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# Mutations in the Cone-Rod Homeobox Gene Are Associated with the Cone-Rod Dystrophy Photoreceptor Degeneration

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## Summary

**Crx is a novel paired-like homeodomain protein that is expressed predominantly in retinal photoreceptors and pinealocytes. Its gene has been mapped to chromosome 19q13.3, the site of a disease locus for autosomal dominant cone-rod dystrophy (CORDII). Analysis of the proband from a family with autosomal dominant CORD revealed an Arg41Trp substitution in the third residue of the CRX homeodomain. The sequence change cosegregated with the disease phenotype and was not detected in 247 normal controls. Recombinant CRX homeodomain containing the Arg41Trp substitution showed decreased DNA binding activity. Analysis of another 169 CORD probands identified three additional CRX sequence variations (Arg41Gln, Val242Met, and a 4 bp deletion in codons 196/7) that were not found among the controls. This data suggests that mutations in the *CRX* gene are associated with photoreceptor degeneration and that the Crx protein is necessary for the maintenance of normal cone and rod function.**

## Introduction

Differentiation and maintenance of neuronal functions requires appropriate regulation of complex patterns of gene expression. In the retina, an increasing number of the transcription factors that regulate tissue-specific gene expression are being identified (Swaroop et al., 1992; Freund et al., 1996). Genetic analysis of these factors is beginning to shed light on their function in vivo. For example, identification of a null mutation in the mouse of the paired class homeodomain gene *Chx10* suggests that it is involved in the proliferation of neuroretinal precursors and is necessary for the generation of differentiated bipolar cells (Burmeister et al., 1996). Similarly, knock out of the POU homeodomain gene *Brn-3b* indicates that it plays an important role in the development of some subclasses of retinal ganglion cells (Erkman et al., 1996; Gan et al., 1996). Knock out of the *Rx* homeodomain gene, which results in a failure of eye formation, demonstrates that *Rx* is a key molecule in the early stages of eye development (Mathers et al., 1997).

Using a yeast one-hybrid screen with "baits" from the promoter region of the gene for the rod visual pigment rhodopsin (Kumar et al., 1996), we recently identified a novel paired-like homeodomain protein, Cone-Rod Homeobox (Crx), which has characteristics suggesting that it may play a significant role in the regulation of photoreceptor gene expression (Chen et al., 1997). Crx belongs to the otd/Otx family, members of which are important in the development of anterior head structures and in cell lineage determination (Finkelstein and Boncinelli, 1994). Crx appears to be one of the earliest known markers of photoreceptor identity. Its expression begins as early as embryonic day 12.5 in the mouse, which is the time of cone cellular determination, and increases dramatically at postnatal day 3, which is near the time of maximal rod genesis (Carter and LaVail, 1979). In the adult, it is expressed predominantly in cone and rod cells. In vitro studies with the recombinant Crx protein have demonstrated that it binds specifically to the Ret 1, BAT-1, and Ret 4 sites in the *rhodopsin* proximal promoter region, elements that were previously implicated as being important in regulation of *rhodopsin* gene expression (Morabito et al., 1991; Yu and Barnstable, 1994; Chen and Zack, 1996; DesJardin and Hauswirth, 1996; Yu et al., 1996). Crx transactivates the expression of *rhodopsin* and a number of other photoreceptor-specific genes in cultured cells. It acts synergistically with the bZIP transcription factor neural retina leucine zipper (NRL) (Swaroop et al., 1992), which demonstrates an overlapping pattern of expression with *Crx* during retinal development (Liu et al., 1996), and has itself been implicated in *rhodopsin* regulation (Kumar et al., 1996; Rehemtulla et al., 1996).

To examine the role of Crx in vivo, we undertook a genetic approach. We cloned the human *CRX* gene and determined that it mapped to chromosome 19q13.3 (Chen et al., 1997), the site of one of several autosomal dominant cone-rod dystrophy loci (CORDII) (Evans et al., 1994; Gregory et al., 1994). The CORDs are a clinically and genetically heterogeneous group of diseases that exhibit early loss of central visual acuity and color vision, with progressive loss of peripheral vision. This

