Homozygous Deletion Related to Alu Repeats in *RLBP1* Causes Retinitis Punctata Albescens

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Purpose. Retinitis punctata albescens (RPA) is an infrequently occurring form of autosomal recessive (and rarely dominant) retinal dystrophy featuring early-onset severe night blindness and tiny, dotlike, white deposits in the fundus. RPA is associated mostly with mutations in *RLBP1* and occasionally in *RHO*, *RDS*, and *RDH5*. In this study, mutations were sought in *RLBP1*, which encodes the retinol binding protein CRALBP in patients with typical RPA.

Methods. Clinical investigation included funduscopy, visual field testing, electroretinogram recording, and adaptometry. The 7 coding exons (3–9) of *RLBP1* and the 15th (last) exon of *ABDH2* were PCR amplified and sequenced. Long-distance PCR and cloning of genomic DNA were performed to characterize the deletion.

Results. The study involved a 24-year-old Moroccan patient with typical RPA, born of first-cousin parents. He carried a 7.36-kb homozygous deletion encompassing the last 3 exons of *RLBP1* (7, 8, and 9) and part of the intergenic region between *RLBP1* and *ABHD2*, which lies downstream of *RLBP1*. This deletion abolishes the retinal binding site of CRALBP. The telomeric breakpoint of the deletion (in *RLBP1* intron 6) is embedded in an Alu element, whereas the centromeric breakpoint (in the intergenic region) lies between two Alu elements placed in the opposite orientation.

Conclusions. Because of the high density of Alu elements in *RLBP1*, a systematic search should be made for deletions in this gene when one or both alleles lack point mutations, in the case of RPA or flecked retinal dystrophy. (*Invest Ophthalmol Vis Sci.* 2006;47:4719–4724) DOI:10.1167/iovs.05-1488

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The cellular retinaldehyde-binding protein (CRALBP) belongs to the CRAL-TRIO family whose members bind lipid ligands in a hydrophobic domain. It binds the vitamin A derivatives 11-*cis* retinol and 11-*cis* retinal, with more affinity for the aldehyde form. As such, CRALBP is a key actor in the visual cycle, the multistep process that starts with all-*trans* retinal, the product of the activated rhodopsin, and ends with 11-*cis* retinal, the chromophore that binds opsins to regenerate rhodopsin and cone photopigments. CRALBP is found in the retina, specifically in the retinal pigment epithelium and the Müller glial cells, where it accelerates the rate of the isomerization to 11-*cis* retinol.[1] Accordingly, mice lacking CRALBP have considerably delayed dark adaptation.[2] CRALBP is also found in the ciliary epithelium, iris, cornea, pineal gland, and in some oligodendrocytes of the optic nerve and brain,[3,4] where its function remains unclear.

In human, mutations in *RLBP1*, the gene encoding CRALBP, have been found in various types of retinal dystrophies—namely, retinitis punctata albescens (RPA) in most cases,[5–8] autosomal recessive retinitis pigmentosa,[9] Bothnia dystrophy,[10–12] Newfoundland rod–cone dystrophy,[13] and fundus albipunctatus.[14] Although there is an apparent phenotypic heterogeneity, the clinical presentation is in fact quite well characterized and helps in directing the molecular diagnosis that prompts the search for *RLBP1* mutations. Clinical features are night blindness from infancy with elevated threshold in adaptometry, progressive loss in visual acuity due to macular degeneration, the presence of tiny white deposits and patches of atrophy in peripheral retina contrasting with the absence or scarcity of pigment deposits, and predominant rod over cone involvement. This condition is in fact appearing as a subtype of autosomal recessive retinitis pigmentosa, leading after several decades to severe visual loss.

In this study, we describe a patient with typical RPA who carries a homozygous 7.36-kb deletion that includes the last 3 exons of *RLBP1*. We show that this deletion occurred in a portion of the genome that is rich in Alu sequences.

