

Functional analysis of cone–rod homeobox (CRX) mutations associated with retinal dystrophy

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Received December 3, 2001; Revised and Accepted February 8, 2002

Mutations in the photoreceptor transcription factor cone–rod homeobox (CRX) have been identified in patients with several forms of retinal degenerative disease. To investigate the mechanisms by which these mutations cause photoreceptor degeneration, CRX constructs representing eleven known mutations, as well as a set of C-terminal deletions, were generated and tested for their ability to activate a rhodopsin–luciferase reporter in a transient cell transfection assay. To further define functional domains, several Gal4dbd–Crx fusions were similarly tested using a Gal4 response element containing heterologous promoter. This analysis demonstrated that the C-terminal region, between amino acids 200 and 284, is essential for CRX-mediated transcriptional activation. Consistent with this, four mutants carrying C-terminal truncations demonstrated significantly reduced transcriptional activation. Confirming the importance of the homeodomain (HD), four of the five mutants carrying HD missense mutations displayed altered transactivating activity, either decreased (three) or increased (one). *In vitro* protein–DNA binding assays (EMSAs) with CRX–HD peptides representing the three HD mutants with decreased transactivating activity, indicated that the alteration was due to reduced, but not abolished, DNA binding to CRX targets. Taken together, these results support the hypothesis that CRX mutations involved in human photoreceptor degeneration act by impairing CRX-mediated transcriptional regulation of the photoreceptor genes. However, a clear relationship between the magnitude of biochemical abnormality and degree of disease severity was not observed, suggesting that other genetic and environmental modifiers may also contribute to the disease phenotype.

INTRODUCTION

Cone–rod homeobox (CRX) is an otd/Otx-like homeodomain transcription factor that is predominantly expressed in the rod and cone photoreceptors of the retina (1,2). The CRX protein consists of 299 amino acids and is highly conserved among different mammalian species. It contains an otd/Otx-like paired homeodomain (HD) near the N-terminus followed by glutamine rich (Gln), basic, WSP, and Otx-tail domains that share homology with Otx1 and Otx2 (Fig. 1). It is known that the HD of CRX is responsible for the DNA-binding and nuclear localization of the CRX protein (1,3), while the C-terminal region, including the WSP and Otx-tail domains, is involved in transcriptional activation (4). *In vitro* protein–DNA binding assays and transient cell transfection studies in HEK293 cells demonstrated that CRX binds to and activates the promoters of

a number of photoreceptor-specific genes, including rhodopsin, β -phosphodiesterase (PDE), arrestin (SAG), and interphotoreceptor retinoid-binding protein (IRBP) (1). CRX also exerts its role in regulating photoreceptor gene expression by interacting with the neural retina leucine zipper protein (NRL), an AP-1 like transcription factor expressed in rod photoreceptor cells (5). CRX and NRL form a physical interaction via their DNA-binding domains (6), and transactivate the rhodopsin promoter in a synergistic fashion (1). Demonstrating the importance of CRX function *in vivo*, mutant mice that are homozygous for a null *Crx* allele (*crx*^{−/−}) do not develop functional photoreceptor outer segments and undergo a slow retinal degeneration (7). Microarray and Northern analyses of the *crx*^{−/−} mouse retina revealed reduced or lost expression of many photoreceptor-specific genes before the onset of degeneration,

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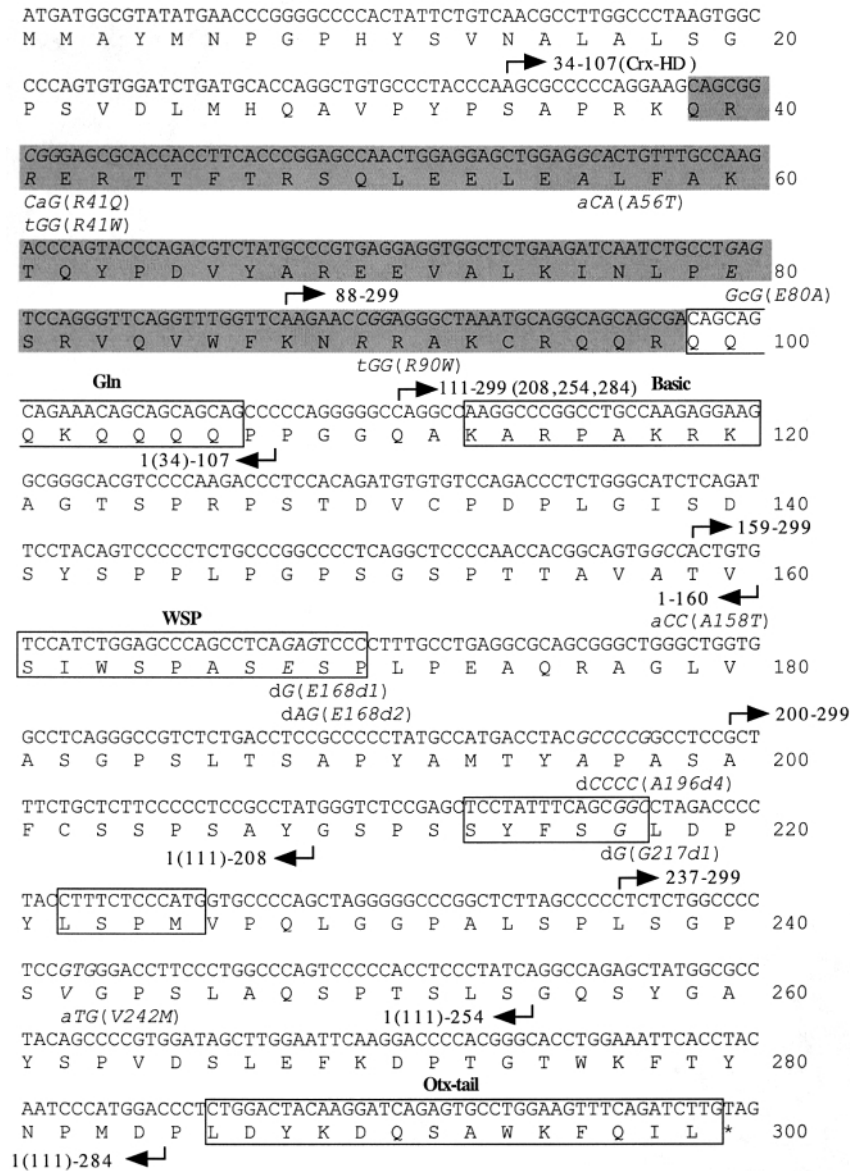


Figure 1. CRX mutations and deletions. Nucleotide and amino acid sequences of human CRX are shown to indicate sequence changes for eleven CRX variants and the corresponding N- or C-terminal end position of each bovine Crx deletion described in the text. The homeodomain is marked by the shaded box. All other domains that share homology with Otx1 and Otx2 are labeled by the open boxes. Changed codons are labeled by italicized letters for the wild-type and small letters (missense mutations) or d (deletions)/ i (insertions) for the mutants. Arrows pointing to the right represent N-terminal deletions, while those pointing to the left represent C-terminal deletions. Numbers adjacent to each arrow represent the N- and C-terminal position in amino acids for each deletion.

