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Crx, a Novel *otx*-like Homeobox Gene, Shows Photoreceptor-Specific Expression and Regulates Photoreceptor Differentiation

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Summary

We have isolated a novel *otx*-like homeobox gene, *Crx*, from the mouse retina. *Crx* expression is restricted to developing and mature photoreceptor cells. CRX bound and transactivated the sequence TAATCC/A, which is found upstream of several photoreceptor-specific genes, including the opsin genes from many species. Overexpression of *Crx* using a retroviral vector increased the frequency of clones containing exclusively rod photoreceptors and reduced the frequency of clones containing amacrine interneurons and Müller glial cells. In addition, presumptive photoreceptor cells expressing a dominant-negative form of CRX failed to form proper photoreceptor outer segments and terminals. *Crx* is a novel photoreceptor-specific transcription factor and plays a crucial role in the differentiation of photoreceptor cells.

Introduction

The neural retina is an exquisitely sensitive light detector, utilizing photoreceptor cells to carry out phototransduction, a series of signal amplifications that enable detection of a single photon of light (Dowling, 1987). The cascade is initiated by the capture of light by 11-*cis* retinal, a chromophore bound by the opsin proteins; rhodopsin in rod photoreceptors and cone opsins in cone photoreceptors. The proteins that carry out phototransduction are found within an elaborate and highly specialized membranous structure, the photoreceptor outer segment. This structure appears to be relatively fragile, degenerating in response to many environmental and/or genetic perturbations (e.g., see LaVail et al., 1987; Dryja and Li, 1995).

While the physiology of photoreceptors has been well studied, the molecular mechanisms that control photoreceptor differentiation remain poorly understood. Several laboratories have characterized *cis*-acting elements and binding activities in the upstream regions of photoreceptor-specific genes (Chen and Zack, 1996). Transgenic mice carrying promoter-reporter gene fusions have led to the identification of sequences capable of conferring photoreceptor-specific expression (Saha et al., 1992). One candidate *trans*-acting factor has been identified molecularly, the neural retina leucine-zipper (NRL) (Swaroop et al., 1992). NRL has been shown to up-regulate transcription from reporter constructs carrying a rhodopsin promoter in cell lines and in primary retinal cell cultures (Kumar et al., 1996; Rehemtulla et al., 1996). NRL is not, however, photoreceptor specific in the developing retina (Swaroop et al., 1992; Liu et al., 1996), and it is not yet clear how NRL activity contributes to the timing and specificity of rhodopsin expression and rod development.

Another route to the identification of *cis*-acting elements important in the regulation of photoreceptor-specific genes is through the study of humans who are color blind. Blue cone monochromacy is a disease in which an individual has no functional red or green cones. In some forms of this disease, there are deletions upstream of the clustered red and green opsin genes on the X chromosome (Nathans et al., 1989). These deletions encompass a common area, with the smallest deletion being 579 bp in size. Interestingly, this 579 bp region contains a conserved 37 bp core sequence that contains an OTX homeobox–binding site. This site has been observed upstream of many other opsin genes from disparate species, including the *rh3* gene of Drosophila (Fortini and Rubin, 1990), and upstream of other photoreceptor-specific genes (Kikuchi et al., 1993). No protein that binds to this site in photoreceptors has been identified.

No photoreceptor-specific transcription factors have been reported, despite the suspicion that at least one such transcription factor might act through the common sequence elements found upstream of photoreceptor-specific genes. We report here the isolation and characterization of a novel homeodomain protein, CRX. CRX is expressed specifically in the developing and adult retina in photoreceptor cells. We found that CRX binds to a conserved site in the upstream region of many photoreceptor-specific genes, including the OTX site that is deleted in human blue cone monochromats, and is capable of transactivating constructs that carry this site. In addition, we present data indicating that CRX transactivation activity is necessary for the formation of outer segments and terminals of rat photoreceptor cells in vivo. *Crx* is also required for the survival of photoreceptors. In the report by Freund et al. (1997, this issue of *Cell*), mutations in human *Crx* are reported to be associated with degeneration of photoreceptors in individuals with cone-rod dystrophy 2.

Results

CRX Belongs to the OTX Family of Homeodomain Proteins

In a degenerate RT-PCR–based screen aimed at identifying members of several gene families expressed in the developing retina, three novel paired-type–related homeobox-containing genes were isolated. This screen and one of these genes (*rax*) have been described elsewhere (Furukawa et al., 1997). The second novel, paired-related homeobox-containing gene, designated *Crx* (for cone-rod homeobox-containing gene) is the subject of this report. A *Crx* cDNA was isolated from a mouse retinal cDNA library and was sequenced (Figure 1A). Sequence analysis revealed a complete open reading

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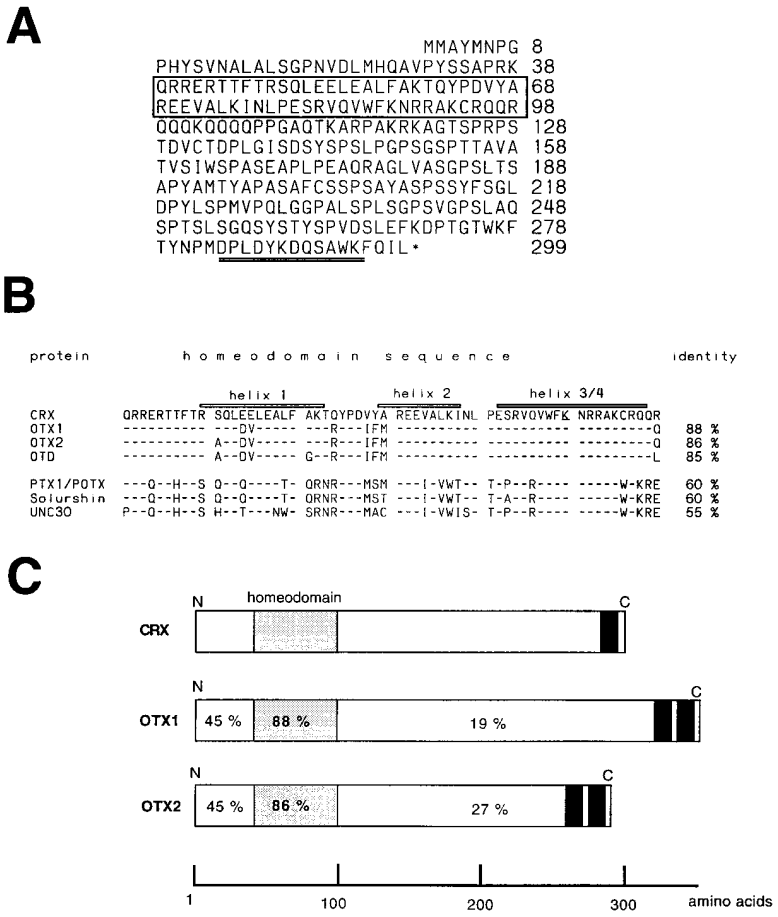

Figure 1. The Deduced CRX Amino Acid Sequence and Comparison with Other OTX-Related Homeodomain Proteins

(A) The deduced amino acid sequence of the mouse *Crx* cDNA. The cDNA contains a single, long open reading frame. Boxed amino acid sequence corresponds to the homeodomain. Double underline indicates the "OTX tail", which is observed in the C terminus of OTX family members.

