

Isolation and Characterization of a Zebrafish Homologue of the Cone Rod Homeobox Gene

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PURPOSE. To isolate and characterize a zebrafish *Crx* homologue. Mammalian *Crx* genes are expressed specifically in photoreceptors and pinealocytes, regulate photoreceptor gene expression, are necessary for normal photoreceptor differentiation, and when mutated cause a variety of photoreceptor degenerations.

METHODS. A zebrafish retinal cDNA library was screened with a human *CRX* cDNA probe. Radiation hybrid mapping, Northern blot analysis, in situ hybridization, and transient transfection studies were performed using standard methods.

RESULTS. Based on amino acid sequence comparisons, zebrafish *crx* shows 50% identity with human *CRX*, and 85% identity in the homeodomain. A phylogenetic analysis indicates that zebrafish *crx* is most closely related to the mammalian *Crx* proteins, and more distantly related to the *Otx* proteins. Zebrafish *crx* maps between 49.6 and 54.5 cm from the top of linkage group LG05C, a map position consistent with the location of the mouse and human *CRX* genes. Northern blot analysis and in situ hybridization indicate that zebrafish *crx* is expressed in the retina and pineal gland. In adult zebrafish, *crx* is expressed by both rods and cones in the outer nuclear layer, and in cells in the outer zone of the inner nuclear layer, in the region occupied by bipolar cells. Similar to mammalian *Crx*, zebrafish *crx* interacts with neural retinal leucine zipper (Nrl) to activate, although weakly, rhodopsin promoter activity.

CONCLUSIONS. Based on molecular phylogeny, chromosomal location, expression pattern, and ability to activate rhodopsin promoter activity in transient transfection assays, zebrafish *crx* appears to be an orthologue and functional homologue of mammalian *Crx*. (*Invest Ophthalmol Vis Sci.* 2001;42:481-487)

In recent years, a number of transcription factors have been identified that appear to be important players in the regulatory gene cascade that controls retinal development.^{1,2} One of these, *Crx*, is a member of the *otd/Otx* family of paired-like homeobox genes.³⁻⁶ The available mammalian *Crx* sequences

(human, bovine, mouse, and rat) indicate that the protein is highly conserved, with 99% overall identity and 100% identity in the paired-like homeodomain. *Crx* expression is largely restricted to photoreceptor and pineal cells. It is the earliest known marker of photoreceptor identity in the developing retina. Its expression increases dramatically in mice at postnatal day 3, the approximate time of initiation of rhodopsin expression and outer segment morphogenesis. *Crx* binds to and transactivates the promoters of rhodopsin and several other photoreceptor and pineal-specific genes.^{3,5,7-9} It acts synergistically with the bZip transcription factor neural retina leucine zipper (Nrl).^{3,10} Mutant alleles of the *CRX* gene are associated with a variety of retinal degenerations, including cone-rod dystrophy, Leber congenital amaurosis, and retinitis pigmentosa.^{4,11-13}

Our understanding of retinal development has benefited from studies of a number of complementary animal model systems (for reviews see References 2,14-16). A particularly powerful and increasing popular model is the zebrafish retina.¹⁷⁻²⁸ Among the advantages of this model are the rapid rate of embryonic development, the relative ease of identifying developmental mutants, the ability to modulate gene expression, the continuation of retinal neurogenesis in adult fish, and the ability of neural retina to regenerate in the adult.

To extend these comparative evolutionary studies and also to take advantage of zebrafish genetics and the ability to manipulate gene expression in these animals, we have been attempting to clone and characterize zebrafish homologues of retinal transcription factors that have been identified in other systems.^{18,29} Here we report the cloning and initial characterization of a zebrafish *crx* gene. Supplemental information related to this study is available on-line at <http://www.ummz.umich.edu/birds/crx/>

MATERIALS AND METHODS

Isolation of Zebrafish *crx* cDNA

A λ ZAPII adult zebrafish retinal cDNA library (kindly provided by James Hurley) was screened at low stringency with a 897-bp probe (*Bam*HI and *Eco*RV fragment) derived from the open reading frame (ORF) of human *CRX*. Resultant clones were sequenced in both directions using dideoxy sequencing reactions and a CEQ2000 automated DNA sequencer (Beckman, Berkeley, CA). Resultant sequences were compared with the available public databases using the BLAST algorithm at the National Center for Biotechnology Information.³⁰

