The Rx-like Homeobox Gene (Rx-L) Is Necessary for Normal Photoreceptor Development

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PURPOSE. The retinal homeobox (Rx) gene plays an essential role in retinal development. An Rx-like (Rx-L) gene from Xenopus laevis has been identified. The purpose of this study was to analyze the function of Rx-L in the developing retina.

METHODS. DNA-binding properties of Rx-L were analyzed by electrophoretic mobility shift assay (EMSA), with in vitro-translated proteins and radiolabeled oligonucleotide probe. The Rx-L expression pattern was analyzed by in situ hybridization using whole or sectioned embryos and digoxigenin-labeled antisense riboprobes. Rx-L loss of function was studied by using antisense morpholino oligonucleotides targeted to the Rx-L translation initiation site. Embryos injected with control or Rx-L morpholinos were analyzed at stage 41 or 45.

RESULTS. Rx-L shares homology with Rx at the homeo-, OAR, and paired-like homeodomain, and a C-terminal orthopedia-aristaless-rx like homeodomain, and a C-terminal orthopedia-aristaless-rx like homeodomain. Rx-L loss of function resulted in a decrease in the length of both rod and cone outer segments. Rx-L knockdown by antisense morpholino oligonucleotides resulted in a decrease in the length of both rod and cone outer segments.

CONCLUSIONS. The results suggest that Rx-L functions to regulate rod and cone development by activating photoreceptor-specific gene expression. (Invest Ophthalmol Vis Sci. 2006;47:4245–4253) DOI:10.1167/iovs.06-01677

The retina is a multilayered neural tissue and is derived from the inner surface of the optic vesicle through cell migration and cell differentiation.1,5 Retinal development begins when the diencephalon evaginates during the neural tube stages of embryogenesis and contacts the presumptive lens ectoderm. A complex set of reciprocal cell and tissue interactions ensues, resulting in the thickening and invagination of the retinal neuroepithelium to form the eye cup. The cells of the retinal neuroepithelium undergo expansion, cell cycle exit, stratification, and differentiation, and ultimately form the mature neural retina. The processes of retinal cell specification and differentiation are regulated by many transcription factors (reviewed by Marquardt6). The earliest developmental processes of specification involve the actions of a set of transcription factors known as the eye field transcription factors (EFTFs).7 These include Pax6, ET, Six3, Optx2, Tlx, and Lhx2. These genes form a genetic network that is largely conserved from flies to humans.6 Many of the EFTFs are mutually independent for initiation or maintenance of expression. Proper expression and function of these gene products is essential for normal eye development. Another member of this group of EFTFs is the retinal homeobox gene, Rx.7 Rx is expressed in the anterior neural plate and the presumptive eye fields, coinciding with the initiation of expression of other EFTFs but before the onset of morphologic signs of retinal development, and continues throughout retinal maturation and differentiation.8–11 In zebrafish and Xenopus laevis, misexpression of Rx results in the formation of ectopic retinal tissue at the expense of the forebrain.8–12,13 Inhibition of Rx expression or function results in severe deficiencies in eye development in several vertebrate species.8,12–17 This body of work indicates that Rx is essential for normal eye development.

Rx gene products share the following conserved domains with other aristless-related homeobox gene family members: an N-terminal octapeptide domain, a highly conserved paired-like homeodomain, and a C-terminal octapeptide domain.24

Some vertebrates encode a Rx-related gene product that functions as a stronger transcriptional activator than Rx, such as chicken Rxl,23,31 and human QRX.24 Mice do not appear to encode a Rxal/QRx-like gene.24 The Rxl and QRX gene products share similar homeodomains and OAR domains with Rx but do not encode an octapeptide domain.23,24–31 Regions outside of these two domains share little identity. As the case with Rx, QRX and Rxl have been shown to bind PCE-1

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elements.\textsuperscript{23-24,31} It has been suggested that QRX also binds the BAT and Ret4 sites.\textsuperscript{24} QRX can activate a rhodopsin promoter reporter construct by co-operating with CRX and NRL. However, investigations of Rxl/QRX-like gene product function in retinal development have been hampered by the absence of loss-of-function data. We report the identification of a new member of the Rx family, Rx-L (Rx-like), from \textit{Xenopus laevis}. We show that it is orthogonal or analogous to QRX and Rxl in structure, expression, and molecular function and that it plays an important role in modulating the expression of opsin genes and photoreceptor development.