(B) Amino acid sequence alignment of the CRX homeodomain and other OTX-related homeodomains. Residues identical to CRX protein are indicated by dashes. The lysine residue at the ninth position of the recognition helix (helix 3/4) is underlined.

(C) The amino acid homologies between the mouse OTX1 and OTX2 proteins and the mouse *Crx*. The homeodomain (shaded) and the OTX tail (black) are shown.

frame for a novel gene belonging to the *otx* family of homeobox genes. Using the first ATG, the deduced open reading frame encodes a 299–amino acid polypeptide with a predicted mass of 32 kDa.

The predicted amino acid sequence of CRX shows a high degree of homology to mouse OTX1 and OTX2 (Simeone et al., 1993). The homeodomain of the predicted CRX protein has 88, 86, and 85% identities with the mouse OTX1, OTX2, and *Drosophila orthodenticle (otd)* (Finkelstein et al., 1990) homeodomains, respectively (Figure 1B). Further, the CRX homeodomain has a lysine at the ninth position of the recognition helix, a feature shared by other OTX and OTX-related homeodomain proteins (Figure 1B). As well, the CRX homeodomain is 55%-60% identical to the homeodomains of a series of other OTX-related proteins, such as Ptx1/Potx (Lamonerie et al., 1996; Szeto et al., 1996), Solurshin (Semina et al., 1996), and UNC30 (Jin et al., 1994) (Figure 1B).

Interestingly, the *Crx*-deduced protein contains a peptide with the sequence DPLDYKDQSAWK in the carboxyl terminus (Figures 1A and 1C). This motif with a slight variation is also observed in the carboxyl termini of OTX1 and OTX2, each of which contains two tandem copies of this repeat. We have therefore named this motif the "OTX tail". The zebrafish, Xenopus, and chick OTX proteins also contain OTX tails at their C termini (Li et al., 1994; Mori et al., 1994; Bally-Cuif et al., 1995; Blitz and Cho, 1995; Pannese et al., 1995), but the Drosophila otd protein does not contain this motif. Elucidation of the function of the OTX tail awaits future analyses.

Finally, amino acid sequence conservation between CRX, OTX1, and OTX2 is observed in regions outside of the homeodomain and OTX tail (Figure 1C). This includes the conservation of 38 amino acids in the amino termini of the proteins. As well, amino acid sequences on the carboxyl side of the homeodomains of these proteins show a low degree of homology among CRX, OTX1, and OTX2 (Figure 1C). The homology between CRX and the OTX proteins in several regions indicates that CRX is a new member of the OTX family of proteins.

Crx Expression Correlates with Photoreceptor Cell Development

To examine the tissue specificity of *Crx* expression, RNA from the adult mouse retina and nine other adult mouse tissues was analyzed on a Northern blot using the mouse *Crx* cDNA as a probe. A single abundant transcript of 3.0 Kb was detected in the mouse retina, and no signal was observed in the other tissues examined. (Figure 2A). In situ hybridization was performed on developing and adult mouse eye sections (Figure 2B–2L). *Crx* transcripts were first detected in the retinae of E12.5 embryos, localized to the outer aspect of the neural retina, corresponding to the prospective photoreceptor layer (Figure 2B). At this stage of retinal development, genesis of cone photoreceptors is increasing while rod genesis has not yet occurred at a significant level (Carter-Dawson and LaVail, 1979). At E15.5, after the completion of cone genesis and the initiation of rod genesis (Carter-Dawson

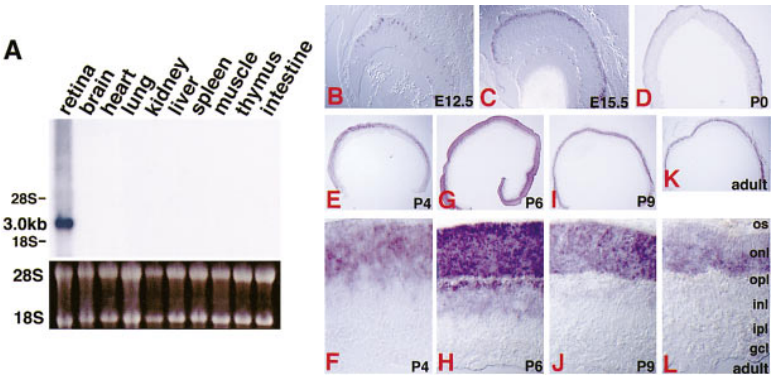

Figure 2. *Crx* Expression in the Developing and Mature Retina

(A) A Northern blot of RNA from adult mouse tissues. The upper panel shows the hybridization signal obtained with a mouse *Crx* cDNA probe. The lower panel shows EtBr staining of RNA. Each lane contains 10 ug of total RNA. The *Crx* transcript is about 3.0 Kb.

(B–L) Tissue sections were processed for in situ hybridization using a probe of mouse *Crx*. (B) E12.5 eye has the *Crx* signal in the outer layer of the neural retina. (C) E15.5 eye shows a slight increase of *Crx* expression in the outer layer of the retina. (D) P0 retina displays the *Crx* signal in the prospective photoreceptor layer. (E and F) P4 retina. (G and H) P6 retina. (I and J) P9 retina. (K and L) Adult retina. Higher magnification of (F), (H), (J), and (L) are shown in (E), (G), (I), and (K), respectively. The *Crx* signal is strongest at P6 and was found in prospective or mature photoreceptors at all times. gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; opl, outer plexiform layer; onl, onl; os, outer segment.

and LaVail, 1979), a moderate level of expression was observed, which was also restricted to the prospective photoreceptor layer (Figure 2C).