Northern Blot Analysis and In Situ Hybridization

Zebrafish (*Danio rerio*) were obtained from a local pet store and kept at 28.5°C in a 14-hour light/10-hour dark cycle. To harvest retinal tissues, fish were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO), and then chilled on ice. For Northern blot analysis, total RNA was extracted using Trizol reagent (Gibco-Life Technologies, Rockville, MD) and separated on a 1% agarose 0.7-M formaldehyde gel. Each lane contained 10 μ g of total RNA, which was

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transferred to the nylon membrane by passive capillary transfer and probed with α -³²P-labeled, random primed *crx* cDNA.

Zebrafish embryos were collected shortly after spawning and maintained at 28.5°C. To prevent development of melanin pigmentation, 0.2 mM 1-phenyl-2-thiourea (PTU; Sigma) was added to the water at 12 hours past fertilization (hpf). Adult tissues and embryos at various stages were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C or 1 hour at room temperature.

In situ hybridization on wholemounts and cryosections was performed as described²⁷ using digoxigenin (DIG)-labeled RNA sense and antisense probes. Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Roche Molecular Biochemicals, Indianapolis, IN) was used as the color substrate. No signals were detected with the sense RNA probes.

All procedures using fish were performed in accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University Committee on Use and Care of Animals in Research at the University of Michigan.

In Vitro Transient Transfection

The pcDNA-*crx* expression construct was generated by cloning the *crx* ORF into the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA). Varying amounts of the pcDNA-*crx* expression construct (0.1–1.0 μ g) were cotransfected with pBR130-*luc* (bovine rhodopsin promoter/luciferase reporter, 5.0 μ g) with and without pED-*bNrl* (1.0 μ g). The pCMV-*LacZ* plasmid was included to normalize transfection efficiency. Calcium phosphate-mediated transient transfection, luciferase, and β -galactosidase assays were performed as previously described,³¹ except that transfections with glycerol shock were performed with 50% confluent, 10-cm plates of the human embryonic kidney 293 cell strain grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, and 1% penicillin-streptomycin (Gibco) and harvested 48 hours after transfection. Each transfection experiment was performed in triplicate.

Molecular Phylogenetic and Character Analysis

We analyzed the phylogenetic relationship of *DreCrx* with two *Crx* and six *Otx* genes. The genes with their GenBank accession numbers are: human: *HsaCrx* (AF024711); mouse: *MmuCrx* (U77615), *MmuOtx1* (X68883),³² and *MmuOtx2* (X68884)³²; zebrafish: *DreOtx1* (D26172), *DreOtx2* (D26173), and *DreOtx3* (D226174); and amphioxus, *BfOtx* (AF043740). The alignment³³ and details of the Wilkinson character analysis³⁴ can be viewed at <http://www.ummz.umich.edu/birds/crx/>

Radiation Hybrid Mapping

A zebrafish radiation hybrid panel was analyzed by the polymerase chain reaction (PCR) method, according to the directions provided by the supplier (Research Genetics, Huntsville, AL). Primers that did not cross-react with rodent genomic DNA were designed from the 3'-untranslated region of *crx* (forward primer: 5'-TGAAGACTGTTCCTCT-GTAATCC; reverse primer: 5'-CAGATTCCCCTCCAGTTATGTGC). The results were submitted for analysis to the Tübingen map of the zebrafish genome (<http://www.map.tuebingen.mpg.de/>).

RESULTS

Isolation of a Zebrafish *crx* Homologue

Screening an adult zebrafish retinal cDNA library with a human *CRX* cDNA probe under low-stringency conditions identified several cDNA clones, the longest of which was 2.4 kb. Analysis of the 2.4-kb clone revealed an 843-bp ORF that encoded a protein with a paired-like homeodomain. Database searches demonstrated that the encoded protein was a novel member of the *otd/Otx/Crx* family. Based on the data shown below, we named this zebrafish cDNA *crx*. (In accord with the convention

established by the Zebrafish Nomenclature Committee [<http://zfsh.uoregon.edu/index.html>], zebrafish genes cloned as homologues of mammalian genes are given the same name and abbreviation, in all lowercase, italic letters.) The amino terminal end of the deduced protein sequence contains a paired-like homeodomain that is 95% identical with mammalian *Otx2* proteins and 85% identical with mammalian *Crx* proteins (Fig. 1A). Similar to the other known *Crx* and *Otx2* sequences, zebrafish *crx* has a WSP box in the middle of the sequence, which is 55% identical with mammalian *Crx* (Fig. 1B), and it shares a conserved *Otx*-tail (75% identity with mammalian *Crx*) at the carboxyl terminal (Fig. 1C). Additional sequences (YFS-GLDPYLSPL and ALSPL) that are conserved among all known *Crx* proteins are also found in zebrafish *crx*, but not in *Otx2* family members (Fig. 1D).