suggesting that CRX, either directly or indirectly, is a significant regulator of photoreceptor gene expression (8). The human CRX gene maps to chromosome 19q13.3, the site of a disease locus for an autosomal dominant cone-rod dystrophy (CORD2) (1,9). Genetic studies have demonstrated that CRX mutations are associated not only with CORD (9-14), but also Leber congenital amaurosis (LCA), an early-onset severe form of retinal dystrophy (12,15-19). So far, genetic screens of patients with retinal dystrophy have identified eighteen variants of CRX (Fig. 4A). Of these variants, half contain base pair insertions or deletions that lead to either a null

allele of CRX (P9i1) or C-terminal truncated forms of CRX. The other half represent single amino acid changes, including five changes in the HD. Two of the variants, A158T and V242M, were found in some normal individuals, and therefore presumably represent rare benign variants (13,16). Although our understanding of CRX genetics is clearly increasing, many questions remain. Why are CRX mutations associated with different forms of retinal dystrophy and with such variable age of onset? What is the molecular mechanism by which mutated forms of CRX lead to photoreceptor degeneration? Is there a correlation between the disease

severity and the degree of functional abnormality of the mutants? Several studies have begun to address these issues by providing initial characterization of individual CRX mutations. In vitro protein-DNA binding studies with the R41W and R90W CRX mutants showed that these sequence changes lead to a significant reduction in CRX DNA-binding activity (13,18). The same two mutations also reduce the ability of CRX to interact with NRL (6). In addition, it has been reported that a 12 bp deletion mutant, L146d12, reduces CRX's ability to activate the rhodopsin promoter (20). However, systematic analyses of the many genetically identified CRX mutations have not been reported. In this paper, we present in vitro protein-DNA binding and transient cell transfection data on eleven of the CRX mutants, including the previously characterized R41W and R90W alleles. We demonstrate that all of the CRX variants that co-segregate with a disease are altered, to varying degrees, in their ability to regulate rhodopsin promoter activity. Using deletion and heterologous promoter analysis, we also provide evidence that the C-terminal portion of CRX (amino acid residues 200–284) is important for CRX-dependent transcriptional activation. Consistent with this, all C-terminal truncation mutants showed reduced trans-activating activity. However, comparison of the biochemical data with available clinical information does not demonstrate a simple relationship between functional activity and severity of clinical phenotype.

RESULTS

The CRX C-terminal region, between amino acids 200 and 284, is important for transcriptional activation

In order to provide a basis for interpreting the data with the human mutations, efforts were first made to map the CRX regions critical for transcriptional activation using transient transfection assays in HEK293 cells. Two approaches were used. In the first, various C- and N-terminal portions of bovine Crx were fused to the Gal4 DNA-binding domain (Gal4dbd). The ability of these Gal4dbd-Crx fusions to activate a luciferase reporter, pFA-luc (Stratagene) that contains five Gal4 binding sites upstream of a minimal promoter was then tested. This heterologous promoter system allowed measurement of the transactivation activity of the various pieces of CRX regardless of the presence or absence of CRX's DNA binding domain (HD). As expected, fusion constructs containing the N-terminal portion of Crx, including Gal4dbd-Crx1–107, Gal4dbd-Crx34–107 and Gal4dbd-Crx1–160 did not produce any detectable transactivation activity compared to the Gal4dbd empty vector (data not shown), while a fusion construct that contains the C-terminal portion of Crx between the residues 111–299 (Gal4dbd-Crx111–299) resulted in a dramatic increase in reporter activity by more than 1000-fold (1255-fold in average). This result confirms the previous

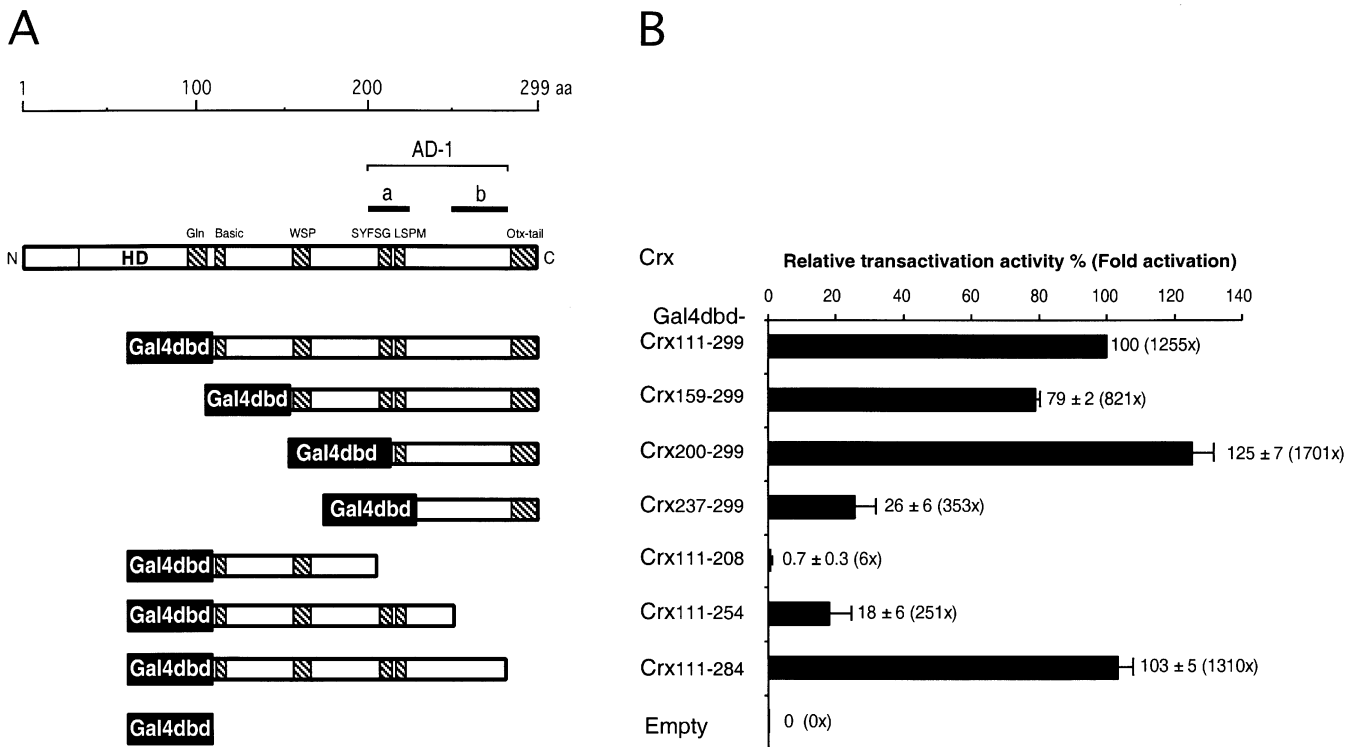


Figure 2. Transient transfection assays with Gal4dbd-Crx fusions and a Gal4-responsive luciferase reporter. (A) Diagram of CRX and Gal4dbd-Crx constructs carrying various C-terminal portion of bovine Crx (residues 111–299) fused to the Gal4 DNA-binding domain (Gal4dbd, amino acid residues 1–174) in the pFA-CMV vector (Stratagene). (B) Transient transfection assays. HEK293 cells cultured on 6-well plates were transfected with 100 ng of each fusion construct shown in (A) and 2 μ g of the pFR-Luc reporter (Stratagene). Fold activation was calculated relative to the luciferase units (after normalization with the internal control) obtained from the empty vector pFA-CMV (0-fold). The results are presented as mean values of transactivation activity (fold activation) relative to that obtained for Gal4dbd-Crx111–299 (100%). The error bars represent standard error of mean (SEM) from four independent experiments.