Materials and Methods

Clinical Investigations

A standard ophthalmic examination (refractometry, visual acuity, slit-lamp examination, applanation tonometry, and funduscopy) was performed. Fluorescein angiography was performed, and visual fields were tested with a Goldmann perimeter using targets V_{4e}, IV_{4e}, and II_{4e}. A full-field ERG was performed according to ISCEV (International Society for Clinical Electrophysiology of Vision) recommendations. Dark adaptometry was performed with a Goldmann-Weekers apparatus and a test seen with an angle of 11°, placed at a distance of 30 cm from the eyes and centered on the point of fixation. Patients were light adapted for 5 minutes at 2100 asb before dark adaptation for 30 minutes.

TABLE 1. Primer Sequences

Amplified Exon	Primer Name	Sequence (5′→3′)	Temp. (°C)	Amplicon Size (bp)
RLBP1 primers				
3	3S	GCCTCGGGTGATTCTGATGC	56	271
	3AS	AAGGAGGGAGGGAGAGGGAA		
4	4S	TCTGAGCAGGCCCATTTCCC	56	280
	4AS	CAGGAGAGAGAATGCAGTCA		
5	5S	CTCATCACCTGTGTGTCCTG	62	350
	5AS	GCCAGGATGAGAGCGGATAG		
6	6S	TTCTGAGTCCCACTAGGAGG	56	338
	6AS	ATTGAGGGCCCAGTAGAGGC		
7	7S	TGACTCTCCCCTCAGGACCT	56	310
	7AS	CCATGAAAGGAGGCCCAGCC		
8	8S	CAGGGAATGAGTGGGAGCCT	62	253
	8AS	GTGTGAGGAGGGCTCAGGTG		
9	9S	GCCCCTTTCCTCCCTCAACC	56	313
	9AS	TTCCTAGCCTTGGGTCCAGG		
ABHD2 primers				
15	15F	TCTGCACCTCCTGTCCTGGA	50	329
	15R	TGAAACAGGGGGTGAGGGGA		
RLBP1-ABHD2 primers				
a	2F	CTCTTCTAGTAAGGCTTTGCCA	50	200
	2R	ATGCTAATGTGGACGTTGGGAG		
b	3F	TGTGAAGCTGAGCACGTCAGAT	50	260
	3R	TTCTGAGGAAGAAGCCATAGG		
c	4F	GCCAACTCCACAGAAGGAAAGC	50	220
	4R	GGGACTACAGGCGCATACCACT		
d	3F	TGTGAAGCTGAGCACGCAGAT	50	8551
	5R	GTCTCTGAGTCCCACTAGG		
e	3F	TGTGAAGCTGAGCACGTCAGAT	50	1662
	5aR	GATGTGCCAGGGCAGCTGGA		
f	3F	TGTGAAGCTGAGGACGTCAGAT	54	330
	6R	TTGGGAGAACTTTGGCATG		
g	7F	AGGTCTCTGAGTCCCACTAGGAG	55	8695
	7R	CAGAATCCCTGCCTTATCCCA		

Mutation Screening

PCR Reactions. Informed consent of the patient and of his unaffected brother were obtained, in accordance with the Declaration of Helsinki, and the genomic DNA was extracted by using a standard salting out procedure.[15] The seven coding exons 3 through 9 of *RLBP1* (GenBank accession no. NM_000326; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and the 15th (last) exon of *ABDH2* (NM_007011) were amplified in a 25-μL volume containing 3 mM $MgCl_2$, 200 μM dNTPs, 10 picomoles of forward and reverse primers (Table 1), 100 to 150 ng of DNA, and 0.5 U of *Taq* DNA polymerase (Promega Madison, WI) in the appropriate buffer. After the denaturation step at 94°C for 5 minutes, the amplification was performed for 35 cycles at 94°C for 30 seconds, at the appropriate annealing temperature for 30 seconds (Table 1), and at 72°C for 1 minute, ending with a final extension step at 72°C for 10 minutes.

Amplicons were run on 2% agarose gels in 1× TAE (Tris-acetate-EDTA) buffer to check for the quality and specificity of the PCR reaction. The same PCR program was applied to search for the deletion in 50 control Moroccan individuals, with primers 3F and 6R (Table 1).

