**QRX**, a novel homeobox gene, modulates photoreceptor gene expression

Qing-liang Wang¹,², Shiming Chen⁵, Noriko Esumi², Prabodh K. Swain⁶, Heidi S. Haines⁸, Guanghua Peng⁵, B. Michele Melia², Iain McIntosh¹, John R. Heckenlively⁹, Samuel G. Jacobson¹⁰, Edwin M. Stone⁸, Anand Swaroop⁶,⁷ and Donald J. Zack¹,²,³,⁴,*

¹Program in Human Genetics and Molecular Biology, McKusick–Nathans Institute of Genetic Medicine, ²Department of Ophthalmology, ³Department of Molecular Biology and Genetics, ⁴Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD, USA, ⁵Department of Ophthalmology and Visual Science, Washington University School of Medicine, St Louis, MO, USA, ⁶Department of Ophthalmology and Visual Sciences and ⁷Human Genetics, University of Michigan, Ann Arbor, MI, USA, ⁸Department of Ophthalmology, University of Iowa College of Medicine, Iowa City, IA, USA, ⁹Jules Stein Eye Institute, University of California–Los Angeles School of Medicine, Los Angeles, CA, USA and ¹⁰Department of Ophthalmology, University of Pennsylvania, Philadelphia, PA, USA

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A novel paired-like homeobox gene, designated as *Qrx*, was identified by a yeast one-hybrid screen using the bovine *Rhodopsin* promoter Ret-1 DNA regulatory element as bait. *Qrx* is preferentially expressed in both the outer and inner nuclear layers of the retina. Its homeodomain is nearly identical to that of Rx/Rax, a transcription factor that is essential for eye development, but it shares only limited homology elsewhere. Although *Qrx* and Rx/Rax show similar DNA binding properties *in vitro*, the two proteins demonstrate distinct target selectivity and functional behavior in promoter activity assays. QRX synergistically increases the transactivating function of the photoreceptor transcription factors Crx and NRL and it physically interacts with CRX. *Qrx* is present in the bovine and human genomes, but appears to be absent from the mouse genome. Nonetheless, a 5.8 kb upstream region of human *QRX* is capable of directing expression in presumptive photoreceptor precursor cells in transgenic mice. These results indicate that Qrx may be involved in modulating photoreceptor gene expression. In addition, the finding of rare heterozygous *QRX* sequence changes in three individuals with retinal degeneration raises the possibility that *QRX* may be involved in disease pathogenesis.

**INTRODUCTION**

The neuroretina has been studied as a model of neuronal function, development and degeneration (1). Photoreceptor cells, consisting of rods and cones, carry out phototransduction and pass their signals onto bipolar, retinal ganglion cells, and eventually to higher centers within the brain to generate vision. An interesting challenge in developmental neurobiology is deciphering the mechanisms that orchestrate how the many specialized retinal cell types develop and differentiate from a limited number of common progenitor cells (2–4).

Retinal development is a function of differential gene regulation, defined by precise temporal and spatial patterns of gene expression. Significant progress has been made in deciphering some of the complex regulatory network that functions to define the profile of gene expression in particular retinal cells. Among the *cis*-acting DNA elements involved are Ret-1/PE-C1, Ret-4, BAT-1 and NRE (5–10); the *trans*-acting transcription factors include CRX, NRL, RX/RAX, PAX6 and CHX10 (8,9,11–17). Rx/Rax, which belongs to the Q50 type paired-like class of homeodomain proteins, is one of the earliest expressed transcription factors in the developing eye, appears to be functionally conserved and is essential for normal eye development within vertebrates (15,18–22). Rx/Rax has also been implicated in determining the fate of Müller cells (19) and regulating photoreceptor-specific gene expression (23).
Mutations of a number of the transcription factors involved in the proliferation and differentiation of retinal cells can give rise to human retinal disease. As an example, Crx, the cone rod homeobox gene, is responsible for controlling the differentiation of photoreceptor cells by modulating the expression of photoreceptor genes, including Rhodopsin and Crx itself (12,13,24–26). Mutations of CRX have been found to cause cone-rod dystrophy (14,27), Leber congenital amaurosis (28,29) and retinitis pigmentosa (30). Another important factor in the regulation of Rhodopsin and in maintaining normal photoreceptor function is the neural retina leucine zipper protein, Nrl, which acts synergistically with Crx through a direct interaction with Crx’s homeodomain (12,31,32). Missense mutations of NRL can also cause autosomal dominant retinitis pigmentosa (33). Nrl is preferentially expressed in the rod photoreceptors (34), and its loss in mice results in the failure of rod photoreceptor development and an excess of functional S-cones (35).

Here, we report the identification and characterization of a novel retinal homeobox gene, designated as Qrx (Q50-type retinal homeobox gene), which shares a nearly identical homeobox with Rx/Rax, plays a role in modulating the expression of Rhodopsin and possibly other photoreceptor genes, and may be associated with retinal degeneration.

RESULTS

Qrx encodes a novel paired-like homeodomain protein

A bovine retinal cDNA-Gal4 activation domain fusion library was screened in a yeast one-hybrid assay using a bait that contains the bovine Rhodopsin promoter Ret-1/PCE-1 element (−148 to −126 bp, referred to as Ret-1 below). In the primary screen of 2.07 million clones, 72 positives were identified by growth on histidine-deficient medium plates. After plasmid recovery, retransformation, and testing for both growth on histidine-deficient medium plates and positive activity in a β-galactosidase assay, 55 of these clones were confirmed as ‘true’ positives. Partial sequence analysis of these clones revealed that 42 clones matched Chx10 (36), five matched Vx1 (a Chx10 homologue) (37–39), and eight represented novel sequences. Among the eight, two encoded the same 184 amino acid residue Q50-type paired-like homeodomain containing protein, which we designate as Qrx. In an independent screen using the BAT-1 element as bait (−112 to −84 bp), three additional Qrx clones were identified. Clones encoding the human QRX orthologue were obtained by screening a cDNA library with the bovine Qrx cDNA as probe.

