Published in final edited form as:

Gene. 2010 November 1; 467(1-2): 13–24. doi:10.1016/j.gene.2010.07.005.

REGULATION OF RETINAL HOMEBOX GENE TRANSCRIPTION BY COOPERATIVE ACTIVITY AMONG CIS-ELEMENTS

Reyna I. Martinez-de Luna^{1,*}, Holly E. Moose^{2,*}, Lisa E. Kelly³, Srivamsi Nekkalapudi³, and Heithem M. El-Hodiri^{1,2,3,4,&}

¹Graduate Program in Molecular, Cellular, and Developmental Biology, College of Biological Sciences, The Ohio State University, Columbus, OH

²Integrated Biomedical Science Graduate Program, College of Medicine, The Ohio State University, Columbus, OH

³Center for Molecular and Human Genetics, The Research Institute at Nationwide Children's Hospital, Columbus, OH

⁴Department of Pediatrics, College of Medicine, The Ohio State University, Columbus, OH

Abstract

The Retinal Homeobox (Rx/rax) gene is essential for the development of the eye. Rx is among the earliest genes expressed during eye development, beginning in the prospective eye fields in the anterior neural plate. Additionally Rx expression persists in retinal progenitor cells and in differentiated photoreceptors. We have isolated and characterized a 2.8 kb genomic DNA fragment that regulates expression of Rx in the developing and maturing retina. We have discovered and characterized cis-acting elements that function to specifically control spatial and temporal Rx expression during retinal development. We have found that the regulation of Rx2A promoter activity requires cooperative interactions between positive and negative regulatory elements. Further, a highly conserved genomic element containing SOX, OTX, and POU transcription factor binding sites is necessary but not sufficient for promoter activity in retinal progenitor or stem cells. Finally, a putative binding element for forkhead transcription factors is necessary for promoter activity and can cooperate with other cis-acting elements to drive Rx2A promoter activity.

Keywords

transcription; promoter; Retinal homeobox; Retinal anterior homeobox; Rx; Rax; retina; transgene; Xenopus

© 2010 Elsevier B.V. All rights reserved.

[&]Address correspondence to: Heithem El-Hodiri, PhD, Center for Molecular and Human Genetics, The Research Institute at Nationwide Children's Hospital, 700 Children's Drive, Columbus, OH 43205, Telephone: 614-722-2868, Fax: 614-722-2817, Heithem.El-Hodiri@NationwideChildrens.org.

^{*}These authors contributed equally to this work and are listed alphabetically.

⁺Current addresses: R.I.M. – Department of Ophthalmology, SUNY Upstate Medical University, Syracuse, NY; H.E.M. – College of Optometry, The Ohio State University, Columbus, OH.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

The neural retina is initially specified by a self-regulating network of transcription factors (Chow and Lang, 2001; Zuber et al., 2003). In each vertebrate system investigated, these transcription factors are expressed in retinal progenitor cells during retinal neurogenesis. The *retinal homeobox (Rx/Rax)* gene is one such factor. Normal expression of the *Rax* gene product begins in the anterior neural plate at a time before eyes are morphologically distinct (Casarosa et al., 1997; Chuang et al., 1999; Furukawa et al., 1997; Mathers et al., 1997). *Rax* continues to be expressed in photoreceptors of the mature neural retina, although it is generally downregulated in differentiated cells (Furukawa et al., 1997; Mathers et al., 1997; Nelson et al., 2009; Pan et al., 2010; Perron et al., 1998). In amphibians and fish, *Rax* expression is maintained in the ciliary marginal zone (CMZ), the region of the peripheral neural retina that contains retinal progenitor cells, and photoreceptors.

A number of studies have demonstrated the importance of *Rax* in normal eye development. Loss of *Rax* function results in lack of eye formation (anophthalmia) in fish and mice (Loosli et al., 2003; Loosli et al., 2001; Mathers et al., 1997; Winkler et al., 2000), demonstrating that *Rax* is important in the initial events in eye specification. Knockdown of *Rax* early in *Xenopus* development results in micro- or anophthalmia (Bailey et al., 2004) while knockdown in the mature retina results in photoreceptor degeneration (Pan et al., 2010). Conversely, overexpression of *Rax* produces extra retinal tissue (Andreazzoli et al., 1999; Chuang and Raymond, 2001; Mathers et al., 1997). Ectopic retinal tissue forms with apparently normal laminar structure, suggesting that *Rax* plays a role in the early decisions of anterior neural tissue to become retinal tissue, which consists of retinal progenitor cells competent to produce the neural retina. The zebrafish *Rx1* and *Rx2* genes induce the formation of ectopic retinal tissue at the expense of forebrain cells, suggesting a role for *Rax* in the early specification of optic primordia. The overexpression of *Rax* also induces the upregulation of other eye field genes, including *Pax6* and *Six3*, while decreasing expression of *Otx2*. This reiterates that *Rax* acts early in eye specification by affecting expression of downstream genes in retinal progenitor cells that have themselves been shown to be necessary for eye development. Together, these studies clearly demonstrate the importance of *Rax* in early eye specification and development. Even with these studies demonstrating the critical importance of *Rax* function in eye field specification, the molecular and genetic events leading to *Rax* transcription initiation, throughout stages of retinal progenitor cell development, have not been clearly established.

Two *Rax* genes, *Rax1A* and *Rax2A*, have been identified in *X. laevis* (Mathers et al., 1997). The presence of the two genes is most likely a result of the partial genome duplication that occurred within the species (Bailey et al., 2004). The expression patterns of these two genes are indistinguishable and the coding region sequences exhibit considerable identity (LEK and HME unpublished). For the purpose of this document, *Rax* expression refers to the combined expression patterns of *Rax1A* and *Rax2A*. Previous work has shown that a 3.4 kb *Rax1A* regulatory sequence can direct transgene expression during retinal development and in the mature retina (Zhang et al., 2003). This *Rax1A* promoter is organized into two distinct regions: the distal portion of the promoter is capable of transcriptional activation in the anterior neural plate, while the proximal region is necessary and sufficient for activation of transcription in later stages of eye development. However, this does not represent a complete *Rax* promoter since the *Rax1A* promoter is not active in the CMZ. In this paper, we describe the identification and characterization of a 2.8 kb fragment of the *X. laevis* *Rax2A* promoter. We identify promoter regions that are necessary for correct spatial and temporal activity in the developing and mature retina, including the CMZ.