To determine the 3′ and 5′ sequences flanking the deletion, we used long-distance PCR with primers 7F and 7R (Table 1) to amplify a 1.7-kb fragment (instead of the 8.695-kb wild-type fragment) in a 20-μL volume containing 500 μM dNTPs, 6 picomoles of forward and reverse primers, 100 to 150 ng of DNA, and 1.5 U of PCR mixture (Expand Long Template; Roche, Basel, Switzerland) in the appropriate buffer. After the denaturation step at 95°C for 2 minutes, the amplification was performed for 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 4 minutes, ending with a final extension step at 68°C for 10 minutes. Amplicons were analyzed on 1% agarose gels, TA-cloned (Invitrogen, Groningen, The Netherlands) and PCR-screened.

Sequencing. Clones and purified PCR products (QIAquick PCR purification Kit; Qiagen, Hilden, Germany) were sequenced in both directions (BigDye Terminator Cycle Sequencing Ready Reaction kit ver. 1.1; Prism 310 or 3130 capillary sequencer; Applied Biosystems, Foster City, CA). Sample sequences were aligned to the wild-type ones and analyzed with the collection and sequence analysis software package (Applied Biosystems).

RESULTS

Case Report

The patient was a 24-year-old Moroccan man, born of first-cousin parents. He became aware of night blindness at the age of 6. At age 12, he noticed some difficulties in far sight. At the time of the examination, he had severe reading impairment and photophobia, but he did not mention difficulties in moving about by himself outside. His visual acuity was 10/200 OD with +1.50(−1.25; 75°) and counting fingers OS with +2.25(−1.25; 120°). The fundus showed many tiny white dots around the fovea and beyond the vascular arcades (Figs. 1B, 1C), whereas that of his unaffected brother was normal (Fig. 1A). The retinal vessels were slightly narrowed. There were no pigment deposits, and the optic discs were not pale. Fluorescein angiography showed a cystoid macular edema (Fig. 1D). In Goldmann perimetry, there was some degree of peripheral visual field loss that predominated in the right eye, and an absolute central scotoma that was larger in the left eye (Figs. 1E, 1F). A full-field electroretinogram with the patient wearing contact lenses did not detect any rod responses, but highly attenuated mixed rod–cone responses and pure cone responses at 30-Hz flickers were still recordable (Fig. 1G). Dark adaptometry

