

A *CRX* Null Mutation Is Associated with Both Leber Congenital Amaurosis and a Normal Ocular Phenotype

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PURPOSE. To identify and characterize new cone rod homeobox (*CRX*) mutations associated with the Leber congenital amaurosis phenotype.

METHODS. The human *CRX* gene was sequenced in 74 consecutive patients carrying the diagnosis of Leber congenital amaurosis.

RESULTS. Two mutations were identified in *CRX* that cause frameshifts and predict severe truncations of the encoded protein. One of these, a 1-bp insertion, spares only nine N-terminal amino acids, removing the homeodomain, WSP motif, and conserved OTX domain at the C terminus. Of the *CRX* mutations described in the literature, this is the first that convincingly represents a null allele of the gene. Although the patient heterozygous for this null allele is affected with Leber congenital amaurosis, it was surprising that her father, who had normal vision, was heterozygous for the same mutation.

CONCLUSIONS. These results strongly suggest that haploinsufficiency of *CRX* is not sufficient to cause a retinal disorder. Loss of function alleles of *CRX* appear to cause Leber congenital amaurosis through a recessive or multigenic mechanism. (*Invest Ophthalmol Vis Sci.* 2000;41:2076–2079)

Leber congenital amaurosis (LCA; MIM 204000) is an inherited, severe dysfunction of the retina involving both rods and cones.¹ It is characterized by blindness noted at birth or shortly thereafter.² The disorder is also accompanied by an absent or extremely attenuated electroretinogram (ERG) that indicates a near absence of neural responses to light. The retina may appear morphologically normal at birth. However, a variety of pigmentary changes may develop over a period of months to years, some of which appear similar to retinitis pigmentosa. There may also be localized structural defects such as a macular coloboma.³ A number of disorders in which patients manifest a congenital retinal dystrophy in association with other neurologic or systemic manifestations have phenotypic overlap with LCA but are recognized as separate entities. These include medullary cystic kidney disease or nephronophthisis (Senior-Loken syndrome, Mendelian Inheritance in Man [MIM] 266900), cone-shaped epiphyses of the hands and cerebellar ataxia (Mainzer-Saldino syndrome, MIM 266920), vermis hypoplasia, oculomotor disturbances, and neonatal respiratory problems (Joubert syndrome, MIM 243910). Other

manifestations also observed in association with LCA include psychomotor and mental retardation, autistic behavior, hydrocephalus, epilepsy, cardiomyopathy, and sensorineural hearing loss.

It is estimated that LCA accounts for 10% of childhood blindness. Although LCA is generally thought to be inherited in an autosomal recessive fashion, some autosomal dominant pedigrees have been reported.^{4,5} The disease is genetically heterogeneous, and to date six independent loci for LCA have been linked to chromosomes 1p31,^{6,7} 6q11-16,⁸ 14q24,⁹ 17p13.1,¹⁰ 17p13.3,¹¹ and 19q13.¹² Mutations have been reported in the *RPE65* gene on 1p31,^{6,7,11} the *AIP1* gene on 17p13.1, and the *GUCY2D* gene on 17p13.3¹¹ in families in which the autosomal recessive mode of transmission has been observed. In contrast, mutations in the *CRX* gene have followed both the dominant^{5,10} and recessive¹³ modes of inheritance.

In this report we describe novel mutations in the *CRX* gene of patients with LCA, and we discuss the possible mechanisms of action underlying these changes.

MATERIALS AND METHODS

Patients and Families

Blood or DNA samples were obtained from the Johns Hopkins Center for Hereditary Eye Diseases collection. All affected individuals and family members gave written informed consent in accordance with institutional guidelines. All research procedures were in accordance with the Declaration of Helsinki. In 32 of the 74 families, an autosomal recessive mode of inheritance could be inferred based on affection status of siblings or a family history of consanguinity. In the remaining, the proband was the only known affected individual. All probands were examined and met the diagnostic criteria for Leber congenital amaurosis: profound visual loss at birth or noted within