2. MATERIALS AND METHODS

2.1 Animals

Female and male *X. laevis* were purchased from NASCO. Egg laying, in vitro fertilization, and embryo culture were carried out as described previously (Sive et al., 2000). Developmental stages were determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

2.2 Isolation of the Rax2A regulatory region

We isolated a 2.8 kb genomic fragment of the *Rax2A* regulatory sequence by three rounds of linker-mediated PCR (LM-PCR) according to manufacturer's protocol (Universal Genome Walker Kit, Clontech) (Moritz et al., 2002; Siebert et al., 1995). Each round of LM-PCR involved two amplification reactions using nested primers. Initial primers were designed to be specific the *Rax2A* 5'-UTR. The primers used for LM-PCR are listed in Table S1. Round 1: primers 1134 & 1135; round 2: primers 1118 & 1119; round 3: primers 1178 & 1179. Primers 1182 & 1183 were used to amplify the intact 2.8 kb *Rax2A* promoter (corresponding to the aggregate products of all 3 rounds of LM-PCR) from *X. laevis* genomic DNA. The sequence of the LM-PCR product has been submitted to GenBank (Accession number HM222418).

2.3 Generation of transgenes

A detailed list of the transgenes used in this study is provided in Figure 3. The 2.8 kb *Rax2A* LM-PCR product was inserted into the BamHI and EcoRV sites of pBS-GFP (Zhang et al., 2003) to make *Rax2A_{2.8}/GFP*. Transgene constructs 6–10 were linearized versions of *Rax2A_{2.8}/GFP* (see Table S2). Constructs 11–14, 16, 18–21, 24, and 26–31 were generated by PCR using Platinum Pfx DNA polymerase (Invitrogen). All primers sets and templates used for PCR for each of these constructs are detailed in Tables S2 and S3. PCR conditions were as follows: 94°C for 2 min, 94°C for 15 sec, 55°C for 30 sec, 68°C for 1 min or 2 min depending on the length of the fragment for 20 or 30 cycles. All PCR products were TA cloned into pCR2.1 (Invitrogen) and sequenced to confirm that point mutations were not introduced during PCR. Subsequently the amplified fragments were subcloned into pBS-GFP or HSP-GFP (Zhang et al., 2003). Constructs 17, 23 and 25 were generated using restriction enzyme sites present in the *Rax2A* promoter. The restriction enzymes used are listed in Table S2. The corresponding fragments were then subcloned into pBS-GFP or HSP-GFP.

Constructs 2–5 were generated by site-directed mutagenesis (Quick Change XL Site-Directed Mutagenesis Kit, Stratagene) using primers and templates as listed in Tables S2 and S3. PCR conditions for mutagenesis were as follows: 95°C for 30 sec plus 18 cycles: 95°C for 30 sec, 55°C for 1 min, 68°C for 11 min. Products of mutagenesis reactions were confirmed by sequencing or restriction enzyme digestion using a restriction site introduced during PCR.

2.4 Transgenesis

Transgenic *X. laevis* embryos were generated by intracytosolic sperm injection (ICSI) (Sparrow et al., 2000). Sperm nuclei were isolated as previously described (Kroll and Amaya, 1996) using digitonin (10 mg/ml) instead of lysolecithin. DNA transgenes were released from their vector by restriction enzyme digestion (detailed in Table S2), and purified from agarose gels using the Gene Clean kit (QBiogene).

2.5 Section in situ hybridization

In situ hybridization was performed on 8µm retinal sections as previously described (Chuang et al., 1999; Shimamura et al., 1994; Viczian et al., 2003). To prepare the GFP antisense riboprobe, pBS-GFP was linearized with EcoRI and the probe transcribed with T7 RNA polymerase (Ambion).

2.6 Immunohistochemistry

Stage 41 transgenic embryos were fixed in MEMPFA (MOPS-EGTA-MgSO₄-paraformaldehyde) for 1 hour (Sive et al., 2000), dehydrated in methanol for 1 hour, embedded in paraffin and sectioned at 8 µm as previously described (Pan et al., 2006).

Immunohistochemistry was performed as described previously (El-Hodiri et al., 1997). Mouse anti-Myc (Sigma) was used as primary antibody at a 1:1,000 dilution in blocking solution.

3. RESULTS

3.1 Identification and conservation of the Rax2A regulatory sequence

We isolated a 2.8 kb genomic region upstream of the Rax2A coding sequence and found that this fragment could direct expression of a green fluorescent protein (GFP) cassette (Rax2A_{2.8}/GFP) in the developing eyes of transgenic *X. laevis* embryos (Figure 1). GFP expression was detected in the anterior neural plate of developing embryos and in the eyes, ventral forebrain and pineal gland of tailbud stage embryos. At st 41, the exterior of the retina is covered by the retinal pigmented epithelium (RPE); transgene expression can barely be seen through the RPE but is evident through the clear lens at the center of the eye (Fig 1F). Generally, the Rax2A_{2.8}/GFP transgene is expressed in the areas in which endogenous *X. laevis* Rax is expressed in early eye development (Mathers et al., 1997).

Comparison of the 2.8 kb Rax2A region with the previously characterized Rax1A regulatory region (Zhang et al., 2003) by percent identity plot (PIP) between Rax2A and Rax1A reveals stretches of high conservation separated by regions with more divergent sequence (Fig 2A – highly conserved regions shown in red). A distal 326 bp region (Rax2A: –2360 to –2035) shares 92% identity between the two sequences, followed by a shorter 123 bp segment sharing 93% identity (Rax2A: –1897 to –1775), and a 272 bp region sharing 95% identity (Rax2A: –456 to –84). The first of these regions is highly conserved among vertebrate Rax genes and was termed Ultra Conserved Element (UCE) 3. Two additional UCES were identified [HME unpublished and (Pan et al., 2010)]. UCE3 corresponds to the conserved noncoding sequence (NCS) that was independently identified as an enhancer of the human RAX gene (Danno et al., 2008). It is also apparent that regions of divergence exist between the two sequences: a 172 bp region (Rax2A: –2559 to –2387) is present in the Rax2A regulatory region that is not present in the Rax1A sequence. Interestingly, this region is present in a region of *X. tropicalis* upstream of the Rax coding region (data not shown). Lastly, PIP analysis predicts an inversion event in the region corresponding to Rax2A –1252 to –1347 compared to Rax1A (not shown). This region also contains a predicted JH12 repetitive DNA element (Rax2A: –1024 to –1329) [(Meyerhof et al., 1990) and <http://www.repeatmasker.org>].