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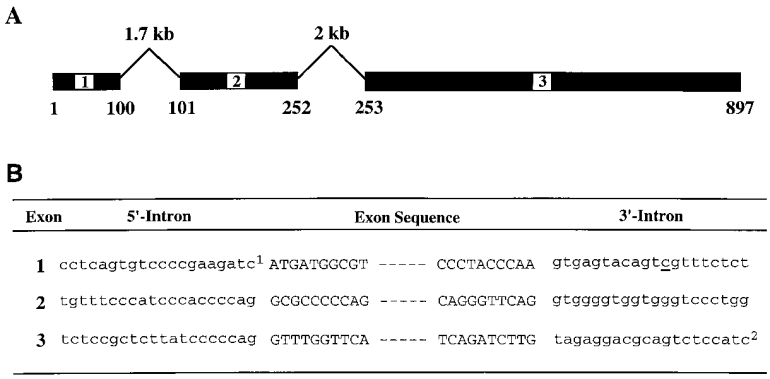


Figure 1. Genomic Structure of the Human *CRX* Gene

(A) Schematic diagram showing the relative size and position of the three exons that contain the *CRX* coding region. Note that there is an additional exon upstream of exon 1 that contains only 5′-untranslated region sequence, but its exact borders have not been defined. It is not shown in the figure.

(B) Intron–exon border sequences and flanking 5′- and 3′-untranslated region sequences for the three coding region exons. The C/T polymorphism in intron 1 is underlined (see text). [1]5′-untranslated sequence. [2]3′-untranslated sequence.

phenotype is clinically distinct from retinitis pigmentosa, in which the initial pathology is largely in the midperipheral retina rather than in the macula, and the loss of rod photoreceptor function precedes that of cones. In their end stages, however, these diseases are often difficult to distinguish. The clinical phenotype associated with CORDII generally involves an early onset macular lesion and a severe clinical course, usually resulting in blindness before the age of 50 (Evans et al., 1995). Unlike some other forms, CORDII is not known to be associated with systemic or other manifestations.

Here, we report the identification of a putative disease-causing mutation (Arg41Trp) in the homeodomain of the CRX protein in a CORD pedigree with an apparently autosomal dominant mode of inheritance and demonstrate that the substitution causes a decrease in DNA binding activity. Analysis of additional probands revealed three other sequence changes in the *CRX* gene that may also be associated with the disease phenotype. We present a clinical description of the Arg41Trp family and discuss the implications of our results for the role of the CRX protein in the development and maintenance of normal photoreceptor function.

## Results

### Arg41Trp Substitution Cosegregates with Disease in a CORD Family

As a first step for mutation analysis, the human *CRX* gene was isolated and its genomic organization determined (Figure 1A). The 897 bp *CRX* coding region is contained in three exons, with the homeodomain split between exons 1 and 2. The intron–exon boundaries are consistent with known splice consensus sequences (Mount, 1982) (Figure 1B).

Since we did not have available any CORD families in which the disease locus had been mapped to 19q13.3, we screened a cohort of 170 probands from families with the diagnosis of CORD for *CRX* mutations. The sequence analysis revealed a proband with a single nucleotide change in codon 41 (CGG to TGG) in one allele, resulting in an Arg41Trp substitution in the third residue of the homeodomain (Figure 2A). The extended pedigree of this family (#H0992) is shown in Figure 2B. The codon 41 sequence change cosegregated with the disease in the pedigree through eight meioses (Figure 2C), providing a lod score of 2.1 (data not shown), and was not observed in 247 normal controls (55 individuals were tested by direct sequencing and restriction digest and 192 by single-strand conformational polymorphism [SSCP] analysis; data not shown). In addition, it should be noted that all otd/Otx-like proteins contain an arginine at the third residue of the homeodomain, and the only homeodomains containing a tryptophan at this location are an atypical subgroup consisting of the H2.0, Hlx, HB24, and Hal-hlx proteins (Duboule, 1994).

### Recombinant Homeodomain Containing the Arg41Trp Substitution Demonstrates Decreased DNA Binding Activity

To examine the possible functional consequences of the Arg41Trp substitution, we compared the DNA binding activity of recombinant wild-type CRX homeodomain–glutathione S-transferase fusion protein (CrxHD–GST) with equivalent protein containing the Arg41Trp substitution ($CrxHD_{Arg41Trp}$–GST) (Figure 3A). We had previously demonstrated that CrxHD–GST binds to the Ret 1, BAT-1, and Ret 4 sites in the *rhodopsin* proximal promoter region (Chen et al., 1997). In an electrophoretic mobility shift assay (EMSA), $CrxHD_{Arg41Trp}$–GST demonstrated significantly reduced binding to the Ret 1 and BAT-1 sites (Figures 3B and 3C). Its binding to the Ret 4 site was barely detectable (Figure 3D). These findings are consistent with the Arg41Trp substitution being a functionally important mutation.

