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## Autoregulation of retinal homeobox (*rax*) gene promoter activity through a highly conserved genomic element

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### Abstract

The Retinal homeobox (*rax*) gene is expressed in vertebrate retinal progenitor and stem cells and is essential for retinal development. In frogs, *rax* is expressed in the ciliary marginal zone (CMZ), a region containing retinal progenitor and stem cells at the anterior of the eye. Little is known regarding regulation of *rax* transcription and regulation of transcription of *rax* targets. We found that three ultra-conserved genomic elements (UCEs) flanking the *rax* coding region regulate expression of a *rax* promoter-GFP transgene in *Xenopus* tadpoles. One of these elements, UCE1, regulates expression of the transgene in the dorsal CMZ. UCE1 contains a Rax binding site, PCE-1. We demonstrate that *rax* regulates expression of the transgene through the PCE-1 site found in UCE1. Therefore, *rax* transcription in the CMZ is controlled, in part, by autoregulatory mechanisms.

### Keywords

retinal homeobox gene; ciliary marginal zone; retinal progenitor cells; genomic element; *Xenopus*

## INTRODUCTION

The retinal homeobox (*rax*) gene is essential for vertebrate retinal development [reviewed in (Bailey et al. 2004)]. *rax* is among the first genes to be expressed in the prospective retina at the anterior neural plate (Casarosa et al. 1997; Mathers et al. 1997; Zuber et al. 2003). Its expression continues in undifferentiated retinal neuroepithelium and is generally down-regulated as retinal neurons differentiate, with expression persisting in photoreceptors (Wang and Harris 2005; Pan et al. 2010). In fish and amphibian tadpoles, *rax* expression persists in retinal progenitor cells residing in the ciliary marginal or germinal zone (CMZ or CGZ, respectively) of the mature retina (Chuang et al. 1999; Wang and Harris 2005; Pan et al.

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2010). *Rax* is an eye field transcription factor (EFTF), expressed early in eye field specification and development (Zuber et al. 2003). Many EFTFs can activate expression of other EFTFs.

The molecular mechanisms by which *rax* expression is regulated are not completely worked out. We previously reported the identification and partial characterization of the *Xenopus laevis rax.S* and *rax.L* (Rx1A and Rx2A) and *Xenopus tropicalis rax* gene promoters (Zhang et al. 2003; Martinez-de Luna et al. 2010; Pan et al. 2010). The *rax* transcriptional unit is flanked by highly conserved genomic elements that we refer to as ultra-conserved elements (UCEs) (Danno et al. 2008; Pan et al. 2010). Two UCEs are found in the 5'-flank of the *rax* transcribed region. Here, we describe the identification and preliminary characterization of UCE1, located in the 3'-flank of the *rax* transcribed region. It has been demonstrated that Sox2 and Otx2 are sufficient to initiate expression of *rax* through UCE3 in naïve *Xenopus* ectoderm (Danno et al. 2008). Outside of Otx2 and Sox2, trans-acting factors regulating *rax* expression are largely unknown. Here, we report that *rax* is capable of autoregulation through UCE1, resulting in regulation of *rax* transcriptional activity in the dorsal portion of the CMZ.

## MATERIALS AND METHODS

### Animals

We have complied with or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) in the use of animals for the studies described here.

### Identification and isolation of UCE1

UCE1 was identified as a highly conserved genomic region adjacent to the *Xenopus tropicalis rax* transcribed region by analysis of the *rax* locus using the UCSC Genome Browser (<http://genome.ucsc.edu/>), spanning scaffold 228:557470–557770 [Aug. 2005 (JGI 4.1/xenTro2) Assembly]. UCE1 was amplified from *X. tropicalis* genomic DNA using gene-specific primers F: 5' – CGCGTTGTTGGGATCCCAGTAAATATGC – 3'; R: 5' – GATCAGATCTCTATCCAGTCCGACCCTGTAGGAATAC – 3'. The R primer contains a BglII restriction enzyme recognition sequence (underlined). Amplified DNA was cloned into pCRII Topo (TOPO TA Cloning Kit, Invitrogen) to make pCRII-UCE1 and verified by DNA sequencing.