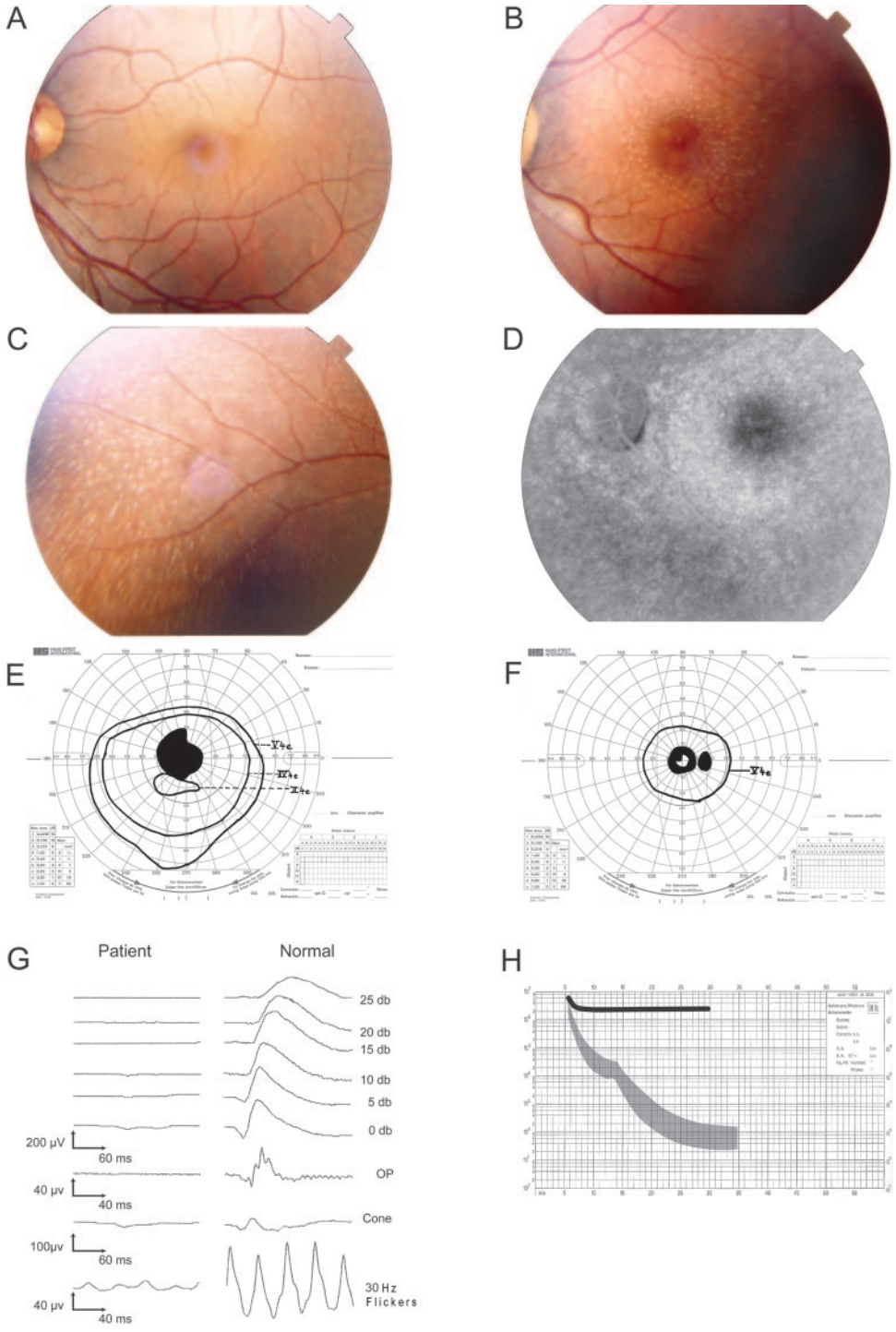

FIGURE 1. Clinical analysis. (**A**–**C**) Fundus photographs of the unaffected brother (**A**) and the patient, showing tiny, white, dotlike deposits on the macula (**B**) and in the retinal periphery (**C**). (**D**) Fluorescein angiogram showing macular edema. (**E**, **F**) Goldmann perimetry showed an absolute central scotoma with moderately reduced peripheral isopters in the left eye (**E**), severely reduced in the right eye (**F**). (**G**) ISCEV ERG recording of the patient and a normal control subject showing that the patient retained a slight cone activity whereas rod activity was barely detectable at the highest light intensity (0 dB). (**F**) Adaptometry. *Shaded area*: normal range. The patient curve (*top* of the diagram) reveals a minute cone adaptation (slight inflection of the curve at the beginning) but no rod adaptation until 30 minutes.

testing did not detect any rod adaptation after 30 minutes (Fig. 1H).

Mutation

The observation of a patient with RPA prompted us to screen the *RLBP1* gene. No mutations were detected in the first four coding exons (3–6). However, exons 7, 8, and 9 could not be amplified in the patient, whereas they could in the unaffected patient's brother (not shown), suggesting that the patient carried a large homozygous deletion. PCR-based DNA walking on 20 kb downstream of *RLBP1* exon 6 indicated that the deletion started in *RLBP1* intron 6 and ended downstream of the *ABHD2* gene, which lies 14.1 kb downstream of *RLBP1* in the opposite orientation (Fig. 2). Using long-distance PCR, we found a 7361-bp deletion associated with the insertion of a C (Fig. 2). This deletion was absent in 100 control Moroccan chromosomes (not shown). It spans the last 3 exons of *RLBP1* and part of the intergenic region situated between *RLBP1* and *ABHD2* which encodes the androgen regulated a/b hydrolase II (Fig 2). A search for intragenic homologous sequences revealed that the region is rich in Alu repeats oriented in both directions. We found that the telomeric breakpoint of the deletion (in *RLBP1* intron 6) is embedded in one Alu element, including the 26 nucleotide core sequence containing the