Human and bovine Qrx are 91% identical at the amino acid level, with 100% conservation in the homeodomain (Fig. 1A). The homeodomain begins at residue 27. A putative monopartite nuclear localization signal (NLS), PKKHHRR, that is homologous to the SV-40 T antigen NLS (CPKKKRRK) (40) is located four amino acids N-terminal to the beginning of the homeodomain. In addition, the Qrx homeodomain contains a sequence (QNRRAKWRRQE) that is homologous to a Crx sequence (KNRRAKCRQQR) that has been reported to be important for its nuclear localization (41).

BLAST analysis (42,43) indicated that the closest homologue of Qrx is Rx/Rax, to which it is 93% identical in the homeodomain. Outside the homeodomain, however, the only other region of significant homology is within the OAR domain, which is part of the C-terminal tail, in which a 20 amino acid stretch shows ~60% identity. The OAR domain has been suggested to be involved in both transcriptional activation (44) and repression (45), but its role in Rx/Rax is unknown. Besides Qrx, 14 other Rx/Rax or Rx/Rax-related genes from nine different species have been identified to date. Amino acid sequence comparison and phylogenetic analysis indicate that human and bovine Qrx are the most distantly related members of the family and the most homologous member is the Drosophila Rx/Rax (Fig. 1D). Rx/Rax from human, mouse and rat seem to be more closely related to each other than to other members of the family. Rx1/Rax1 from zebrafish, astyanax and chicken are clustered together with the Rx2 from oryzias and zebrafish, while the chicken Rx/Rax2 (C-Rx2) is most homologous to the xenopus Rx and Rx2.

Qrx appears to be deleted from the Mus musculus genome

Multiple efforts were made to clone a murine (Mus musculus) Qrx ortholog, including screening of murine (M. musculus) retinal cDNA and genomic libraries with a human QRX cDNA probe, screening an arrayed murine genomic library with a human QRX cDNA and oligomer probes, and a reverse transcription PCR (RT–PCR) approach with several degenerate primers based on areas of conservation between the human and bovine sequences. Although several murine Rx/Rax clones and a number of other sequences were obtained, no clone representing a murine Qrx ortholog was identified, suggesting the possibility that there might not be a murine Qrx.

To further address this issue, we pursued both experimental and bioinformatic comparative genomic approaches. Southern blot analysis was carried out with genomic DNA from eight species [human, bovine, M. musculus (C57BL6 and 129SvJ), Mus castaneus, Mus spretus, rat, zebrafish and Drosophila] using a human QRX coding region fragment as the probe. The human QRX probe detected the Qrx gene in both human and bovine as a single band (data not shown). In addition, several weak bands were identified in M. castaneus, M. spretus, zebrafish and Drosophila, but not in M. musculus or rat. Although the identities of these other bands are unknown, they could represent orthologs of QRX or some other homologous gene. However, since the murine Rx/Rax gene was not identified under the hybridization conditions employed, they are unlikely to represent Rx/Rax orthologs.

Bioinformatic comparison of the human QRX locus on chromosome 19p13.3 (see mapping data below) to the conserved syntenic region in M. musculus (10C1, 79 kb from the centromere) provided additional evidence to support the absence of a Qrx gene in mouse. The murine region (derived from the publicly available NCBI Human–Mouse Homology Map and the Celera database, which was generated with data from the 129X1/SvJ, DBA/2J and A/J strains, with reported 6-fold coverage and >99% accuracy) shows significant overall conservation with the human region (Fig. 1E) (46–48).
The genes flanking QRX in human (the centromeric ZN-FINGER RNA BP and MATK and the telomeric MRP154, and APBA3) are preserved in mouse in terms of position and orientation. However, the interval between them is only 2 kb in mouse compared to 10 kb in human, and sequence analysis does not reveal evidence of any homeobox-like sequence, nor even any substantial open reading frame, within the interval. In addition, BLAST analysis of human QRX against mouse genome databases did not reveal any significant matches except to the homeodomain itself.
**Qrx** is preferentially expressed in outer and inner nuclear layers of retina

The expression pattern of Qrx in adult tissues was examined by northern blotting (Fig. 2A) and RT–PCR (data not shown) analyses. Among the tissues tested with northern blotting [retina, retinal pigment epithelium (RPE), brain, heart, liver, muscle and testis], Qrx is only expressed in the retina, with a single abundant transcript of ~2 kb. However, by RT–PCR analysis, Qrx expression was also detected weakly in the brain, testis and spleen. Based on RT–PCR analysis of the earliest bovine embryonic retinal tissue available, Qrx expression during development is evident as early as 4.5 months gestation (data not shown). For comparison, bovine Rhodopsin is minimally detectable at 5.2 months gestation and is highly expressed by 6.3–7.4 months gestation (7,49). By in situ hybridization analysis, Qrx is expressed in cells of the outer nuclear (ONL) and inner nuclear layers (INL), with stronger staining in the ONL (Fig. 2B and C). The expression of QRX in adult human retina has a similar pattern, which overlaps largely with that of RX/RAX (data not shown).