\section*{Methods}

We confirm that this research adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

\subsection*{Embryo Injection}

Embryos were obtained by in vitro fertilization.\textsuperscript{32} Morpholinos (MOs; 0.1 or 0.25 mM) and green fluorescent protein (GFP) RNA (0.05 \textmu g/\mu l) were co-injected into one prospective dorsal blastomere at the four-cell stage in a 10-nl volume. Embryos with GFP expression in only one eye were selected for further studies. The Rxl antisense MO (\texttt{5′-CTAGAAACAATCCCGTTGCGAGAC-3′}), was designed by the manufacturer (Gene-Tools, Philomath, OR; \url{http://www.gene-tools.com}) to target a region including the translation start codon (in italic), 16 nucleotides upstream, and 5 nucleotides downstream. As a control, an MO with random sequence was obtained from Gene-Tools. Capped RNAs for microinjection were prepared (mMessage mMachine kits; Ambion, Austin, TX). RNAs were purified by gel filtration chromatography (RNA Mini Columns, Roche Diagnostics, Indianapolis, IN) to remove unincorporated nucleotides and cap analogue.\textsuperscript{33} Rx and Rxl RNAs were prepared from plasmids pSP64T/Rx1A and pCS2/Rxl, respectively.

\subsection*{DNA Constructs}

The RxL expressed sequence tag (EST; XL073a16) was obtained from The National Institute of Basic Biology, Japan (\url{http://xenopus.nibb.ac.jp}) and will be referred to as pBS/Rx1A. The coding region (CDS) was amplified from the EST by using specific primers, RxL CDS F and R containing \textit{EcoRI} and \textit{XhoI} sites, respectively (F: \texttt{5′-GATCGAATTCCTGCGACTAGT-3′}; R: \texttt{5′-GATCCAGATCTGGTCGACTG-3′}; restriction enzyme sites used for subcloning are in italic). The amplified product was subcloned into pCS2\textsuperscript{34} using corresponding restriction sites to prepare pCS2/RxL. To prepare pRl-GFP, cDNA encoding the final 39 nucleotides of the 5′-untranslated region (UTR) and the first 38 amino acids of Rxl after the start codon, which includes the target site for RxL MO, were amplified (primers: RxL CDS F and the first 38 amino acids of RxL after the start codon, which includes the target site for RxL MO, were amplified (primers: RxL CDS F, above, and RxL SGP R: \texttt{5′-GATCCAGATCTGGTCGACTG-3′} – untranslated region (UTR), digested using \textit{EcoRI} and \textit{SpeI} (recognition sites were incorporated into PCR primers, in italic) and cloned in-frame with the GFP coding sequence in the super GFP (SGP) plasmid using \textit{EcoRI} and \textit{XhoI} sites.\textsuperscript{35} Amplification errors were minimized by using a high-fidelity polymerase (Platinum Pfx; Invitrogen, Carlsbad, CA). Integrity of the amplified products was ensured by sequencing (DNA Sequencing Core, Columbus Children's Research Institute [CCRI]). For preparation of in situ hybridization probes, the Rx1A coding region was liberated from pSP64T/Rx1A using \textit{BglII} and \textit{EcoRI}, and ligated with a cloning vector (pBlueScriptII KS; Stratagene, La Jolla, CA) digested with \textit{BanHI} and \textit{EcoRI}, resulting in the plasmid pBS/Rxl. XOP-Luc was prepared by inserting the \textit{BamHI-BglII} fragment of pXOP-EGFP (the gift of Barry Knox)\textsuperscript{36} into the \textit{BglII} site of pGEl3 (Promega, Madison, WI). The Pax6 plasmid (pCS2/Pax6) was the gift of Yi Rao,\textsuperscript{37} and the Rx plasmid (pSP64T/Rxl1A) was the kind gift of Milan Jamrich.\textsuperscript{8}