observation, using a rhodopsin-luciferase reporter, that the transactivation domain of CRX is located in a region C-terminal to the homeodomain (HD) (4). More importantly, this three-magnitude transactivation provides a reliable measurement for mapping the CRX activation domain (AD). The results of such an analysis, with the data shown relative to the Gal4dbd-Crx111-299 fragment, are presented in Figure 2. Deletion of up to 199 N-terminal amino acid residues (see constructs Gal4dbd-Crx159-299 and Gal4dbd-Crx200-299) did not lead to a significant change in transactivation activity compared to that obtained with Gal4dbd-Crx111-299 ($P = 0.068$ and 0.072 , respectively). However, removing another 37 N-terminal amino acids (Gal4dbd-Crx237-299 compared to Gal4dbd-Crx200-299) led to a 75% decrease in transactivation activity ($P < 0.005$), indicating that the region between amino acids 200-237, designated as 'a', is important for transactivation. Similarly, deletion of the C-terminal region from 285-299 (Gal4dbd-Crx111-284), which includes the conserved Otx-tail, had little effect on transactivation activity ($P = 0.8349$), but deletion of 30 additional amino acids (Gal4dbd-Crx111-254) led to a 82% decrease in activity ($P < 0.003$). This suggests that the region adjacent to the Otx-tail (residues 255-284), designated as 'b', is important for transactivation. A significant change in activity was also observed with fusion construct Gal4dbd-Crx111-208 as compared to Gal4dbd-Crx111-254 ($P = 0.02$), suggesting that

there are functionally important sequences throughout the 200-284 region (designated as AD-1).

The heterologous promoter analysis reported here suggests a model for the transactivation domain(s) of CRX that is slightly different from that suggested by previous studies (4). This prior work, which was based on transient transfections with constructs that utilized the CRX homeodomain for providing DNA binding activity (as opposed to the heterologous Gal4dbd), implicated the Otx-tail and WSP domains as being responsible for significant transcriptional activity. In order to address this apparent discrepancy, we decided to re-analyze a series of Crx C- and N-terminal deletions for their ability to activate a rhodopsin promoter-luciferase reporter, pBR130-luc, either on their own or in combination with NRL. As previously observed, wild-type Crx activated reporter activity 3-6 fold on its own, and 60-90 fold in cooperation with NRL (data not shown). The transactivation activity of each C-terminal deletion of Crx relative to that of the wild-type is shown in Figure 3. Consistent with the heterologous promoter analysis, removal of the Otx-tail at the C-terminus (Crx1-284) did not significantly reduce Crx-dependent transactivating activity with either Crx alone (88% of the wild-type level, $P = 0.35$) or with Crx plus NRL (82% of the wild-type level, $P = 0.67$). In contrast, deletion of 45 residues at the C-terminus (Crx1-254) resulted in a dramatic decrease of activity to 37% (Crx alone; $P = 0.00003$) and 43% (Crx plus NRL; $P = 0.001$)

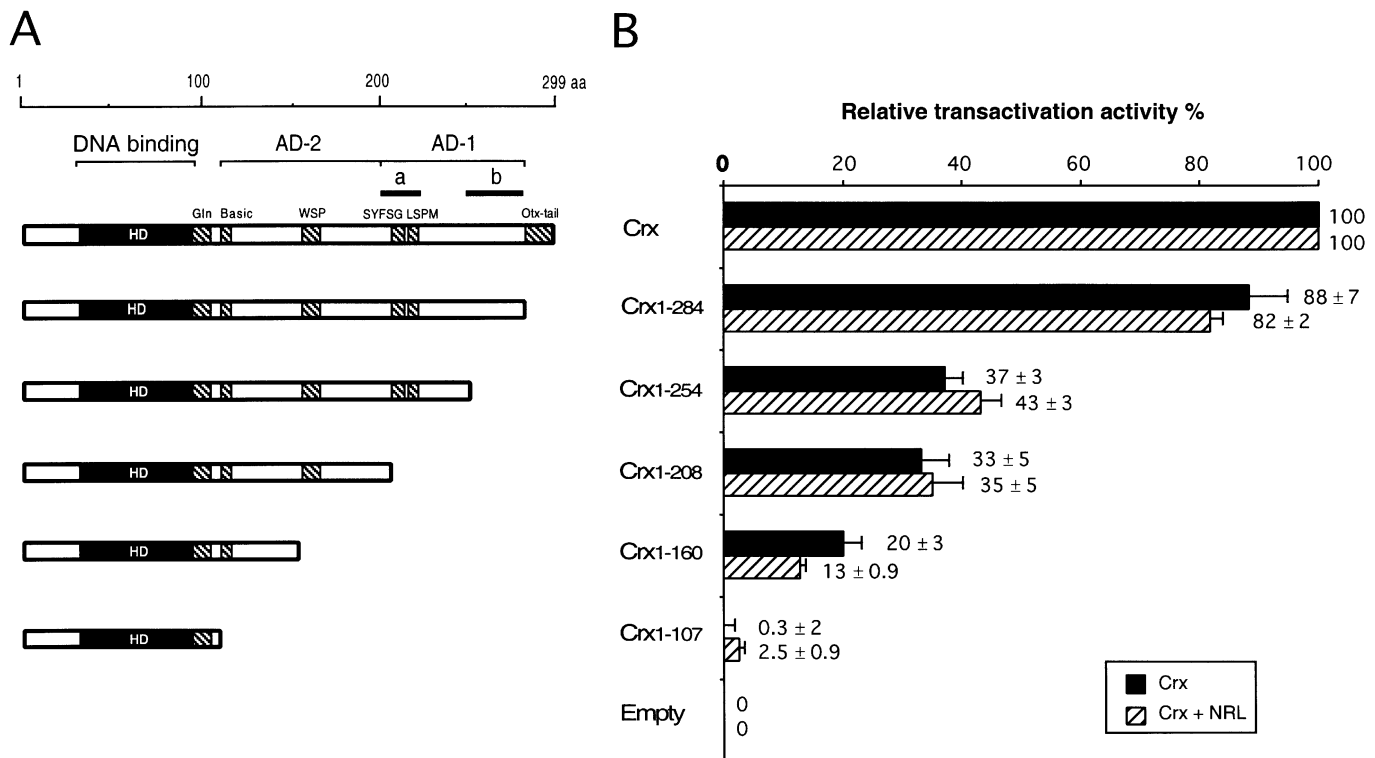


Figure 3. Transient transfection assays with C-terminal deletions of Crx and a rhodopsin promoter-luciferase reporter. (A) Schematic representation of the bovine Crx expression constructs carrying serial C-terminal deletions in the pcDNA3.1/HisC vector (Invitrogen). (B) Transient transfection assays: HEK293 cells cultured on 6-well plates were co-transfected with 100 ng of each Crx deletion construct shown in (A) in the absence or presence of 100 ng of pMT-NRL, and 2 μ g of the rhodopsin-luciferase reporter pBR130-luc. Fold activation is calculated relative to the luciferase value from the empty vector pcDNA3.1/HisC. The mean values of relative transactivation activity are presented by black (Crx alone) and hatched bars (Crx plus NRL) compared to that of the full-length Crx (100%). Error bars represent SEM from four independent experiments.