A human QRX ‘promoter’ fragment is sufficient to drive GFP expression in presumptive photoreceptor precursors in transgenic mice

Given the absence of Qrx in mice, we were interested in whether the human QRX promoter would still function in mice (i.e. whether the regulatory information was evolutionarily conserved). Transgenic mice were generated using a 8.1 kb genomic DNA fragment containing the human QRX locus (including 5.8 kb upstream sequence). To aid expression studies, an IRES-EGFP cassette was added to the downstream end of the genomic fragment. Analysis of postnatal day 1 retinas from four out of five analyzed independent transgenic lines revealed strong GFP expression in cells in the outer region of the neuroblast layer, presumptive developing photoreceptor cells of the forming outer nuclear layer (Fig. 2D and E). Also of potential interest is moderate GFP expression in isolated cells in the middle part of the neuroblast layer, perhaps migrating progenitor cells. These results suggest not only the strong conservation of the upstream regulatory complexes between human and mouse retina gene expression but also the likelihood of the involvement of QRX in the development and function of photoreceptors.

Qrx and Rx/Rax bind to both the Ret-1 and BAT-1 elements

Electrophoretic mobility shift assay (EMSA) and DNase I footprint analyses were carried out to characterize the DNA binding properties of QRX. Since the full-length QRX fusion protein was insoluble, recombinant QRX homeodomain GST fusion protein (QRX-HD-WT) containing six amino acids N-terminal to the homeodomain and five amino acids C-terminal to it, was expressed and purified from bacteria. As expected based on the initial one-hybrid results, QRX-HD-WT binds to both Ret-1 and BAT-1 oligos in EMSA (Fig. 3A). With larger amounts of QRX-HD-WT protein, faint additional bands of lower mobility were also evident (Fig. 3A, lanes 3 and 4), possibly due to the binding of QRX-HD-WT multimers to the DNA target. The binding specificity of the fusion protein to Ret-1 and BAT-1 sites was assessed by cold competitor assay. When wild-type cold DNA oligomer was added to either Ret-1 (Fig. 3A, lanes 5 and 6) or BAT-1 binding reactions (Fig. 3A, lanes 17 and 18), the binding of QRX-HD-WT to the labeled probes was competed away by the cold competitor. When the putative binding site in Ret-1 was mutated (MUT for Ret-1), the oligomer could no longer compete effectively with the probe (Fig. 3A, lanes 7 and 8). In the case of the BAT-1 site, when only one of the two putative QRX-HD-WT binding sites within the element was mutated, the cold competitor could still compete away the binding (Fig. 3A, MUT-1 and MUT-2, lanes 19–22). When both sites in the BAT-1 element were mutated (MUT-3), the mutant competitor showed minimal, if any, ability to compete with the labeled wild-type probe (Fig. 3A, lanes 23 and 24). A non-target sequence (NTS) cold competitor derived from another region of the Rhodopsin promoter showed no competition for the labeled Ret-1 probe (Fig. 3A, lanes 9 and 10). In addition to the EMSA analysis with the purified QRX homeodomain GST fusion protein (QRX-HD-WT), we also examined the interaction of native Qrx from bovine retinal nuclear extract with both Ret-1 and BAT-1 using an affinity purified rabbit polyclonal anti-Qrx antibody (6760) in the supershift assay. It appears that the anti-Qrx antibody largely eliminated the interaction of retina-enriched factors, presumably including both Qrx and Qrx-Crx complex in the retinal nuclear extract, with both Ret-1 and BAT-1 (Fig. 3B, lanes 3, 4 and 11, 12).
whereas it did not alter the non-specific interaction present in the 293 cell nuclear extract (Fig. 3B, lanes 7, 8, and 15, 16). In addition, the pre-immune serum (Fig. 3B, lanes 5 and 13) from the corresponding rabbit did not show any blockage of the binding activity. This is not surprising because the amino acids used as the peptide antigen (codon 88 to 107) to generate the anti-Qrx antibody (6760) are located in close vicinity to the C-terminus of the Qrx homeodomain (codon 27–86) and are likely to be required for the DNA binding activity of Qrx.

To further explore the DNA binding properties of QRX in the context of the Rhodopsin promoter, and to compare it with other factors implicated in Rhodopsin regulation, DNase I footprinting analysis was performed with a probe spanning the Rhodopsin promoter from −225 to +70 bp. Consistent with the EMSA results, the Ret-1 and BAT-1 sites were efficiently protected by QRX-HD-WT (Fig. 4, lanes 1–5 and 14–18). As might be predicted from the high degree of homology between the QRX and Rx/Rax homeodomains, the Rx/Rax homeodomain pattern of protection is essentially identical to that of QRX (Fig. 4, lanes 6–9 and 19–22). However, the binding affinity of Rx/Rax may be somewhat lower in that 4-fold more protein was required to achieve a similar degree of protection. Of potential significance, the protection pattern of QRX and Rx/Rax is similar to, but distinct from, that of Crx (12) (Fig. 4, lanes 10–13 and 23–26). The Ret-1 and BAT-1 protected sequences are overlapping but not identical, and Crx also protects the Ret-4 site. In addition, the patterns of hypersensitive sites are also distinct, presumably reflecting different DNA-protein conformations (Fig. 4, see arrows and arrowheads).
QRX specifically transactivates the Ret-1 element in the presence of Crx and NRL.

To assess the transcriptional activity of QRX, we utilized a transient transfection assay with HEK293 cells. Using the bovine Rhodopsin promoter, we employed previously to assess the transactivating activity of Crx and Nrl (8,9), we did not observe significant transactivating activity with QRX, either alone or in combination with Crx and/or NRL (data not shown). To develop a potentially more sensitive assay, and one that would be specific for individual DNA binding sites, thereby eliminating potential neighboring negative regulatory elements, luciferase reporter constructs were generated in which multimers of the Ret-1, BAT-1, Ret-4 or NRE/Ret-4 element were placed upstream of a minimal CMV promoter. Co-transfection of a QRX expression plasmid with each of these promoter elements again revealed minimal transactivating activity. This result was distinct from that with Rx/Rax, which could activate the BAT-1 construct (Fig. 5B) and has been reported to activate a Ret-1/PCE-1 site from the Arrestin promoter (23).