### Clinical Characterization of Arg41Trp CORD Family

The core clinical features in pedigree #H0992 are normal vision during childhood and young adult years, with development of progressive vision loss centrally beginning at 35–50 years of age. The deceased father (II-2, Figure 2B) had been diagnosed in 1957 with 20/200 acuities from "presenile macular degeneration" that began in his mid-30's. He reported that his father (I-1) and two brothers were also affected, consistent with autosomal dominant inheritance. The three affected siblings (III-1, III-7, and III-9) reported first noticing vision problems between 37 and 45 years of age. All three had early and patchy atrophy of the retinal pigment epithelium (RPE) in the macula and beneath the arcade vessels at 20°–40° peripherally in the region of highest rod density (Figures 4A and 4B). Visual field testing revealed relative blind spot regions (scotomas) within the area of central vision

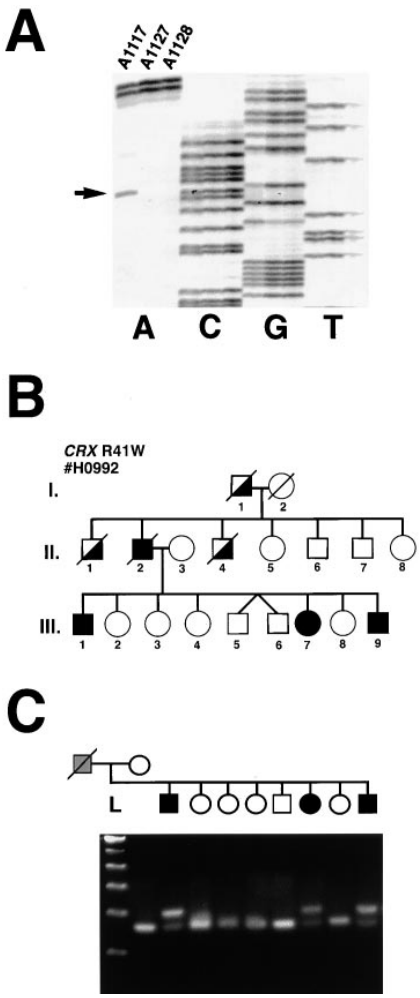


Figure 2. Arg41Trp Substitution Cosegregates with the CORD Phenotype in Pedigree #H0922

(A) Sequence analysis of *CRX* exon 2. The sequence of the antisense strand in the region of the C-to-T transition is shown. The A numbers on top indicate the proband number. The nucleotide substitution is detected in patient A1117 and is indicated by an arrow.

(B) Pedigree #H0992 through three generations. Solid squares and circles indicate affected males and females, respectively.

(C) Segregation of Arg41Trp mutation in the pedigree. Since the transition from C to T resulted in the loss of restriction site for FauI ($CCCGC(N)_4/GGGCG(N)_6$; produced by SibEnzyme, Novosibirsk, Russia, and obtained from New England Biolabs), the amplified products of *CRX* exon 2 were digested with FauI and analyzed on a 2% agarose gel. Solid squares and circles indicate affected males and females, respectively. The gray square represents the deceased father, who was legally blind during the later part of his life and presumably carried the mutation. The affected individuals carry both a normal and a mutated allele. L shows 100 bp ladder of DNA markers.

(Figure 4C). Color vision was abnormal (Table 1). Rod psychophysical threshold was elevated by 0.5 log units at some points in the macula, but rod thresholds were normal at multiple peripheral points for all three. Widespread cone disease was indicated by delayed photopic flicker implicit timing on full-field electroretinogram (ERG) testing (Figure 4C, triple arrows).