Zebrafish *crx*, a Member of the *Crx* Gene Family

To test the hypothesis of *Crx* monophyly (genes that are hypothesized to be derived from a common ancestor), we used the program MEAWILK to perform a statistical evaluation (Wilkinson support) based on the amino acid alignment (available at <http://www-personal.umich.edu/~fred/>). We evaluated four alternative, hypothesized monophyletic groups: 1) *DreCrx*, *MmuCrx*, *HsaCrx* (95, 199, 34, 0); 2) *DreCrx*, *MmuOtx1*, *DreOtx1*, *DreOtx3* (67, 115, 25, 0); 3) *DreCrx*, *DreOtx2*, *MmuOtx2* (78, 123, 21, 0), and 4) *DreCrx*, *BfOtx* (81, 114, 24, 2.7). Numbers in parenthesis indicate the number of characters (i.e., amino acid residues) with Wilkinson support for the hypothesis, the sum of weights of those characters, their percentage of the total possible weight, and the percentage of random hypotheses with comparable statistics, respectively. These values indicate that the best-supported hypothesis places *DreCrx* in a monophyletic group with mouse and human *Crx*.

We also developed a phylogenetic hypothesis to describe the proposed relationships of these nine members of the *Otx/otd/Crx* family based on the amino acids in the alignment. As shown in Figure 2, the most parsimonious tree contains three gene clades: *Otx2*, *Otx1/3* and *Crx*. Zebrafish *crx* (*DreCrx*) is monophyletic with the *Crx* clade, with 96% bootstrap support and seven steps until the node collapses (see <http://www.ummz.umich.edu/birds/crx/> for additional explanation). Therefore, based on both Wilkinson and phylogenetic evidence, we are confident in our assessment that *DreCrx* is an orthologue of the mammalian *Crx* genes.

Zebrafish *crx* Maps to Linkage Group 05

To determine whether *crx* might be a candidate gene for known or future zebrafish mutations, its chromosomal position was mapped by radiation hybrid analysis. This analysis on the Research Genetics/Tübingen mapping panel placed *crx* between 49.6 and 54.5 cM from the top of zebrafish linkage group LG05, with a lod score of 18.16. The two nearest markers were AF006488 (zebrafish *deltab*) and fa28d05.1/AA606175 (an EST with sequence similarity to the M chain of human creatine kinase). A comparison of linkage relationships of genes mapped on a human radiation hybrid panel (LocusLink, National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/locuslink>) and the orthologous zebrafish markers located on a meiotic mapping panel³⁵ (John Postlethwait, personal communication) suggested that the map position of zebrafish *crx* on LG05 is syntenic with human *CRX* at 19q13.3 and mouse *Crx* at 7.85 cM. For example, human muscle creatine kinase M chain (CKMM/AA606175) maps to chromosome 19q13.2-13.3 and the orthologous mouse gene maps to 74.5 cM. Other closely

A. Homeodomain

Consensus	QRRERTTFTR.QLD.LEALFAKTRYPDIFMREEVALKINLPESRVQVWFKNRRRAKCRQQQ
Dre-cruxT...I....T.....
Hsa-CRXS..EE.....Q...VYA.....R
Mnm-CrxS..EE.....Q...VYA.....R
Rno-CrxS..EE.....Q...VYA.....R
Dre-otx2A...V.....
Hsa-OTX2A...V.....
Mnm-Otx2A...V.....
Xla-Otx2A...I.....