The peak period for rod photoreceptor birthdays is around the time of birth of the animal (Carter-Dawson and LaVail, 1979; Nir et al., 1984). The neonatal period is also the time when photoreceptor-specific gene expression can be observed in the developing photoreceptor layer for a number of genes. During postnatal retinal development, *Crx* also showed expression in the photoreceptor layer (Figures 2D–2L), with expression throughout the prospective photoreceptor layer at postnatal day 4 (P4) (Figures 2E and 2F) and peak expression at P6 (Figures 2G and 2H). This pattern correlates with the rapid increase in cells expressing rhodopsin and other phototransduction genes between P6-P8 (Hicks and Barnstable, 1987; Ahmad et al., 1990; Ni et al., 1992; Stepanik et al., 1993). Around P6, the outer plexiform layer (OPL) is visible and leads to the separation of developing rods into two groups, those in the ONL (ONL) and those in the inner nuclear layer (INL). Similarly, *Crx* expression was observed throughout the newly established ONL, as well as in a subset of cells in the INL, presumably corresponding to developing rods trapped on the vitreal side of the OPL (Young, 1985) (Figure 2H). At P9, the intensity of the *Crx* signal was slightly decreased but was discretely localized to the ONL (Figure 2I and 2J). Expression of *Crx* mRNA persisted in mature photoreceptors in the adult retina (Figures 2K and 2L).

DNA Binding Activity of CRX Protein

The *otx* gene family has a lysine at position 51 of the homeodomain that confers DNA binding specificity for the sequence motif TAATCC/T (Treisman et al., 1989; Hanes and Brent, 1991). We searched for this motif in available upstream regions of photoreceptor-specific genes, focusing on sequences in regions that are conserved among species. The consensus sequence (TAA TCC/T) and a variant sequence (TAATCA) were found in several species in photoreceptor-specific upstream regions, including the interphotoreceptor retinoid-binding protein (IRBP)(Liou et al., 1991), rhodopsin (Zack et al., 1991; Kumar et al., 1996), cone opsin (Saha et al., 1992), and arrestin (Kikuchi et al., 1993) (Figure 3A). Although the fifth base (C) of the OTX recognition sequence, 5′TAATCC/T 3′ is known to be important for DNA binding specificity, the sixth base (C/T) has less importance (Hanes and Brent, 1991). CRX was tested for binding to these sequences using the electrophoretic mobility shift assay (EMSA). As DNA probes, the OTX-oligos (TAATCC/T), the OTX(A)-oligo (TAATCA), and the Ret1-oligo were used (Figure 3B). *Ret1* (PCE 1) is one of the putative *cis*-acting DNA regulatory elements of the rhodopsin promoter (Morabito et al., 1991), and its core consensus sequence (5′ TAATTG 3′) is found in many opsin promoter sequences (Kikuchi et al., 1993).

A fusion protein between glutathione S-transferase (GST) and the homeodomain of CRX was incubated with radio-labeled DNA probes (Figure 3C). The CRX homeodomain showed strong binding both to the OTX-oligo and to the OTX(A)-oligo (Figure 3C, lanes 1–6). The Ret1 probe, however, demonstrated a shifted band of lower intensity (Figure 3C, lanes 7–9), indicating that CRX bound less strongly to the Ret1 oligo than it did to the OTX- and OTX(A)-oligos.

The specificity of CRX binding to the OTX consensus sequence was assessed by a competition experiment. The addition of cold competitor of the identical oligo resulted in a dose-dependent inhibition of the DNA binding activity of CRX (Figure 3D, lanes 2–4). Binding was not, however, inhibited by competition with an oligo that was mutated in the OTX consensus sequence (Figure 3D, lanes 5–7).

CRX Transactivates the *Irbp* Promoter

To assay the activity of CRX as a transcriptional activator, a CRX expression construct was cotransfected into NIH3T3 cells with various reporter constructs, and assays were conducted during the period of transient expression (Figure 4). First, reporter plasmids containing the *Cat* gene under the control of the thymidine kinase (*tk*) minimal promoter linked to either five repeats of the OTX, OTX(A), or Ret1 core sequences (Figure 4A) were tested. CRX showed transactivation activity over control from reporter constructs carrying the OTX consensus–binding site (Figure 4B, lanes 1 and 2). CRX also showed significant transactivation activity from the promoter containing the TAATCC binding repeat (Figure 4B, lanes

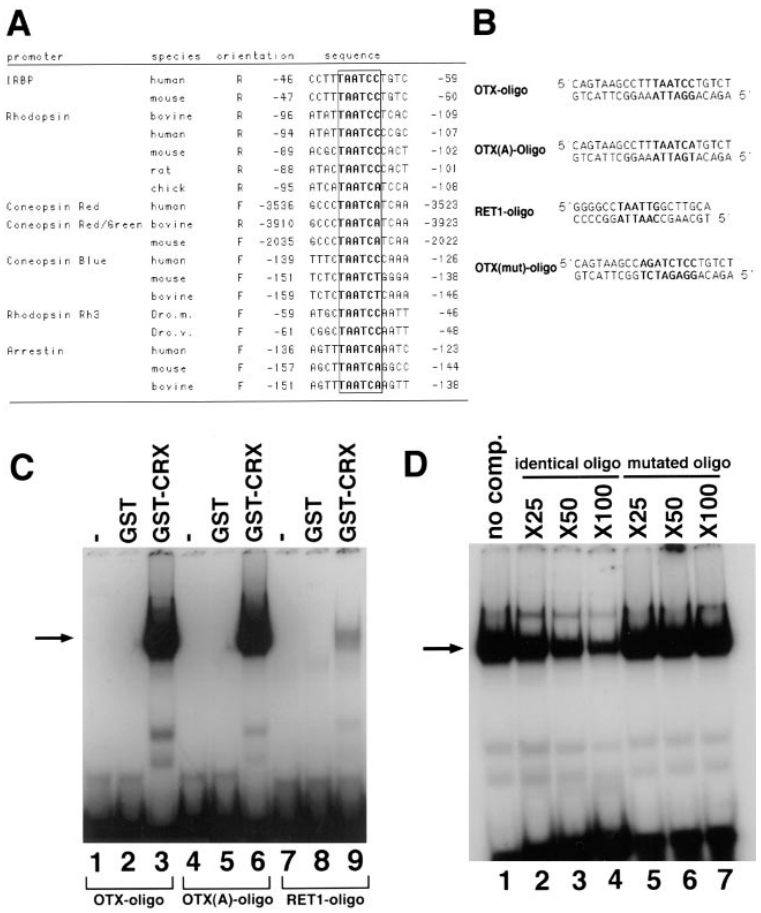

Figure 3. EMSA of CRX Protein

(A) The OTX-binding consensus sequence is contained in the upstream regulatory regions of photoreceptor-specific genes. OTX binding consensus sequences are in bold type and are boxed in the 5′ flanking sequences for *Irbp* (Liou et al., 1991), opsin (Takao et al., 1988; Morabito et al., 1991; Zack et al., 1991), red and green opsins (Saha et al., 1992), blue opsin (Chiu and Nathans, 1994), Drosophila opsin (Fortini and Rubin, 1990), and arrestin (Kikuchi et al., 1993). "Orientation" indicates that the sequence is shown in forward orientation (F) or reverse orientation (R) with respect to the direction of transcription.

