁻¹² We analyzed to identify mutations in the *PRPF3*, *PRPF8*, and *PRPF31* genes in 150 index patients with RP who were members of adRP-carrying Spanish families previously excluded for mutations in the *RHO*, *peripherin/RDS*, *RPI*, *NRL*, and *CRX* genes. Nine different mutations were found in patients with

adRP, which means that approximately 5% of adRP-affected families in Spain are carriers of a mutation in one of these genes related to the spliceosome complex.

The spliceosome is a large RNA-protein complex that catalyzes the pre-mRNA splicing in the cell.¹⁶ More than 40 precursor mRNA-processing proteins (prp) have been identified in yeast to date, and several of these proteins have been demonstrated to be vital for the spliceosome assembly in vitro.¹³ The formation of the major spliceosome complex involves the stepwise assembly of four small nuclear ribonucleoproteins particles (snRNP) U1, U2, U4/U6, and U5 and many non-snRNP splicing factors on a pre-mRNA complex. The individual snRNP particles interact during the splicing cycle in a highly dynamic manner. For example, at the start of the splicing cycle U4 and U6 snRNP are tightly associated by extensive RNA-RNA base pairing, forming a single particle termed U4/U6. This complex associates with U5 snRNP to form the particle termed U4/U6-U5 tri-snRNP. After splicing, this particle is dissociated to the snRNP level and must be reassembled from U4, U6, and U5 snRNP to take part in a new round of the splicing process. The products of the three pre-mRNA splicing-factor genes linked to adRP, *PRPF8*, *PRPF31*, and *PRPF3*, are components of the U4/U6-U5 tri-snRNP complex (reviewed in Ref. 16 and references therein).

A human protein of 61 kDa encoded by *PRPF31* has been characterized as a component of U4/U6-U5 tri-snRNP.¹⁴ In vitro experiments demonstrate that the 61-kDa protein is es-

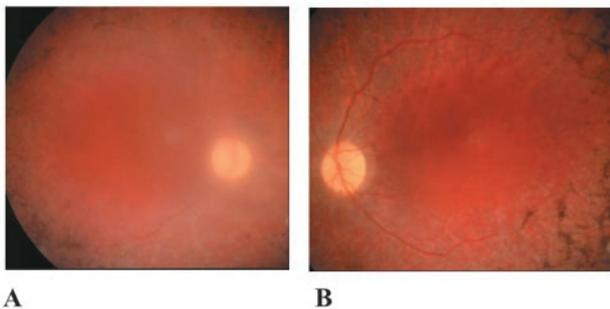
TABLE 4. Clinical Features of adRP Families Carrying the *PRPF31* Mutation

Family	Subject	Age (y)	Onset of NB	Onset of ↓ VF	Onset of ↓ VA	Present VF	Present VA	ERG	Fundus	Cataract
M-368	II-1	79	20	20	60	10°	≪0.1	NR	RP	56
	II-3	70	20	20	50	10°	≪0.1	NR	RP	NA
	III-2	43	5	5	40	10°	≪0.1	NR	RP	NA
	III-6	38	12	12	NA	10°	1	NR	RP	NA
M-637	I-1	66	18	24	55	10°	Cd	NR	RP	55
	II-1	41	NA	NA	NA	N	1	N	N	NA
	II-2	40	10	20	—	10°	0.7	NR	RP	NA
	II-3	24	NA	NA	NA	—	1	N	N	NA
S-240	II-1	70	20	20	50	—	—	NR	RP	NA
	III-1	30	6	12	20	—	—	NR	RP	NA

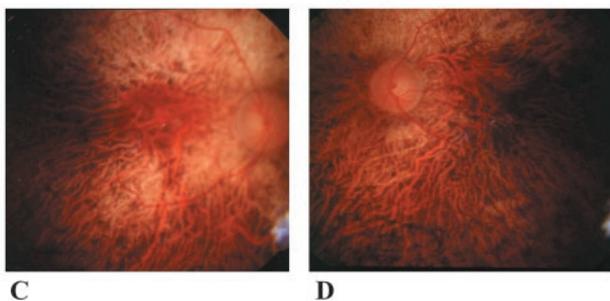
Abbreviations are as in Table 4.

Carrier of mutation in the *PRPF8* gene. adRP SJD1

Patient IV.2 at age of 30.



Patient III.4 at age of 65.



Carrier of mutation in the *PRPF31* gene. adRP M368

Patient III.6 at age of 38.

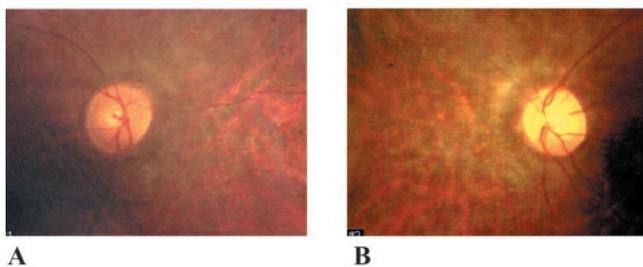


FIGURE 3. Fundus images of patients carrying mutations in *PRPF8* and *PRPF31* showing attenuated arterioles, a waxy pallor optic disc, areas of atrophy, and diffuse peripheral bone spicules. Fundus images of patient IV-2 of family SJD-1 also shows vitritis in both eyes.