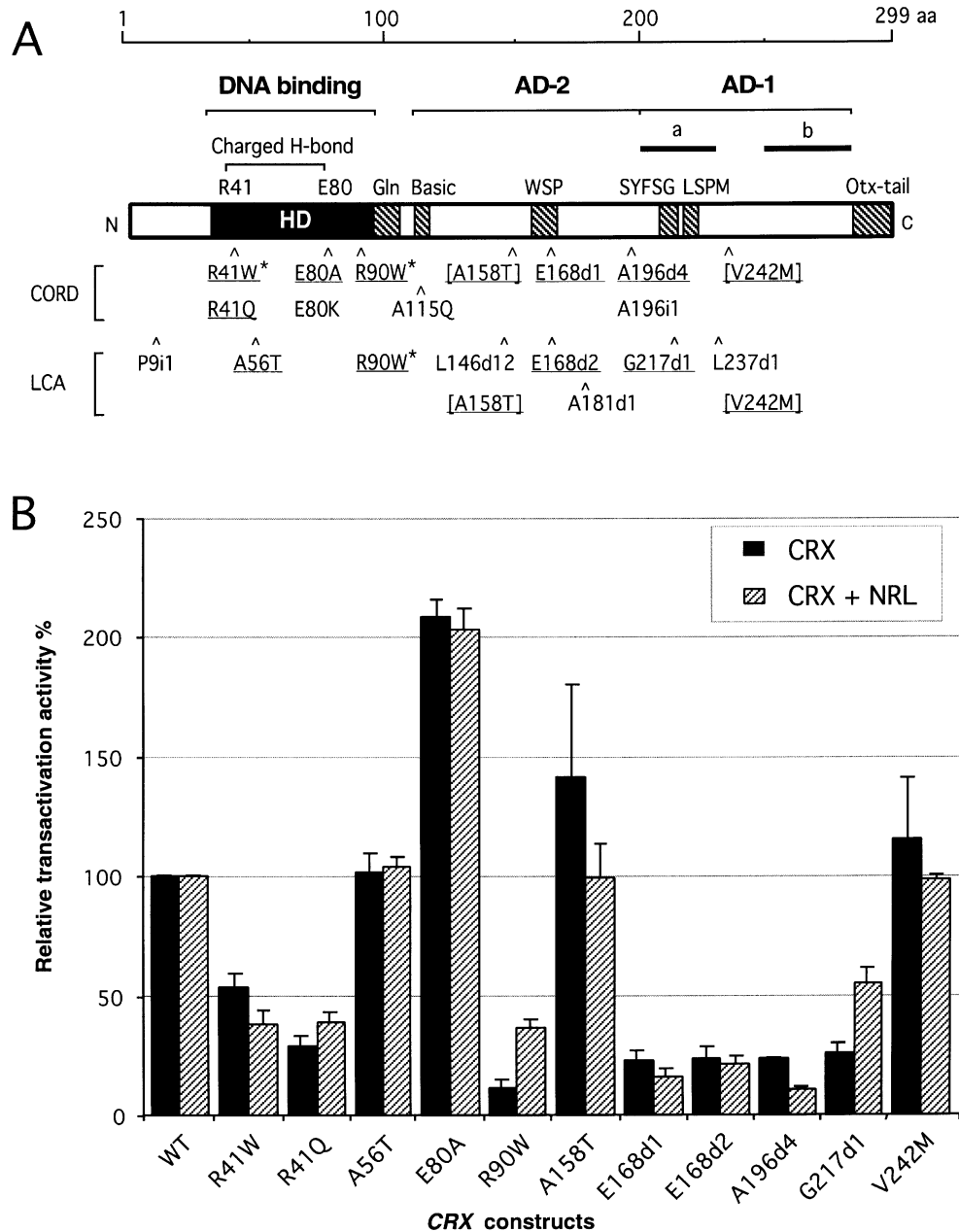


Figure 4. Transient transfection assays with CRX constructs harboring genetically identified mutations. (A) Schematic diagram showing the location of human CRX mutations. Top panel represents the CRX protein and various domains (filled blocks) that are homologous to regions in Otx1 and Otx2. Letters and numbers below represent mutations identified by genetic studies of patients with autosomal dominant cone rod dystrophy (CORD) and Leber congenital amaurosis (LCA): Numbers indicate positions (in amino acids) of the mutations; capital letters represent amino acid residues; d and i represent deletions and insertions in base pairs, respectively. Mutations underlined represent those that have been analyzed for functional defects described in this manuscript, including the two (labeled with *) reported previously. A158T and V242M are shown in brackets because they have been also found in normal individuals. (B) Transient transfection assays: HEK293 cells cultured on 100 mm plates were transiently transfected with 1 μ g of the indicated CRX expression vector, either alone or in combination with 1 μ g of pMT-NRL, and 5 μ g of the rhodopsin luciferase reporter pBR130-luc. The relative transactivation activities (from three independent experiments) were calculated and presented as described in Figure 3.

of the wild-type level. To verify these results, the same experiment was carried out using another rhodopsin-luciferase reporter, pBR250-luc, that contains three CRX binding sites (Ret-1, BAT-1 and Ret-4) instead of the two in pBR130-luc (BAT-1 and Ret-4). We also tested three different DNA preparations for each Crx deletion construct. In all the cases,

the same results were maintained (data not shown), suggesting the sequences adjacent to the Otx-tail, between amino acid residue 254–284, play a major role in CRX-mediated transcriptional activation of the rhodopsin promoter.

Consistent with the previous report (4), a second region contributing to CRX transcriptional activity is located between

amino acid residues 107–208, designated as AD-2. This region contains the WSP domain and flanking sequences. As shown in Figure 3, significant differences in the activity were observed for Crx1–107 versus Crx1–160 ($P = 0.0006$ for Crx alone and 0.0013 for Crx + NRL), and Crx1–160 versus Crx1–208 ($P = 0.032$ for Crx, and 0.014 for Crx + NRL). However, AD-2 may play a more minor role compared with AD-1 (between 200–284) because in the heterologous promoter analysis the sequence between 111–208 only stimulated reporter activity by 6-fold in average (less than 1% of the activity observed with the entire 111–299 region). In addition, all the constructs carrying N-terminal truncated forms of Crx that lack the functional HD (Crx88–299, Crx111–299, Crx160–299 and Crx200–299, Crx237–299), as well as the construct containing only the HD and its flanking Q-rich region (Crx34–107), failed to show any transactivation activity on their own or in combination with NRL (data not shown). This is consistent with the previous observation that the CRX HD is necessary and sufficient for binding to the CRX targets, and suggests that both the homeodomain and the activation domain(s) of CRX are required for CRX to function either independently or in synergy with NRL.