(B) Nucleotide sequences of oligonucleotides used as labeled probes or competitors in EMSAs.

(C) Radio-labeled oligonucleotide probes were incubated without additional protein (lanes 1, 4, and 7), with GST protein (lanes 2, 5, and 8) or with GST-CRX fusion protein (lanes 3,6, and 9). Arrow indicates a specifically shifted band.

(D) The same unlabeled OTX-oligo was coincubated as a competitor at increasing molar excess with respect to the labeled probe (25-, 50-, and 100-fold molar excess in lanes 2–4, respectively). A mutated OTX-consensus oligo was included as a competitor at increasing molar excess with respect to the labeled probe (25-, 50-, and 100-fold molar excess in lanes 5–7, respectively).

3 and 4), although the control with no CRX gave higher background in these experiments (Figure 4B, lane 3). Transactivation activity was also observed using constructs containing the Ret1 sequence. However, this activity was weaker compared with either of the OTX consensus sites (Figure 4B, lanes 5 and 6).

The ability of CRX to activate transcription from the promoter for IRBP, a photoreceptor-specific protein, was also tested. Expression of IRBP mimics that of *Crx* temporally (Liou et al., 1994). Analysis of transgenic mice carrying *Irbp* promoter constructs has revealed that 123 bp of the 5′ flanking region of the human *Irbp* gene is sufficient for photoreceptor specific-expression in vivo (Bobola et al., 1995). In a DNaseI footprinting analysis, only the OTX-binding site was protected specifically by nuclear extracts from retinoblastoma cell lines, which express IRBP. A reporter construct containing the −123 to +18 bp region of the *Irbp* promoter was cotransfected with the CRX expression construct. The results of this experiment (Figure 4B, lanes 7 and 8) demonstrate that CRX is capable of significant transcriptional activation from reporter constructs carrying the *Irbp* promoter fragment. In addition, when the CRX-binding site was mutated using the same mutations used in the previous analysis of *Irbp* promoter (Bobola et al., 1995) (Figure 4A), a significant reduction in the transcription activity was observed (Figure 4B, lane 9). The remaining transcription activity was likely due to the presence of a Ret1-like site in this promoter (Figure 4A).

Forced Expression of CRX Affects Retinal Cell Differentiation In Vivo

To test the effects of CRX overexpression on development of retinal cells in vivo, retroviruses expressing CRX were injected into the developing retina. Infection of the rat retina at P0 with viruses carrying reporter genes alone results in clusters of clonally related cells ranging in size from 1 to 22 cells and containing rod photoreceptors, amacrine and bipolar interneurons, and Müller glial cells in various combinations (Turner and Cepko, 1987). Approximately 75% of these clones contain only rods, while the remaining clones contain exclusively non-rods or combinations of rods and non-rods. If CRX expression were sufficient to instruct the rod cell fate, the percentage of rod-only clones should increase in retinae infected with a retrovirus expressing CRX. Alternatively, CRX alone may not be sufficient for rod determination, but CRX expression may be sufficient to block non-rod cell fates. This possibility would be supported by an absence or a decrease of non-rod cells in clones infected by the CRX-expressing virus.

Retinal progenitor cells at P0 were infected in vivo with LIA or LIA/CRX virus (Figure 5A). After retinal development was complete, infected retinae were subsequently stained for alkaline phosphatase (AP) activity, and clonal analysis was performed by reconstructing serially sectioned retinae. Identification of cell types was determined by the characteristic morphologies and locations of terminally differentiated cells (Figures 5B–5E).

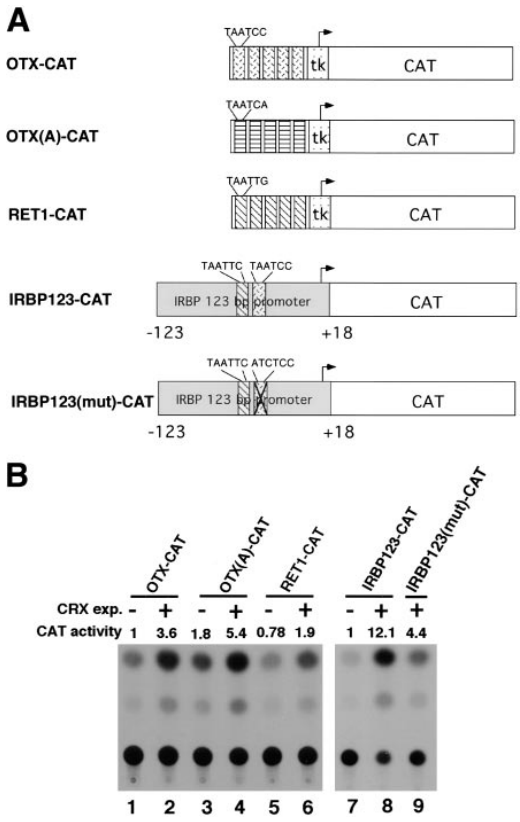

Figure 4. Transcriptional Analysis of CRX Protein

(A) Reporter constructs for a transactivation assay. The CAT reporter plasmids contained the *tk* promoter linked to five repeats of the OTX consensus sequence (pOTX-CAT), TAATCA sequence (pOTX(A)-CAT), or Ret1 sequence (pRET1-CAT). The CAT reporter plasmid without the *tk* promoter was linked to the *Irbp* 123 bp promoter (−123–+18 bp) and to its mutated promoter at the OTX-binding consensus site, termed as pIRBP123-CAT and pIRBP123 (mut)-CAT, respectively.

(B) CAT reporter plasmids, pOTX-CAT (lanes 1 and 2), pOTX(A)-CAT (lanes 3 and 4), pRET1-CAT (lanes 5 and 6), pIRBP123-CAT (lanes 7 and 8), and pIRBP123(mut)-CAT were transfected into NIH3T3 cells with either control vector pME18S (*SR*α promoter) alone (lanes 1, 3, 5, and 7) or pME18S-CRX plasmid (lanes 2, 4, 6, 8, and 9). The cells were harvested 2 days later, and CAT assays were performed. Loading was normalized by reference to levels of β-gal activity derived from the cotransfected pSVβ plasmid. These experiments were carried out three times. CAT activities were measured using a phosphoimager and the values were averaged.

