Retinal Degenerations with Truncation Mutations in the Cone-Rod Homeobox (CRX) Gene

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PURPOSE. To define the phenotypes of retinal degenerations associated with mutations in the gene encoding CRX (cone-rod homeobox), a photoreceptor-specific transcription factor.

METHODS. Heterozygotes with the E168 [Δ1 bp], E168 [Δ2 bp], or G217 [Δ1 bp] CRX gene mutation were studied clinically, with visual function tests, including rod and cone perimetry and electro-retinography (ERG), and with optical coherence tomography (OCT).

RESULTS. Clinical diagnoses included autosomal dominant cone-rod dystrophy in one family (E168 [Δ1 bp] mutation) and simplex Leber congenital amaurosis in two families (E168 [Δ2 bp], G217 [Δ1 bp] mutations). In the family with the E168 [Δ1 bp] mutation, two siblings had relatively mild disease expression in the third decade of life. The central retinas of these two patients had profound loss of rod and short wavelength cone function; long/middle wavelength cone thresholds were elevated at fixation, but there were greater paracentral than central abnormalities. Peripheral retinal dysfunction was evident by psychophysics and by maximum amplitude loss for rod- and cone-isolated ERG photoreceptor responses. OCT cross-sectional reflectance images showed decreased central retinal thickness consistent with photoreceptor loss. An additional member of this family (E168 [Δ1 bp] mutation) and two other patients (representing E168 [Δ2 bp] and G217 [Δ1 bp] mutations) had a severe phenotype with retina-wide loss of function and islands of function remaining only in the temporal periphery.

CONCLUSIONS. Truncation mutations in CRX are associated with retinopathies that share phenotypic features but vary in disease severity. The disease mechanism could involve abnormal photoreceptor development compounded by a disturbance in the maintenance of photoreceptors in the mature retina. (Invest Ophthalmol Vis Sci. 1998;39:2417-2426)

Transcription factors are regulatory proteins that control gene expression during embryonic development and in the adult organism, and mutations in genes that encode transcription factors have been implicated in human disease.1-3 CRX (cone-rod homeobox) is the first photoreceptor-specific homeodomain transcription factor to be identified.4-6 It is involved in the regulation of rhodopsin and other photoreceptor-specific genes. A critical role for CRX in the human photoreceptor has been emphasized by the finding that patients with inherited retinal degeneration can show mutations in the CRX gene, located on chromosome 19q. The first clinical subtype of retinal degeneration to be associated with CRX gene mutations was cone-rod dystrophy (CRD).5,7 More recently, de novo mutations in CRX have been found in early-onset severe retinal degenerations categorized clinically as Leber congenital amaurosis (LCA).8

To increase understanding of the role of CRX in these human diseases, the phenotype of patients with different heterozygous truncation mutations in the CRX gene was analyzed with tests of retinal function and structure.

METHODS

Subjects

Patients included in this study were from three different families (Fig. 1). Results of molecular genetic investigations of the families leading to the identification of the CRX gene mutations have been reported.6,8 Informed consent for all procedures was obtained from subjects after the nature of the studies had been explained fully. The research procedures were in accordance with institutional guidelines and the Declaration of Helsinki.
FIGURE 1. Pedigrees of the patients in this study. Filled symbols, affected; open symbols, unaffected; +/—, heterozygote for the CRX (cone-rod homeobox) mutation listed above each pedigree; +/+,, wild type for CRX.

Phenotype Analysis

Psychophysical Tests. Static threshold perimetry in the dark- and light-adapted states was performed using a modified automated perimeter and techniques described previously.9-12 Full-field tests on a 12° grid and profiles of sensitivity at 2° intervals were measured with stimuli of 500 nm and 650 nm in the dark-adapted state, 600 nm in the light-adapted state (for long/middle wavelength [L/M] cone function), and 440 nm on a yellow background (for short wavelength [S] cone function). In patients without central fixation, testing was performed in one of two ways: with the eye pointing straight and monitored continuously for this position; or the preferred eccentric fixa-
tion locus was determined, and the results were shifted by the number of degrees from the anatomic fovea to the eccentric locus. In two patients with severe visual loss and no detectable function using our conventional stimuli for static perimetry, vertical scans performed in the superior retina (from 0° to 15° eccentric) were digitally fused with the central scans to extend the range covered. Longitudinal motion artifacts originating from micron-scale eye and head motions were compensated for by alignment of the longitudinal reflectivity profiles (LRPs) making up each OCT.12 The OCT thickness was quantified automatically as the depth between the first (vitreal) and the last (scleral) major transi-
tions of the OCT signal. The transitions were defined as the extremes of the first derivative of the LRPs. The first transition is believed to correspond to the vitreoretinal interface, and the last transition corresponds to the scleral boundary of the outer retina choroid complex (ORCC). The specific origins of which are currently unknown but are believed to include the retinal pigment epithelium (RPE) and the choriocapillaris. A seven-
sample moving median filter was used on the thickness measures to avoid outliers produced by speckle noise contained in the interferometric signal.