Taken together, the deletion and heterologous promoter analyses suggest that multiple regions in the C-terminal portion of CRX contribute to CRX's transactivating activity. AD-1, located in the region 200–284, contains two sub-regions, 'a' (200–237) and 'b' (255–284), and plays a major role in transactivation. In contrast, AD-2, located between amino acids 107–208, plays a more minor role in transactivation.

The ability of CRX to activate the rhodopsin promoter is altered by disease-associated mutations

To determine how the CRX mutations identified in human disease alter CRX function, we measured the ability of eleven CRX mutants to activate the rhodopsin promoter either on their own or in combination with NRL, using co-transfection assays in HEK293 cells. The eleven mutations shown in Figure 1 (also in Fig. 4A, underlined) were generated by site-directed mutagenesis. The resulting mutant constructs were tested with the bovine rhodopsin promoter–luciferase reporter pBR130-luc. Fold activation was calculated for wild-type CRX and each of the mutants compared to samples that received only the empty expression vector, and then these values were converted into 'relative transactivation activity' using the fold stimulation of the wild-type construct as 100% (Fig. 4B). Of the eleven mutants tested, seven (R41W, R41Q, R90W, E168d1, E168d2, A196d4, and G217d1) showed a significant reduction (11–55% of wild-type level; $P < 0.03$), while one (E80A) showed a significant increase, in CRX's ability to activate the rhodopsin promoter either alone or in combination with NRL. Four of the seven defective mutants carry base pair insertions or deletions that result in C-terminal truncated forms of CRX. This is consistent with the observation described earlier that the C-terminal region 200–284 of CRX is critical for transactivation. The three other mutants with reduced transactivating activity (R41W, R41Q, and R90W) carry missense mutations in the homeodomain. Interestingly, E80A, one of the HD missense mutants, showed about a 2-fold increase in transactivation activity (208%, $P < 0.0000001$ and 203%, $P < 0.0005$, respec-

tively, for CRX and CRX plus NRL). In contrast, three out of the eleven mutants tested (A56T, A158T, and V242M) did not show any significant change in CRX activity ($P > 0.05$). This finding, together with the observation that none of the three have been reported to co-segregate with disease and that two of them (A158T and V242M) have been reported in normal individuals (13,16), strongly suggests that A56T, V242M and A158T are not disease-causing mutations.

The DNA binding activity of CRX is altered by three of the homeodomain mutations

Since it has been established that the CRX homeodomain is necessary and sufficient for binding to CRX targets (1,4), it seemed likely that at least some of the mutations located in the homeodomain would result in defects in DNA binding activity. To test this possibility, electrophoretic mobility shift assays (EMSAs) were carried out using three CRX target sites in the rhodopsin promoter as probes and purified CRX-HD peptides (amino acids 34–107, including HD and flanking sequences) containing each of the five HD mutations. Previous EMSA studies already demonstrated that GST-tagged CRX-HD proteins carrying R41W and R90W are defective in DNA binding (13,18). As the GST-tag is large in size and can form homodimers, to avoid any unexpected influence from the tag, we decided to use untagged CRX-HD peptides for quantitative measurements of the three untested (R41Q, A56T and E80A) and two previously tested (R41W and R90W) mutants. To accomplish this, GST-tagged CRX-HD proteins were expressed and purified (Fig. 5A), and then equal amounts of each protein were treated with an excess amount of thrombin to remove the GST tag. After complete digestion was confirmed by SDS-PAGE (data not shown), the resulting untagged CRX-HD peptides were analyzed by EMSAs (Figs 5B–D).

With relatively large amounts of purified protein (≥ 3 ng), wild-type CRX-HD peptide produced two shifted bands with the BAT-1 probe, which contains two potential CRX-binding sites (Fig. 5B). Based on separate experiments with mutated BAT-1 probes containing a single CRX site (data not shown), it appears that the lower band represents binding activity from a single CRX-HD molecule (monomer), while the higher band represents the binding from two CRX-HD peptides (dimer) to the same probe. Compared to wild-type CRX-HD peptide, the three mutant peptides R41W, R41Q, and R90W showed significantly reduced DNA-binding activity. Dimeric binding to the BAT-1 probe was not observed with these mutants even when using protein amounts as high as 6 ng, and higher amounts of protein were required to produce observable monomeric binding—6 ng, 6 ng, 3 ng, and 0.75 ng for R41W, R90W, R41Q and wild-type, respectively. In contrast, two other HD mutants, E80A and A56T, did not show detectable defects in binding to the BAT-1 probe. Similar results were obtained with Ret-4 and Ret-1 probes (Figs 5C and D), respectively, though these two probes contain only a single, low affinity CRX binding site. To more accurately assess the relative binding behavior, shifted bands were quantitated by phosphor-imager analysis (Fig. 5E). Overall, the severity of the binding defects can be ranked R41W > R90W > R41Q, with E80A and A56T behaving similarly to wild-type.