Patient III-1 had sought help for vision problems at age 45, but by age 52, his acuities were still 20/20 for both eyes. His macula showed punctate pigmentary abnormality of the RPE and early mild atrophy that extended into the nasal retina. His ERG was the least affected of the three, with normal b-wave amplitudes for scotopic rod and photopic cone conditions (Figure 4C). However, his ERG showed abnormalities: his cone 30 Hz flicker implicit time (from flash to response peak) was marginally prolonged to 31 ms, and the white scotopic responses to the bright flash were "electronegative" (Figure 4C, arrow). These changes suggested diffuse rod and cone disease. III-1 was tested again 5 years later at age 57, and progression was noted: acuities had dropped slightly to 20/30 in both eyes, his central blind spots were not larger but were more dense (now to the V4e target), and his photopic cone response had become electronegative like III-9, even though the 30 Hz flicker implicit time and amplitude had not changed (data not shown).

### Other *CRX* Mutations May Also Be Associated with CORD

The screening of the CORD cohorts identified four additional probands with sequence changes in one of their *CRX* alleles (Figure 5). One of them also showed a nucleotide change in the Arg41 codon, but in this proband, a G-to-A transition resulted in an arginine-to-glutamine substitution. Among a compilation of 346 homeodomain sequences (Duboule, 1994), none had a glutamine residue at this position. Unfortunately, no other members from the Arg41Gln family, nor from the families below, were available for clinical or DNA testing.

The three other changes that were identified were a GCC-to-ACC change in codon 158, resulting in an Ala-to-Thr substitution; a 4 bp deletion involving codons 196 and 197, resulting in a frame shift and premature termination; and a GTG-to-ATG change in codon 242, resulting in a Val-to-Met substitution (Figure 5). All of the patients with these sequence changes had clinical and electrophysiological findings consistent with CORD. The Arg41Gln, Val242Met, and 196/197 deletion alleles, using the same SSCP protocol that was used to identify them, were not found in a group of 192 normal controls. The Ala158Thr substitution, however, was observed in 2/110 control chromosomes. The only other sequence variation detected was a common polymorphism, a C-to-T substitution at the +12 position in intron 1 (see Figure 1), which was present in approximately 25% of both normal and patient chromosomes.

### Discussion

Previous studies implicated Crx as a potentially important regulator of photoreceptor gene expression both during development and in the adult retina (Chen et al., 1997). However, since this data was mostly in vitro in nature, it did not directly address the role of Crx in vivo. In this report, we describe the identification of five potentially mutant alleles of the human *CRX* gene that are associated with CORD, thereby providing strong evidence that the CRX protein is indeed required for the maintenance of normal rod and cone function in vivo.

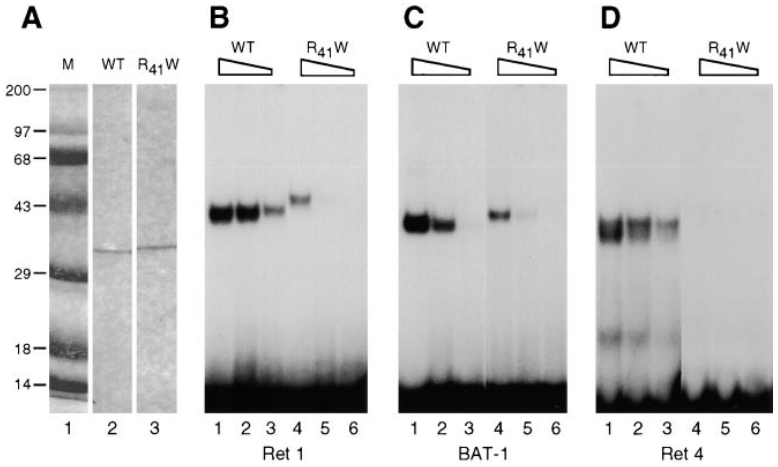


Figure 3. Arg41Trp Substitution Is Associated with Decreased DNA Binding Activity

(A) Protein gel demonstrating purification of CrxHD–GST and $CrxHD_{Arg41Trp}$–GST fusion peptides. Lane 1 contains protein molecular weight markers (BRL); lanes 2 and 3 each contain 100 ng of the indicated protein; the numbers adjacent to the markers represent the apparent molecular weights (kilodaltons). (B–D) EMSAs with CrxHD–GST (lanes 1–3) and $CrxHD_{Arg41Trp}$–GST (lanes 4–6) fusion peptides. The $^{32}P$-labeled probes used are indicated on the bottom of each figure. The amount of fusion protein used for lanes 1 and 4, 2 and 5, and 3 and 6 were 10, 2, and 0.4 ng, respectively, for (B); 2, 0.2, and 0.02 ng, respectively, for (C); and 60, 40, and 20 ng, respectively, for (D).