In the LIA/CRX-infected retinae, no abnormal cell types were observed. However, the cellular composition of clones infected with LIA/CRX was clearly altered relative to clones infected with LIA (Table 1). First, the LIA/CRX clones exhibited a statistically significant increase in the percentage of clones containing rod photoreceptors only (from 75.5% to 84.6%) (Table 1 and Figure 5F). Second, the LIA/CRX virus–infected retinae were remarkable for an almost complete absence of amacrine interneurons in virally infected clones (Table 1, Figure 5G). The percentage of clones containing amacrines was reduced from 4.0% to 0.55%. As well, the percentage of clones containing Müller glia was markedly reduced (from 5.07% to 1.53%) (Table 1, Figure 5G). Finally, the percentage of clones containing bipolar interneurons appeared to be unaltered in LIA/CRX-infected clones (Table 1, Figure 5G).

Transactivation Activity of CRX Is Necessary for Rod Outer Segment Formation

To determine whether CRX is required for rod differentiation, an LIA vector carrying a variant of CRX, CRX-EnR, was made (Figure 6A). The homeodomain of CRX was fused to the repressor domain of the Drosophila engrailed protein, producing a fusion protein which should block transcription activation by CRX (Badiani et al., 1994; Conlon et al., 1996). To examine the ability of CRX-EnR to impair transcription activation by CRX, transcription assays were performed following cotransfection of CRX and CRX-EnR expression plasmid into NIH3T3 cell lines (Figure 6B). The CRX-EnR itself was unable to activate transcription (Figure 6B, lane 3), and it interfered with transcription activation by wild-type CRX in a dose-dependent fashion (Figure 6B, lane 4–7).

The LIA/CRX-EnR virus was used to infect P0 rat retinae, and infected eyes were examined at P21. As a control, an LIA virus that expressed the engrailed repressor domain alone (LIA/EnR) was used. The retinae infected with LIA/EnR developed rods (Figures 6C and 6E), amacrine cells, and Müller cells (data not shown) that appeared normal in terms of morphology and location. However, the number of bipolar cells was reduced almost to zero. This effect on bipolar cells confounded our ability to draw conclusions from a quantitative analysis of the clonal composition of the LIA/CRX-EnR–infected retinae. The LIA/CRX-EnR virus-infected retinae exhibited a dramatic phenotype of photoreceptor differentiation relative to either LIA-infected or LIA/EnR-infected retinae (Figures 6D and 6F). While the LIA/EnR-infected retinae showed normal rod photoreceptors (Figures 6C and 6E), the LIA/CRX-EnR–infected cells failed to form terminally differentiated rods. LIA/CRX-EnR–infected cells were located in the outer nuclear layer (ONL) yet only rarely formed outer segments or terminals (i.e., the axonal endings of rod cells located in the outer plexiform layer) (Figures 6D and 6F). Over 1000 ONL cells, resulting from multiple retinae from several litters of infected animals, were observed to have this phenotype. The few cells that formed any outer-segment-like structures exhibited abnormal outer segments and/or terminals. The LIA/CRX-EnR virus–infected retinae exhibited normal amacrine and Müller cells. As in the LIA/EnR retinae, bipolar cells were again reduced to near zero.

To distinguish whether the photoreceptor phenotype of LIA/CRX-EnR–infected retinae was due to a failure of morphogenesis or due to degeneration of outer segments and terminals that may have formed normally, retinae infected by the same viruses were harvested at P14, the earliest age when significant outer segment formation would have occurred in the majority of rod photoreceptors. Although LIA/EnR virus–infected retinae showed normal morphology of rod photoreceptor cells with outer segments, the LIA/CRX-EnR virus–infected retinae harvested at P14 showed a very similar phenotype to those harvested at P21 (Figures 6G–6H).

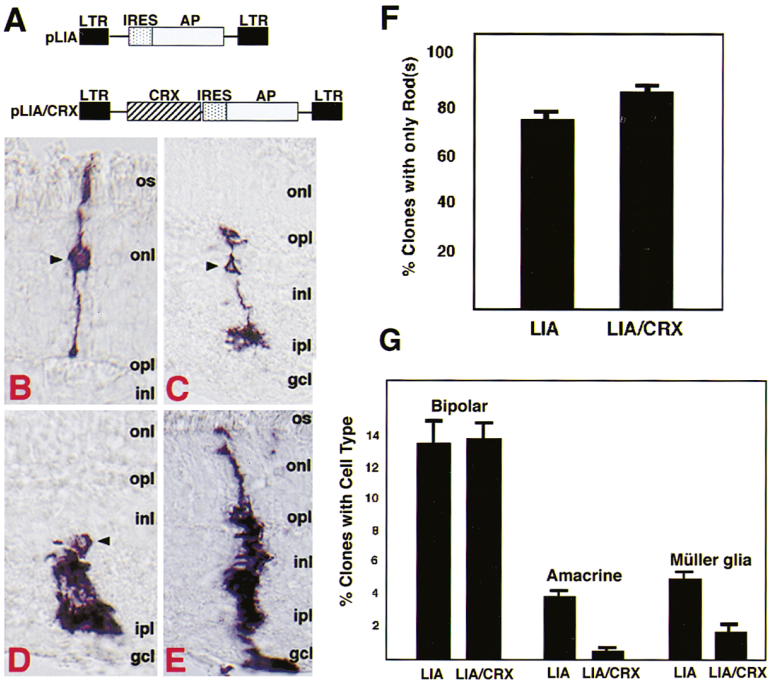

Figure 5. Transduction of CRX by a Retrovirus Vector

(A) Viral constructs used to express *Crx*. pLIA was derived from MMLV and was designed to express a marker gene, *AP*, through an IRES sequence and another gene under the control of the *LTR* promoter.

(B–E) Examples of retinal cell types from 20 μm frozen sections of pLIA-infected retinae. Four cell types were observed. (B) Rod photoreceptor, (C) bipolar cell, (D) amacrine cell, (E) Müller glial cell. Cells infected with LIA/CRX showed morphologies indistinguishable from cells infected with LIA. Arrow head indicates cell body. gcl, ganglion cell layer; ipl, inner plexiform layer; inl, inner nuclear layer; opl, outer plexiform layer; onl, ONL; os, outer segment.

(F) The percentage of rod-only clones out of the total number of infected clones by LIA or LIA/CRX virus. The standard error is represented by the error bar.

(G) The percentage of clones containing at least one bipolar cell, amacrine cell, or Müller glial cell out of the total number of clones infected by LIA or LIA/CRX virus (from Table 1).

These data indicate that formation of outer segments did not occur in presumptive rods infected by the LIA/CRX-EnR virus.