3.2 Spatial and temporal specificity of Rax expression are determined by regions within the Rax2A regulatory sequence

We performed deletion analysis of the Rax2A promoter to gain insight into the regulatory mechanisms governing Rax transcription. We generated 5' and internal deletions of the Rax2A regulatory sequence, and tested the ability of these constructs to drive transgene expression in transgenic *X. laevis* embryos.

3.2.1 Analysis of 5'-end promoter deletions—To characterize the activity of the Rax2A promoter, 5' deletions were generated, fused to the GFP expression cassette, and introduced into transgenic *X. laevis* embryos (Figure 3, constructs 6–15). This analysis revealed regions of the Rax2A promoter that are necessary for spatial and temporal specificity of the Rax2A promoter sequence (Figure 4, Table). The –2359 fragment drove GFP expression indistinguishable from the full-length construct (Figure 4 A–C). Deletion to –2160 bp resulted in loss of GFP expression in the anterior neural plate (not shown); GFP expression was observed

in the optic vesicles beginning at early tailbud stage and persisting through tadpole stages (Figure 4D–F). This deleted region contains the UCE3 element discussed above. In addition to the loss of expression in the anterior neural plate, ectopic expression was observed in the dorsal hindbrain beginning at stage 24 (Figure 4D–F, red arrowheads) and persisted to tadpole stages. Both loss of anterior neural plate expression and ectopic expression in the dorsal hindbrain were observed in transgenic embryos prepared using constructs containing successive 5' deletion to –944 of the Rax2A promoter (Figure 4G–L). Additionally, expression, both ectopic and in regions of endogenous Rax expression, became progressively weaker in these transgenics (Figure 4G–L). Collectively, this data suggests that a cis-acting element that provides spatial and temporal specificity to the Rax2A promoter is located between –2160 and –2359 bp. Additionally, the data suggests that additional cis-elements that regulate the strength of expression are present between –2160 and –944 bp of the Rax2A promoter.

As discussed above, deleting to –944 bp resulted in ectopic hindbrain expression, very weak forebrain expression and retinal expression from stage 24 onward (Figure 4L). Further deletion to –818 resulted in loss of visible GFP transgene expression (not shown). Analysis of the sequence between –944 and –818 revealed a putative forkhead binding elements (FBE). We found that addition of the FBE to –818 restored transgene expression in the eyes of developing embryos (Figure 4M). However, the FBE did not direct activity of a heterologous (heat shock protein gene) promoter in the eyes (not shown), suggesting that the FBE itself was not sufficient for specifying promoter activity during eye development. We conclude that the FBE at –847 represents a functional cis-acting element that confers expression in the eye in cooperation with the –818 minimal fragment of the Rax2A promoter.

3.2.2 Analysis of internal deletions—We next characterized the Rax2A regulatory region by generating a series of internal deletions (Figure 3, constructs 17–22). Since FBE + Rax2A₈₁₈/GFP, but not Rax2A₈₁₈/GFP alone, was expressed in the eye, we reasoned that there may be cooperative functional interactions between Rax2A₈₁₈/GFP and other parts of the Rax2A promoter. We generated internal deletion constructs where successively smaller portions of the distal Rax2A promoter were fused to Rax2A₈₁₈/GFP (constructs 17–21). All internal deletions lack the critical region between –944 and –818 bp that contains the functionally defined putative FBE regulatory element. Results are shown in Figure 5 and summarized in Table. The shortest internal deletion, lacking –818 to –1208 bp, drove GFP transgene expression in a pattern similar to that of the full-length construct (Figure 5A, B). In contrast, we found that internal deletions spanning from –1703 to –2400 bp resulted in ectopic GFP expression in a domain ventral to the cement gland at the neural plate and neural tube stages (Figure 5C, F, G). Ectopic transgene expression was first observed at neural plate stages in a region ventral to the cement gland and persisted throughout development (Figure 5D, E, G). Surprisingly, the ventral domain of ectopic GFP expression seemed to include the developing heart region (Figure 5G, H). Additionally, ectopic transgene expression was observed in the developing brain (Figure 5F, G – red arrowheads). Taken together, these results suggest that additional elements between –818 and –2400 are necessary for proper spatial and temporal specificity of the Rax2A promoter. Since deletion of this region results in ectopic ventral transgene expression, we reasoned that it function as a repressor of ventral expression; thus, we term this region the Central Repressive Element.

3.2.3 Analysis of HSP constructs—To further characterize the function of the Rax2A regulatory region, we produced a series of constructs in which the Rax2A minimal fragment (–818 to 0 bp) was replaced with the heat shock protein gene (HSP) minimal promoter (Figure 3, constructs 23–26, 28, 29). Results are shown in Figure 6 and summarized in Table. Adding the distal fragments from –2823 to –1703, –1823 or –2136 to the HSP minimal promoter results in transgenic embryos that transiently express GFP in the optic cups (Figure 6A–C).

These transgenes were expressed only from stage 24 to stage 28 (Figure 6A–C), stages when the optic vesicles form and invaginate to become the double-layered optic cup (Grant et al., 1980). In contrast, Rax2A 2400- HSP, lacking UCE3, does not drive observable GFP expression in embryos (data not shown). These data suggest that the distal portion of the promoter is sufficient to initiate transgene expression in Rax expression domains, but that more proximal regions are necessary to sustain the expression during later stages of development.

We had determined that the FBE could restore GFP expression during late stages. For this reason, we tested whether the FBE element could sustain late expression of the transiently expressed hsp transgenes. Adding the FBE to 2136- HSP resulted in sustained expression of GFP from neurula stages until stage 41 (Figure 6D and E). However, addition of the FBE to 2400- HSP did not result in detectable expression of GFP (data not shown). This result suggests functional cooperation between the FBE and regulatory elements present in the distal 2136 promoter fragment (which contains UCE3) to sustain promoter activity until late developmental stages.

3.3 Conserved elements within UCE are necessary for spatial specificity of Rax expression

As discussed above, UCE3 is a highly conserved region in the distal region of the Rax2A promoter that has been previously reported to be sufficient for activation of Rax expression by cooperative activities of Sox2 and Otx2 (Danno et al., 2008). Additionally, UCE3 contains a conserved putative binding site for the neural subfamily of POU domain transcription factors (Figure 2C). We tested the ability of conserved predicted transcription factor binding sites within this region to regulate the expression of the GFP transgene (Figure 7 and Table).