The multimer reporters demonstrated a number of specific patterns in terms of their abilities to be activated by the various transcription factors that were tested. When used alone, Crx activated the Ret-1 and Ret-4 constructs (Fig. 5A and D), and NRL selectively stimulated the NRE/Ret-4 containing reporter (Fig. 5C). However, when QRX was co-transfected with Crx and NRL, it further stimulated the expression of the Ret-1 construct by more than 10-fold over the effect of Crx and NRL combined in a dose dependent manner (Fig. 5E). The level of activation achieved with QRX was significantly higher (5-fold more) than what could be achieved simply by increasing the amount of Crx or NRL (data not shown). The transactivating effect peaks at 0.3 μg of QRX expression plasmid (Fig. 5E), which is relatively low for this type of assay. Furthermore, as little as 0.1 μg of QRX expression plasmid can achieve more than 5-fold activation. This transactivation activity of QRX is binding site specific, even though as noted above QRX binds to both the Ret-1 and BAT-1 sites, its ability to stimulate the activity of Crx and NRL is only observed with Ret-1 (BAT-1 data not shown). Rx/Rax also stimulates the combined activity of Crx and NRL, but only to about 50% the level of QRX (Fig. 5F). In order to dissect the relative contributions of Crx and NRL interactions in the observed QRX stimulation, QRX was tested with either of the two factors individually. QRX stimulates Crx alone significantly, to ~50% the activity seen with Crx and NRL, whereas QRX stimulation of NRL alone is only minimal (Fig. 5F), suggesting that Crx is the key contributor in this three factor interaction.
QRX physically interacts with CRX, but not NRL

The finding that Crx is essential for most of the transactivating function of QRX suggested that the QRX protein might physically interact with Crx, and that this interaction might be responsible for the observed synergy between the factors. To test this hypothesis, we performed co-immunoprecipitation and GST pull-down analyses. An anti-CRX antibody precipitated radioactively labeled in vitro transcribed and translated QRX protein only in the presence of CRX (Fig. 6A, lane 1). The same antibody did not yield precipitate when used with the empty QRX expression vector (data not shown), nor did it precipitate QRX in the absence of CRX (Fig. 6A, lane 5). In addition, using recombinant proteins expressed in HEK 293 cells, QRX (QRX-Xp) was co-immunoprecipitated with Flag tagged CRX (CRX-Flag) (Fig. 6B, lane 1).
Consistent with these results, we found in a yeast two-hybrid assay that the Crx homeodomain interacts with Qrx (data not shown). In contrast, QRX failed to interact with NRL in a GST pull-down assay (Fig. 6C, lane 4). This is in contrast to the finding that both Rx and Crx interact with NRL (Fig. 6C, lanes 8 and 12), and that deletion of the transactivation domain containing amino-terminus of NRL (GdN), which has been shown to be involved in its interaction with Crx (32), reduced its interaction with both Rx and Crx (Fig. 6C, lanes 7 and 11).

Mapping of QRX to human chromosome 19p13.3, and identification of QRX sequence variants in cone-rod dystrophy and age-related macular degeneration patients

Human QRX was mapped to chromosome 19p13.3 by radiation hybrid mapping, in which it was closely linked to the marker WI 6480 with a lod score of >3. The result was confirmed by somatic cell hybrid mapping to chromosome 19 and subsequently also by data from the human genome project. The cosmid F16403, which spans the entire QRX cDNA fragment, also maps to the same
To test the hypothesis that defects of QRX and its protein product could result in retinal disease, we screened a cohort of patients with retinopathies for QRX mutations: 322 cone-rod dystrophy (CORD), 107 Leber congenital amaurosis (LCA), 92 age-related macular degeneration (AMD), 14 autosomal recessive retinitis pigmentosa (ARRP) and 14 autosomal dominant retinitis pigmentosa (ADRP) patients, as well as a pool of 94 normal individuals. Three different heterozygous sequence variations were identified in patients with retinal disease that would be expected to alter the QRX protein (Table 1). In contrast, no amino acid altering sequence variations were observed in the pool of 94 normal control individuals. A 67-year-old patient with the clinical diagnosis of AMD was found to harbor a heterozygous G to A change at codon 87, which would be expected to result in a change from arginine to glutamine (CGG to CAG in Fig. 7A). This replacement of a bulky, positively charged amino acid by a neutral one occurs at the C-terminal border of the homeodomain (Fig. 1A), and suggested that it might affect the protein’s DNA binding activity. When this possibility was directly tested using a GST tagged recombinant QRX-HD-R87Q mutant protein, we found the R87Q alteration to cause an apparent increase in binding affinity for the Ret-1 and BAT-1 elements (Fig. 7C and D). This increase was not due to different amounts of the mutant or wild-type proteins used in the assay (Fig. 7B).

A 78-year-old patient with macular degeneration and electroretinographic evidence of peripheral retinal involvement (which we interpret as CORD) was found to harbor a missense change in codon 137 (GGC to CGC). This change would be expected to result in a change of arginine to glycine (Fig. 1A). Since this region of the gene contains multiple repeats of the CCGGGG sequence, insertion or duplication of the CCGGGG sequence at multiple positions between codons 137 and 141 could result in the same final sequence. For simplicity, we designate this mutation as 140P_141Gdup (50). The same sequence change was also identified in the proband’s mother and two siblings. Although these relatives are said to have ‘normal’ vision, they all live in Pakistan and are not available for ophthalmologic exam and testing. Hence, the possibility that they have mild CORD cannot be excluded. If the relatives in fact do not have CORD, then this would suggest either incomplete penetrance, perhaps due to modifier or environmental influence, or that the 140P_141Gdup sequence change is not disease-associated.