Several lines of evidence suggest that at least some of the identified *CRX* sequence changes are mutations that are causally related to the CORD phenotype: (1) several families with CORD have been mapped to 19q13.3 (Evans et al., 1994; Gregory et al., 1994), the site of the *CRX* gene. The fraction of CORD cases that are linked to 19q13.3, however, is unknown. (2) In pedigree #H0992, there is perfect cosegregation of the Arg41Trp allele with the clinical phenotype through eight meioses. (3) The Arg41Trp mutation causes a charge and hydrophobicity change at a conserved and potentially important site, the third residue of the homeodomain. The Crx homeodomains are completely conserved between mouse, cow, and man. Furthermore, the arginine residue itself is conserved between all members of the otd/Otx family, and in a compilation of 346 homeodomains, a basic amino acid (Arg or Lys) is present in 312 of the proteins (Duboule, 1994). Based on

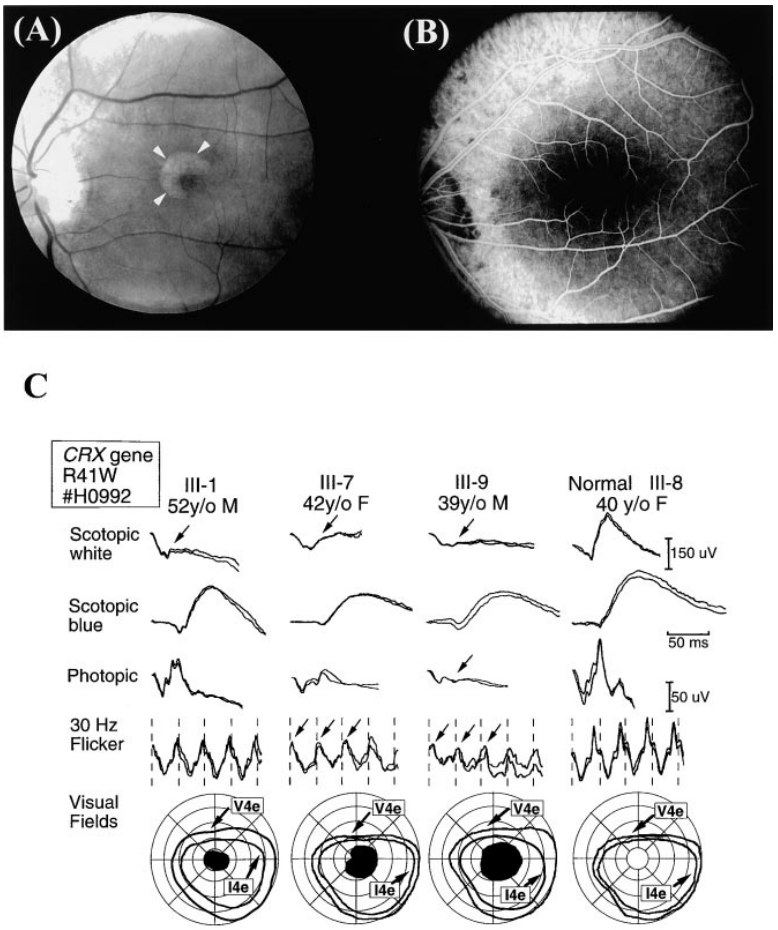


Figure 4. Clinical Characterization of Patients from Pedigree #H0992

(A) Fundus photograph of the right eye of III-9, a 39-year-old male, shows a crescent of perifoveal RPE atrophy from 3- to 7-o'clock position and extensive RPE atrophy underneath the macular arcade vessels (right and bottom of photograph). The arterioles are narrowed.

(B) Fluorescein angiogram of macula of the right eye of III-9 shows extensive RPE atrophy along the macular arcade vessels (right and bottom of photograph) causing a "window defect" through which the bright choroidal fluorescence is evident. This is the rod-dense region of the retina between 20°–40° from the fovea.