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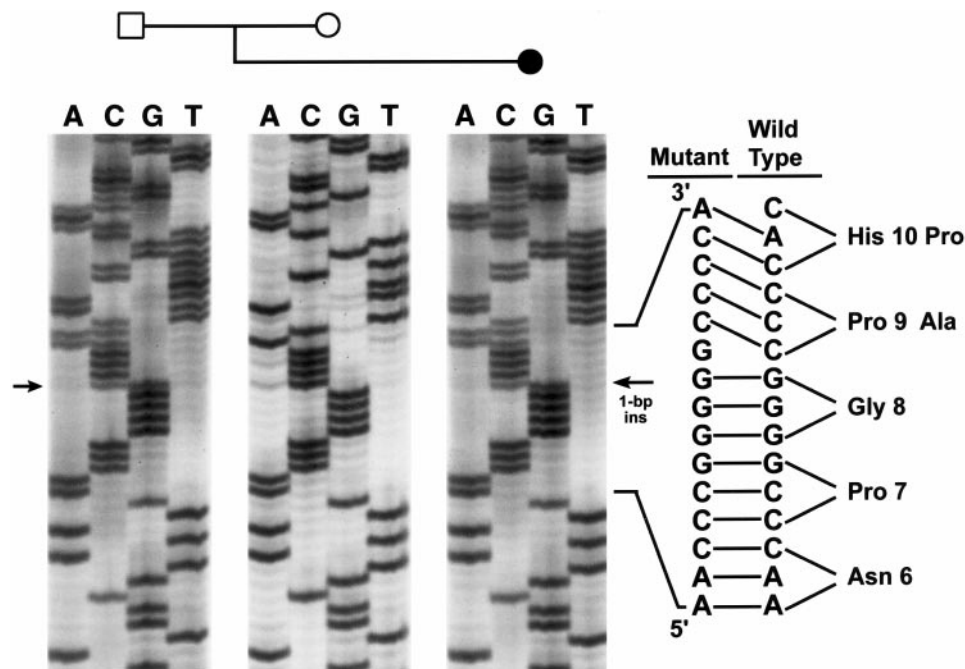


FIGURE 1. Direct sequence of exon 1 in the region surrounding codon 9 of *CRX* in the affected proband and parents. The P9ins1bp mutation is present in both the father and the proband, whereas in the mother two normal *CRX* alleles are present.

the first year of life, pendular nystagmus, and an extinguished or extremely attenuated photopic and scotopic ERG. The family members were also examined clinically, and one individual underwent the same ERG evaluation. All ERGs were performed using an International Society for Clinical Electrophysiology of Vision standard technique in accordance with institutional guidelines.

Mutation Analysis

Genomic DNA was prepared from peripheral blood by phenol-chloroform extraction or using the Qlamp blood kit (Qiagen, Santa Clarita, CA) according to manufacturer's instructions. Polymerase chain reaction (PCR) amplification was obtained from genomic DNA using previously published sets of primers.¹⁴ After PCR amplification, fragments were analyzed on a 8% polyacrylamide gel and visualized by ethidium bromide staining. To sequence the amplicons individually, these were separated on a 1.6% low-melting-point agarose, and the bands were excised and treated with β -agarase and subsequently phenol-chloroform purified. The purified products were used for direct manual sequencing using a cycle sequencing kit with ³³P-labeled dideoxy nucleotides (Thermo Sequenase; Amersham, Life Sciences, Arlington, IL), according to the manufacturer's instructions. To further confirm the mutations, the gel purified amplicons were cloned into the pCR 2.1-TOPO vector, using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. For each of the two probands, 10 different amplicon subcloned fragments were sequenced at the Johns Hopkins University core facility, using the ABI 100, version 3.2 automated sequencer (ABI prism; Applied Biosystems, Foster City, CA).

RESULTS

We sequenced the human *CRX* gene in 74 patients with the diagnosis of LCA. Analysis revealed the presence of two previ-

ously unreported heterozygous mutations in two unrelated patients; one was a 1-bp insertion c.25insG (GenBank accession number AF024711) causing a frameshift in codon 9 (P9ins1bp; Figs. 1 and 2A) and the second a 1-bp deletion, c.709delC, causing a frameshift in codon 237 (L237 Δ 1bp; Fig. 2B). No missense, nonsense or splice-site mutations were identified in this group. Both mutations cause frameshifts that predict significant changes in protein structure (Fig. 3).