RESULTS

Clinical Characteristics

Figure 1 shows the three pedigrees of patients in this study, and Table 1 lists clinical data. There were three heterozygotes with the E168 [Δ1 bp] CRX gene mutation: a mother (F1-1) and her two children (F1-2, F1-3). F1-1’s parents had no visual symptoms throughout their lives; they died at 79 and 82 years of age. By history, the siblings F1-2 and F1-3 noted abnormal night vision in childhood and later experienced visual acuity change; peripheral vision has not been a problem to date. F1-1 recalled night vision and visual acuity disturbances in her teenage years with progression to severe central and peripheral vision loss over ensuing decades.

On examination, F1-2 and F1-3 had decreased visual acuity; this was progressive over a 4-year interval in F1-2 but not F1-3. Severe visual acuity loss was present in F1-1. There was myopia and astigmatism in all three patients. A Farnsworth D-15 panel showed a tritan axis of confusion in both eyes of F1-2 and F1-3. Ophthalmoscopy was normal at age 21 years in F1-3 and at ages 24 and 28 years in F1-2. At age 25, F1-3 had a granular appearance to the foveal region. F1-1 had an atrophic macular lesion surrounded by a ring of better-preserved RPE, mid- and far peripheral pigmentary retinopathy (depigmentation, bone spiculelike pigment, and chorioretinal atrophy), and attenuated retinal vessels (see Fig. 4A). Based on the presence of maculopathy and relatively better preserved peripheral retinas in all three affected members of this family, a cally based model of phototransduction.12,14,18

Optical Coherence Tomography. Cross-sectional retinal reflectivity profiles were obtained with optical coherence tomography (OCT; Humphrey Instruments, San Leandro, CA). The principles of the instrument have been described.12,13,19,20 In patients with central fixation, vertically oriented scans (15° in extent) were obtained centered at fixation. Vertical “serial sections” were also obtained by laterally offsetting the scan from fixation at 0.5° intervals up to 2.5° in temporal and nasal directions. In a patient with no central vision, several vertical scans were obtained through the atrophic macular lesion at different lateral offsets. In all patients, the tomogram displaying the deepest foveal pit was chosen to represent the scan through the anatomic fovea. In healthy subjects, only scans through fixation were used. Vertical scans performed in the superior retina (from 0° to 15° eccentric) were digitally fused with the central scans to extend the range covered. Longitudinal reflectivity profiles were obtained with optical coherence tomography (OCT). The OCT thickness was quantified automatically as the depth between the first (vitreal) and the last (scleral) major transitions of the OCT signal. The transitions were defined as the extremes of the first derivative of the LRPs. The first transition is believed to correspond to the vitreoretinal interface, and the last transition corresponds to the scleral boundary of the outer retina choroid complex (ORCC). The specific origins of which are currently unknown but are believed to include the retinal pigment epithelium (RPE) and the choriocapillaris. A seven-sample moving median filter was used on the thickness measures to avoid outliers produced by speckle noise contained in the interferometric signal.
clinical diagnosis of CRD, autosomal dominantly inherited, was made (Table 1).

F2-1, a heterozygote for the E168 [Δ2 bp] CRX mutation with parents who did not carry the mutant allele (Fig. 1), had nystagmus and visual inattention at 1 month of age. At the time, ophthalmoscopic examination was reported to be normal, and a subsequent ERG showed no detectable signals. A diagnosis of LCA was made. Examinations in the first decade of life reported markedly reduced visual acuities, but some peripheral function. Cycloplegic retinoscopy revealed minimal refractive errors. The fundus examination at age 22 years, the patient had very limited vision. There was markedly reduced visual acuity, but some peripheral function remained; retinoscopy showed myopia and astigmatism. Ophthalmoscopy revealed an atrophic macular lesion, pigmentary retinopathy (depigmentation and bone spiculelike pigment) in the periphery, and attenuated retinal vessels (see Fig. 4B).