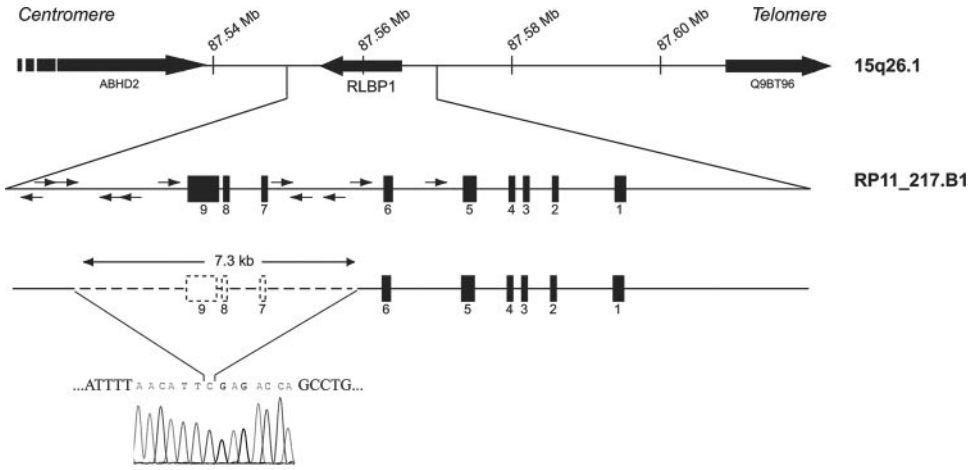

FIGURE 2. Diagram showing the position of the genes in the region of *RLBP1*. Magnified region corresponding to clone RP11_217.B1 shows the intron–exon structure and the Alu repeats (*arrows*) and their orientation. Electrophoregram spanning the deletion is shown. Note the insertion of a C.

A

Centromeric

```
ggcgtggtggcgcatgcctgtaatcccagctactcaggaggctgaggcaggagtatcgcttgaactcgggaagtg
caggttgcggtaaactgagatctcaccattgcactccagcctgggcaacaaaagcgaaactccgtctcaaaaaaa
aaaaaaaaaaaaaaaacaaacaaacaaacaaaaaaaaaaatcagatatccccagaaaggaagttgttatgg
gcaccaaatggttgtcataaaattcacgttttgttgtccccttcccatagctggtggccttcaaaagagtattt
tattgtctctctccaataatgtgggggccatttgtaggaatcacacacacaggcagctgggattttgtgaagctg
agcacgtcagattttaacatt▶▶ggtagtcttcagacacgttttcccaaaagcggcaccaccttctcaggcaagg
tctggcccagccccaccccagctaaccctcctgccccagcttgtccctggaggggcagtgtcagggcacggccagcag
ggggcggcgcgagcaca
```

Telomeric

```
ggacttttaataaattgtgttggcacaactagatagcattatctaaaattagtttgggccaggtgcggtgtctt
atcctgtaatcccagcactttgggaggccgaggtgggcggatcacttgagctcaggagttt◀◀gagaccagcctg
gccaacatgggaaaccccgtctctactaaaaatacaaacattagctgggcatggtggcgtgcctctgtaatccca
gctacttggaaggctgaggcttgaggatcgctcgaacccaggaggcggaggttgcagtgagctaagatcatgcca
ctgcactccagcctgggtgaccagagcgagactccatctcaaaaataaataaataaaataaaataagtttt
```

B

```
Intergenic :  tgaagctgagcacgtcagattttaacatt-ggtagtcttcagacacgttttcccaaaag
              :::::::::::::::::::::::::::::  :  :     : : :     :    :::
Mutated    :  tgaagctgagcacgtcagattttaacattcgagaccagcctggccaacatggtgaaacc
                 :: :: ::: : :: :    :  :: ::::::::::::::::::::::::::::
Intron 6   :  gtgggcggatcacttgagctcaggagttt-gagaccagcctggccaacatggtgaaacc
```