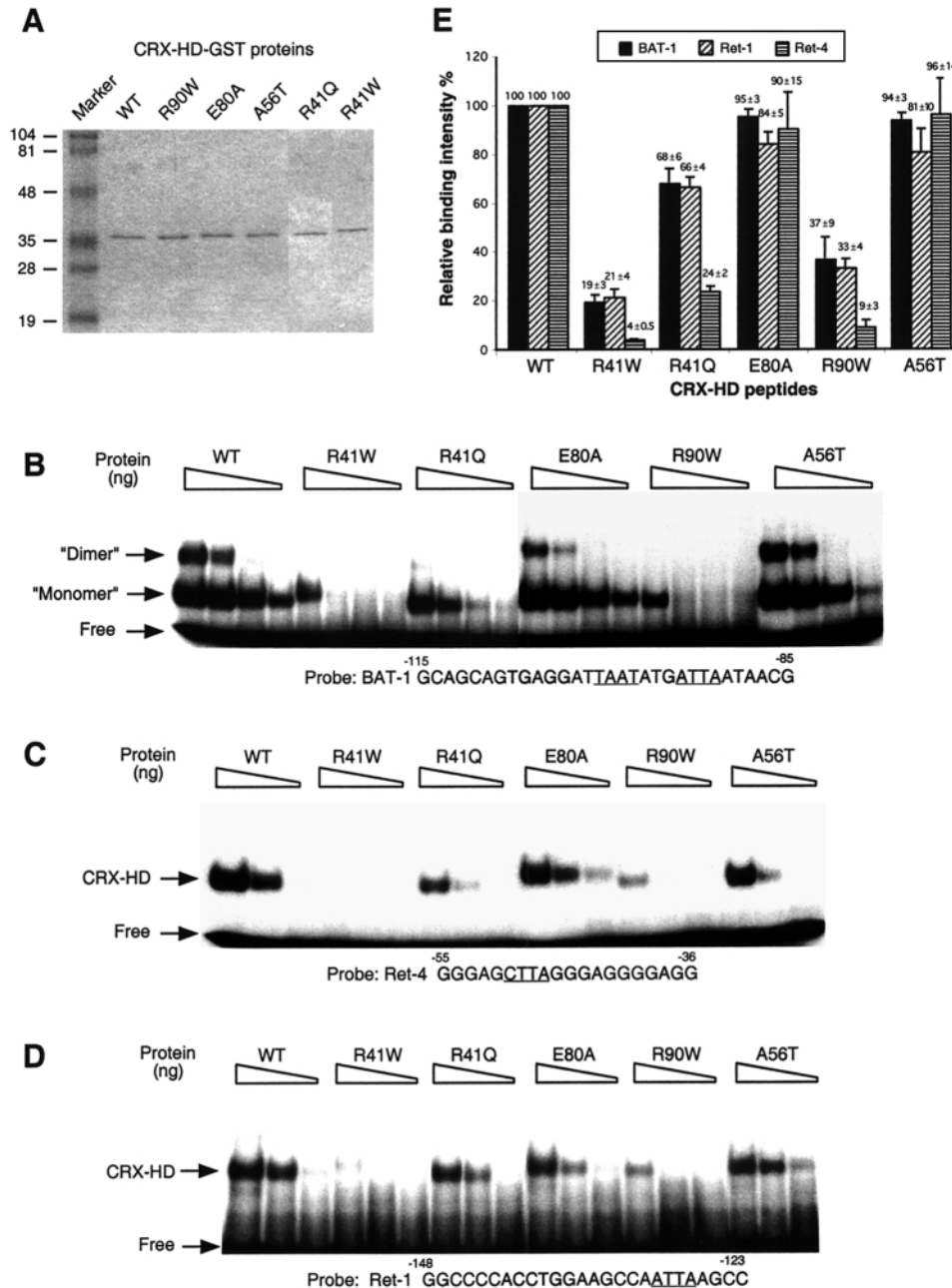


Figure 5. In vitro protein–DNA binding assays with purified CRX-HD peptides carrying five HD mutations. (A) A SDS–PAGE demonstrating purification of six GST-tagged CRX-HD proteins. Each CRX-HD–GST protein was expressed in BL21 *E. coli* cells and purified using glutathionyl-agarose beads (Pharmacia). 100 ng of each purified CRX-HD–GST protein was analyzed by 11% SDS-PAGE. The left lane (Marker) was loaded with pre-stained protein markers (BioRad), with the apparent molecular weight (kilodaltons) shown on the left. (B–D) EMSAs using purified CRX-HD peptides and 32 P-labeled probes containing rhodopsin promoter elements. The probes were BAT-1 for (B), Ret-4 for (C), and Ret-1 for (D). The sequence of each probe is shown at the bottom of each panel. The GST tag of each CRX-HD–GST protein was removed prior to the EMSA reactions. The amount of the HD peptide used for each set was approximately 6, 3, 1.5, and 0.75 ng for (B), 9, 3, and 0.9 ng for (C), and 10, 2.5, and 0.63 ng for (D), respectively. (E) Phosphorimager analyses of the EMSA results. The intensity of the shifted band at the highest protein concentration in each set shown in (B–D) was measured and analyzed by using the Storm 860 PhosphorImager System (BioRad). For panel (B), the 'monomeric' band was used for quantification. The results are presented as mean intensity relative to that of the wild-type HD peptide (100%). The error bars represent SEM from three independent measurements.

Nuclear localization of CRX is not affected by the five homeodomain mutations

Since the homeodomain of CRX is known to be involved in the nuclear localization of CRX, we examined mutant forms of

CRX carrying each of the five HD mutations for their subcellular location in transfected HEK293 cells using immunocytochemistry with a polyclonal antibody to CRX (21). As observed with the wild-type CRX protein, all five HD

Table 1. Biochemical defects and clinical phenotypes associated with eleven CRX mutations

Mutations	Biochemical Defects (% WT)					Clinical Phenotypes			References
	Transactivation		DNA-binding			Disease type ^a	Onset (years)	Co-segregation	
	CRX	CRX + NRL	BAT-1	Ret-1	Ret-4				
R41W	53	38	19	21	4	adCORD	~40	+	13
R41Q	29	39	68	66	24	adCORD	50–60	+	12–14
A56T	102	104	94	81	96	adLCA	0–10	ND	16
E80A	208	203	95	84	90	adCORD	10–20	+	9,12
R90W	11	37	37	33	9	adCORD/arLCA	~40/0–10	+	18
(A158T)	142	100	ND	ND	ND	adCORD/adLCA	late/0–10	– ^b	13,16
E168d1	23	16	ND	ND	ND	adCORD	10–20	+	10
E168d2	24	22	ND	ND	ND	adLCA	0–10	de novo	15
A196d4	24	11	ND	ND	ND	adCORD	adult	ND	13
G217d1	26	55	ND	ND	ND	adLCA	0–10	de novo	15
(V242M)	119	98	ND	ND	ND	adCORD/adLCA	late/0–10	– ^b	13

^aad – autosomal dominant, ar – autosomal recessive.

^bThe sequence changes have been found in normal individuals.

mutants are localized to the nucleus of transfected cells (data not shown), suggesting these HD mutations do not have a detectable effect on the nuclear localization of CRX. We also analyzed the expression of Crx deletion constructs using an antibody to Xpress, the N-terminal epitope tag for recombinant Crx (data not shown). Consistent with a previous study using GFP–CRX fusion proteins (3), we found that the region between amino acid residues 35 and 107 is required for CRX nuclear localization.

Lack of correlation between disease severity and in vitro functional activity

To assess if there is a correlation between disease severity and the degree of biochemical abnormality associated with CRX mutations, we combined the data from the above functional analysis with reported clinical phenotypes as shown in Table 1. No simple qualitative nor quantitative relationship between the disease phenotype and the degree of biochemical abnormality was evident. As one example of this lack of correlation, both E168d2 and E168d1 demonstrate a similar degree of biochemical abnormality, yet E168d1 causes CORD and E168d2 causes LCA, a much more severe disease.

DISCUSSION

Multiple transactivation domains are located in the C-terminal portion of CRX

We have used two different transient transfection systems to map the transactivation domain(s) of CRX. One system, which utilizes the rhodopsin promoter, has the advantage that it utilizes a biologically relevant template together with CRX's endogenous DNA-binding domain. A potential concern with this approach, however, is that induced deletions/mutations in CRX could alter transactivating activity indirectly by altering DNA binding affinity. To address this concern, the Gal4dbd–Crx fusion system provides heterologous DNA binding sites

and a corresponding heterologous DNA-binding domain to help separate DNA-binding from transactivating activity. Both approaches yielded essentially the same result, suggesting that the major transactivation domain(s) is located between amino acid residues 200–284 (AD-1). Interestingly, during the process of establishing conditions for carrying out a two-hybrid screen for Crx interacting partners, the same transactivation domain was identified in yeast (Xu and Chen, unpublished data).