F3-1, a heterozygote for the G217 [Δ1 bp] CRX mutation with parents who did not carry the mutant allele (Fig. 1), had nystagmus and strabismus and apparent visual difficulties at age 2 months. An ERG at 11 months of age was described as subnormal in response to a white flash, dark-adapted, and not detectable for flicker. Our examination at ages 7 and 9 years showed nystagmus, markedly reduced visual acuities, eccentric fixation, and some retained peripheral function. Cycloplegic retinoscopy revealed minimal refractive errors. The fundus had a granular appearance throughout.

**Peripherial Retinal Function**

ERGs were detectable by a standard protocol in three of the five patients (Fig. 2A). The two younger patients with the E168 [Δ1 bp] CRX mutation and the patient with the G217 [Δ1 bp] mutation had abnormally reduced rod-, mixed cone-rod-, and cone-isolated ERG amplitudes. B-wave-to-a-wave amplitude ratios for the mixed cone-rod response were normal in the patients (1.1, 1.2, and 1.0 for F1-3, F1-2, and F3-1, respectively; normal mean ± 1 SD, 1.8 ± 0.4). Rod- and cone-isolated photoresponses from the two siblings with the E168 [Δ1 bp] CRX mutation shown in Figure 2B were analyzed for the “P3” component using models of phototransduction. Rod and cone maximum amplitudes were abnormally reduced in both patients. Cone maximum amplitude was ~40% of the normal mean in the two patients; rod maximal amplitudes were 22% and 43% of normal mean in F1-3 and F1-2, respectively (at ages 25 and 28, respectively). The sensitivity of rod photoresponses was within normal limits in both cases. Cone sensitivity was normal in F1-2 and borderline in F1-3.

The distribution across the retina of rod- and cone-mediated dysfunction in the three patients with recordable ERGs (F1-2, F1-3, and F3-1) is shown in Figure 3. The perimetric results in the two patients with the E168 [Δ1 bp] CRX mutation were similar. With kinetic perimetry, there was a normal extent of peripheral visual field to the V-4e target; with I-4e, the fields had decreased peripheral extent and paracentral relative scotomas. Static perimetry showed that L/M cone sensitivities were abnormally reduced. S cone function was not detectable in F1-3 and F3-1; F1-2 had detectable function at only a few paracentral loci (see Fig. 5). Rod sensitivity was not measurable at fixation and mildly reduced in the midperiphery. In the far periphery, rod sensitivity loss was more pronounced in Fl-2. The young patient with the G217 [Δ1 bp] CRX mutation had only peripheral function on kinetic perimetry; rod and L/M cone sensitivity was abnormal but detectable in this retinal region.

Dark adaptation, after 99% and 15% bleaching exposures, was performed in F1-2 and F1-3 (data not shown). The rod-cone break after the larger bleach and time to recovery of final threshold after both bleach exposures were normal. The

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**Table 1. Clinical Characteristics of Patients**

<table>
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<tr>
<th>Patient</th>
<th>Age at Visit (yr)</th>
<th>Visual Acuity*</th>
<th>Refraction</th>
<th>Clinical Diagnosis</th>
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<td></td>
<td></td>
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<td>LE</td>
<td>RE</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>50</td>
<td>LP</td>
<td>HM</td>
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<td>20/30</td>
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<td>20/60</td>
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<tr>
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<td>20/50</td>
<td>20/60</td>
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<tr>
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<td>5/200</td>
<td></td>
<td>-5.25 + 1.75 × 130</td>
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* Best corrected visual acuity.
† With cycloplegia.
slopes of the second and third components of recovery were normal, although the final rod thresholds were abnormally elevated at both loci tested in the patients. The shape of the rod dark adaptation function fell within normal limits when the patient curves were vertically shifted at all time points to compensate for the loss of rod sensitivity.