B. WSP Box

Consensus	APVSIWSPASISP
Dre-cruxV...T.L..
Hsa-CRX	.T.....E..
Mnm-Crx	.T.....EA.
Rno-Crx	.T.....E..
Dre-otx2
Hsa-OTX2
Mnu-Otx2
Xla-Otx2

C. Otx Tail

Consensus	DCLDYKDQ.S-WK
Dre-cruxQA...
Hsa-CRX	.P.....SA...
Mnm-Crx	.P.....SA...
Rno-Crx	.P.....SA...
Dre-otx2T.S...
Hsa-OTX2T.S...
Mnm-Otx2T.S...
Xla-Otx2T.S...

D. Crx sequences

Consensus	YFSGLD--PYLSP	ALSPL
Dre crux--.....
Hsa-CRX--.....
Mnm-Crx--.....
Dre-otx2	..G.M.CGS..T.	Q...M
Hsa-OTX2	..G.M.CGS..T.	T...M
Mnm-Otx2	..G.M.CGS..T.	T...M

FIGURE 1. Comparison of amino acid sequences of zebrafish *crx* with other vertebrate Crx and Otx2 proteins. Deduced amino acid comparisons are shown for the (A) homeodomain, (B) WSP box, (C) Otx tail, and (D) Crx-specific sequences of zebrafish (*Danio rerio*, *Dre crux*, *Dre otx2*), human (*Homo sapiens*, *Hsa CRX*, *Hsa OTX2*), mouse (*Mus musculus*, *Mmu Crx*, *Mmu Otx2*), and *Xenopus laevis* (*Xle otx2*) proteins.

linked markers on human chromosome 19 include Fc (gamma) binding protein (D84239) and vaccinia-related kinase (VRK3/AB031052) at 19q13. On a meiotic mapping panel from homozygous diploid zebrafish embryos, an expressed sequence tag (EST; fb18c01/A1416123) with sequence similarity to *vrk3* and an EST (zehn1245/AI617215) with sequence similarity to Fc (gamma) binding protein are both located on linkage group 05 (John Postlethwait, personal communication). There are no currently mapped zebrafish mutations near the *crx* locus.

Zebrafish *crx* Expressed Specifically in the Neural Retina and Epiphysis

Northern blot analysis indicated that among the five adult zebrafish tissues we tested (eye without the lens, brain, heart, liver, and skeletal muscle), the *crx* probe detected a single band of approximately 2.4 kb that was restricted to the eye, although when the film was overexposed as shown in Figure 3, RNA from liver revealed a very faint band.

We next used in situ hybridization to determine the cell-type specificity and pattern of *crx* expression during development and in adult zebrafish. Expression of *crx* was first detected in the epiphysis (primordium of the pineal gland) in embryos at 17 hpf (data not shown). Pineal expression was strong at 24 to 31 hpf (Figs. 4A, 4C), and it persisted up to at least 50 hpf, the oldest age at which the brain was examined (data not shown).

Expression of *crx* in the retina was first observed at 24 hpf in a few cells in the ventronasal region immediately adjacent and nasal to the choroid fissure (data not shown). At this stage, all cells in the presumptive neural retina are mitotically active retinal progenitors.²² The site at which *crx* expression first appears corresponds to the location of the earliest differentiating neurons, retinal ganglion cells, which appear a few hours later,^{22,28} and the first photoreceptors, which do not begin to become postmitotic until approximately 43 hpf^{22,27} and do not begin to express opsin until approximately 48 hpf.²⁷ By 31 hpf, the expression domain of *crx* was confined to a localized patch of undifferentiated, retinal progenitor cells in ventronasal retina (Figs. 4B, 4C). With further development, the *crx* expression domain gradually expanded into the dorsotemporal retina (Figs. 4C, 4D, 5A). As differentiation of the retina progressed, *crx* became restricted to the outer layers of the retina (Fig. 5B), so that by 52 hpf the differentiating ganglion cell layer was devoid of staining (Fig. 5E). In the retina of adult zebrafish, *crx* continued to be expressed in the outer nuclear layer by both rods and cones (Fig. 5F), which have distinct morphologies in the zebrafish retina.^{36,37} Unidentified cells in the outer zone of the inner nuclear layer, in the region occupied by bipolar cells, also express *crx* (Fig. 5F).