The R87Q, G137R and 140P_141Gdup sequence variants were expressed and tested in vitro to assess their effect on the interaction between QRX and CRX, and their functional consequence in the context of other transcription partners in vivo. In co-immunoprecipitation analyses, the G137R and 140P_141Gdup proteins demonstrated decreased but not absent interaction with CRX, while the R87Q protein did not show decreased interaction (Fig. 6A). In transient transfection assays, all three sequence variants demonstrated subtle but statistically significant (P < 0.0001) and reproducible

locus. By comparing the sequence of the human QRX cDNA to the cosmid F16403, the genomic structure of QRX was determined. The QRX cDNA is composed of three exons, with the coding region encoded by exons 2 and 3 (Fig. 1B). All the intron–exon boundary sequences match the canonical GT-AG consensus sequence (Fig. 1C).

Figure 6. QRX physically interacts with CRX, which also interacts with NRL, and QRX variants demonstrate reduced interaction between QRX and CRX (A) In vitro transcribed and translated 35S-labeled QRX (WT and mutants) is co-immunoprecipitated with CRX using an anti-CRX antibody (lanes 1–5). Both the G137R (lane 3) and the 140P_141Gdup mutation (140PGins in lane 4) resulted in reduced amount of QRX being precipitated suggesting decreased interaction between CRX and these mutant QRX proteins. However, the R87Q mutation resulted in little, if any, change compared to the wild-type (lane 2). In the absence of CRX, QRX is not precipitated (lane 5). Lanes 6–9 demonstrate that an approximately equal amount of each input protein was used. (B) Recombinant QRX (QRX-Xp) synthesized in HEK293 cells is co-precipitated with CRX-Flag. Immunoprecipitation using anti-Flag-M2 resin containing either CRX-Flag, or its vector control, with QRX-Xp was performed as described in experimental procedures. Lanes 1, 2, 4 and 5 are elutes (20 μl) from the resin with the indicated protein extracts; lanes 3, 6 and 7 are the inputs (10 μl) as indicated. Lanes 1, 2, 3 were probed with anti-Xpress (Invitrogen, 1:1000) and goat anti-mouse IgG-HRP and lanes 5, 6, 7 with anti-CRX P261 antibody (1:250) and anti-rabbit IgG-HRP. Arrows with labeling indicate the running positions for QRX-Xp and CRX-flag. (C) GST pull-down assays with in vitro transcribed and translated 35S-labeled QRX (QRX*), Rx/Rx (Rx*) or Crx (Crx*). QRX* did not show evidence of binding to columns with GST-NRL (GNRL, lane 4), GST-NRL with transactivation domain deleted (GdN, lane 3) nor with GST alone (lane 2). In contrast, both Rx and Crx interact with GNRL (lanes 8 and 12) and GdN (lanes 7 and 11) but not with GST alone (lanes 6 and 10).
difference from the QRX-WT in their ability to transactivate the Ret-1 element in the presence of Crx and NRL (Fig. 5G). At the DNA concentration that showed peak QRX-WT activity (0.3 μg/plate), R87Q and 140P_141Gdup resulted in increased transactivation activity of 30 and 50%, respectively, while G137R demonstrated 40% reduced transactivating activity at a concentration of 1 μg/plate.

DISCUSSION

The transcriptional network implicated in the regulation of photoreceptor gene expression is becoming increasingly complex, and now includes Crx (12–14,51), Nrl (8,9,31,32,34,52), Erx (53), Rx (15,16,19,21,23), Mash1 (17,54), Mok2 (55), nuclear receptor NR2E3 (35,56), thyroid hormone receptor beta 2 (57), the Sp family (58), and, at least in Drosophila, Pax6 (59–61). The combinatorial interplay of these factors is important not only for orchestrating normal development and function, but also in mediating retinal pathology, as shown by reports that mutations in many of their genes have been associated with photoreceptor disease (14,27,33). Here, we have presented the characterization of a new transcription factor, Qrx, which appears to be involved in modulating the expression of photoreceptor specific genes and may play a role in retinal degeneration. Based on its structure, Qrx is a member of the Q50-type paired-like homeobox gene family, and its closest homolog is Rx/Rax. Rx/Rax and its related genes have been identified from nine different species, and some species express as many as three distinct family members (15,21,62).

Unfortunately, however, relatively little functional information is available for these Rx/Rax-related genes, and whether Qrx represents a true ortholog of any of them is unclear. One finding, however, of potential relevance to Qrx is that expression of a putative dominant negative allele of a chicken Rx/Rax-related gene, cRaxL (C-Rax1), causes a decrease in expression of early photoreceptor markers (63). An interesting, and puzzling, aspect of the evolution of Qrx is that it appears to be absent from the M. musculus genome. Qrx seems to have been deleted from the M. musculus genome during evolution, rather than having evolved more recently than the branch point of M. musculus. Although it had been thought that the order Rodentia, to which M. musculus belongs, is more distantly related to the primate lineage than the order Cetartiodactyla, to which bovine belongs (64), more recent molecular phylogenetic analysis indicates that M. musculus is a closer relative to human than is bovine (65,66). If so, a mechanism explaining the absence of a murine Qrx ortholog as being due to more recent evolution would necessitate an independent origin for...
the gene in human and bovine ancestors, and this seems unlikely given the high degree of nucleic acid sequence homology between the bovine and human orthologs. A more likely mechanism involves a small deletion mediated by recombination between the flanking repetitive elements. Of potential significance, multiple genes immediately flanking Qrx are conserved between mouse and human, but there are local disruptions of the nearby synteny (Fig. 1E). Perhaps related to these findings, compared to the human genome, the mouse genome is evolutionarily more volatile (67), giving mice the ability to rapidly adapt to changing environments. It is therefore interesting that, as demonstrated by the transgenic experiments, the regulatory information from the human QRX promoter region is still largely recognized by the mouse. As a practical side benefit, a QRX promoter/GFP transgene may turn out to be a useful early marker of developing photoreceptor cells.