(C) Retinal function testing on three affected siblings. ERG responses to scotopic bright white flashes (10 cd-s/$m^2$, dark-adapted condition) are primarily from rods and rod pathway neurons; arrow highlights "electronegative" shape form loss of positive-going b-wave that did not even return to baseline. Blue scotopic flash (0.014 cd-s/$m^2$) elicits the rod system signature maximal b-wave that is normal for III-1 but abnormal for III-7, -9. Photopic flash (10 cd-s/$m^2$ flash on 43 cd/$m^2$ light-adapting background) and 30 Hz flicker elicit responses primarily from cone pathway neurons; single arrow highlights b-wave loss, and triple arrows indicate delayed flicker implicit timing. Visual fields show central relative blind spots of ⩾30° tested with the Goldmann perimeter I4e target but with retention of full peripheral fields to both target sizes.

Table 1. Clinical Summary of Affected Members of Pedigree H0992

| Pedigree Number | Age at Dx. | Visual Acuity | Visual Fields (Goldmann I4e) | Color Vision (D15 test) | Refraction | Electroretinogram | | | Dark-Adapted Thresholds |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Photopic b-wave | Scotopic b-wave | 30 Hz Flicker | |
| II-2 | 41 | 20/200 | Central scotoma OU | — | −0.25 + 0.50 × 90 | — | — | — | — |
| | | 20/200 | | | +0.50 | | | | |
| III-1 | 52 | 20/20 | 30° central scotoma OU | 11 Tritan errors OD | +1.50 + 1.50 × 95 | Normal; | Normal; | Slight delay; | NL periphery. |
| | | 20/20 | Full periphery | 5 Tritan errors OS | +1.75 + 0.75 × 80 | 73 μV | 240 μV | 70 μV | 0.6 log unit elevated centrally. |
| III-7 | 40 | 20/30 | 35° central scotoma OU | 6 Tritan errors OD | +1.75 + 1.50 × 90 | Reduced; | Reduced; | Reduced; | NL periphery. |
| | | 20/30 | Full periphery | 4 Tritan errors OS | +1.50 + 1.00 × 95 | 35 μV | 155 μV | 40 μV Delayed | 0.5 log unit elevated centrally. |
| III-9 | 39 | 20/40 | 50° central scotoma OU | 13 Random errors OD | Plano | Very reduced; | Reduced; | Reduced; | NL periphery. |
| | | 20/25 | Full periphery | 9 Tritan errors OS | +0.50 | 10 μV | 150 μV | 30 μV Delayed | |

the crystal structure of engrailed, homeodomain Arg-3 (equivalent to Arg-41 in the CRX protein), which is part of the N-terminal arm, makes contact with the DNA minor groove (Kissinger et al., 1990). In Drosophila melanogaster paired, which is probably more relevant to CRX due to its greater similarity in primary sequence, the crystal structure suggests that Arg-3 is involved in homeodomain dimerization, interacting intermolecularly with the beginning of helix II (Wilson et al., 1995). In addition, the Arg-3 residue (equivalent to CRX Arg-41) also forms a charged hydrogen bond with homeodomain Glu-42 (equivalent to CRX Glu-80) (see Figure 5), which itself is 100% conserved among paired class homeodomains. (4) As predicted based on the paired crystal structure, the recombinant CRX homeodomain containing the Arg41Trp substitution demonstrates decreased binding activity for the *rhodopsin* promoter (Figure 3). (5) Arg41 is also altered in another family, but in this case to a glutamine, which as with the tryptophan substitution represents a change in net charge. (6) The codon 196/7 deletion causes loss of the CRX tail, a potentially important region that is conserved among Otx proteins (Chen et al., 1997). (7) Four of the CORD-associated sequence changes were not identified among normal control chromosomes. It should also be noted that the temporal and spatial expression of the *Crx* gene during mouse retinal development and its biochemical properties (Chen et al., 1997) are consistent with the hypothesis that mutation in the human *CRX* gene could cause both cone and rod degeneration in affected patients. Taken together, the data indicates that the Arg41Trp CRX substitution and possibly several of the other sequence changes are likely to be disease-causing mutations.