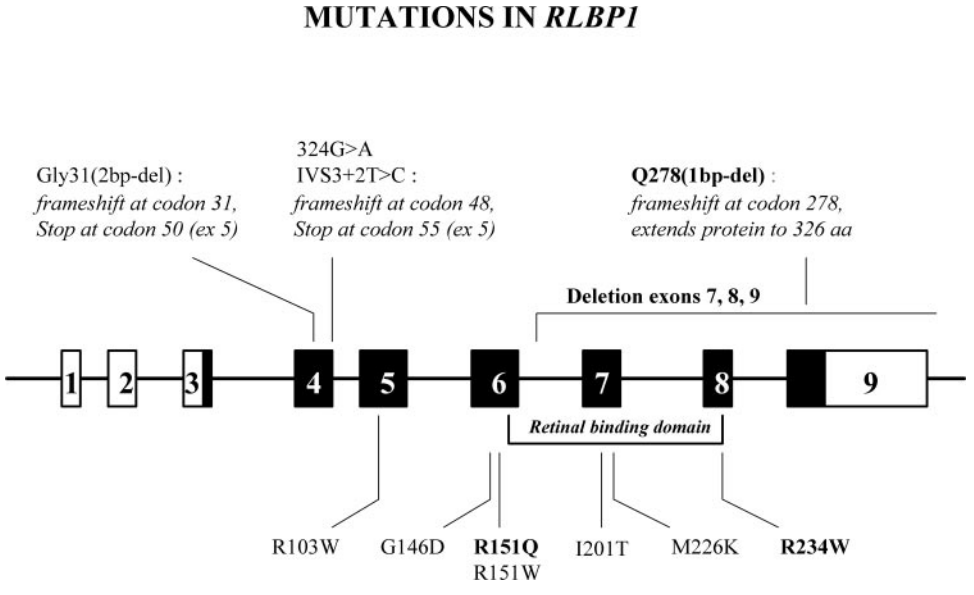

FIGURE 3. (**A**) Breakpoints (*double arrowheads*) and flanking sequences are shown. *Gray*: Alu repeat sequences; *black*: core sequence; *double-underscored italic*: pentanucleotide chi-like sequence. The centromeric breakpoint is at distance from the Alu repeat, with the presence of four isolated chi-like sequences downstream. The telomeric breakpoint is embedded in an Alu repeat. (**B**) The mutated sequence is aligned with intergenic (centromeric) and intron 6 (telomeric) sequences. *Underscore*: the three identical nucleotides; *black*: the inserted c in the mutated sequence.

FIGURE 4. Currently and previously reported mutations in *RLBP1*. *Numbered boxes*: exons (shown in scale); *open boxes*: uncoding exons; *dark boxes*: coding exons. Introns are not shown in scale. Truncating mutations are shown at the *top* of the exons, missense mutations are shown at the *bottom*. *Bold*: homozygous mutations.

TABLE 2. Comparison of the Presently and Previously Reported Phenotypes from Patients with *RLBP1* Mutations

Age (y)	Mutation	Disease Type	Night Blind	Visual Acuity OD/OS	Yellow-White Dots in Fundus	Macula	Pigment Deposits	Patches of Atrophy	Adaptometry	ERG
Truncating mutations										
7	R151W Gly31(2-bp del)	RPA [7]*	Yes	20/30 : 20/25	Mainly in midperiphery	~Normal	Few clumps in periphery	No	ND	Undetectable rod and reduced cone responses
19	IVS3 + 2T → C M226K	RPA [5]	Yes	?	Around fovea and in midperiphery	~Normal	No	No	Elevated threshold after 45°	Rod loss>cone loss
24	Exons7_9del	RPA [this study]	Yes	10/200 : CF	Around fovea and in midperiphery	Cystoid edema	No	No	No rod dark adaptation at 30°	Unrecordable except for 30-Hz flickers
52	Q278(1-bp del)	RPA [5]	Yes	?	Few	Perifoveal depigmentation	Few in periphery	Yes	Elevated threshold after 45°	Rod loss>cone loss
8-68	324G>A IVS3 + 2T → C	NFRCD (13)	Yes	~Normal to LP	Around fovea and in midperiphery	Beaten-bronze atrophy	No	Yes	Raised 4–4.5 log units in teens	Rod loss>cone loss to flat in aged patients
Missense mutations										
10	R234W	BD [11]	Yes	20/50; 20/100	No	Normal	No	No	Moderate thresh. elev. after 40°	Rod loss>cone loss
11	R234W	BD [11]	Yes	20/20; 20/20	No	Normal	No	No	Moderate thresh. elev. after 40° but normal after 20 h	Rod loss >cone loss but normal after 20 h
11	R151Q	FA [14]	Yes	20/40 OU	Over the whole fundus	Normal	No	No	ND	Rod loss >cone loss
14	G146D I201T	RPA [6]	No	20/20; 20/20	In midperiphery	Normal	No	Beginning	ND	Rod loss >cone loss but normal implicit times
18	R234W R103W	RPA [6]	Yes	0.25; 0.13	In midperiphery only	Degeneration	No	Beginning	ND	Rod and cone ERGs unrecordable
~35	R151Q	ARRP [9]	Yes	?	Over the whole fundus	Degeneration	No	?	ND	Flat
8–71	R234W	BD [10,12]	Yes	10/10 to LP	Around fovea and in midperiphery	Degeneration	In advanced stages	Yes	Raised 4 log units	Severely reduced rod and rod cone responses