The absence of murine orthologs for human genes, and vice versa, is not rare. It has been estimated to occur for as many as 3–5% of genes in both genomes (48). Furthermore, homeobox genes appear to have a higher than average frequency of such variation. In human, of the 302 homeobox loci we identified from a NCBI Locuslink search, 139 harbor already characterized mouse orthologs. The other 163 represent novel homeobox genes predicted from their nucleotide sequences or expressed sequence tag (EST) clusters. Within the group of previously characterized 139 homeobox genes, nine (6.5%) are absent from the mouse genome based on the search of gene symbols, NCBI HomoloGene data and nucleotide sequence BLAST of both the NCBI and the Celera databases. Analysis of the known murine homeobox genes revealed a similar frequency for which human orthologs could not be identified. These ‘missing’ homeobox genes are likely to be important contributors in the processes that account for the biological, morphological, and functional difference between mouse and man. In the eye, for example, variation in properties such as the number of types of cones, the relative frequency of cones and rods and the presence or absence of a macular region could partly be determined by differences in the pattern of homeodomain proteins expressed in the retina.

The absence of a murine Qrx has made functional and developmental analysis more difficult. Nonetheless, several lines of evidence do provide insights into the role of Qrx within the retina. Although results of transient transfection experiments must always be interpreted with caution in terms of their relevance to the in vivo situation, the data presented suggest that Qrx may play a role in modulating the expression of Rhodopsin and perhaps other photoreceptor genes. While QRX did not demonstrate significant transactivating activity on its own, when used in combination with Crx and NRL, it stimulated reporter gene expression more than 10-fold. This effect was observed only with the Ret-1 site and not with the Ret-4 or BAT-1 sites. In the same assay with the same site, Rx/Rax showed less than half the activity demonstrated by QRX, even though in the DNA binding assays it demonstrated essentially identical sequence specificity. Rx/Rax was the most potent activator of the BAT-1 reporter construct. These findings are noteworthy since although homeodomain proteins demonstrate in vivo specificity, they often show significant promiscuity in in vitro binding studies and cell transfection assays. A schematic model of Rhodopsin regulation that integrates these results, as well as previous studies, is shown in Figure 8. An unusual aspect of the model is that NRL is suggested to function by two mechanisms. With its already identified binding site, NRE, Nrl is shown to function in the standard manner as a DNA binding transcription factor (9). However, we suggest that with the Ret-1 site, NRL functions more as a co-activator or modulator, acting through protein–protein interaction with Crx without its direct DNA binding. This hypothesis is based on the finding that the addition of NRL with the Ret-1 reporter approximately doubles the activity observed with Crx and QRX alone (Fig. 5F), yet NRL does not demonstrate detectable binding to the Ret-1 sequence. NRL is shown as interacting directly with Crx, but not with QRX, based on the co-immunoprecipitation and GST pull-down assays shown in Figure 6. A second potential co-activator shown in the model is p300/CBP, as has been reported previously that it can both physically interact with Crx and enhance its transactivating function (68). An additional level of complexity, not indicated in Figure 8, is that negative acting transcription factors may bind the Rhodopsin promoter and repress expression in non-photoreceptor retinal cells or modulate Rhodopsin promoter activity even in rods (51,52). Since Qrx does not exist in mouse genome, presumably its activity in regulating Rhodopsin expression could be compensated by other factors, including Rx, in mouse.

The identification of QRX sequence changes in patients with retinal diseases is also consistent with a role for QRX in photoreceptor function and/or survival. Although the absence of existing or available relatives for the R87Q and G137R probands, as well as the presence of 140P_141Gdup in the proband’s presumably normal relatives, make it difficult to conclude that any of the identified sequence changes truly represent disease-causing mutations, the nature and positions of the sequence changes and the biochemical studies with the mutated proteins, are all supportive of this possible association. Each of the sequence changes led to an alteration that could be detected in our functional assays. The G137R mutation resulted in decreased interaction with CRX and decreased transactivation activity, the 140P_141Gdup led to decreased interaction with CRX but increased transactivation, and the R87Q mutation increased both transactivation and DNA binding activity. The possibility that increased transcription factor activity can cause eye disease is not novel, having been reported previously with both NRL (33) and PAX6 (69). Although this suggestion that different, and sometimes opposite, types of transcription factor alterations can result in similar ocular phenotypes may at first seem paradoxical, it probably reflects the growing consensus that the retinal gene expression network is so tightly regulated that a variety of different perturbations can all result in retinal degeneration.

**MATERIALS AND METHODS**

**Yeast one hybrid assay**

Library screening was performed essentially as described in Chen et al. (12) except the target elements used here were Ret-1 (five copies) or BAT-1 (three copies).
Radiation hybrid mapping

The MIT Genebridge radiation hybrid panel was analyzed using primers corresponding to human QRX cDNA sequences (forward: 5′-CAGTCTGGCCAGCCACCTC-3′, reverse: 5′-GGCTGGAGTGCAGCAGTGTG-3′) that amplify the 494 bp PCR products. Analysis of the resulting PCR pattern was submitted to the database (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper), which indicated close linkage to the marker WI6480 with a LOD score of more than 3.

Northern blot analysis

Total RNA was isolated from different fresh bovine tissues (RPE, retina, brain, heart, liver, muscle and testis) using the TRIZOL reagent (Invitrogen). Approximately 10 μg of total RNA from each tissue was used. After UV crosslinking, the membrane was hybridized to a probe generated from the full-length bovine Qrx cDNA followed by washing and overnight autoradiography.