\subsection*{Luciferase Assay}

RNA encoding Rx or Rxl (100 pg) and XOP-Luc reporter plasmid (25 pg) was injected into \textit{Xenopus laevis} embryos (one blastomere at the two-cell stage). As an internal control, pRLtk, a plasmid containing a \textit{Renilla} luciferase expression cassette under the control of the thymidine kinase promoter, (50 pg) was co-injected. For each set of effectors and reporters, 20 embryos were injected and cultured to approximately st 10.5. Embryos were divided into three groups of at least three embryos each, and lysed in PLB (20 \textmu l/embryo; Stop ’n Glo Dual Luciferase Assay Kit; Promega). Lysates were clarified by centrifugation\textsuperscript{37} and firefly, and \textit{Renilla} luciferase activities were measured using 20 \textmu l of lysis, according to the manufacturer’s instructions. Firefly luciferase levels were normalized against \textit{Renilla} luciferase levels. The statistical significance of the results was determined with the Student’s \textit{t}-test.

\subsection*{Electrophoretic Mobility Shift Assays}

Proteins were synthesized in vitro (Quick TNT Linked In Vitro Transcription/Translation Kit; Promega) and verified by SDS-PAGE. Electrophoretic mobility shift assays using 3 \mu l of in vitro translated Rx or Rlx were performed as described previously\textsuperscript{15} using a radiolabeled PCE-1 probe\textsuperscript{16} and unlabeled PCE-1, BAT-1,\textsuperscript{19} and Ret-4\textsuperscript{20} oligonucleotide competitors. Rx and Rlx were synthesized from plasmids pSP64T/Rx1A and pCS2/RxL, respectively.

\subsection*{Immunohistochemistry}

Stage (st)-41 or -45 tadpoles having GFP expression in only one eye were fixed in 4\% parafomaldehyde (MEMFA)\textsuperscript{32} at room temperature for a hour, dehydrated with 100\% methanol for at least 1 hour, embedded in paraffin, and serially sectioned at 8 \mu m. Staining of paraffin-embedded sections with antibodies was performed as previously described.\textsuperscript{36} Primary antibodies were used at the following dilutions: mouse anti-rodopsin (RetP1; Biomeda, Foster City, CA) 1:50; mouse anti-islet 1, 1:50 (39.4D5; Developmental Studies Hybridoma Bank [DSHB], University of Iowa). Biotinylated-peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA) was used at 5 \mu g/mL.

\subsection*{Quantification of Rod Length}

To determine the effect of Rx-L knockdown on rod length, we measured the width of the photoreceptor layer in digital images of injected or un.injected retinas immunostained with RetP1 antibody. Using image management software (Photoshop; Adobe Systems, Mountain View, CA), bars were drawn across the photoreceptor layer of the injected and uninjected retina of the same embryo. Each bar was drawn so that it spanned the region of RetP1 staining, along the schlerovitreal axis (parallel to the long axis of the rods, perpendicular to the retinal pigmented epithelium). Six or seven bars were drawn per section, at approximately equal intervals around the retina (Fig. 5E). The bars were drawn at similar positions of each uninjected and injected retina pair. The length of each bar was determined by the software. Bar lengths from injected retinas were normalized against the lengths of corresponding bars from uninjected retinas and averaged. In the example illustrated in Figure 5E, the lengths of bars a–g and a′–g′ would be determined and the following ratios would be calculated and averaged: a/a′, b/b′, ..., g/g′. The average normalized bar lengths from RxL and control MO oligonucleotide-injected retinas were compared by a Student group \textit{t}-test.

\subsection*{In Situ Hybridization}

Wholemount and section in situ hybridizations were performed with digoxigenin-labeled antisense riboprobes, as described previously.\textsuperscript{52} Sections in situ were performed on 8-\mu m sections of paraffin-embedded, parafomaldehyde-fixed embryos.\textsuperscript{59} Rx antisense probe was prepared from pBS/Rxl1A linearized with \textit{EcoRI} and transcribed with T7. Rx sense probe was prepared from pBS/Rxl1A linearized with \textit{BanHI} and transcribed with T3. To prepare RxL antisense probe, we linear-
Results

Sequence Analysis of Rx-L

We identified an EST encoding an Rx-like gene product by BLAST search of *Xenopus* ESTs. Figure 1 shows the alignment between *Xenopus laevis* Rx1A with Rx-L. The overall identity is 62% and similarity is 75%. The homeodomains of Rx-L and Rx1A are nearly identical, with only one mismatch (97% amino acid identity and 98% similarity). The C-terminal OAR domains of the two protein sequences also share high homology. The region between two domains is relatively dissimilar, except for the Rx-domain, which shows moderate identity and similarity. Rx-L does not encode an octapeptide motif, found near the N terminus of Rx and many related proteins.