Discussion

We have isolated and characterized a novel member of the *otx* homeodomain gene family, *Crx*. *Crx* is expressed exclusively in photoreceptor cells in the neural retina. In the developing retina, *Crx* expression begins at approximately E12.5. As very few rods have been generated by this point, *Crx* most likely is expressed by cone photoreceptors. It is most highly expressed between P4 and P9, when rod photoreceptor differentiation is maximal. By EMSA, CRX exhibited specific binding to conserved DNA sequences found upstream of several photoreceptor-specific genes, and CRX was able to activate transcription from the *Irbp* minimum promoter in

Table 1. Clonal Composition Following Infection of the Retina with Retroviruses Encoding *Crx*[a]

Litter #	Virus	# and % of Clones Containing at Least One: Rod	Bipolar	Amacrine	Müller	UINL[b]	# and % of Rod-Only clones
1[c]	LIA/CRX	106/110	12/110	1/110	0/110	1/110	96/110
		96.3	10.9	0.91	<0.91	0.91	87.2
2	LIA	269/279	46/279	14/279	13/279	8/279	197/279
		96.4	16.5	5.0	4.6	2.9	70.6
2	LIA/CRX	259/265	43/265	1/265	9/265	5/265	229/265
		97.7	16.2	0.34	3.8	1.9	86.4
3	LIA	251/251	27/251	8/251	10/251	2/251	199/251
		100	11	3.2	4.0	0.80	79.3
3	LIA/CRX	248/250	31/250	1/250	2/250	6/250	212/250
		99	12.4	0.40	0.80	2.4	84.8
4	LIA	283/286	34/286	11/286	19/286	4/286	219/286
		99.0	12.0	3.8	6.6	1.4	76.6
4	LIA/CRX	200/205	34/205	0/205	2/205	5/205	164/205
		97.5	16.6	<0.49	0.98	2.4	80.0
Totals	LIA	803/816	107/816	33/816	42/816	14/816	615/816
	LIA/CRX	813/830	120/830	3/830	13/830	17/830	701/830
Avg. ± SE	LIA	98.2 ± 1.0	13.8 ± 1.6	4.0 ± 0.43	5.07 ± 0.64	1.7 ± 0.51	75.3 ± 2.1
	LIA/CRX	97.6 ± 0.48	14.0 ± 1.2	0.55 ± 0.13	1.53 ± 0.82	1.9 ± 0.31	84.6 ± 1.4

[a] Each line represents the clonal composition of one retina, with the exception of the LIA/CRX entry of litter 4, which is the sum of all clones in two retinae.

[b] UINL refers to cells that were in the inner nuclear layer but which were unidentifiable owing to a lack of defining processes.

[c] The control LIA-infected retinae of litter 1 were not scored, as the density of clones was too high to permit an accurate assignment of clonal boundaries.

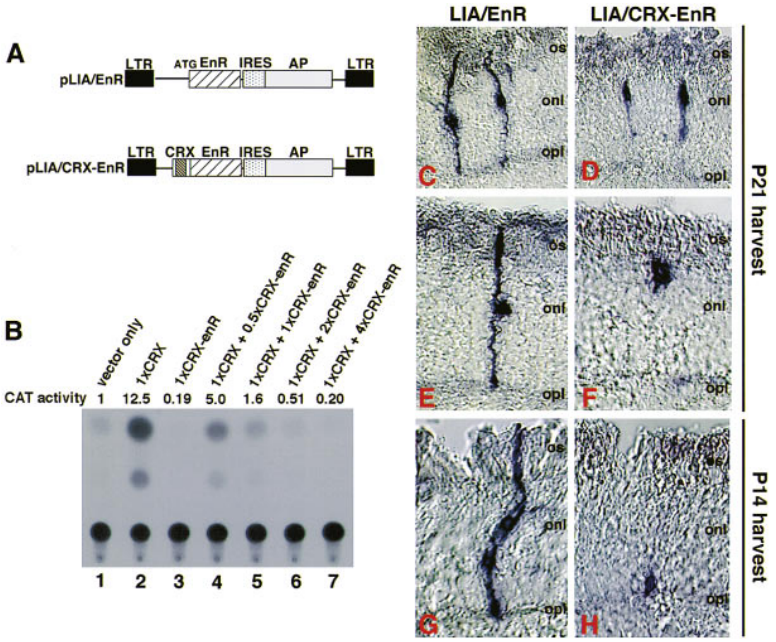

Figure 6. Morphology of Cells Transduced by Dominant Negative CRX Virus

(A) Viral constructs for expressing CRX-EnR and EnR proteins. Slashed box of the pLIA/CRX-EnR indicates the homeodomain of CRX.

(B) Repression of transcription activation by a dominant-negative CRX-EnR construct. A plasmid encoding the wild-type CRX causes activation of transcription of the *Irbp* promoter (pIRBP123-CAT) (lane 2). A plasmid encoding a fusion construct of the CRX homeodomain with the Drosophila engrailed repressor domain (CRX-EnR) causes no activation (lane 3), and it inhibits activation by CRX in a dose-dependent manner (lanes 4–7). Loading was normalized by reference to levels of β-gal activity derived from the cotransfected pSVβ plasmid. This experiment was carried out three times. Relative CAT activities were measured using a phosphoimager and the values were averaged.

(C–H) Morphologies of ONL cells from frozen sections (20 mm) of P21 retinae infected at P0 by pLIA/EnR (C and E) or pLIA/CRX-EnR (D and F) are shown. (G and H) Morphologies of ONL cells in P14 retinae infected at P0 by pLIA/EnR (G) or pLIA/CRX-EnR (H) are shown. Note that outer segments were not present in ONL cells transduced with CRX-EnR, although a high percentage of labeled cell bodies were observed in the ONL following infection with LIA/EnR or with LIA/CRX-EnR. opl, outer plexiform layer; onl, ONL, os, outer segment.

a CRX-binding site–dependent manner. In addition, a dominant-negative form of CRX inhibited rod outer segment and rod terminal formation in vivo. Misexpression of CRX in vivo increased the frequency of rod-only clones and was sufficient to block the development of some types of nonphotoreceptor cells. These data show that *Crx* plays a key role in photoreceptor development and can affect the development of other retinal cells in rats in vivo. In humans, *Crx* is crucial for photoreceptor survival, as mutations in *Crx* lead to photoreceptor degeneration and the early onset of blindness in individuals with cone-rod dystrophy 2 (Freund et al., 1997).

Crx and Photoreceptor-Specific Gene Regulation

A number of genes are expressed exclusively in photoreceptor cells. Most of these genes are directly involved in phototransduction, such as the opsin genes, while others are less directly involved, such as the gene encoding IRBP. The transcriptional regulation of some of these genes has been investigated using several approaches, including EMSA, cell lines transfected with reporter constructs, in vitro transcription reactions using retinal extracts, and transgenic mice (Chen and Zack, 1996). These studies have revealed several *cis*-acting regulatory regions and, in some cases, have led to the definition of specific regulatory sequences. As discussed below, some of these sequences are conserved among several vertebrate species, as well as in Drosophila. In particular, an OTX consensus site has been found in the upstream region of several photoreceptor-specific genes.