The disease expression in the two most severely affected patients (heterozygotes for El68 [Δ1 bp] and El68 [Δ2 bp] CRX mutations) is compared in Figure 4. As described above, F1-1 and F2-1 had atrophic macular lesions on ophthalmoscopy and undetectable ERGs to standard stimuli. Kinetic perimetry revealed islands of peripheral function in both patients; F2-1 also detected stimuli in the pericentral retina. Static perimetry indicated that the visual function in these islands was severely reduced in sensitivity compared with normal.

Central Retinal Function and Structure
Serial studies were performed in two patients with the El68 [Δ1 bp] mutation to characterize the central retinal disease in its mildest expression (Fig. 5). Fundus kinetic scotometry showed relative paracentral defects that increased in size in both patients in an interval of 4 years. Rod defects spanned the central 15° to 20° in both patients at the two ages tested; at loci between 10° and 30° eccentric to fixation, rod sensitivity approached the normal limits. L/M cone sensitivity was slightly reduced at fixation, but there were pronounced paracentral losses that increased at the later time points; between 10° and 30°, L/M cone sensitivity approached normal. S cone function (measured only at the later ages) was not detectable in F1-3, and only present at a few loci in F1-2.

Structural correlates of the central retinal dysfunction were examined with OCT in the three patients with the El68 [Δ1 bp] mutation (Fig. 6). A comparison of pseudocolor images from vertical OCT scans of fovea and paracentral superior retina (Fig. 6A) shows that the overall depth of the OCT signal appears thinner in F1-2 than that of a representative normal subject. F1-1 also shows a thinner signal than normal in the superior retina, but more centrally the scans appear thicker because of the high reflectivity deep to the retina. OCT scans from F1-3 (data not shown) were similar to those of F1-2 except centrally where there was a somewhat increased reflectivity originating from deep layers.

Thickness of the OCT scans was quantified by measuring the depth between the major vitread transition and the major sclerad transition at each individual LRP (inset, Fig. 6B). There is a slight reduction in OCT thickness at eccentricities of...
FIGURE 3. Kinetic and static perimetry data in the three patients with mildest disease expression (F1-2, F1-3, and F3-1). I-4e and V-4e isopters are shown for kinetic perimetry (top). Gray areas are relative scotomas to I-4e target, and black areas denote the physiological blind spot. Static perimetry, performed with monochromatic stimuli in the dark- or light-adapted states, is displayed as gray scales of cone (middle) and rod (bottom) sensitivity losses; scales have 16 levels of gray, representing 0 (white) to 3 (black) log units of loss. Physiologic blind spot is shown as a black square at 12° in the temporal field. N, nasal; T, temporal; I, inferior; S, superior field.

approximately 10° to 15° and pronounced reduction from 5° to 7° toward fixation (Fig. 6B). These "structural" findings correlate well with the locations of central rod and L/M-cone dysfunctions (Fig. 6C).

How does the shape of the OCT signal change in the patients? Figure 6D shows average LRP at three loci (I, II, III; Fig. 6A) from the affected members of the family with the E168 (Δ1 bp) CRX mutation overlaid on representative normal data. Scans from the normal subject and the patients show two prominent peaks: one near the vitreoretinal interface and another in the outer retina, the ORCC. The exact cellular localization of the origin of OCT reflections that give rise to the ORCC is currently not known. Therefore, LRP s have been (arbitrarily) aligned by the scleral transition of the ORCC. At location I, 10° to 15° eccentric, F1-3 and F1-2 show an ORCC that is nearly normal; a reduction in OCT thickness may be the result of a loss of the deep valley and the small peak vitread to it. In F1-1, OCT results were similar with an additional loss of width of the ORCC. At location II, the area of the worst visual function for F1-3 and F1-2, there is greater reduction of OCT thickness and reduction of ORCC width. The vitread part of the LRP of F1-1 is similar to that of her daughters; however, on the scleral side, there are large reflections presumably originating from choroidal structures revealed by the RPE atrophy in the macula. Foveally (location III) the ORCC is a double-peaked structure in normal subjects, whereas F1-3 and F1-2 show only a single peak. The signal from F1-1 is complicated by the large amount of backscatter originating deep to the retina.