Similar Rx-like gene products, RaxL and QRX, have been identified in chick and humans, respectively. Rx-L is also similar to QRX and RaxL (Fig. 1B). Of note, the Rx domains of the Rx-like proteins are more similar to each other than they are to that of Rx.

Expression of Rx-L in *Xenopus* Retina

The expression of Rx-L during early *Xenopus* eye development was analyzed by in situ hybridization and RT-PCR (Fig. 2). Rx-L is not expressed at neurula stages, and extremely faint Rx-L expression can be detected in the early tailbud embryo stage (Figs. 2A, 2B), whereas Rx is expressed robustly in the developing eye in these stages (Figs. 2G, 2I). In late tailbud stages, Rx-L expression is observed in the developing eye, and becomes excluded from the developing lens (Figs. 2C–E), as is the case for Rx (Figs. 2I–K). No staining was observed when embryos are probed using sense control riboprobes encoding Rx-L (Fig. 2F) or Rx (Fig. 2L). These results suggest that Rx-L is expressed in the developing retina but that Rx-L expression begins later than that of Rx.

The spatial pattern of Rx-L expression was analyzed in further detail by in situ hybridization using sections of *Xenopus* tadpoles. At st 36 and 38, when photoreceptors have
begun differentiating and the nuclear layers of the retina are becoming defined, Rx-L expression was found primarily in the peripheral and outer portion of the central regions of the neural retina (Figs. 2M, 2N), including the ciliary marginal zone (CMZ), where retinal progenitor cells are found at this stage.44 The expression of Rx-L in the central retina is comparable to that of rhodopsin, which is restricted to cells in the differentiating photoreceptor layer (Fig. 2S). At these stages, Rx is also expressed in the CMZ and the outer neural retina (Figs. 2P, 2Q). Rx is expressed in a thicker layer than Rx-L and rhodopsin in the central neural retina, comprising the developing outer nuclear layer and at least a portion of the inner nuclear layer. At st 41 when retinal cell types have become morphologically defined, Rx-L expression can no longer be detected in the central retina (Fig. 2O). Residual expression can be detected in the CMZ. By contrast, Rx is expressed in the photoreceptor layer; intermittently in the inner nuclear layer, and robustly in the CMZ (Fig. 2R). Staining was not observed when Rx or Rx-L sense control riboprobes were used (Figs. 2S, 2T). These results suggest that Rx-L expression is restricted to the differentiating photoreceptor layer and the CMZ in the developing retina at st 36 to 38, when photoreceptor differentiation is initiated.

To pinpoint the initiation of Rx-L expression, we performed RT-PCR using RNA isolated from whole embryos at different stages (Fig. 2V). Low levels of Rx-L expression can be detected as early as st 21 (neural tube/early tailbud stage) and increases by st 25 and 30. Rx expression is detectable at st 13 to 14, the late gastrula/early neural plate stages45 when Rx-L expression is not detectable. Rx-L expression continues through st 38, when photoreceptors are differentiating. Taken together, these results indicate that Rx-L is expressed during stages of retinal development and differentiation, after the initial specification of the eye, until retinal maturation.

### Binding of the PCE-1/Ret1 Site

The DNA-binding properties of the Rx-L gene product were studied by EMSA using a radiolabeled PCE-1 oligonucleotide probe and synthetic protein. As has been reported for Rx,19 Rx-L bound the PCE-1 oligonucleotide. A shifted band was observed when the PCE-1 probe was incubated with Rx-L protein, indicating interaction between Rx-L protein and the PCE-1 oligonucleotide (Fig. 3A). The radiolabeled complex was not observed in the presence of an excess of unlabeled wild-type but not mutated PCE-1 oligonucleotide, indicating that binding of Rx-L to the PCE-1 probe was specific. It has been suggested that QRX binds other conserved elements found in the rhodopsin promoter containing core homeodomain binding motifs, such as BAT and Ret4.24 As reported previously, Rx and Rx-L had less affinity for the BAT element, as the BAT competitor decreased the binding between Rx-L and the PCE-1 probe but failed to completely compete for binding (Fig. 3A, lanes 6, 11), indicating that neither Rx-L nor Rx bind appreciably to the RET-1 element. Taken together, our data suggest that the primary binding site for Rx-L and Rx is the PCE-1 site.