This finding that the same activation domain is functional in yeast and mammalian cells suggests that the transactivation mechanism utilized by CRX is evolutionarily highly conserved. As with other transcription activators, this mechanism could involve interactions with general transcription factors and co-activators in the basal transcriptional machinery (22,23), antagonizing general repressors and/or opening chromatin configuration (24–26). Consistent with this, a general transcription co-activator protein, P300/CBP, has been shown to interact with CRX and potentiate CRX transactivation function in mammalian cells (27). The CRX region(s) that interacts with P300/CBP is located, at least in part, within the CRX AD-1 (27). However, it should be kept in mind that the transfection assays described here were performed in HEK293, a non-photoreceptor cell line. Since CRX is mainly a photoreceptor transcription factor, a unique cell-type specific mechanism, such as interaction with a photoreceptor-specific co-activator, could be involved in CRX-mediated transactivation in vivo. An example of such a cell-type specific interaction is the cooperation between CRX and NRL (1). Furthermore, the activity, and even the structure and interfaces involved, of the CRX-AD may vary among different promoters. For example, consistent with previous data (4), the WSP domain and its N-terminal flanking region (AD-2) appear to contribute to CRX's ability to activate the rhodopsin promoter either on its own or in cooperation with NRL. However this region plays only a minimal, if any, role in activating Gal4 responsive promoter when fused with Gal4 DNA-binding domain. Thus, the role of WSP domain might be specific to the rhodopsin promoter and perhaps a subset of other promoters. Transfection analysis utilizing primary retinal cultures from Crx null mice,

or transgenic mouse studies in the $crx^{-/-}$ background, would provide a more desirable way to approach these issues. Unfortunately, however, the ability to perform such studies is limited by the difficulty in efficiently transfecting primary retinal cells and the difficulty of assessing limited quantitative effects in transgenic mice due to the noise contributed by potentially large transgene integration site (position) effects.

The Crx deletion and fusion protein results indicate that the region flanking the Otx-tail, but not the tail per se, is important for CRX transactivation function. This conclusion differs from previously published data that suggested that the tail itself was functionally important (4). The explanation for this discrepancy is not clear. It is unlikely to be due to gross differences in the expression levels of the Crx deletions as, consistent with the previous study, immunocytochemical analysis of the transfected 293 cells (utilizing the Xpress epitope tag on the recombinant Crx) demonstrated that all the C-terminal deletion mutants shown in Figure 3 were appropriately expressed and localized to the nucleus (data not shown). There were some technical differences between the studies. Different methods for cell transfection (LipofectAmine by Chau et al. versus calcium phosphate in this study) and different internal controls for transfection efficiency (CAT assays by Chau et al. versus dual luciferase assays in this study) were used. This may affect the assays, as we have observed better linearity with calcium phosphate than with liposome-based transfection methods. Future studies, perhaps using missense mutations within the tail rather than deletions, will be required to resolve this issue.

Mechanism(s) by which CRX mutations cause photoreceptor degeneration

The finding that all of the CRX mutations tested that are known to co-segregate with retinal disease result in detectable defects in the ability of CRX to bind or/and activate the rhodopsin promoter is consistent with prior data and lends further support to the hypothesis that disease-causing CRX mutations act by interfering with the normal control of photoreceptor gene expression. Interestingly, although most of the mutants were associated with decreased transcription factor function, one of the mutants (E80A) demonstrated increased trans activating activity. Although E80 is part of the homeodomain, since the mutant protein appears to both bind DNA and be transported to the nucleus normally, this finding suggests the interesting possibility that the altered homeodomain might directly or indirectly affect one of the transactivating domains. This finding with E80A is reminiscent of the retinitis pigmentosa-associated NRL mutation S50T, which also shows increased activity (28), and suggests the not unlikely scenario that normal retinal function requires a careful balance of gene expression and that overexpression as well as underexpression can lead to retinal degeneration. At least in the case of rhodopsin, overexpression as well as underexpression have in fact both been shown to cause photoreceptor degeneration (29,30).

The finding that mutation of one allele of the CRX gene is sufficient to cause photoreceptor degeneration raises the question of whether the cell death involves a haplo-insufficiency or a dominant negative mechanism, or perhaps components of both. The report that heterozygous mice carrying one null Crx allele ($Crx^{+/-}$) demonstrated normal

photoreceptor function at six months of age appears to argue against the haplo-insufficiency model (7). However, defects in photoreceptor function were observed with the $Crx^{+/-}$ retina at earlier ages (1–2 months old; perhaps, due to a delayed development of photoreceptor function) and it has not been reported whether older $Crx^{+/-}$ mice develop a photoreceptor degeneration phenotype. Furthermore, human mutant CRX alleles have not yet been tested in the background of the Crx null mouse. Therefore, although informative, the mouse studies do not at this point provide definitive information about the genetic mechanisms involved.

In order to further explore these issues, we performed two types of in vitro analysis. First, EMSAs were carried out to test if the presence of a mutant CRX-HD peptide, R41Q, or R41W, could affect the binding of the wild-type CRX-HD to the BAT-1 probe. No detectable effect on wild-type CRX-HD binding was observed in the presence of these mutant peptides (Chen and Zack, unpublished data), suggesting that these HD mutations do not have a dominant-negative effect on the CRX DNA-binding activity. Second, transient transfection assays were performed to test if a mutant form of CRX could affect the transactivation activity of the wild-type CRX when HEK293 cells were co-transfected with an equal amount of the two expression vectors. None of the seven mutants tested, including four HD mutants (R41W, R41Q, E80A and R90W), and three C-terminal truncation mutants (E168d1, E168d2 and A196d4), demonstrated a significant dominant-negative effect on the transactivation activity of the wild-type CRX (Li and Chen, unpublished data). However, it should be noted that, given the complexity of the in vivo situation, these in vitro results do not preclude the possibility of a dominant negative component. Future studies employing knock-in mice carrying the various mutated CRX alleles may help in better understanding these issues.

Possible explanations for the lack of phenotype–genotype correlation

We compared the clinical phenotype of the patients in this study with the biochemical phenotype of their mutant proteins to test the hypothesis that the mutations associated with more severe biochemical abnormalities would be associated with more severe clinical phenotypes. Unfortunately, however, no clear phenotype–genotype correlations could be discerned. A number of factors could contribute to this difficulty. These include general factors such as the complexity of biology and genetics, as well as environmental interactions, which often can obscure phenotype–genotype relationships. More specific factors include limitations in our in vitro assays since at best they give only an approximation of in vivo transcriptional behavior. As one relatively small example related to technical issues, we used HD peptides rather than full-length CRX protein in the EMSAs. This may have affected the sensitivity and perhaps also the quantitative relationship of the DNA-binding results, as we have noticed that even the presence or absence of a tag on a fusion protein can affect EMSA results. We were able to detect 'monomeric' versus 'dimeric' complexes with the BAT-1 probe using untagged CRX-HD peptides, but not with GST-tagged peptides, and untagged peptides bind significantly better to the 'low affinity' Ret-4 site

than do tagged peptides. As an additional example, in addition to the inherent limitations of all transient transfection promoter studies, our studies were performed in non-photoreceptor cells so the potential effects of interactions with photoreceptor-specific regulatory molecules could not be explored.

Another level of complexity is added by potential modifier genes, as well as environmental factors. Such factors appear to be important in CRX-associated disease. In a recent study it was reported that a null CRX allele, P9i1, is associated with severe LCA in a child and a normal ocular phenotype in the unaffected father (31). Among the many potential genetic modifiers are the genes encoding CRX interacting proteins, such as NRL (6), phosducin (32), and ataxin-7 (21), as well as the factors that post-translationally modify these proteins (33). Although many questions remain, it is very clear that much more needs to be learned about the molecular mechanisms regulating photoreceptor gene expression before we will fully understand how specific mutations in transcription factors such as CRX lead to various forms of retinal degeneration.