Deleting the POU site within UCE results in ectopic GFP expression in developing neural tissue (Fig 7A,B). The ectopic expression appears to be pan-neural in nature as opposed to the focal expression in the dorsal hindbrain observed in other constructs. The ectopic expression was first observed in early tailbud stage embryos and persisted through all stages examined. Interestingly, the ectopic expression of this transgene is similar to the expression pattern of the POU domain transcription factor Oct 1 (POU1F1) (Kiyota et al., 2008). This data suggests that the POU site contributes specificity to the Rax2A promoter, including regional specificity to within the developing brain, perhaps through the repressive activity of Oct1.

Mutating the SOX site alone is sufficient to produce ectopic expression of the transgene in the hindbrain region, albeit in a small percentage of embryos (15%, n=12, Figure 7G,H). Expression in the eyes appears indistinguishable from the full-length construct. However, mutating both the SOX and OTX sites of UCE (construct 5) increases the frequency of ectopic expression to that observed in Rax2A 2160 transgenics (100%, n=12 construct 5 vs. 100%, n=24, construct 7) (Figures 4 D–F, 7 I–K). This data suggests that the SOX and OTX sites within UCE are required for spatial specificity within the forebrain, resulting in eye-specific expression. The OTX and SOX sites act cooperatively as the construct harboring mutations in both sites results in a higher frequency of embryos with ectopic hindbrain expression than either single mutation alone.

3.4 Rax2A promoter activity in the mature retina

We next characterized activity of the Rax2A promoter in the mature retina, where Rax is normally expressed in the photoreceptors, throughout the CMZ, and sporadically in the INL. We examined expression of the Rax2A₂₈₂₃/GFP transgene by *in situ* hybridization on retinal sections and found that it is expressed in the PRL, INL, and most of the CMZ at stage 41 (Figure 8A), similar to the expression pattern of endogenous Rax gene. The transgene was expressed in all but the distal portion of the CMZ, where the least differentiated retinal progenitor cells, the retinal stem cells, are found (Perron et al., 1998).

We next analyzed activities of some of the previously described Rax2A/GFP transgene constructs in the mature retina (Figure 8 and Table). The expression pattern of Rax2A₂₁₆₀/GFP, the longest of the deletion constructs lacking UCE3, is similar to the expression of the full-length promoter construct in the photoreceptor layer and INL but has diminished expression in the CMZ (Figure 8B). Further deletion results in weakened expression in the PRL as well (Rax2A₁₇₆₄/GFP, Figure 8C). Rax2A₉₄₄/GFP, the shortest construct to exhibit eye expression, is not expressed in the INL, CMZ, or most of the photoreceptor layer (Figure 8D). Instead, this transgene is expressed in a region between the central retina and the CMZ. This region may represent newly differentiated or differentiating photoreceptors derived from the CMZ.

We also analyzed the expression of transgenes in which the putative OTX and SOX cis-regulatory elements were mutated in the context of the full-length promoter. Mutation of the OTX site contained within UCE3 results in an expression pattern similar to Rax2A₂₈₂₃/GFP (Figure 8E). Mutation of both the SOX and OTX elements abolished expression of the transgene in the INL and CMZ but not the photoreceptor layer (Figure 8F). Deletion of UCE3 similarly results in loss of expression in the CMZ and INL (Figure 8G), confirming that the SOX and OTX sites are necessary for UCE3 regulation of Rax2A promoter activity.

4. DISCUSSION

4.1 Comparison of Rax promoters

The *Xenopus laevis* Rax1A and Rax2A gene products are nearly identical and are expressed in apparently indistinguishable patterns (HME unpublished). The identification and characterization of a 3.4 kb Rax1A regulatory region has been described (Zhang et al., 2003); here we have presented a preliminary characterization of a 2.8 kb Rax2A regulatory region. Several lines of evidence suggest that Rax1A and Rax2A are similarly regulated. First, the Rax1A and Rax2A regulatory regions are similar and are sufficient to direct expression of a GFP transgene in regions of endogenous Rax expression throughout development. Second, the distal regions of the Rax1A and Rax2A promoters are necessary for Rax expression in the anterior neural plate and portions of the proximal regions work in concert with a minimal promoter to drive expression in the maturing retina. Both Rax1A and Rax2A are both expressed throughout the entire CMZ. However, the Rax1A promoter does not drive transgene expression in the CMZ of the mature retina (Zhang et al., 2003), whereas the Rax2A promoter presented here drove transgene expression in all but the distal-most region of the CMZ. Both promoters contain the conserved UCE3 sequence that is necessary for CMZ activity of the Rax2A promoter and includes conserved POU, SOX, and OTX motifs. This suggests that a region other than UCE3 is involved in directing expression in the retinal progenitor cells of the CMZ. Our analysis revealed additional differences between Rax1A and Rax2A or *X. tropicalis* Rax, including deletions, inversions, and insertions. Additional work is required to determine if one or more of these differences underlie the lack of Rax1A promoter activity in the CMZ and whether these differences involve cis-acting elements that function in cooperation with UCE3.

4.2 Role of UCE3

UCE3, a ~250 bp genomic element conserved across vertebrate species, has been shown to be necessary for Rax expression (Danno et al., 2008). We found that UCE3 is necessary but not sufficient for activation of transgene expression in the retinal progenitor cells of the CMZ in the mature retina. Additionally, UCE3 contains conserved cis-acting elements that bind transcription factors in the SOX and OTX families of transcription factors and that Otx2 and Sox2 are sufficient for activation of Rax transcription in naïve ectoderm (Danno et al., 2008). However, it seems that this cannot explain the observed expression of Rax in the CMZ as no known OTX transcription factor is expressed in the CMZ (Decembrini et al., 2006). It is likely

that a transcription factor with similar DNA binding specificity, such another member of the OTX a related family, may bind this site.

We also observed that UCE3 also contains a conserved binding element for members of the POU family of transcription factors. Cooperative activity between POU and SOX transcription factors has been found to be involved in regulating gene transcription, including the nestin and FGF4 genes (Ambrosetti et al., 1997; Ambrosetti et al., 2000; Dailey and Basilico, 2001; Nishimoto et al., 1999; Tanaka et al., 2004). Indeed, the combination of these three cis-acting element motifs has been termed the “highly conserved non-coding DNA region” (HCNR) (Bailey et al., 2006); it has been suggested that SOX and POU elements serve to drive expression in neural stem cells with specificity provided by a homeodomain transcription factor binding element. Further, the POU domain transcription factor POU3f2 regulates the transcription of Chx10 in retinal progenitor cells (Rowan and Cepko, 2005). It will be interesting to identify the trans-acting factors that bind the OTX and POU sites in UCE3 and determine their functional interactions.