### Rx-L as a Transcriptional Activator

To investigate the function of Rx-L, we generated a DNA reporter construct, XOP-Luc, containing a minimal XOP promoter and a luciferase expression cassette. The XOP promoter contains four highly conserved transcriptional binding sites: PCE-1, BAT1, NRE, and Ret4.20 The ability of Rx and Rx-L to activate the XOP-Luc reporter was assayed in Xenopus embryos (Fig. 3B). When Rx RNA was co-injected with the XOP reporter construct, there was approximately a 1.5-fold increase in XOP-Luc reporter activity; however, injection of a similar amount of Rx-L RNA elicited a 4.2-fold increase in reporter activity. Rx-L activated expression of the reporter construct to a significantly greater degree than did Rx (P < 0.0097), indicating that it is a stronger activator than Rx.

### Effect of the Knockdown of Rx-L

To study the function of Rx-L in Xenopus retinal development, an antisense MO oligonucleotide targeted to the Rx-L translation start (Rx-L MO) was used to knock down Rx-L expression in Xenopus embryos. The specificity of the Rx-L MO was assayed in two ways. First, the Rx-L MO inhibited in vitro translation of Rx-L in a dose-dependent manner, whereas a control MO oligonucleotide (ctl MO) did not affect Rx-L translation (Fig. 4A). To further confirm the efficacy of the Rx-L MO
in *Xenopus* embryos, we made a DNA construct (Rx-L-GFP) containing the 5’-end of the Rx-L cDNA, including the MO target, fused in-frame with a GFP expression cassette. Every embryo injected with Rx-L-GFP exhibited GFP expression (Fig. 4C). Co-injection of Rx-L-GFP with control (ctl) MO at different concentrations (Figs. 4D, 4E) did not affect GFP expression. The Rx-L MO knocked down the GFP expression to nearly undetectable levels at both concentrations (Figs. 4F, 4G). From these results, we determined that the Rx-L MO could specifically knock down the translation of Rx-L.

Next, we knocked down expression of Rx-L, by injecting Rx-L MO unilaterally into *Xenopus* embryos along with a GFP lineage tracer. No outward changes were observed in these embryos (Figs. 4H–N). Injected tadpoles with normal ocular appearance and unilateral GFP expression were fixed and analyzed. No obvious histologic changes were observed in these retinas (Figs. 4O–V). The effects of injection of the Rx-L MO on rod photoreceptors were analyzed by immunostaining with RetP1 (anti-rhodopsin) antibody (Figs. 5A–D). No changes in Ret-P1 immunostaining were observed in retinas of ctl MO–injected embryos (compare Figs. 5A, 5B). Retinas of Rx-L MO–injected embryos appeared to have shorter outer segments (compare Figs. 5C and 5D). In two independent experiments, the rods of Rx-L MO–injected eyes were significantly shorter than those of ctl MO–injected eyes. In one experiment (Fig. 5F), the rods of MO-injected eyes were 72.13% ± 9.01% the length of those of the contralateral uninjected side, whereas rods of ctl MO–injected eyes were 72.13% ± 9.01% the length of those of the contralateral uninjected side, whereas rods of ctl MO–injected eyes were 72.13% ± 9.01% the length of those of the contralateral uninjected side, whereas rods of ctl MO–injected eyes were 72.13% ± 9.01%
Injected eyes were nearly the same length (103.38% ± 10.55%). In a second, independent experiment, we found that rods of MO-injected eyes were 78.06% ± 10.66% the length of those of the contralateral uninjected side, whereas rods of ctl MO–injected eyes were nearly the same length (100.34% ± 3.66%). In both cases, Rx-L MO injection resulted in a significant decrease in rod length compared with the ctl MO (experiment 1: \( P < 0.00013 \); experiment 2: \( P < 0.00014 \)). The rhodopsin RNA level was also studied directly by in situ hybridization using a rhodopsin antisense riboprobe (Figs. 5A–5D). We found that rhodopsin expression was reduced in retinas injected with the Rx-L MO (compare Figs. 6C, 6D). The control MO had no apparent effect on rhodopsin expression (compare Figs. 6A, 6B). We verified these results by quantita-