To compare the expression of *crx* and *otx2*, we examined embryos at 49 to 52 hpf and found *otx2* expression in retinal pigmented epithelium (RPE), the retinal germinal zone at the

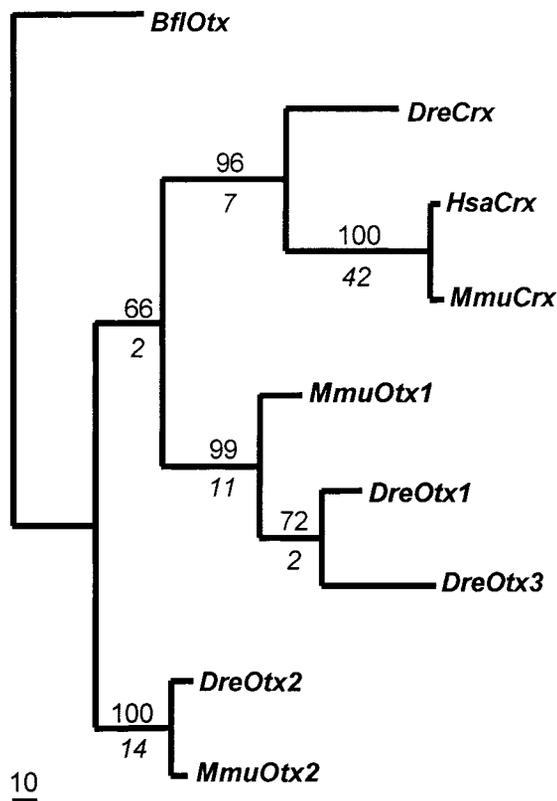


FIGURE 2. Hypothesized phylogeny of Crx and Otx proteins. The most parsimonious phylogenetic tree using an exhaustive search in PAUP (phylogenetic analysis using parsimony; <http://www.lms.si.edu/PAUP/about.html>) for nine genes from four taxa (human, *Homo sapiens*, Hsa; mouse, *Mus musculus*, Mmu; zebrafish, *Danio rerio*, Dre; and amphioxus, *Branchiostoma floridae*, Bfl). Bootstrap percentage values based on 10,000 branch and bound repetitions are given above each branch,⁴⁹ and decay indices (the number of parsimony steps before the node collapses)⁵⁰ from TreeRot,⁵¹ are in italics below the branch. The tree was rooted with an outgroup, the amphioxus Otx protein (BflOtx). The bar indicates a scale of 10 parsimony steps.

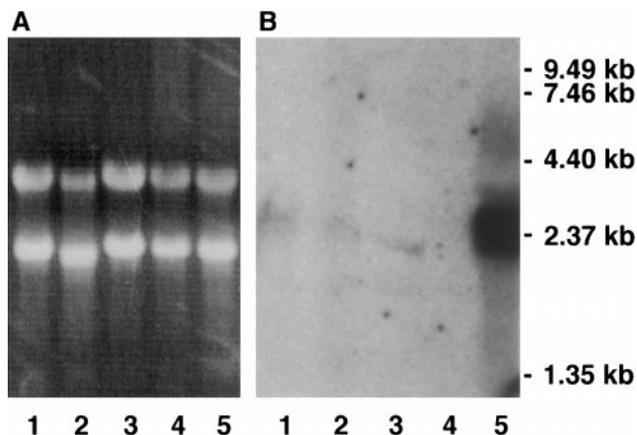


FIGURE 3. Northern blot analysis of *crx* expression in adult zebrafish. (A) Ethidium bromide-stained membrane, indicating that an approximately equal amount of RNA was present in each lane. (B) Northern blot analysis of zebrafish total RNA (10 µg/lane) extracted from lane 1: liver; lane 2: heart; lane 3: muscle; lane 4: brain; and lane 5: eye without lens.