Interestingly, deletion of the POU site within UCE resulted in ectopic transgene expression throughout the neural tube from neural plate stages on, suggesting that this cis-regulatory element acts with repressive function. Oct-1 (POU1f1) is a POU domain transcription factor that is expressed throughout the neural plate. The expression of Oct-1 at early tailbud stages is strikingly similar to the ectopic expression of the GFP transgene construct containing a mutated POU site (Kiyota et al., 2008). Functionally, Oct-1 acts as a repressor until the recruitment of additional co-factors allow conformational changes in the protein and transcriptional activation results (Ryan and Rosenfeld, 1997). For these reasons, Oct-1 is a POU domain transcription that serves as a candidate trans-acting regulator of the Rax2A promoter. Future work will be needed to determine if Oct-1 binds the Rax2A promoter *in vitro*, and whether POU3f2 or Oct-1 are located at the Rax2A locus *in vivo*.

4.3 The forkhead binding element (FBE)

We identified a putative forkhead binding element (FBE) in the proximal part of the Rax2A promoter. This element was conserved between the *X. laevis* Rax1A and Rax2A and *X. tropicalis* Rax promoters. Elimination of the FBE extinguishes transgene expression and addition of the FBE to the minimal 818 bp promoter drives weak transgene expression from optic vesicle stage and onward. Several forkhead transcription factors are expressed in the mature retina (Moose et al., 2009). FoxO3 is expressed in differentiating cells in the mature retina while FoxN2, FoxN4, and FoxM1 are expressed primarily in progenitor cells in the mature retina (Kelly et al., 2006; Moose et al., 2009). Both FoxO3 and FoxN4 can both bind the FBE *in vitro* (HEM and HME, unpublished) and we expect that FoxN2 to bind the FBE as well, due to the high degree of similarity between FoxN2 and FoxN4. There are reports of additional forkhead transcription factors expressed in mature retinal neurons (Moose et al., 2009), but the *Xenopus* orthologs have not been reported to be expressed in the retina.

4.4 Regulation of promoter specificity

Our analysis of the Rax2A promoter revealed that it contains a repressive cis-element in the central region we termed the Central Repressive Element. Expression of the transgene in ventral regions of the embryo was not observed in 5' end deletions lacking the Central Repressive Element, suggesting that ventral transgene expression may require positive acting elements in the distal part of the promoter and are normally repressed by the Central Repressive Element. If the Central Repressive Element acts alone, similar ectopic expression of GFP should have been observed with the 5' end deletions that lack the central region of the promoter. Our data suggests that the distal Rax2A promoter contains a region, termed the Distal Activator Element, that activates Rax expression in a broad domain and that the Central Repressive Element

represses expression in the ventral regions of the embryos, refining expression specifically to the developing eyes. Further dissection of the promoter is needed to identify specific cis-regulatory elements responsible for regulating Rax transcription within the Central Repressive and Distal Activating Elements. Interestingly, a control element was recently identified in the zebrafish *six3* promoter that functions similarly to the Central Repressive Element; that is, deletion of the element results in expression in the ventral region of the embryo (Chao et al.). These findings are summarized in a model (Figure 9).

In our analysis of Rax2A promoter internal deletion constructs, we found that most of the central region of the promoter is not required for its activity in the developing eyes. In these constructs, successively smaller distal promoter fragments were fused to the -818 distal fragment, which had no observable activity alone. All of the internal deletion constructs we tested were expressed from neural plate stage onwards. However, these distal fragments drove transgene expression only transiently when fused to the hsp minimal promoter. Transient expression of GFP commenced at stage 24 and was extinguished by stage 28, at the early stages of retinal histogenesis. Addition of the FBE restored expression throughout retinal development. This suggests that functional interactions between the FBE and the distal promoter are necessary for full promoter activity. However, the FBE was not necessary for activity of the internal deletions that included distal promoter fragments and the -818 minimal promoter, suggesting that the -818 fragment may contain elements, possibly FBEs, that can interact with the distal promoter to enhance promoter activity throughout retinal development, as the FBE does in the HSP constructs. Indeed, we could identify several putative FBEs in the -818 fragment (HEM and HME, unpublished) that may interact with the distal promoter and function redundantly with the FBE at -847.

We hypothesize, based on the expression of the internal deletion constructs, that the region distal to the UCE contains an activation domain (the Distal Activating Element). When the Central Repressive Element was deleted, this distal fragment of the Rax2A promoter drove transgene expression in ventral regions of the embryo. However, constructs containing the DAE upstream of the hsp minimal promoter did not exhibit ectopic ventral expression, suggesting that -818 fragment of the Rax2A promoter and the hsp minimal promoter have different capacities for transcriptional activation. This is consistent with the idea that the -818 fragment may contain forkhead binding elements or additional cis-acting elements that are not active in the context of the -818 alone but functionally interact with distal elements. This is also consistent with findings concerning Rax1A promoter constructs (Zhang et al., 2003).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS

ANP	anterior neural plate
bp	base pairs
CMZ	ciliary marginal zone
CRE	central repressive element
DAE	distal activator element
FBE	forkhead binding element
GCL	ganglion cell layer
GFP	green fluorescent protein

ICSI	intracytosolic sperm injection
INL	inner nuclear layer
LM-PCR	linker-mediated polymerase chain reaction
MEMPFA	MOPS-EGTA-MgSO ₄ -paraformaldehyde
NCS	non-coding sequence
PCR	polymerase chain reaction
PIP	percent identity plot
PRL	photoreceptor layer
RPE	retinal pigmented epithelium
UCE	ultra-conserved element
UTR	untranslated region

Acknowledgments

The authors thank Kathryn Moore for critical reading of the manuscript, Yi Pan for assistance in preparation and microinjection of transgenes and Carlos Alvarez for advice on multi-species promoter comparisons.

ROLE OF THE FUNDING SOURCE

This work was supported by NIH EY015480 to HME and Prevent Blindness Ohio Young Investigators Student Fellowship Awards for Female Scholars in Vision Research to HEM and RIM. These study sponsors played no role in study design, collection, analysis, and interpretation of the data, the writing of the report, or the decision to submit the report for publication.