The *Irbp* upstream region has been found to have a 123 bp sequence that is well conserved between human and mouse (Bobola et al., 1995). This sequence contains the OTX binding motif and was found to confer photoreceptor-specific expression in transgenic mice. The sequence, TTAATC, which overlaps with the OTX consensus motif in the −123–+18 bp sequence of the *Irbp* gene, is known to be essential for the photoreceptor specific expression (Bobola et al., 1995). In the present study, we demonstrate that *Crx* binds to the TTAATC sequence and that *Crx* activates transcription from reporter constructs carrying the previously characterized −123–+18 regulatory sequence from the *Irbp* gene (Figures 3 and 4). In addition, previous studies have shown that *Irbp* expression begins on embryonic day 13 (E13) in mouse at about the same time as *Crx*. As well, *Irbp* expression increases rapidly in the neonatal period, when *Crx* expression also is found at high levels (Liou et al., 1994). Taken together, these data strongly suggest that *Crx* is involved directly in the regulation of *Irbp* expression.

The rhodopsin promoter of several species also has been characterized. Several regulatory elements upstream of rhodopsin, including the Ret1–4 (Morabito et al., 1991; Kikuchi et al., 1993; Yu et al., 1993; Chen and Zack, 1996), NRE (Swaroop et al., 1992; Kumar et al., 1996; Rehemtulla et al., 1996) and Eopsin-1 (Ahmad, 1995) sites, have been identified in several laboratories using a variety of methods. The bovine rhodopsin sequence between −222 and +70 bp has been found to be sufficient to direct photoreceptor-specific gene expression in transgenic mice (Zack et al., 1991). An OTX consensus site (at −105–−100 bp for bovine and −98–−93 bp for mouse) is included in this region; however, the significance of this site has not been tested. Interestingly, it was recently reported that the −130–−84 bp region of bovine and mouse rhodopsin contains a

strong positive regulatory element for transcription by chick retinal extracts (Kumar et al., 1996).

Regulation of the cone photoreceptor visual pigment genes has also been well studied. The human retina has three cone visual pigments, referred to as the blue, green, and red opsins (Nathans et al., 1986). The regulation of the cone pigment genes has been studied using transgenic mice. A region between 3.1 kb and 3.7 kb upstream of the human red opsin was found to be essential for photoreceptor-specific gene expression (Wang et al., 1992). This region, which was found to be deleted in some patients with human blue cone monochromacy (Nathans et al., 1989), contains three CRX-binding sites. Sequences in this region are highly conserved among humans, cows, and mice. In particular, a 200 bp region showed 70% identity, including a core 37 bp sequence of perfect identity (Wang et al., 1992), and was proposed to have an essential function for photoreceptor-specific gene expression. In the 37 bp core sequence, an OTX consensus site was found. These observations raise the possibility that CRX has a direct role in regulation of opsin genes. We propose that the absence of red/green cone-mediated vision in blue cone monochromats who lack this region is due to the absence of the CRX-binding sites.

Five rhodopsin genes, *Rh1–5*, have been isolated and characterized from Drosophila (Chou et al., 1996). Studies of mutations localized to the regulatory regions of *Rh1–4* have led to the identification of a rhodopsin core sequence (RCS I) that is necessary for photoreceptor-specific activity (Fortini and Rubin, 1990). The RCS I sites of *Rh1*, *-2*, and *-4* contain a Ret1 site, and *Rh3* contains an OTX consensus site (Figure 3A). It was shown that these two sequences were equivalent in terms of function in vivo in experiments in which one was exchanged for the other (Fortini and Rubin, 1990). Interestingly, it has also been reported that the Drosophila *otx* homolog, *otd*, is required for photoreceptor cell morphogenesis during pupation (Vandendries et al., 1996). Further analysis of the function of *otd* in Drosophila photoreceptor cell development should be interesting, as might the results of a directed search for a *Crx* homolog in Drosophila.

The Ret1 (PCEI) site, originally identified by DNaseI footprint analysis on the rat rhodopsin promoter (Morabito et al., 1991), contains a homeobox consensus sequence (TAATTG). It has been found in the promoter regions of numerous vertebrate and Drosophila photoreceptor-specific genes (Kikuchi et al., 1993). The Ret1 site binds to a putative 40 kDa factor, which has been partially purified (Yu and Barnstable, 1994). Our results show that CRX, with a predicted molecular mass of 32 kDa, binds to and transactivates from this sequence, but weakly in vitro. These observations might suggest that CRX is not likely to be the factor that most tightly binds to the Ret1 site. However, as cited above, the Ret1 sequence and the OTX consensus sequence are functionally equivalent in Drosophila, and thus CRX might interact with the Ret1 site in vertebrate opsin genes.

Roles of *Crx* in Retinal Cell Differentiation

Studies in both Drosophila and vertebrate neural development argue that cell fate determination is achieved through interactions between both extrinsic and intrinsic factors. Further, a currently favored model is that the competence of neural precursors to respond to given extrinsic cues is defined in part by the expression of a temporally changing set of transcription factors (Zipursky and Rubin, 1994; Cepko et al., 1996). To examine the role that *Crx* may play in retinal cell fate determination, we forced expression of *Crx* in multipotent progenitors at P0 in the rat retina in vivo, using retrovirus-mediated gene transfer. Forced expression of *Crx* resulted in a significant increase of clones with only rods, in an almost complete absence of amacrine interneurons, and a marked decrease in glial cells. The presence of non-photoreceptor cells in clones infected with virus expressing *Crx* argues that *Crx* expression alone is not sufficient to instruct the rod cell fate. However, the near complete absence of amacrine cells in virally infected clones suggests that *Crx* expression is either sufficient to block multipotent progenitors from choosing the amacrine cell fate or it results in the death of those that do. Additional experiments are required to reveal the mechanisms underlying these changes in clonal composition.

The biochemical analysis presented here indicates that CRX can work as a transcription activator. To investigate the significance of CRX transactivation activity in vivo, a virus expressing a dominant-negative CRX construct, in which the homeodomain of CRX was fused with the repressor domain of the Drosophila engrailed protein, was used to infect P0 rat retina. The infected clones showed that the differentiation of rod outer segments and terminals was dramatically impaired (Figures 6C–6H). This phenotype is presumably mediated through CRX-binding elements in promoter regions of various genes involved in rod differentiation. Interestingly, this idea is reinforced by the report that mice homozygous for a mutation in the rhodopsin gene failed to form an outer segment (Humphries et al., 1997). However, due to the somewhat promiscuous DNA binding activity of the CRX homeodomain, analysis of mice with targeted disruption of *Crx* will be important in confirming this notion.