In the case of P9ins1bp, the predicted protein is without all three conserved motifs: homeodomain, the WSP motif, and the OTX tail, a conserved motif near the C-terminus. Only the first nine amino acids of the 299-amino acid *CRX* protein remain unchanged by the frameshift, whereas the remainder of the protein is replaced by an out-of-frame polypeptide of 69 amino acids.

A very different situation is provided by the L237 Δ 1bp frameshift mutation. In this case, the predicted protein does not have 21% of the C-terminal tail, including the conserved OTX tail motif and C-terminus. This frameshift eliminates the original stop codon. The missing amino acids are replaced by a new C-terminus that determines a 369-amino acid protein derived from an alternate reading frame and the normal 3' untranslated region. These mutations were not found in the *CRX* gene of 208 control individuals. These two patients also were screened for mutations in *GUCY2D* and *RPE65*, but the results were negative.

Sequence analysis demonstrated that the c.709delC mutation was absent in both parents, who had normal visual function, indicating its de novo origin in association with the disease. Ophthalmoscopic examination of the 18-month-old proband was unremarkable; however, the ERG was abolished.

In contrast, sequence analysis showed that the father of the proband with the c.25insG allele also carried this insertion mutation. It should be underscored that in both cases parentage was tested and confirmed. A surprising finding was that the father who carried the c.25insG mutation did not show any

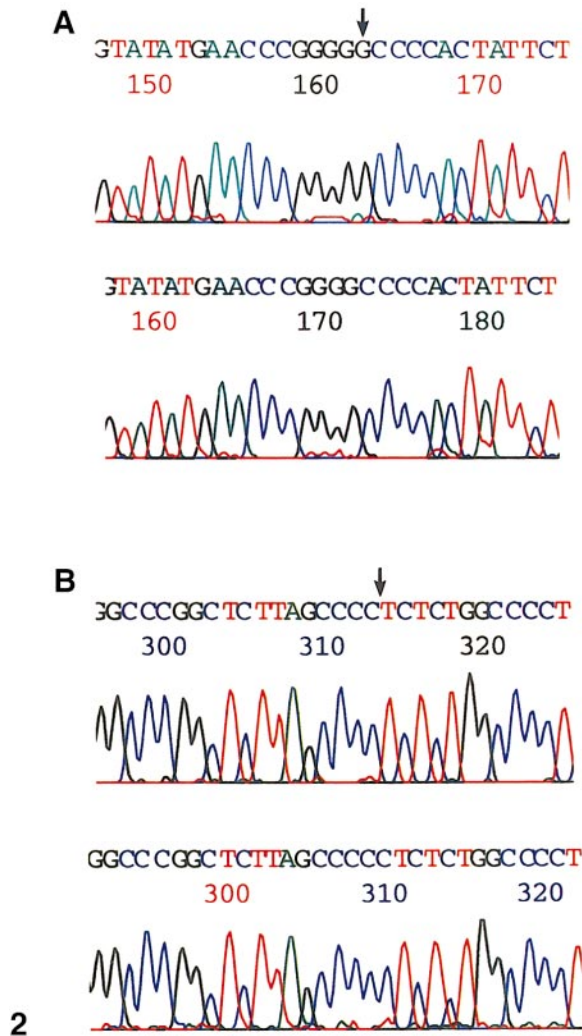


FIGURE 2. (A) Direct sequence of exon 1 in the region surrounding codon 9, obtained after subcloning the amplicon of the affected proband and unaffected father. Note the insertion of one G in the mutated allele (*top*) in comparison with the normal sequence (*bottom*). (B) Direct sequence of exon 3 in the region surrounding codon 237 in cloned DNA derived from the proband. Note the deletion of one C in the mutated allele (*top*) in contrast with wild-type (*bottom*).

significant clinical abnormalities. Ophthalmologic examination of the fundus, fluorescein angiography, and color testing all produced normal results. ERG results revealed normal scotopic responses and only a very mild reduction in the photopic flash responses. This ERG profile is also observed in a significant percentage of normal individuals in the same age group. Another surprising feature is the finding that, since birth, the proband had had severe bilateral sensorineural hearing loss for all frequency ranges.