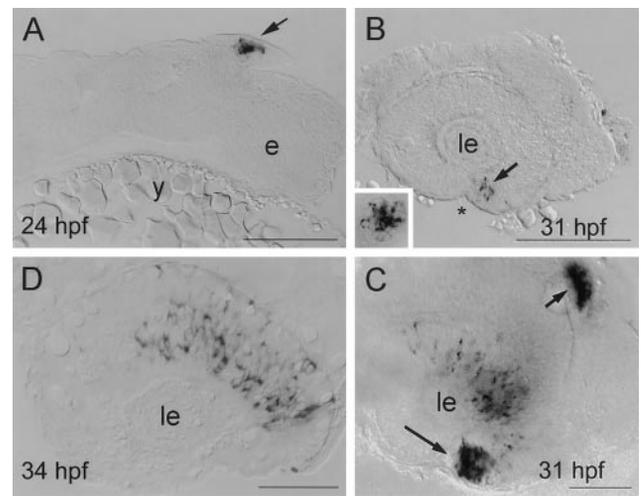


FIGURE 4. In situ hybridization analysis of *crx* expression in zebrafish embryos. Embryos were processed intact for in situ hybridization, then examined as wholemounts (C) or sectioned sagittally (A, B, and D). Nasal is to the right in all panels. (A) Expression of *crx* in the epiphysis (arrow) at 24 hpf. e, eye; y, yolk. (B) Expression of *crx* in the retina at 31 hpf (arrow); choroid fissure (*). Inset: A ventral view of *crx* expression as seen in a wholemount. le, lens. (C) Lateral view of an embryo at 31 hpf showing expression of *crx* in the ventronasal retinal patch (large arrow) and the epiphysis (small arrow). (D) Expression of *crx* in the nasal retina of a 34-hpf embryo. Bars, (A) 50 µm; (B) 25 µm; (C, D) 12.5 µm.

ciliary margin, the inner nuclear layer (INL) of the retina, and the forebrain and midbrain (Fig. 5D). Of particular note is that *otx2* was completely absent from the developing outer nuclear layer (ONL; Fig. 5D), in contrast to the high levels of *crx* expressed in those regions (Fig. 5C).

Zebrafish *crx* and Bovine *Nrl* Act Synergistically on the Proximal Rhodopsin Promoter

We showed previously that mammalian Crx acts synergistically with Nrl to transactivate the rhodopsin promoter.³ To test whether zebrafish *crx* has similar activity in this assay, we transiently expressed zebrafish *crx* in human embryonic kidney cells (293) together with a construct containing the bovine rhodopsin proximal promoter region (RPPR) fused to a luciferase reporter gene. In these experiments, pcDNA-*crx* by itself demonstrated only minimal transactivating activity (less than 50% increase even at the highest concentrations; Fig. 6A). However, when pcDNA-*crx* was cotransfected with pED-*bNrl*, reporter activity increased up to threefold compared with pED-*bNrl* alone, and the fold increase in stimulation showed a dose-dependent relationship with the amount of pcDNA-*crx* (Fig. 6B). Although this degree of activation is small, and considerably less than that observed with mammalian Crx, it was seen reproducibly in our assay system.

DISCUSSION

In this article, we describe the identification and characterization of a zebrafish ortholog of Crx. The only Crx genes identified to date are mammalian (human, bovine, mouse, and rat).³⁻⁶ Phylogenetic molecular analysis indicates that the Crx genes are a divergent subgroup of the Otx family.³⁸ In contrast to the high degree of sequence conservation (96% overall identity) among mammalian Crx homeodomain proteins, zebrafish *crx* exhibits only moderate overall amino acid sequence homology with the mammalian proteins: 49% identity over the entire amino acid sequence and 85% identity in the homeodo-