Experimental Procedures

Isolation of Mouse *Crx* cDNA

The strategy used to screen for homeobox genes expressed in the retina was previously described (Furukawa et al., 1997). The *Crx* cDNA fragment obtained in the screening was used as a probe for a mouse P0-P3 eye cDNA library (Yang et al., 1993). The longest clone isolated from this library (approximately 1.6 Kb) was subcloned into pBluescript (Stratagene) and analyzed.

Northern Analysis and In Situ Hybridization

RNA was extracted from adult Swiss-Webster mouse tissues. Ten micrograms of total RNA from each tissue was used for Northern blots, using nucleotides 928–1594 of the *Crx* cDNA as a probe. The full-length cDNA of *Crx* was used as a probe for in situ hybridization (Furukawa et al., 1997).

Protein Preparation and EMSA

For EMSA, a homeodomain peptide of CRX was produced by cloning a 302 bp SmaI fragment containing the homeobox sequence into the SmaI site of pGEX4T1 (Pharmacia) and overexpressing it in E. coli. It was purified using Glutathione Sepharose 4B (Pharmacia). EMSA was performed as described (Wilson et al., 1993).

Transactivation Assays

For expression in NIH3T3 cells, the entire *Crx* cDNA was subcloned into pME18S (Dr. K. Maruyama, Tokyo Medical and Dental University) driven by the *SR*α promoter. For the CRX-EnR fusion construct, a 302 bp XmaI fragment (amino acids 7–108) containing the *Crx* homeobox was fused to the EnR domain. To make the CRX-EnR fusion, the XmaI *Crx* fragment, an EcoRI-XmaI linker encoding amino acids 2–6 with a NcoI site at the methionine of position 2, an XmaI-ClaI linker, and a ClaI-NotI (partial digestion) 930 bp fragment of the EnR (Conlon et al., 1996) were ligated into a shuttle vector, pSLAX21 (Morgan and Fekete, 1996). The ClaI site inside this construct was designed so that the entire CRX-EnR fusion could be excised as a 1.3 kb ClaI fragment from pSLAX21/CRX-EnR. The oligonucleotide sequences for the XmaI-ClaI linker was: (top strand) 5′ CCGGGCATC GACGGTAC, (bottom strand) 5′ CGGTACCCTCGATGC. The 1.3 kb CRX-EnR ClaI fragment was blunt ended and cloned into pME18S. CAT reporter constructs were made by inserting a HindIII-BamHI fragment containing the OTX-binding consensus site or its derivatives into the HindIII-BamHI site either of pBL-CAT2 (Luckow and Schütz, 1987) for pOTX-CAT, pOTX(A)-CAT, and pRET1-CAT, or of pBL-CAT3 (Luckow and Schütz, 1987) for pIRBP123-CAT and pIRBP(mut)123-CAT. The HindIII-BamHI fragments used for these constructs were created by annealing the following complementary oligonucleotides: for pOTX-CAT, (top strand) 5′ AGCTTCAGGATTAA AGGGCAGGATTAAAGGCAGGATTAAGGCAGGATTAAAGGCAGGA TTAAAGGG, (bottom strand) 5′ GATCCCCTTTAATCCTGCCTTTAA TCCTGCCTTTAATCCTGCCTTTAATCCTGCCTTTAATCCTGA; for pRET1-CAT, (top strand) 5′ AGCTTGCCAATTAGGCCCCGCCAATTA GGCCCCGCCAATTAGGCCCCGCCAATTAGGCCCCGCCAATTAG GCCCCG, (bottom strand) 5′ GATCCGGGGCCTAATTGGCGGGGC CTAATTGGCGGGGCCTAATTGGCGGGGCCTAATTGGCGGGGCC TAATTGGCG. The GGATTA sequence in the top strand of pOTX-CAT was replaced with TGATTA in pOTX(A)-CAT. For pIRBP123-CAT, two oligonucleotides corresponding to −123–- 58 bp and to −57–+18 bp within the promoter sequence of human *Irbp* were annealed with the two complementary oligonucleotides that were designed to make HindIII and BamHI ends. The pIRBP123(mut)-CAT is identical to pIRBP123-CAT except for the mutations in the promoter sequence between −48 and −52 indicated in Figure 4A. NIH3T3 cells on 10 cm dishes were transfected with 10 ug of the expression vector with or without the *Crx* cDNA insert, plus 7.5 ug CAT reporter vector, and 2.5 ug of the β-gal expression vector (pSVβ, Clontech). After 2 days, cells were harvested, and CAT activity was measured according to Sambrook et al. (1989).

CRX Retroviral Construction

An EcoRV-HincII *Crx* cDNA fragment containing the entire *Crx* coding region was subcloned into the SnaBI site of the pLIA virus vector (pLIA/CRX). In addition, the 1.3 kb ClaI fragment of CRX-EnR described above was cloned into pLIA to make pLIA/CRX-EnR. A ClaI-BamHI 930 bp EnR fragment was cloned into the EcoRI-BamHI site of the pSLAX21 with a NcoI-ClaI linker. A 1.0 kb ClaI fragment containing EnR was cloned into the SnaBI site of pLIA. To produce virus, the plasmids were transfected along with a helper plasmid into a subline of the 293T cell line (Dr. Martine Roussel, St. Jude Children's Research Hospital, Memphis); supernatant was collected every 6–8 hr starting at 24 hr posttransfection and was concentrated (Cepko and Pear, 1997).

Viral Injection and Clonal Analysis

In vivo infection of retinae was carried out by injection of virus into P0 rat eyes (C/D, Charles River Laboratories) (Turner and Cepko, 1987). Infected retinae were dissected after 3 weeks, fixed, and stained for AP (Fields-Berry et al., 1992). Retinae were mounted and serially cryosectioned at 20 um to visualize infected clones. The cellular composition of each clone was determined through reconstruction from camera lucida drawings and/or photographs (Turner and Cepko, 1987). All clones were scored in LIA/CRX infected retinae. Owing to a higher titer leading to a greater number of clones in LIA-infected retinae, only ≈250 clones in continuous sections were scored. As shown in Table 1, injected animals from the same litter were compared.

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GenBank Accession Number

The GenBank accession number of the CRX amino acid sequence reported in this paper is U77615.

Note Added in Proof

Crx was independently isolated by Chen et al. using a yeast one-hybrid screen and the rhodopsin upstream region: Chen, S., Wang, Q.-L., Nie, Z., Sun, H., Lennon, G., Copeland, N.G., Gilbert, D., Jenkins, N.A., and Zack, D.J. (1997). Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron, in press.