REFERENCES CITED

- Ambrosetti DC, Basilico C, Dailey L. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol Cell Biol* 1997;17:6321–6329. [PubMed: 9343393]
- Ambrosetti DC, Scholer HR, Dailey L, Basilico C. Modulation of the activity of multiple transcriptional activation domains by the DNA binding domains mediates the synergistic action of Sox2 and Oct-3 on the fibroblast growth factor-4 enhancer. *J Biol Chem* 2000;275:23387–23397. [PubMed: 10801796]
- Andreazzoli M, Gestri G, Angeloni D, Menna E, Barsacchi G. Role of Rx1 in Xenopus eye and anterior brain development. *Development* 1999;126:2451–2460. [PubMed: 10226004]
- Bailey PJ, Klos JM, Andersson E, Karlen M, Kallstrom M, Ponjavic J, Muhr J, Lenhard B, Sandelin A, Ericson J. A global genomic transcriptional code associated with CNS-expressed genes. *Experimental cell research* 2006;312:3108–3119. [PubMed: 16919269]
- Bailey TJ, El-Hodiri H, Zhang L, Shah R, Mathers PH, Jamrich M. Regulation of vertebrate eye development by Rx genes. *Int J Dev Biol* 2004;48:761–770. [PubMed: 15558469]
- Casarosa S, Andreazzoli M, Simeone A, Barsacchi G. Rx1, a novel Xenopus homeobox gene expressed during eye and pineal gland development. *Mech Dev* 1997;61:187–198. [PubMed: 9076688]
- Chao CH, Wang HD, Yuh CH. Complexity of cis-regulatory organization of six3a during forebrain and eye development in zebrafish. *BMC Dev Biol* 10:35. [PubMed: 20346166]
- Chow RL, Lang RA. Early eye development in vertebrates. *Annu Rev Cell Dev Biol* 2001;17:255–296. [PubMed: 11687490]
- Chuang JC, Mathers PH, Raymond PA. Expression of three Rx homeobox genes in embryonic and adult zebrafish. *Mech Dev* 1999;84:195–198. [PubMed: 10473141]
- Chuang JC, Raymond PA. Zebrafish genes rx1 and rx2 help define the region of forebrain that gives rise to retina. *Dev Biol* 2001;231:13–30. [PubMed: 11180949]

- Dailey L, Basilico C. Coevolution of HMG domains and homeodomains and the generation of transcriptional regulation by Sox/POU complexes. *J Cell Physiol* 2001;186:315–328. [PubMed: 11169970]
- Danno H, Michiue T, Hitachi K, Yukita A, Ishiura S, Asashima M. Molecular links among the causative genes for ocular malformation: Otx2 and Sox2 coregulate Rax expression. *Proc Natl Acad Sci U S A* 2008;105:5408–5413. [PubMed: 18385377]
- Decembrini S, Andreazzoli M, Vignali R, Barsacchi G, Cremisi F. Timing the generation of distinct retinal cells by homeobox proteins. *PLoS Biol* 2006;4:e272. [PubMed: 16903786]
- El-Hodiri HM, Shou W, Etkin LD. xnf7 functions in dorsal-ventral patterning of the *Xenopus* embryo. *Dev Biol* 1997;190:1–17. [PubMed: 9331327]
- Furukawa T, Kozak CA, Cepko CL. rax, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. *Proc Natl Acad Sci U S A* 1997;94:3088–3093. [PubMed: 9096350]
- Grant P, Rubin E, Cima C. Ontogeny of the retina and optic nerve in *Xenopus laevis*. I. Stages in the early development of the retina. *J Comp Neurol* 1980;189:593–613. [PubMed: 7381043]
- Kelly LE, Nekkhalapudi S, El-Hodiri HM. Expression of the forkhead transcription factor FoxN4 in progenitor cells in the developing *Xenopus laevis* retina and brain. *Gene Expr Patterns*. 2006
- Kiyota T, Kato A, Altmann CR, Kato Y. The POU homeobox protein Oct-1 regulates radial glia formation downstream of Notch signaling. *Dev Biol* 2008;315:579–592. [PubMed: 18241856]
- Kroll KL, Amaya E. Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* 1996;122:3173–3183. [PubMed: 8898230]
- Loosli F, Staub W, Finger-Baier KC, Ober EA, Verkade H, Wittbrodt J, Baier H. Loss of eyes in zebrafish caused by mutation of chokh/rx3. *EMBO Rep* 2003;4:894–899. [PubMed: 12947416]
- Loosli F, Winkler S, Burgdorf C, Wurbach E, Ansgor W, Henrich T, Grabher C, Arendt D, Carl M, Krone A, et al. Medaka eyeless is the key factor linking retinal determination and eye growth. *Development* 2001;128:4035–4044. [PubMed: 11641226]
- Mathers PH, Grinberg A, Mahon KA, Jamrich M. The Rx homeobox gene is essential for vertebrate eye development. *Nature* 1997;387:603–607. [PubMed: 9177348]
- Meyerhof W, Stalder J, Koster M, Wirthmuller U, Knochel W. Sequence analysis of the upstream regions of *Xenopus laevis* beta-globin genes and arrangement of repetitive elements within the globin gene clusters. *Mol Biol Rep* 1990;14:17–26. [PubMed: 2345547]
- Moose HE, Kelly LE, Nekkhalapudi S, El-Hodiri HM. Ocular forkhead transcription factors: seeing eye to eye. *Int J Dev Biol* 2009;53:29–36. [PubMed: 19123124]
- Moritz OL, Peck A, Tam BM. *Xenopus laevis* red cone opsin and Prph2 promoters allow transgene expression in amphibian cones, or both rods and cones. *Gene* 2002;298:173–182. [PubMed: 12426105]
- Nelson SM, Park L, Stenkamp DL. Retinal homeobox 1 is required for retinal neurogenesis and photoreceptor differentiation in embryonic zebrafish. *Dev Biol* 2009;328:24–39. [PubMed: 19210961]
- Nieuwkoop, PD.; Faber, J. Normal Table of *Xenopus laevis* (Daudin). New York: Garland Publishing, Inc.; 1994.
- Nishimoto M, Fukushima A, Okuda A, Muramatsu M. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. *Mol Cell Biol* 1999;19:5453–5465. [PubMed: 10409735]
- Pan Y, Martinez-De Luna RI, Lou CH, Nekkhalapudi S, Kelly LE, Sater AK, El-Hodiri HM. Regulation of photoreceptor gene expression by the retinal homeobox (Rx) gene product. *Dev Biol*. 2010
- Pan Y, Nekkhalapudi S, Kelly LE, El-Hodiri HM. The Rx-like Homeobox Gene (Rx-L) Is Necessary for Normal Photoreceptor Development. *Invest Ophthalmol Vis Sci* 2006;47:4245–4253. [PubMed: 17003412]
- Perron M, Kanekar S, Vetter ML, Harris WA. The genetic sequence of retinal development in the ciliary margin of the *Xenopus* eye. *Dev Biol* 1998;199:185–200. [PubMed: 9698439]
- Rowan S, Cepko CL. A POU factor binding site upstream of the Chx10 homeobox gene is required for Chx10 expression in subsets of retinal progenitor cells and bipolar cells. *Dev Biol* 2005;281:240–255. [PubMed: 15893976]