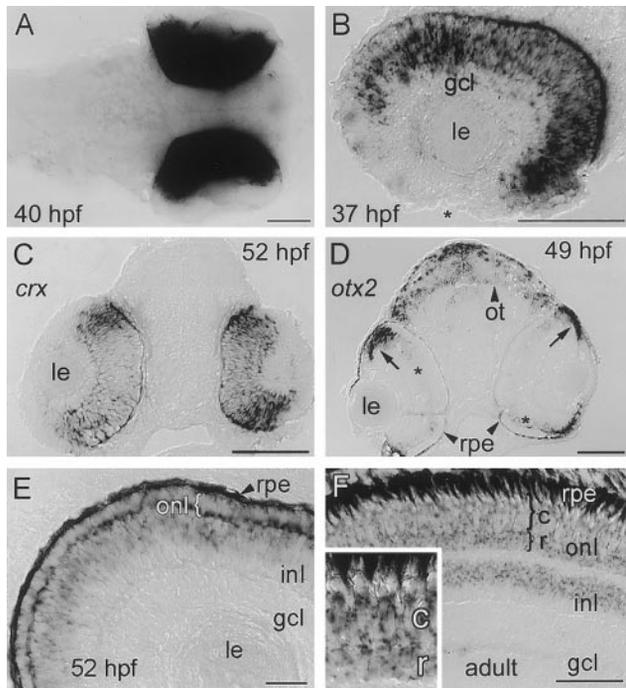


FIGURE 5. Expression of *crx* and *otx2* at later stages of development and in adult zebrafish. Embryos were processed intact for in situ hybridization, then examined as whole mounts (A), sectioned sagittally (B), or transversely (C, D). Adult retina was first cryosectioned and then processed for in situ hybridization (E, F). (A) Ventral view of *crx* expression at 40 hpf. (B) Embryonic eye at 37 hpf; gcl, ganglion cell layer; le, lens. (*), Choroid fissure. (C) An embryo at 52 hpf hybridized with *crx* probe. Dorsal is up. (D) An embryo at 49 hpf hybridized with *otx2* probe. Arrows: Germinal zone at the ciliary margin of the retina; (*), cells in the inner nuclear layer. ot, optic tectum. (E) The eye of an embryo at 52 hpf (without PTU treatment). (F) Retina of adult zebrafish; cones (c) and rods (r). Inset: ONL at higher magnification. Cone nuclei were elongated and formed a single row adjoining the RPE, whereas the rod nuclei were rounded and located vitread to the cones. All photoreceptors expressed *crx*. Bars, (A through D, F) 25 μ m; (E) 50 μ m.

main, compared with 55% overall amino acid identity to human and mouse *Otx2* and 95% identity in the homeodomain. However, in specific regions outside the homeodomain zebrafish *crx* is more similar to mammalian *Crx* genes than to *Otx2*, and a rigorous phylogenetic analysis indicated that zebrafish *crx* is monophyletic with the mammalian *Crx* proteins. Furthermore, the zebrafish gene mapped to a location syntenic with the chromosomal loci of mouse and human *Crx* genes, providing additional evidence to support the designation of this gene as a zebrafish *crx* orthologue.

Consistent with the proposed phylogenetic molecular relationship of zebrafish *crx* and mammalian *Crx* genes, we found that their patterns of expression were more similar to each other^{3-5,39,40} than to any members of the *Otx* gene family.^{41,42} In zebrafish, the onset of *crx* expression at 17 hpf is well after the onset of *otx1*, *otx2*, and *otx3* at approximately 6 to 8 hpf,^{23,43,44} and the pattern of expression of *crx* does not correspond to any of the *otx* genes. *Crx* is first expressed in the zebrafish retinal neuroepithelium in a small patch of progenitor cells in the ventronasal region at 24 hpf, before the onset of neural differentiation, which occurs in the same location.^{22,36,45} In the mouse, *Crx* is first expressed in the retinal neuroepithelium at E12.5, when cone genesis is underway,³ and human *CRX* was first detected in the fetal retina at 10.6 weeks after conception, before the onset of cone genesis.⁴⁰ The onset of expression of zebrafish *crx* in the retinal neuroepithelium at 24 hpf is also well after the initial stages in the formation of the optic primordia (at 10–11 hpf in zebrafish embryos,¹⁸) but well before the first photoreceptors become postmitotic.

Experimental evidence suggests that *crx* may be involved in regulation of photoreceptor differentiation.⁵ Compelling evidence in support of this hypothesis is the association of mutations in the human *CRX* gene with several forms of retinal dystrophies that involve photoreceptor dysgenesis or degeneration.^{4,11-13,46,47} In addition, consistent with the human studies, outer segments do not develop in the photoreceptors in *Crx* null mice generated by gene targeting, and the photoreceptors eventually degenerate.⁴⁸ Expression levels of some photoreceptor-specific genes are reduced in *Crx*^{-/-} mice, whereas other genes are upregulated, suggesting that *Crx* may function as both a positive and a negative regulator of gene expression. These results demonstrate that in mice, *Crx* is not essential for cell fate determination in photoreceptors, but it is