In the terminology used clinically, the earliest phenotype in the Arg41Trp family can be described as a macular degeneration involving both cones and rods that evolves into a "cone-rod dystrophy." The three affected siblings had symptoms primarily from central vision loss due to rod and cone disease in the macula. The reduced acuity and color vision implicate a cone disease, while the considerable RPE changes between 20°–40° peripherally in the region of greatest rod density implicate rod involvement. The ERG of both III-7 and III-9 showed widespread cone and rod involvement on initial examination, and progression was documented in III-1 over a 5 year interval. Although several of the abnormal ERG components originate primarily from neural activity postsynaptic to the photoreceptors (Bush and Sieving, 1994, 1996), it is reasonable to presume that rod and cone disease accounts for these ERG changes, including the 30 Hz flicker delay. The electronegative scotopic and photopic responses suggest defective signaling by the depolarizing bipolar cells of the ON visual pathway (Sieving, 1993), but one cannot know whether this results from pathology of the bipolar cells or from deficient photoreceptor signaling. It is, however, surprising that all three siblings demonstrated normal psychophysical dark-adapted sensitivities in the periphery, as this indicates normal photon catch by the peripheral rod photoreceptors and implies that they are in normal or near normal numbers. It also suggests that their outer segments are of normal length and that they have normal ability to couple quantum catch to closure of the

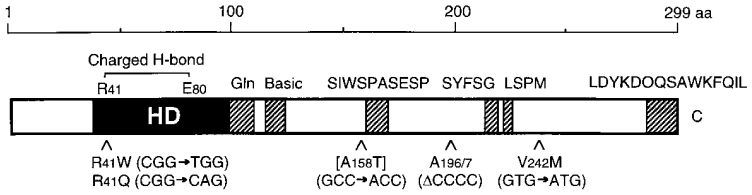


Figure 5. Schematic Diagram Showing the Location of the Predicted Sequence Changes Identified in the *CRX* Gene

The positions of the homeodomain (HD) and other conserved sequences in the protein, representing potentially important regions, are indicated. The Ala158Thr substitution is shown in brackets because it has also been identified in two normal controls. The predicted charged hydrogen bond between Arg-41 and Glu-80 is also shown, since it would be interrupted by the residue 41 substitutions.

G-protein–coupled membrane channels. Overall, the clinical phenotype of this family appears to be less severe than that reported for the original CORDII family (Evans et al., 1995).

The molecular mechanism(s) by which *CRX* mutations cause a dominant phenotype in pedigree #H0992 and perhaps in the other families is not clear. Possible explanations include a dominant negative effect as well as haploinsufficiency, since both over- and underexpression of rhodopsin, one of the targets of CRX activation, can cause photoreceptor degeneration (Olsson et al., 1992; Humphries et al., 1997). For example, since the Arg41Trp mutation causes decreased DNA binding activity, unbound mutant molecules could repress transcription by squelching (Ptashne, 1988). Alternatively, a mutation in the homeodomain could change binding specificity and lead to aberrant CRX binding, or a mutation in the activation domain could create a competitive inhibitor that binds to the appropriate DNA site but no longer activates transcription. Since the CRX and NRL proteins together transactivate *rhodopsin* regulation (Chen et al., 1997), it is also possible that some of the *CRX* alleles alter the interaction of mutant protein with NRL and thereby lower the rate of transcription of the *rhodopsin* gene. Although mutations in the *NRL* gene have not yet been detected (Farjo et al., 1997), it remains a candidate for various retinal degenerations including the CORDs. It is tempting to speculate that certain alleles of *CRX* and *NRL* may also modify the clinical phenotype observed in patients with retinal dystrophies caused by mutations in *rhodopsin* or other photoreceptor-specific genes. As one possibility, alterations in transcription factors that reduce the level of expression of photoreceptor molecules that cause autosomal dominant retinal disease could lead to a less severe phenotype (Olsson et al., 1992) and thereby provide a new avenue for therapeutic intervention.

Several studies have shown that mutations in a single gene can lead to a wide variety of different clinical phenotypes. For example, mutations in the *RDS/peripherin* gene result in RP, CORD, or macular degeneration (Keen et al., 1994; Wroblewski et al., 1994; Nakazawa et al., 1996; Fishman et al., 1997). It, therefore, seems reasonable to hypothesize that mutations in the *CRX* gene might also be associated with retinal diseases other than CORD. Future genetic analyses of other patient populations, as well as mouse knock-out and transgenic studies, will hopefully provide additional insights into CRX's role in vivo, as well as broader understanding of its role in causing human retinal disease.