**Figure 5.** The Rx-L antisense MO oligonucleotide resulted in perturbations in photoreceptor development. (A–D) Analysis of rod photoreceptor morphology visualized by immunohistochemistry with an antibody to rhodopsin (Ret-P1). (E) Diagram of measurements used to analyze changes in photoreceptor layer thickness. (F) Quantification of the effect of micro-injection of control or Rx-L antisense MO oligonucleotide on rod photoreceptor layer thickness. (G–J) Analysis of cone morphology visualized by binding to PNA. (A′–D′) and (G′–J′): high-magnification (100×) views of boxed area indicated in (A–D) and (G–J), respectively. Green bars indicate the width of the photoreceptor layer. All images were captured at 40× magnification. *\( P < 0.00013 \).
We found that injection of Rx-L MO reduced rhodopsin expression by nearly 90% compared with when ctl MO was injected, a statistically significant reduction ($P < 0.002$). Our results suggest that the rhodopsin level of the retina on the MO-injected side of the embryo was much lower than on the uninjected side, suggesting that Rx-L expression is necessary for rhodopsin gene expression and proper rod photoreceptor development.

The red cone opsin (RCO) promoter also contains a conserved PCE-1 element, suggesting that it may be a target of Rx-like transcription factors. We investigated the expression of RCO in Rx-L knockdown embryos by in situ hybridization (Figs. 6E–6H). We found that Rx-L knockdown resulted in a decrease in RCO expression (compare Figs. 6G, 6H). Using quantitative RT-PCR, we found that injection of the Rx-L MO reduced expression of RCO by more than 70% compared with injection of ctl MO, a statistically significant reduction ($P < 0.001$). To determine the effect of Rx-L knockdown on cone development, we used PNA, which binds to the cone outer sheath and labels essentially the entire cell (Figs. 5G–J).

Injection of the Rx-L MO diminished the width of the cone photoreceptor layer as visualized by PNA labeling (Figs. 5I, 5J), whereas injection of ctl MO did not affect PNA labeling (Figs. 5G, 5H). These data suggest that the knockdown of Rx-L reduces expression of RCO and has a deleterious effect on cone development.

The effects of Rx-L knockdown seemed to be specific to photoreceptors. We analyzed the expression of Pax6 in Rx-L MO–injected embryos by in situ hybridization (Fig. 7A–D). In st-41 tadpoles, Pax6 is expressed broadly in the ganglion cell layer and the inner nuclear layer and is unaffected by injection of the Rx-L MO (compare Figs. 7C and 7D). We also analyzed, by immunohistochemistry, the expression of Isl-1, a transcription factor expressed primarily in retinal ganglion cells and to a lesser degree in amacrine and horizontal cells, (Figs. 7E–H). Injection of the Rx-L MO did not noticeably affect the expression of Isl-1 (compare Figs. 7G, 7H). In addition, injection of the Rx-L MO did not affect expression of other homeodomain transcription factors expressed in the developing retina, such as Rx and otx5b (data not shown). These results verify that the effect of Rx-L knockdown on photoreceptors was specific and not the result of a general deficiency in retinal development.

**DISCUSSION**

Rx-L is a newly discovered member of the Rx family. Rx-L and Rx belong to the aristaless-related paired-like homeobox gene...
family. Members of this subfamily of homeobox proteins contain a characteristic homeodomain and a conserved domain of unknown function located near the C terminus, called the aristless or OAR domain. Most members of this family also have an octapeptide motif related to the eh-l sequence at N-terminal region. The octapeptide motif mediates transcriptional repression through interaction with corepressors of the groucho-TLE family.\(^{44,45}\) Our results suggest that Rx-L and Rx have different transcriptional activities. *Xenopus* Rx is a weak activator, whereas Rx-L is a relatively potent activator. This is in agreement with comparisons of transcriptional activities of Rax/RaxL\(^{23}\) and RX/QRX.\(^{24}\) The ability of Rx-L to function as a stronger activator than Rx is largely due to the lack of an octapeptide motif in Rx-L (EH-Hodiri et al., unpublished data, 2006). Rx-L is one of very few members in aristless-related gene family that does not have an octapeptide motif, along with Prx and Otp, which are known to function as transcriptional activators.\(^{46}\)