In situ hybridization

In situ hybridization experiments were performed essentially as previously described (70), except that DIG-labeled bovine Qrx riboprobes were generated by in vitro transcription of either T3 (antisense probe) or T7 (sense probe) promoter driven plasmid vector containing partial bovine Qrx cDNA (191 basepairs), which has been digested with either EcoRI (sense probe) or BamHI (antisense probe). Eight μm thick cryosections of bovine retina, fixed in fresh 4% paraformaldehyde, were used in the hybridization with buffer containing 100 ng/ml of either antisense or sense DIG-labeled riboprobe.

Generation and analysis of the QRX-IRES-EGFP transgenic mice

A polycliniker containing EcoRI–AflII–Smal–AgeI–BamHI sites was inserted into the EcoRI and BamHI sites of the pIRES2-EGFP vector (BD Biosciences #632306). The 8.1 kb human QRX fragment generated by digesting the cosmid F16403 with BspAI and AgeI enzymes was directionally cloned into the Smal and AgeI sites of the above modified pIRES2-EGFP vector. The resulting construct was further digested with AflII to release the 9.6 kb QRX-IRES-EGFP fragment for microinjection into the mouse embryos of SJL/J mice at the Johns Hopkins Transgenic Core Facility. The resulting transgene positive mice were genotyped by PCR analysis and further bred for multiple generations to eliminate the rd1 locus from the background. Out of a total of 12 transgenic founders, nine independent lines were established. GFP expression in the perinatal retinas of five of these nine lines have been analyzed. Ten micrometer cryosections of the eyecups from transgene positive mice were generated for standard histological processing and viewed with a Nikon Eclipse E1000 microscope using DAPI (excitation: 330–380 nm; dichroic mirror: 400 nm; barrier: 435–485 nm) or FITC (excitation: 465–495 nm; dichroic mirror: 505 nm; barrier: 515–555 nm) filter sets.

Expression and purification of GST-tagged QRX-HD-WT, QRX-HD-R87Q, Rx-HD, Crx-HD

Bovine Qrx cDNA fragment corresponding to codons 20–91 or mouse Rx/Rax cDNA fragment corresponding to codons 130–200 was cloned in frame into the BamHI (5′) and EcoRI (3′) sites of the pGEX-4T-2 vector (Pharmacia). Since the amino acid sequence within this region is 100% conserved between human and bovine Qrx and also between human and mouse Rx/Rax, we designate the constructs as QRX-HD-WT and RX-HD, respectively. The QRX-HD-WT was also used as the template for the generation of QRX-HD-R87Q mutant construct using QuikChange site-directed mutagenesis kit (Strategene). CRX-HD was the same as previously described (12). All the expression constructs were transformed and expressed in bacteria strain BL21 (Pharmacia). The GST fusion proteins were purified following protocol provided by Pharmacia. The concentrations and quality of purified proteins were determined by both SDS–PAGE/Coomassie blue staining and spectrophotometry.

Generation of polyclonal anti-Qrx antibody (6760)

Peptide antigens corresponding to the region of codon 88–107 of both human (LESGSGAAPRLPEAP) and bovine (LESGSGAAPRLPEVPALP) Qrx were synthesized according to standard procedure at Genemed Synthesis Inc. The antigens were injected as a mixture into two rabbits according to standard immunization protocol and antisera were obtained and affinity-purified using the peptide antigens.
EMSA

For the study of purified QRX-HD-WT GST fusion protein, fill-in labeled radioactive \(^{32}P\) double stranded short DNA probes (10,000 cpm/µl corresponding to the Ret-1WT (5'-GGCCCCACCTGGAAGCCAATTAAGGC-3') and Ret-1WT (5'-GCAGC AGTGAGGTAAATGTATTAATAACGC-3') elements were used to mix with purified GST-tagged QRX-HD-WT protein in the presence or absence of appropriate amount of non-radioactive competitors. The DNA sequence for the mutant non-radioactive competitors are as following: Ret-1MUT (MUT: 5'-GGCCCCACCTGGAAGCCAATTAAGGC-3'), NTS (non-target sequence: 5'-GGCTCTGCTCTTTCCAGGTGCCCC-3'), BAT-1MUT1 (MUT1: 5'-GCAGCAGTGAGGTGCGATGATTAATAACG GCC-3'), BAT-1MUT2 (MUT2: 5'-GCAGCAGTGAGGTAAATGTATTAATAACGCC-3'). Assays were carried out as described (71). The analysis of native Qrx protein in nuclear extract from bovine retina and the control 293 cell nuclear extract were carried out essentially the same as above except that 4 µg of nuclear extract, either from retina or 293 cells, and 0.1 µg of Poly(dI-dC) were used in each reaction. Nuclear extracts were added 20 min before the addition of 1.5 µl (Fig. 3B, lanes 3, 7, 11, 15) or 4.5 µl (Fig. 3B, lanes 4, 8, 12, 16) of the anti-Qrx antibody (6760). As controls, 1 µl of unpurified preimmune serum (PI) was added instead in lanes 5 and 13 of Figure 3B.

DNase I footprinting

DNase I footprintings were performed essentially the same as previously described (71), except that up to 16 ng of purified GST-tagged QRX-HD-WT, RX-HD, CRX-HD proteins were used to mix with either top (forward) or bottom (reverse) strand labeled DNA probes corresponding to bovine Rhodopsin promoter −225 to +70 bp.

Human subjects

Informed consent was obtained from participants after explanation of the studies. The research procedures were in accordance with institutional guidelines and the Declaration of Helsinki. Clinical ocular examinations and visual function testing were performed in the patients with retinal degenerations that had candidate gene screening.