sential in the generation of the assembly of the tri-snRNP complex, probably functioning as a bridge physically interacting with the U4/U6 and U5 subunits. The three mutations in the *PRPF31* gene detected in Spanish families are deletions-insertions that lead to truncated proteins. Whether these protein variants are stably translated remains to be established, but in any case it is unlikely that these mutants would promote formation of functional tri-snRNP. Consequently, the result may be the decreasing to 50% of the functional U4/U6-U5

tri-snRNP that would lead to lower the rate of splicing in the cell. In the adRP families M368 and M637 reported in this study, two asymptomatic carriers of a mutation in the *PRPF31* gene were identified, suggesting incomplete penetrance in these pedigrees. One of these mutations, 769-770insA, has been reported in a sporadic RP patient,¹⁰ but incomplete penetrance has also been observed in two previously reported adRP pedigrees carrying deletion mutations in *PRPF31*.¹⁰ To explain the incomplete penetrance in several families linked to this RP11 locus, it has been suggested that a high expression of wild-type allele may be able to compensate for the presumable nonfunctioning mutant allele.^{10,22} Thus, these mutations seem to induce a pathogenic mechanism by haploinsufficiency rather than by a mutant dominant negative effect.

A scenario similar to that reported for the *PRPF31* gene may occur with mutations in the *PRPF8* and *PRPF3* genes. Thus, we have detected mutations in the *PRPF8* gene, which encodes a large 2335-amino-acid protein Prp8, the core component of the U5 snRNP.¹³ Three different nonsense mutations due to small deletion-insertions in the coding sequence of exon 42 were detected in adRP-affected Spanish families. In these families, perfect segregation of the mutation with the disease was observed. The three nonsense mutations reported predict a change of the last 37, 15, and 10 amino acids and a shift of the stop codon of 1, 22, and -7 residues, probably causing nonfunctional variant proteins. Whether these protein variants are currently synthesized or remain stable in the cell is not known. The two additional sequence variants detected in the *PRPF8* gene are a single nucleotide substitution. One is a nonsense mutation that eliminates the stop codon (TGA→CGA) of the *PRPF8* gene, predicting until the next in frame (TGA) stop codon, a protein with 41 additional amino acids. The second nucleotide change detected in the *PRPF8* gene sequence produces the change of the basic 2310 Arg to the uncharged polar Gly amino acid in the encoding protein. A previous reported conservative substitution Arg2310Lys had been found in one adRP Dutch pedigree.¹¹

In the *PRPF3* gene we detected in one adRP family the previously reported¹² missense mutation Thr494Met. Only mutation in codons 493 and 494 of the *PRPF3* gene in Danish and British families have been reported so far.¹² These two codons placed in the C-terminal region of the protein are highly conserved, and this region has been suggested to interact with other proteins.^{12,23-24} The mutation in the *PRPF3* gene (T494M) identified in one Spanish family has also been detected in two simplex cases, two English adRP families, and one Danish adRP-affected family, apparently unlinked.¹²

Clinical expression of RP exhibits a high degree of heterogeneity. In attempts to simplify, adRP-carrying families have been classified as type 1 (early onset and diffuse retinal involvement), type 2 (late onset and regional retinal involvement),²⁵ or a variable intrafamilial expression. Thus, mutations in the rhodopsin gene, such as Pro347Leu, represent an extreme of type 1 diffuse adRP phenotype, whereas Pro23His shows a type 2 mild RP phenotype.²⁶ Some mutations in the *RP1* gene causing a truncated protein have been found in adRP-carrying families with variable intrafamilial expression (Carballo M, unpublished results, 2003). Mutations in the pre-mRNA splicing-factor genes *PRPF3* and *PRPF8* identified in adRP-affected Spanish families display a type 1 phenotype. The phenotypes observed in the previously reported families linked to RP13 (*PRPF8*) and RP18 (*PRPF3*) loci are also indicative of type 1 adRP.²⁷⁻³⁰ However, two families with mutations in the *PRPF31* gene have been reported with variable interfamily expression as in the Spanish M368 and M637 families.^{10,22} The phenotype observed for Spanish patients with RP who have mutations in *PRPF8* and *PRPF31* showed early onset, diffuse pigmentary changes, and visual fields with concentric depres-

sion; most fields were constricted to 10°. The ERG is abolished in most patients from the second decade of life. The patient carrying the mutation T494M in the *PRPF3* gene showed a late onset and less severe RP phenotype than the patients with mutations in *PRPF8* or *PRPF31*. Because we have clinically examined only one patient carrying the mutation T494M, limited conclusions can be made about the RP phenotype causing by this mutation in the family.

The intriguing question is why mutations in these systemically expressed pre-mRNA splicing-factor genes can result in a specific retinal disease. It has been proposed that pre-mRNA splicing genes, as well as other retinal expressed genes, may be involved in rate-limiting steps¹⁰⁻¹² in this extremely fast-metabolizing tissue. Alternatively, mutations in the pre-mRNA splicing genes causing adRP may interfere with specific protein-protein interactions that take place specifically in retinal cells or may play an important role in a second class of the recently discovered spliceosome, the U12-dependent spliceosome, that excise AT-AC flanking introns instead of the canonical AG-GT sequences.³¹ Thus, mutations in a family of genes that maintain U12-dependent introns cause neuromuscular and neurologic diseases.³² Future work is needed to discover the pathologic mechanism involved in retinal degeneration induced by mutations of these systemically expressed pre-mRNA splicing genes.

Acknowledgments

The authors thank the families for their participation in this research and Mary P. White and Geoffrey B. Arden for help in the correction of the manuscript.

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