MATERIALS AND METHODS

Expression constructs harboring deletions, mutations and Gal4dbd fusions of CRX

Mammalian expression vectors (pcDNA3.1/HisC [Invitrogen] derivatives) carrying a series of N- and C-terminal deletions of bovine Crx were generated using a PCR-based method as described previously (6). A series of Gal4dbd-Crx fusion constructs were generated in the mammalian expression vector pFA-CMV (Stratagene) by cloning various PCR fragments of the bovine Crx in-frame with the Gal4 DNA-binding domain 1-174 (Gal4dbd) at the BamHI and EcoRI site. Human CRX mammalian expression constructs carrying 11 individual mutations (Fig. 1), as well as the CRX-HD-GST prokaryotic expression constructs carrying two (A56T and E80A) of the five mutations in the CRX homeodomain, were generated using the Quick Change site-directed mutagenesis kit (Stratagene) as described by Swaroop et al. (18). The primers used for mutagenesis were as follows.

- R41Q: 5'-CCCAGGAAGCAGCGGCgGAGCGCACCACTTTC-3'
5'-GAAGGTGGTGCCTCCtGCCGCTGCTTCC-TGGG-3'
- R41W: 5'-CCCAGGAAGCAGCGGtGGGAGCGCACCACTTTC-3'
5'-GAAGGTGGTGCCTCCCaCCGCTGCTTCC-TGGG-3'
- A56T: 5'-CTGGAGGAGCTGGAGaCACTGTTTGCCAA-GAC-3'
5'-GTCTTGCCAAACAGTGtCTCCAGCTCCTCC-AG-3'
- E80A: 5'-CTGAAGATCAATCTGCCTGcGTCCAGGGTT-CAGGTTTG-3'
5'-CCAAACCTGAACCCTGGACgCAGGCAGAT-TGATCTTTCAG-3'
- R90W: Described previously (18)

- A158T: 5'-CCCAACCACGGCAGTGaCCACTGTGTCCA-TCTG-3'
5'-CAGATGGACACAGTGGtCACTGCCGTGGTTGGG-3'
- E168d1: 5'-CCATCTGGAGCCCAGCCTCAAGTCCCCTTTGCCTGAGGCG-3'
5'-CGCCTCAGGCCAAAGGGGACTTGA-GGCTGGGCTCCAGATGG-3'
- E168d2: 5'-CCATCTGGAGCCCAGCCTCAGTCCCCTTTGCCTGAGGCGCAG-3'
5'-CTGCGCCTCAGGCCAAAGGGGACTGAGGCTGGGCTCCAGATGG-3'
- A196d4: 5'-CCTATGCCATGACCTACGGGCTCCGCTTTCTGCTC-3'
5'-GAGCAGAAAGCGGAGGCCCGTAGGTCATGGCATAGG-3'
- G217d1: 5'-CCGAGCTCCTATTTTCAGCGCCTAGACCCCTACCTTTC-3'
5'-GAAAGGTAGGGGTCTAGGCGCTGAAATAGGAGCTCGG-3'
- V242M: 5'-CCTCTCTGGCCCCTCCaTGGGACCTTCCCTGGC-3'
5'-GCCAGGGAAGGTCCCAtGGAGGGGCCA-GAGAGG-3'

Two other HD mutant-GST constructs, CRX-HD^{R41W}-GST and CRX-HD^{R90W}-GST, were described previously (13,18). CRX-HD^{R41Q}-GST was generated using the same strategy as described for CRX-HD^{R41W}-GST except using the R41Q mutant primer 5'-ACGGATCCAGCGCCCCAGGAAGCAG-CaGCGGGAGC-3'. The sequence of each deletion, mutation and Gal4dbd fusion construct was confirmed by sequencing.

Transient cell transfection, luciferase, and immunocytochemistry assays

Transient transfection assays with the mutant human CRX constructs were performed using the calcium phosphate method and analyzed as previously described (1). Transfection assays with bovine Crx deletions and Gal4dbd-Crx fusions were performed using a similar method as for the mutant CRX studies with the following modifications: 35 mm, instead of 100 mm, plates in a 6-well culture plate format were used for culturing cells. Transfections were performed when cells reached 70% confluence. Typically, a total of 2.1 µg of DNA was used for each transfection, including 2 µg of the reporter construct, 0.1 µg of a protein expression construct and 1 ng of the Renilla luciferase reporter pRL-CMV (Promega) as an internal control for transfection efficiency. Dual luciferase assays were performed using the TD-20/20 Luminometer (Turner Designs) and Dual-Luciferase Reporter Assay Kit (Promega) following the manufacturer's instructions. Relative luciferase units were calculated as light units normalized to the value of the internal control. The luciferase reporters used for transfection assays were pBR130-luc (34) for analyzing Crx deletions and pFR-Luc (Stratagene) for analyzing Gal4dbd-Crx fusions, respectively. Each sample was done in duplicates and at least three independent experiments were performed. The significance of the results was analyzed using the Student t-test, assuming two samples equal variance. For

immunocytochemistry, HEK 293 cells were cultured on glass cover slips treated with 0.1 mg/ml poly-D-lysine (Sigma) in 35 mm plates and transfected with 2 µg of a CRX expression vector. Cells were fixed in 4% paraformaldehyde and stained 48 hours later with the anti-CRX antibody P261 (21) at 1:200. Rhodamine Red-labeled goat anti-rabbit (Molecular Probes) at 1:1000 was used as the secondary antibody. The cover slips were then mounted using Vectashield with DAPI (Vector Laboratory Inc). Fluorescence images were obtained using a Olympus BH-2 fluorescence microscope.

Purification of CRX homeodomain and electrophoretic mobility shift assays (EMSA)

Expression and purification of the wild-type and mutant forms of CRX-HD-GST were carried out using essentially the same method as described previously (1). The concentration of each protein preparation was measured using the BioRad Protein Assay Kit II and verified by SDS-PAGE (11% gel). The GST tag was removed from each of the GST-fusion proteins by digesting 1 µg of purified protein with 2 units of thrombin (Sigma) overnight at room temperature in a total volume of 100 µl. Completion of digestion was verified using both SDS-PAGE and EMSAs with undigested GST-tagged proteins as controls. EMSAs were carried out using CRX-HD peptides without the GST tag and ³²P-labeled oligomers containing the Ret-1, Ret-4 and BAT-1 site, respectively, as described in Chen et al. (1). The monomeric shifted bands were quantified by phosphorimager analysis using a Storm 860 PhosphorImager System (Molecular Dynamics) and ImageQuant 5.0 software.

ACKNOWLEDGEMENTS

We thank Belinda McMahan for technical assistance in immunocytochemistry studies. This work was supported by grants from the National Institutes of Health—EY12543 (S.C.), EY09769 (D.J.Z.) and EY02687 (WU-DOVS Core), Macula Vision Research Foundation, Steinbach Foundation, unrestricted funds from Research to Prevent Blindness, Inc. and Fight for Sight, Inc. S.C. is a recipient of a Career Development Award from Research to Prevent Blindness. Q.-L.W. is a recipient of fellowship award from Nights Templar Foundation. D.J.Z. is the Guerrieri Professor of Genetic Engineering and Molecular Ophthalmology.

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