## Experimental Procedures

### Determination of *CRX* Genomic Structure

A set of human genomic GSI P1 high density filters (Genome Systems) was screened by hybridization with a human *CRX* cDNA probe, and resulting positive clones were ordered and prepared according to the supplier's directions. Dideoxy cycle DNA sequencing (ThermoSequenase, Amersham Life Science) was performed directly on the P1 DNA templates using primers corresponding to the human cDNA sequence to determine the intron–exon borders. Approximately 3 μg of template was used per reaction, according to the supplier's protocol. Sequencing and PCR were used to determine the length of introns.

### Expression of Crx Homeodomain and EMSAs

Recombinant CrxHD–GST and $CrxHD_{Arg41Trp}$–GST were expressed, purified, and tested by EMSA as previously described (Chen et al., 1997). Plasmid *$CrxHD_{Arg41Trp}$–GST* was generated the same as previously described for plasmid *CrxHD–GST* except that the 5′ oligomer used (ACGGATCCAGCGCCCCCAGGAAGCAGCGGTGGG AGCGCACC) encoded the Arg41Trp substitution.

### Clinical Analysis

Pedigree #H0992 was identified and studied at the University of Michigan (by P. A. S.). Clinical evaluation and retinal function testing, including ERG recordings, visual fields testing, dark-adapted absolute threshold sensitivity by Goldmann-Weekers determination, and color vision testing, were performed in standard fashion as previously described (Sieving, 1993).

### Mutation Analysis

Individuals were tested for sequence variations in the *CRX* gene either by direct sequencing (method one) or by SSCP analysis (method two). Informed consent was obtained from participants in the study. DNA was prepared from the blood lymphocytes using standard procedures.

For method one, exons and the flanking intronic sequences were amplified from genomic DNA by PCR using primer pairs (see below) derived from the *CRX* sequence (Chen et al., 1997). The products were directly sequenced without purification, using the Sequenase kit with $^{33}$P-labeled terminators (Amersham Life Science), as described (Fujita et al., 1997).

For method two, SSCP was performed essentially as described (Stone et al., 1997). Following electrophoresis, gels were silver stained as previously described (Bassam et al., 1991). PCR products were sequenced using fluorescent dideoxynucleotides on an Applied Biosystems (ABI) model 373 automated sequencer. All mutations were recognized by the approximately equal peak intensity of two fluorescent dyes at the mutant base.

The primer pairs used were as follows: method one: exon 1, 5′CCT CAGTGTCCCCGAAGA3′/5′TGCCAAGAGAAACGACTGTACT3′; exon 2, 5′GGTCCTGTTTCCCATCCC3′/5′ACCCACCACCCCACCTGAAC3′; exon 3A, 5′TTCCCCACTTACCCACCC3′/5′GGACTGTAGGAATCTG AGATGCC 3′; exon 3B, 5′TCCCCAAGACCCTCCACAG3′/5′AGC TCGGAGACCCATAGGC3′; exon 3C, 5′CCTCCGCTTTCTGCTCTT C3′/5′CCCGATGGAGAGAGATGGAG3′.

Method two: exon 1, 5′GTGTCCCCGAAGATCATGATGGCG3′/5′ CAGAGGTCCTCCAAGAGATGAGGCC3′; exon 2, 5′GGATGGAATC

TTGGTCATCCCAC3′/5′CTCTTTGTTCCGGGCAGGCCTC3′; exon 3A, 5′CCTCTTCCCCACTTACCCA3′/5′CACATCTGTGGAGGGTCTTGG3′; exon 3B, 5′CAAGGCCCGGCCTGCCAAG3′/5′GGCGTAGGTCATGG CATAGG3′; exon 3C, 5′AGGGCCGTCTCTGACCTCC3′/5′ATAGCTC TGGCCTGATAGGGA3′; exon 3D, 5′TCCTATTTCAGCGGCCTAG A3′/5′GAATTCCAAGCTATCCACGG3′; exon 3E, 5′TCCCTATCAGG CCAGAGCTAT3′/5′AGAGAGATGGAGACTGCGTCC3′.

Lod score analysis was peformed using M-LINK, part of the LINKAGE program developed by Dr. Jurg Ott.

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**Note Added in Proof**

Mutations in *CRX* associated with CORD were also independently identified by Freund et al., Cell *91*, 543–553 (1997). Interestingly, one of the two changes they identified was a substitution in Glu-80, which is the postulated hydrogen-bonding partner of Arg-41 (see Discussion).