- Ryan AK, Rosenfeld MG. POU domain family values: flexibility, partnerships, and developmental codes. *Genes Dev* 1997;11:1207–1225. [PubMed: 9171367]
- Schwartz S, Zhang Z, Frazer KA, Smit A, Riemer C, Bouck J, Gibbs R, Hardison R, Miller W. PipMaker--a web server for aligning two genomic DNA sequences. *Genome Res* 2000;10:577–586. [PubMed: 10779500]
- Shimamura K, Hirano S, McMahon AP, Takeichi M. Wnt-1-dependent regulation of local E-cadherin and alpha N-catenin expression in the embryonic mouse brain. *Development* 1994;120:2225–2234. [PubMed: 7925023]
- Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA. An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res* 1995;23:1087–1088. [PubMed: 7731798]
- Sive, HL.; Grainger, RM.; Harland, RM. *Early Development of Xenopus laevis: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2000.
- Sparrow DB, Latinkic B, Mohun TJ. A simplified method of generating transgenic *Xenopus*. *Nucleic Acids Res* 2000;28:E12. [PubMed: 10648800]
- Tanaka S, Kamachi Y, Tanouchi A, Hamada H, Jing N, Kondoh H. Interplay of SOX and POU factors in regulation of the Nestin gene in neural primordial cells. *Mol Cell Biol* 2004;24:8834–8846. [PubMed: 15456859]
- Viczian AS, Vignali R, Zuber ME, Barsacchi G, Harris WA. XOt5b and XOt2 regulate photoreceptor and bipolar fates in the *Xenopus* retina. *Development* 2003;130:1281–1294. [PubMed: 12588845]
- Winkler S, Loosli F, Henrich T, Wakamatsu Y, Wittbrodt J. The conditional medaka mutation *eyeless* uncouples patterning and morphogenesis of the eye. *Development* 2000;127:1911–1919. [PubMed: 10751179]
- Zhang L, El-Hodiri HM, Ma HF, Zhang X, Servetnick M, Wensel TG, Jamrich M. Targeted expression of the dominant-negative FGFR4a in the eye using Xrx1A regulatory sequences interferes with normal retinal development. *Development* 2003;130:4177–4186. [PubMed: 12874136]
- Zuber ME, Gestri G, Viczian AS, Barsacchi G, Harris WA. Specification of the vertebrate eye by a network of eye field transcription factors. *Development* 2003;130:5155–5167. [PubMed: 12944429]

Figure 1. Rax2A_{2.8} directs gene expression in the eye fields, ventral forebrain, and pineal gland
 Fluorescent images of Rax2A_{2.8}/GFP transgenic embryos and tadpoles. Stages are indicated in each panel. Views: (A, E) dorsal; (B) anterior; (C, D, F, G) lateral. Expression of GFP is first evident in the anterior neural plate (A), and is present in the developing eye during neurul tube and tailbud stages (B–F). (E) Dorsal view demonstrating eye-specific expression. (G) At tadpole stages, GFP fluorescence is obstructed by the RPE but is readily observed through the clear lens. Abbreviations: B – blastopore, E – eye, VF – ventral forebrain, P – pineal gland, L – lens, R – (RPE). Arrows indicate nonspecific nasal expression.

Figure 2. Conservation and in silico analysis of the Rax2A regulatory region

(A) Percent identity plot (PIP) alignment of the Rax2A promoter with the Rax1A regulatory sequence (Zhang et al., 2004). Segments of DNA with percent identity above 50% are shown. Alignment analysis was performed using the PipMaker program

(<http://pipmaker.bx.psu.edu/pipmaker>), which plots the percent identity of DNA segments between two or more designated sequences (Schwartz et al., 2000). (B) Schematic representation of the Rax2A regulatory region. Scale indicates transcription start to the right. Longest regions of highest identity revealed by PIP analysis (red boxes) and predicted cis-regulatory elements predicted by MatInspector are shown. Yellow: POU Domain Motif. Light Green: OTX Motif. Blue: SOX Motif. Dark Green: Forkhead Motif. Regions of greatest divergence are shown in light blue. Arrows indicate relative locations of highly conserved regions or non-conserved regions between A and B. (C) Alignments showing conservation of predicted transcription factor binding sites within Ultra-Conserved Element 3 (UCE3).

Completely conserved residues are shown in gray. Colored boxes indicate core transcription factor binding motifs. Numbers indicate relative position from proximal end of isolated Rax2A regulatory region.

Figure 3. Deletion constructs produced to investigate transcriptional activity of the Rax2A regulatory region

Deletion name and schematic representation of transgene constructs are shown: black bar, Rax2A sequence; green bar, GFP; gray bar, heat shock protein gene (HSP) promoter. The entire regulatory sequence isolated is shown in schematic form above and below the transgene constructs: red, regions of high conservation with Rax1A sequence, light blue, regions of divergence with Rax1A sequence; blue, predicted inversion event. Vertical dashed lines delineate the edges of UCE3 (ultra-conserved element 3).

Figure 4. The Rax2A promoter contains cis-elements required for proper spatial and temporal gene expression of the GFP transgene

Fluorescent images of tadpoles generated using Rax2A/GFP 5'-deletion transgenes, as indicated on the right side of the figure. (A–C) Rax2A –2394, (D–F) Rax2A –2160; (G–H) Rax2A –1823; (I–J) Rax2A –1208; (K) Rax2A –1090; (L) Rax2A –944; (M) Rax2A –818 +FBE. Embryos are shown at early tailbud stages (~st 24; A, D), mid-tailbud stages (~st 28; B, E, G, I), or tadpole stages (~st 41; C, F, H, J–M). All panels show lateral views except dorsal view in (E). White arrows - regions of endogenous expression; red arrows - ectopic expression.