DISCUSSION

Common Disease Expression in Heterozygotes with CRX Truncation Mutations Despite Different Clinical Diagnoses

There are sufficient shared disease features among the patients in this study to warrant the hypothesis that the phenotypes of these heterozygotes with CRX gene mutations represent a spectrum of severity of a similar process. The disease involves central visual loss as an early symptom, whether manifest in infancy as nystagmus or in adulthood as an acuity disturbance and reduced central visual sensitivity under dim lighting. Retinal function measurements indicate that the disease affects...
rod and cone photoreceptors across the retina in all patients at all stages.

Although the phenotype of patients F1-2 and F1-3 was mild compared with those of the other patients, there was profound loss of rod function in the central retina and a progressive paracentral more than central L/M cone dysfunction. S cone function was virtually undetectable throughout the retina. Abnormalities in rod- and cone-isolated ERG photoresponse maximum amplitudes and perimetry showed that rod and cone photoreceptor outer segment disease was present across the retina, not just centrally.

The disease expression in patients F1-1, F2-1, and F3-1, despite their different ages, mutations, and clinical diagnoses, was similar and far more severe than those in the other two patients. Central vision was lost entirely, and only far peripheral islands were retained. The atrophic macular lesions in patients F1-1 and F2-1 were about the size of the rod and L/M cone defects in the central retina of patients F1-2 and F1-3.
The relatively mild functional abnormalities could thus be the antecedent of the chorioretinal atrophy.

OCT results of the patients with the E168 [Δ1 bp] mutation at different disease stages were consistent with photoreceptor loss progressing to include RPE disease. Overall OCT depth (presumed proportional to retinal thickness) decreased from pericentral to central retina in the two patients with milder phenotype. In regions of mild rod and/or cone dysfunction, there was a nearly normal ORCC waveform. Moderate dysfunction was accompanied by a thinned ORCC with or without some deep backscatter. In areas of unmeasurable function, there was a thinned ORCC with pronounced deep backscatter. The deep backscatter can be interpreted as increased penetration of the probing light through diseased RPE. However, the exact origins of the ORCC and the basis of the observed thinning remain uncertain. The ORCC has been ascribed to the combination of backscatter originating from the RPE and choriocapillaris. Others have hypothesized a contribution to the ORCC from photoreceptor-based backscatter. Our results would be consistent with the latter hypothesis, but further studies of the relation of OCT to retinal microanatomy are obviously needed.

How do the findings in this study compare with those from patients with other CRX mutations? Detailed analyses of phenotype have not been reported to date on other CRX mutations except for a family with the Arg41Trp substitution. Affected members had an onset of visual loss in the fourth decade of life or later. Most of these patients, like F1-2 and F1-3 in this study, had relative central scotomas, early S-cone dysfunction by color vision testing, and abnor-
CRX in the Retina

How Disease Expression May Relate to the Role of CRX in the Retina

CRX, the first photoreceptor-specific transcription factor to be identified, is highly expressed in developing and adult mouse retinas and binds to sites in the rhodopsin, interphotoreceptor retinoid-binding protein, β-phosphodiesterase, and arrestin promoters.\(^{4,6}\) The association of heterozygous CRX gene mutations with "adult-onset" inherited retinal degenerations, specifically autosomal dominant CRD, was taken as evidence that CRX is essential for maintenance of photoreceptors in the mature human retina.\(^{5,7}\) The recent association with "infantile-onset" retinal degeneration, specifically the diagnostic category of LCA, reinforced the notion that CRX may play a critical role in human retinal development.\(^{8}\)

A parsimonious explanation for our findings in human retinal disease associated with CRX truncation mutations is that the pathobiology involves a developmental abnormality of photoreceptors compounded by an alteration in gene expression, leading to slowly progressive photoreceptor death. By this explanation, patients could differ in the degree of developmental retinal defect. The predilection for more severe disease expression in the macula may relate in some way to the complex pre- and postnatal development of this retinal region.\(^{23,24}\) In contrast to the patients with severe phenotype near birth (E168 [A2 bp] and G217 [A1 bp] mutations), those with the E168 [A1 bp] CRX mutation could have been born with subtle abnormalities in their retinas and only after decades of life have clinically overt disease. A component of the rod dysfunction in the central and peripheral retinas of patients F1-2 and F1-3, for example, may thus be like a congenital stationary photoreceptor disorder. There is some evidence for failure of rod morphogenesis in an experimental system that reduced Crx transactivation activity.\(^{4}\) There are other diseases such as blue cone monochromacy in which there is a congenital stationary photoreceptor abnormality and maculopathy in some patients later in life,\(^{25,26}\) or the enhanced S cone syndrome, in which a suspected developmental abnormality of the photoreceptor mosaic can be accompanied by a progressive retinal degeneration.\(^{27}\) Alternatively, embryonic development may not be altered and degeneration is entirely postnatal such as what occurs posthatching in the rd (retinal degeneration) chicken, a naturally occurring model of LCA caused by a RETGC mutation.\(^{28}\)