When only one mutation is reported, it means that the patient is homozygous. In the Disease Type column, the numbers in brackets refer to articles cited in the references. ARRP, autosomal recessive retinitis pigmentosa; BD, Bothnia dystrophy; CF, counting fingers; FA, fundus albipunctatus; LP, light perception; ND, not done; NFRCD, Newfoundland rod-cone dystrophy, RPA, Retinitis punctata albescens.

chi-like recombinogenic pentanucleotide CCAGC, whereas the centromeric breakpoint (in the intergenic region) lies between two Alu elements placed in the opposite orientation (Figs. 2, 3A). Fifty-nine nucleotides upstream of the centromeric breakpoint in the deleted fragment are four copies of the recombinogenic pentanucleotide (Fig. 3A). Alignment of the mutated sequence with that at each breakpoint shows a homology on three nucleotides around the deletion (Fig. 3B).

Discussion

To date, there have been seven pathogenic amino acid changes in six codons reported in *RLBP1* and three truncating mutations, including two frameshifts in exon 4, leading to premature stop codons in exon 5 and one in exon 9 that extends the CRALBP protein from 317 to 326 amino acids (Fig. 4). In our patient, the deletion led to the loss of the C-terminal 142 (over 317) amino acids of CRALBP (i.e., 45% of the protein). Because the deletion involves the last exons of the gene, it is possible that downstream cryptic splice sites are recruited in the intergenic sequence, with the selection of alternative exons, thus resulting in the addition of illegitimate amino acids. Further studies using antibodies against the N-terminal portion of the protein would be necessary to address this question. In any case, the deletion causes the loss of the retinal binding site, which extends from residues 165 to 255,[16] as is true of two of the previously reported truncating mutations.

So far, the phenotypes described in the literature do not show genotype–phenotype correlations that would have distinguished between patients carrying either amino acid changes or protein truncations (Table 2). The phenotype observed in our patient is typical of RPA. The severity of the disease appears to be in the average range. As is usually found in young adults with RPA, signs of photoreceptor loss were not prominent in the fundus, retinal vessels being only moderately attenuated, and, except for the tiny white deposits, there were no lesions in the retinal periphery. The major symptom of the patient was the dramatic decrease in visual acuity, which was due to the macular cystoid edema, a frequent complication in RPA.

Unequal homologous recombination of Alu sequences[17] is a frequent cause of deletions and insertions in the human genome, with some cases reported in X-linked retinoschisis, another type of hereditary retinal dystrophy.[18] The Alu elements are approximately 300 bp in length and make up approximately 10% of the human genome.[19] In the case of *RLBP1*, there are 10 Alu elements from exon 6 to 10 kb downstream, representing 29% of the DNA in this region, much higher than that of the 10% of the whole human genome. The presence of these elements at such a high density is therefore likely to be the primary cause of the deletion. The very short homology (Fig. 3B) between the breakpoint regions may have played a role in the deletion process.

Although this is the first report of a large deletion in this gene, the high density of Alu elements in this region offers multiple possibilities of recombination. Hence, other deletions could be encountered in *RLBP1*. Therefore, we suggest that the search for deletions be systematically tested in *RLBP1* when one or both alleles do not show a point mutation in the case of a suggestive phenotype such as RPA or flecked retinal dystrophy.

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