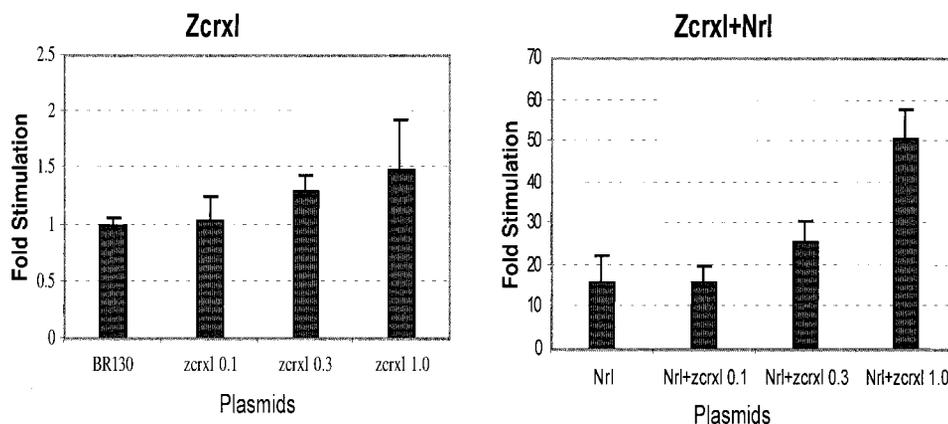


FIGURE 6. Synergistic transactivation on the rhodopsin promoter by *crx* and *Nrl*. Human embryonic kidney 293 cells were transfected with 5 μ g of the bovine rhodopsin proximal promoter-luciferase construct with (left) or without (right) the *Nrl* expression plasmid pED-*Nrl* (1 μ g) and with or without the indicated amount of *zcrxl* expression plasmid pCDNA3-*zcrxl*. Fold stimulation represents the ratio of the corrected luciferase activity with pED-*Nrl* and pCDNA3-*crx* to the activity of bovine rhodopsin proximal promoter-luciferase construct only. The values shown represent the means of three independent experiments performed in triplicate \pm SE.

essential for their proper differentiation.⁴⁸ Although the function of *crx* in the developing zebrafish retina has not yet been demonstrated, the similarities in the pattern of expression and its developmental regulation in zebrafish, compared with mammalian *Crx*, suggest that *crx* may also play a role in the commitment and differentiation of retinal photoreceptors, and perhaps other retinal neurons, in zebrafish. The strong expression of *crx* in the inner nuclear layer, perhaps in bipolar cells, was originally unexpected based on initial reports that mammalian *Crx* appears to be expressed weakly, if at all, in few INL cells.³ However, more recent immunocytochemical data have shown substantial expression of Crx protein in the bipolar cell layer in adult mouse retinas.³⁹

Zebrafish *crx* is also expressed in the epiphysis-pineal gland, beginning at approximately 17 hpf, earlier than its first appearance in the neural retina. In mammals, Crx is thought to be involved in circadian behavior. For example, in *Crx*^{-/-} mice, the gross structure of the pineal gland is normal at 1 month, but expression levels of pineal-specific genes are decreased, and circadian entrainment is affected.⁴⁸ In adult rat pineal gland, *Crx* mRNA in pinealocytes displays a diurnal variation with peak levels in the dark phase.⁹ In contrast, *Crx* shows constant levels of expression in the retina of rats maintained in a 12-hour:12-hour light-dark cycle,⁶ indicating that the regulation of *crx* mRNA expression in the retina differs from the pineal gland in this species. We did not examine the circadian expression of *crx* in adult zebrafish, but our Northern blot analysis showed no detectable *crx* message in adult brain during the light phase of the diurnal cycle.

Mammalian *Crx* can activate transcription of a number of photoreceptor-specific genes.^{3,5} In this study we show that zebrafish *crx* alone did not transactivate gene expression from a bovine rhodopsin promoter construct in a transient transfection assay. However, a small synergistic effect was observed when *crx* was cotransfected with the bovine Nrl transcriptional regulator, which also binds to photoreceptor-specific regulatory elements. The absence of effect with *crx* alone, and the relatively weak synergistic effect with Nrl, may be due to divergence in the structure of the DNA-binding and activating domains of the zebrafish and mammalian genes. Future studies are needed to determine whether the effect we observed is biologically significant.

Taken together, the molecular phylogeny and genomic map location, the comparisons of gene expression patterns, and the ability of *crx* to cooperate with Nrl to activate gene expression from the rhodopsin promoter, all suggest that zebrafish *crx* is orthologous to the mammalian *Crx* genes, and the function of zebrafish *crx* is likely to be more similar to mammalian Crx than to the other Otx family members.

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