Why are Rx-like gene products needed in addition to Rx/ Rax gene products? Perhaps these gene products function to activate expression of PCE-1-containing promoters during development. Because both Rx and Rx-L bind the PCE-1 site and Rx-L is a stronger activator than Rx, the function of the Rx-like family members may be to boost, rather than initiate, promoter activity. There are examples of control of target gene activity during different phases of development by differential expression of transcriptional regulators. For example, in anterior pituitary development, the transcriptional repressor Rpx/ Hesx1 is replaced by the activator Prop-l when pituitary development shifts from specification and patterning to differentiation.\(^{22}\)

Our results indicate that one primary function of Rx-L is to regulate photoreceptor-specific gene expression. Our EMSA results suggest that Rx-L binds to the Rx-L-binding site, a site usually upstream of photoreceptor related genes. QRX was found to bind to the Ret-1 site as well as the PCE-1 site.\(^{24}\) Our data indicate that Rx-L primarily interacts with the PCE-1 site and weakly, if at all, with the Ret-1 site in EMSA. This apparent difference in binding specificity is difficult to understand in light of the high degree of identity between the homeodomains of *Xenopus* Rx-L and human QRX (93.4%). However, it is possible that the few differences between the two proteins result in subtle difference in DNA-binding characteristics.

Our in situ hybridization results show that Rx-L is primarily expressed in the maturing photoreceptor layer in *Xenopus* tailbud embryos. In contrast, QRX is expressed in inner retinal layers in addition to the outer retina.\(^{24}\) This may reflect a real difference between the expression patterns of Rx-like genes in frogs and mammals or may reflect differences in photoreceptor development between these organisms. Chicken Rax-L is expressed in both inner and outer retinal layers during retinal maturation but becomes restricted to the photoreceptor layer by E14.\(^{22}\) Overall, then, Rx-like genes are expressed in developing photoreceptors.

Our results suggest that Rx-L is involved in regulating photoreceptor development by controlling transcription of photoreceptor-specific genes. Studies in chicken demonstrated that expression of a putative dominant negative allele of a chicken Rx-like gene, *cRaxl*, causes a decrease in expression of early photoreceptor markers.\(^{22}\) However, expression of a dominant negative or interfering form of a transcription factor does not necessarily result in loss of function. Mutations in QRX have been found in some human retinal diseases probably due to a role for QRX in photoreceptor function and/or survival,\(^{24}\) although these studies do not conclusively demonstrate that QRX mutations result in these diseases. Mice do not appear to encode a QRX/Rx-L gene,\(^{24}\) making it impossible to study QRX/Rx-L function by gene knockout in mammals. *Xenopus* is thus an ideal animal model to study Rx-L loss of function. Our data suggest that an Rx-L MO to knock down the expression of Rx-L both in vitro and in *Xenopus* embryos, and that Rx-L is required for the normal spatial expression of photoreceptor-specific genes at appropriate levels. Our results also indicate that loss of Rx-L expression leads to defects in photoreceptor development.

Several vertebrate species have the Rx-L gene in addition to the Rx gene, as discussed above. A notable exception seems to be the mouse, whose genome does not encode an Rx-like gene. This suggests that, in mice, Rx can function as both Rx and Rx-like gene products do in other species. Specifically, this suggests that mouse Rx can act as a strong transcriptional activator, at least under some circumstances, despite the presence of an octapeptide motif and high levels of expression of groucho family members in the developing *Xenopus* eye.\(^{48}\) One possibility is that Rx-groucho interactions are regulated at the physical or functional level, to allow mouse Rx to function as a strong transcriptional activator during some periods of development, such as the initial phases of photoreceptor development, to promote increased levels of transcription of Rx-L target genes such as rhodopsin and red cone opsin. Additional experiments are necessary to explore this possibility and determine conditions under which Rx may function as a strong activator.

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