DISCUSSION

We have identified two new heterozygous mutations in the *CRX* gene of patients with LCA. Both sequence changes, c.25insG and c.709delC, cause frameshifts and predict significant protein changes. It is possible that the G-insertion mutation, c.25insG, has arisen de novo from polymerase slippage,

because single-nucleotide DNA stretches show a tendency toward polymorphism.¹⁵ The c.25insG mutation is of special interest because all conserved functional motifs are missing and therefore represents the first convincing example of a *CRX* null allele. The c.25insG allele was also found in the proband's father, who is phenotypically normal. This is in striking contrast to previously described *CRX* mutations^{12,16} found in patients with LCA. In each of these cases, a de novo heterozygous truncation mutation of the *CRX* gene was associated with disease. However, at the protein level, all these earlier published mutations, as well as the c.709delC mutation, leave the DNA-binding homeodomain intact.

The observation that a heterozygous null mutation such as c.25insG has no obvious phenotype eliminates haploinsufficiency as the disease-causing mechanism. However, we cannot exclude the presence of mutations outside the coding regions of the other, maternal *CRX* allele. Such mutations may affect promoter function and splicing or reduce the stability of the mRNA. Any of these possibilities would be consistent with an autosomal recessive model and could account for the dramatic differences in phenotype observed between the father and the proband. Another possible explanation is digenic inheritance (e.g., with the *NRL* gene)—specifically, double heterozygosity of two interacting mutations. In this context, we also screened for mutations in *GUCY2D* and *RPE65* genes known to cause LCA and found no sequence changes. We could also conceive that a variant of the *CRX* promoter produces higher than normal levels of mRNA of the wild-type allele, suppressing disease in the father by boosting CRX protein above a critical threshold. Other explanations for this phenotypic variation include the possibility of somatic mosaicism in the father, genetic imprinting of the wild-type *CRX* allele, or environmental factors that could influence the normal course of retinal development and enhance the effect of the mutant allele.

In contrast, the c.709delC allele will produce a protein that has retained its DNA-binding homeodomain but has lost its OTX-conserved motif and C-terminal domain. This mutation closely fits the category described earlier by Freund et al.¹² for which haploinsufficiency and dominant-negative effects were discussed as possible disease-causing mechanisms. Functional

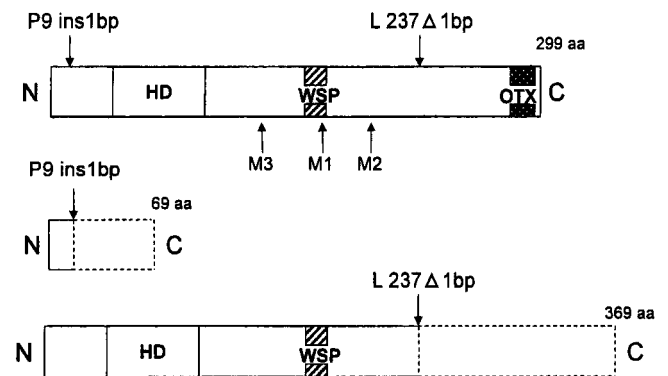


FIGURE 3. The *CRX* protein. *Top*: The three *CRX* conserved motifs are depicted in boxes: homeodomain, WSP domain, and OTX domain; M1, M2, and M3 represent LCA mutations previously reported. Two new mutations (P9ins1bp and L237Δ1bp) that cause frameshifts are indicated by arrows above the schematic. *Bottom*: The predicted protein truncations are indicated by dashed lines, and their sizes are indicated (69 and 369 amino acids).

studies of the PAX6 protein have recently demonstrated that truncation mutations in the C-terminal transactivation region of PAX6 result in dominant-negative mutants.¹⁷ By analogy with the PAX6 studies, retention of the CRX homeodomain in the defective L237Δ1bp protein may allow it to compete for DNA binding sites with the functional wild-type transcription factor. Biochemical studies should give insight to the functional consequences of this mutation. These new findings bring a new perspective to the role of heterozygous CRX mutations in ocular disease and reinforce the need for caution when genetic counseling is requested by patients and their families.

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