Figure 5. Internal deletions of the Rax2A promoter demonstrate additional regulatory regions that provide spatial specificity

Fluorescent images of tadpoles generated using Rax2A/GFP internal deletion transgenes, as indicated on the right side of the figure. (A–B) Rax2A 818–1208; (C–E) Rax2A 818–1236; (F–H) Rax2A 818–2400. Embryos are shown at late neural plate/early neural tube stages (~st 19/20; A, C, F) or tailbud stages (~st 26 – 28; B, D, E, G). Embryo orientation: (A, C, F) anterior views; (B, D, E, G) lateral views. (H) Immuno-labeling of Rax2A 818–2400 transgene expression (brown spots) in the tadpole (st 41) heart (bracket). White arrows - regions of endogenous expression; red arrows - ectopic brain expression; red asterisks – ectopic ventral expression.

Figure 6. Cooperative activity of UCE and the proximal FBE directs gene expression throughout developmental stages

Fluorescent images of tadpoles generated using Rax2A–HSP/GFP transgenes, as indicated on the right side of the figure. **(A)** Rax2A 1703-hsp; **(B)** Rax2A 1823-hsp; **(C)** Rax2A 2136-hsp; **(D–E)** Rax2A 2136 + FBE-hsp. Embryos are shown at tailbud (~st 26 – 28; **A–D**) or tadpole (~ st 41; **E**) stages. All panels show lateral views except anterior view in **D**.

Figure 7. POU, SOX and OTX sites within UCE3 cooperatively regulate Rax expression in the developing eyes of *Xenopus laevis* embryos

Fluorescent images of tadpoles generated using Rax2A/GFP with mutations in UCE3 conserved transcription factor binding sites. Transgenes represented are detailed on the right. (A–B) Rax2A UCE delPOU; (C–E) Rax2A mutOtx (–2179/–1703); (F–H) Rax2A UCE mutSOX; (I–K) Rax2A UCE mutOtx/mutSox. Embryos are shown at tailbud (~st 26 – 28; A, C, D, F, G) or tadpole (~st 41; B, E, H) stages. All panels show lateral views. White arrows, regions of endogenous expression. Red arrows, ectopic expression.

Figure 8. Expression of Rax2A–GFP transgenes in the mature retina

Transgene expression was analyzed by in situ hybridization using a GFP riboprobe and sections of stage 41 transgenic tadpoles. (A, A') Rax2A 2823; (B, B') Rax2A 2160; (C) Rax2A 1764; (D) Rax2A 944; (E) Rax2A UCE3 mutOTX; (F) Rax2A UCE3 mutOtx/mutSox; (G) Rax2A ΔUCE3. A' and B' are high magnification views of A and B, respectively. Red brackets indicate the ciliary marginal zone (CMZ). Red arrowheads indicate examples of transgene expression in the inner nuclear layer. Abbreviations: L – lens; G – ganglion cell layer; I – inner nuclear layer; P – photoreceptor layer.

Figure 9. Model of *Rax2A* transcriptional regulation derived from deletion analysis
Abbreviations: ANP – anterior neural plate; CRE – central repressor element; DAE – distal activator element; DB – dorsal brain; PN – pan-neural.

TABLE 1

Summary of Rax2A–GFP transgene expression

#	Construct Name	Eye Expression			Ectopic Expression	Mature Retinal Expression		
		16/18	24	28	(stage)	CMZ	INL	PR
1	Rax2A 2823	yes	yes	yes	no	yes	yes	yes
2	Rax2A UCE delPOU	yes	yes	yes	DB (20), PN(20)	yes	yes	yes
3	Rax2A UCE mutOTX	yes	yes	yes	no	yes	yes	yes
4	Rax2A UCE mutSox	yes	yes	yes	yes	yes	yes	yes
5	Rax2A mutSox/mutOTX	no	yes	yes	yes	no	yes	yes
6	Rax2A 2359	yes	yes	yes	no	yes	yes	yes
7	Rax2A 2160	no	yes	yes	DB(20)	yes	yes	yes
8	Rax2A 1954	no	yes	yes	DB(20)	ND	ND	ND
9	Rax2A 1823	no	yes	yes	DB(20)	ND	ND	ND
10	Rax2A 1764	no	yes	yes	DB(20)	yes	yes	yes
11	Rax2A 1553	no	yes	yes	DB(24)	ND	ND	ND
12	Rax2A 1208	no	yes	yes	DB(24)	ND	ND	ND
13	Rax2A 1090	no	yes	yes	DB(24)	ND	ND	ND
14	Rax2A 944	no	yes	yes	DB(24)	ND	ND	ND
15	Rax2A 818	no	no	no	no	ND	ND	ND
16	Rax2A 818+FBE	no	yes	yes	no	ND	ND	ND
17	Rax2A 818–1208	yes	yes	yes	no	yes	no	yes
18	Rax2A 818–1553	yes	yes	yes	no	ND	ND	ND
19	Rax2A 818–1703	yes	yes	yes	VM	ND	ND	ND
20	Rax2A 818–2136	yes	yes	yes	VM	yes	no	yes
21	Rax2A 818–2400	yes	yes	yes	VM	ND	ND	ND
22	Rax2A deltaUCE	ND	ND	ND	ND	no	no	yes

#	Construct Name	Eye Expression			Ectopic Expression (stage)	Mature Retinal Expression		
		16/18	24	28		CMZ	INL	PR
23	Rax2A 1208-HSP	yes	yes	yes	no	ND	ND	ND
24	Rax2A 1703-HSP	no	yes	no	no	ND	ND	ND
25	Rax2A 1823-HSP	no	yes	no	no	ND	ND	ND
26	Rax2A 2136-HSP	no	yes	no	no	ND	ND	ND
27	Rax2A 2136+FBE-HSP	yes	yes	yes	no	ND	ND	ND
28	Rax2A UCE-HSP	no	no	no	no	ND	ND	ND
29	Rax2A 2400-HSP	no	no	no	no	ND	ND	ND
30	Rax2A 2400+FBE-HSP	no	no	no	no	ND	ND	ND
31	Rax2A FBE-HSP	no	no	no	no	ND	ND	ND

Abbreviations: DB, dorsal brain; VM, ventral mesoderm; PN, pan-neural; ND, not determined.