Screening of QRX mutations using single-strand conformational polymorphism (SSCP) and automated sequencing

Samples from patients and normal controls were screened in an identical manner for mutations in the 5’UTR and coding sequences of the QRX gene using single-strand conformational polymorphism (SSCP) and sequencing analyses. Four pairs of oligonucleotide primers (exon1/forward: 5'-ACCTTGAGC TAATCTCCCC-3', exon1/reverse: 5'-CACCCCTGAGGG ATCTCTG-3'; exon2/forward: 5'-CCAAGAAGAAGACCG GA-3'; exon2/reverse: 5'-AAAGATGTTGTGTTGGGG G-3'; exon3A/forward: 5'-TCAGACGGAGGTGCTAGACCC-3'; exon3A/reverse: 5'-TCCAGGGGCGACGATG-3'; exon3B/ forward: 5'-CTGGAGTCAGGCTCAGG-3', exon3B/reverse: 5'-CGTTGCTCCCATGACCT-3') were used to amplify all but 90 base pairs of the coding sequence. Mutations using single-strand conformational polymorphism (SSCP) and sequencing analyses. Four pairs of primer pairs and numerous different reaction conditions. The rest of exons 1 and 2 and the first portion of exon 3 (3A) were amplified with the Qiagen Taq DNA Polymerase Kit containing Coenzyme Q. The second portion of exon 3 (3B) was amplified with MasterAmp 2X PCR PreMix L from Epicentre. PCR products were denatured for 3 min at 94°C and electrophoresed on 6% polyacrylamide/5% glycerol gels at 25 W for ~3 h at room temperature. Following electrophoresis, gels were stained with silver nitrate (72). PCR products exhibiting an electrophoretic shift in SSCP analysis were sequenced. Mutations were identified by the approximately equal peak intensity of two fluorescent dyes at the mutant base. The 140P_141Gdup sequence was further confirmed by cloning the mutant PCR products into the pGEM T-EASY vector (Promega) and sequencing multiple clones to identify both the mutant and normal allele sequences. All sequencing was bi-directional.

Generation of target element containing reporter constructs, QRX wild-type and mutant expression constructs and transient transfection assays

Ret-1 (10 copies), BAT-1 (three copies), Ret-4 (four copies) and Ret-4 + NRE (four copies) were individually and directionally cloned into the Xhol site of the modified PGL2-promoter vector (Clontech), in which the SV40 promoter has been substituted by a minimal CMV promoter. QRX wild-type cDNA was in-frame cloned into the BamHI (5') and EcoRI site of the pcDNA3.1HisB vector (Invitrogen). The mutant clones (R87Q, G137R and 140P_141Gdup) were generated using QuickChange site-directed mutagenesis kit (Strategene) using the wild-type QRX construct as the template in the initial PCR reactions. The resulting mutant constructs were sequenced and correct inserts were cut out and pasted into the pcDNA3.1HisB vector to eliminate any potential secondary mutations. The DNA of the resulting subclones were purified twice through CsCl gradient. The Crx and NRL expression constructs, the transfection of 293 cells and luciferase and β-gal assays were essentially as previously described (12).

In vitro translation and GST pull-down assay

QRX, RX/Rax and Crx were translated and radio-labeled with \(^{35}S\)-methionine using coupled in vitro transcription/translation system (Promega) and Glutathione Sepharose bound GST, GST-DNRL and GST-NRL. were prepared as described earlier (32). Equal amounts (~30%) of each in vitro translated protein (QRX, RX/Rax and Crx) were incubated separately with GST, GST-DNRL or GST-NRL (~100 µg of protein). After the final wash, the Glutathione Sepharose bound protein was solubilized in 2 x SDS sample buffer by heating for 5 min at 100°C. Proteins labeled with \(^{35}S\)-methionine were analyzed by SDS–PAGE followed by autoradiography. Along with the pull-down proteins, 10% of the total protein used for binding was analyzed as input to check the translation efficiency of the individual protein.
**In vitro and in vivo expression and co-immunoprecipitation analysis of CRX and wild-type and mutant QRX proteins**

Co-immunoprecipitation experiments using *in vitro* translated wild-type and mutant QRX (\(^{15}S\)-labeled) and CRX were carried out using anti-Crx antibody P261, essentially as described (73) with minor modifications. The coupling time with Protein-A beads was reduced to 3 h instead of overnight, and a cocktail of protease inhibitors (Roche Molecular Biochemicals) was added to each reaction.

In *vivo* expression of recombinant QRX-Xp (Xpress tagged hQRX) and CRX-Flag (Flag-tagged hCRX) were carried out by individually transfecting HEK293 cells cultured on 100 mm plates with 5 μg of hCRX-Flag and hQRX-pcDNA3.1/HisB, respectively. Pull-down assays were performed with the above plates with 5 hQRX) and CRX-Flag (Flag-tagged hCRX) were carried out to each reaction.

3 washes with 1 ml TBS, proteins bound to the resin were eluted with 100 μl of 3x Flag peptide (150 ng/μl in TBS) and analyzed using SDS-PAGE and western blots with primary antibodies specific to CRX (73) or Xpress (the expression tag for hQRX), and secondary goat anti-mouse IgG-HRP (for anti-Xpress) or anti-rabbit IgG-HRP (for anti-CRX), respectively. ECL plus western blotting detection reagents and Hyperfilm for ECL were used for detection.

**Statistical analysis of transient transfection data**

Relative luciferase activities from each experiment were analyzed and compared among transcription factors using a linear mixed model. This is analogous to analysis of variance (ANOVA), except that rather than averaging correlated observations prior to analysis as required by ANOVA, all data points are used in the analysis and the correlation in observations arising from using the same solution mix to transfect two tissue culture plates was modeled directly, using the assumption that the two tissue culture plates were exchangeable experimental units. No formal adjustment for multiple comparisons of transcription factors was made; however, only *P*-values ≤ 0.0025 were considered statistically significant. All analyses were performed using SAS statistical software (SAS Inc.).

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