Could phenotypic variation be accounted for by differences in the CRX mutations? All three mutations cause frame-shifts; E168 [A1 bp], E168 [A2 bp], and G217 [A1 bp] mutations are predicted to lead to losses of 44%, 45%, and 25%, respectively, of the C-terminal tail of the protein and to replacement with 18, 4, and 1 new amino acids, respectively. Present in all the mutant alleles is the homeodomain and deleted is the conserved OTX tail.\(^{29}\) The various mutant alleles could differ from each other in terms of residual transcriptional activity, the ability to possibly interact with other transcription factors such as neural retina leucine zipper,\(^{30,31}\) protein stability, or the capacity to act as dominant-negative regulators. Further experimental studies are needed to explore the biochemistry of the mutant proteins. It is also possible that mechanisms other than the identified CRX mutations cause or contribute to the variation in the expression observed in these patients. Possibilities include undiscovered mutations in CRX or other genes; different genetic backgrounds including different modifier genes,\(^{32}\) subtle alterations in timing of the switching on of the mutant gene,\(^{33}\) and environmental or other factors that could influence the developing retina.\(^{34}\) Of interest, the finding of variable severity in this study is not unique in that almost identical mutations in PAX6, a well-studied transcription factor involved in ocular disease, can cause both mild and severe phenotypes.\(^{35}\)

Clinical Context of the CRX Results: LCA and CRD

Early-onset severe retinal degenerations, collectively known as LCA, are classically considered to be inherited as autosomal recessive traits.\(^{34,36}\) Nonallelic heterogeneity has long been suspected in LCA\(^{37}\) and recently has been proven by the finding of mutations in the RETG\(^{38}\) and RPE65\(^{39,41}\) genes in patients with this diagnosis. Most of the LCA patients with RETG and RPE65 mutations are homozygotes or compound heterozygotes, although there are some unexplained cases of heterozygotes with disease\(^{38,40}\) and a de novo mutation.\(^{41}\) It is of interest that a spectrum of severity of disease expression has recently been noted in patients with RPE65 mutations. Some patients carry the diagnosis of retinitis pigmentosa (RP), whereas others have the diagnosis of LCA.\(^{41}\)

Patients with de novo mutations in the CRX gene (F2-1 and F3-1), if no further mechanism is found to explain their phenotype, might have an autosomal dominant form of LCA. The lack of symptoms of patient F1-1’s parents into late life makes this patient also suspicious but unproven for a de novo mutation. There are multiple generation pedigrees of severe early-onset retinal degeneration in the literature.\(^{42-45}\) It will be important to determine whether CRX gene mutations are associated with the retinal diseases in these families. Screening of larger numbers of LCA patients for CRX mutations should reveal whether there are cases of homozygous mutations or compound heterozygosity in addition to the unexpected presentation in our patients. Until there is further clarification of the role of heterozygous CRX mutations in LCA, caution should be exerted in genetic counseling of these patients and their families.

CRD, the other clinical diagnosis associated with CRX mutations to date, has also been shown to have genetic heterogeneity.\(^{46,47}\) Before the current era of molecular discovery, studies of phenotypes found different patterns of visual loss within the CRD diagnostic category.\(^{48,51}\) Al-
though details of CRD subclassification schemes differ, it is agreed that these diseases all manifest maculopathy and either show relatively equal loss of cone and rod function by full-field ERG b-wave analysis or greater cone dysfunction than rod dysfunction. The patients with recordable ERGs in the present study would fall into the former category. As molecular causation continues to be clarified in patients with the clinical diagnosis of CRD, detailed phenotype studies should tell us whether there are subtle differences in disease expression in different genotypes. Such an approach, combined with in vitro studies and studies in genetically engineered animals, can help increase understanding of the sequence from gene mutation to human retinal degeneration.

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