### Plasmids

UCE1 was liberated from the pCRII TOPO using BamHI and BglII and inserted into the BamHI site of *Tg(rax:GFP)<sup>Hme</sup>* (previously known as tRx<sub>3000</sub>-GFP) (Pan et al. 2010) to make *Tg(UCE1, rax:GFP)<sup>Hme</sup>*. The BglII site was included in the R amplification primer as discussed above. The BamHI site was within the amplified genomic region.

The PCE-1 sites or Nkx sites of UCE1 (pCRII-UCE1) were mutagenized by site-directed mutagenesis using the QuickChange XL Kit (Stratagene) and the following oligonucleotides (top strands are shown; bottom strands are reverse complement oligonucleotides): PCE-1

site: 5'-CTGAGGCAGGATTATTAGGCTCAACACCTAGCTAACGAGGGCAGC-3' Nkx site: 5'-GCGTGAGCAGCTGGATGCTGGTTAAGGAATTAATCACTAATGGG-3'. To mutate both sites, site-directed mutagenesis was carried out on the PCE-1 mutated UCE1 using Nkx mutagenic primers. Singly or doubly mutated UCE1 was then subcloned into desired plasmids as described above.

To prepare the *Tg(UCE1, rax.S:GFP)<sup>Hme</sup>* constructs, wild type or mutated UCE1 was liberated from the pCRII using BamHI and BglII and inserted into the BamHI site of pLITMUS28 (New England Biolabs) to make pL-UCE1. *Tg(rax.S:GFP)<sup>Mjam</sup>* (previously known as Rx1A-GFP), including the 3.4 kb *rax.S* promoter, was liberated from pBS/Rx1A-GFP (Zhang et al. 2003) using SacI and KpnI and ligated into the same sites in pL-UCE1.

### Chromatin immunoprecipitation

*Xenopus tropicalis* embryos were generated by in vitro fertilization and injected with RNA encoding myc-tagged Rax (MT-Rax) in both dorsal animal blastomeres at the 4 cell stage (1 pg per blastomere). Abnormally developing embryos, including those exhibiting the *rax* gain-of-function phenotype [i.e. ectopic RPE (Mathers et al. 1997)] were discarded. Embryos were cultured to st 41 and then fixed and used for ChIP as described previously (Pan et al. 2010). ChIP products were detected by real time PCR using oligonucleotide primers spanning either UCE1 or UCE2.

### Transgenesis

Transgenes were purified from agarose gels using the GeneClean Kit (Bio101) and introduced into *X. laevis* eggs by intra-cytosolic sperm injection (ICSI) (Sparrow et al. 2000; Kelly et al. 2005). Sperm nuclei were prepared as described previously (Kroll and Amaya 1996) but treated with digitonin instead of lysolecithin (Huang et al. 1999).

### In situ hybridization

Section *in situ* hybridization was performed on 8 µm retinal sections processed using either digoxigenin or fluorescein-labeled antisense riboprobes as previously described (Shimamura et al. 1994; Viczian et al. 2003).

### Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed as described previously (Voronina et al. 2004; Pan et al. 2006). The probe used was the PCE-1 site from UCE1 (F: 5'-CTGAGGCAGGATTATTAGGCTCAACACCCAATTAACGAGGGCAGC-3'). Competitors were the UCE1 PCE-1 site, the mouse rhodopsin promoter PCE-1 site (Kimura et al. 2000), of the UCE1 Nkx site (5'-GCGTGAGCAATTAGATGCTGGTTAAGGAATTAATCACTAATGGG-3'). All were annealed to the appropriate reverse complement oligonucleotides to make double-stranded oligonucleotides.

## RESULTS

### The *rax* CDS is flanked by conserved genomic elements

We identified 3 ultra-conserved elements (UCEs) that are conserved across vertebrates and demonstrated that UCE2 and UCE3 are necessary for expression of a GFP transgene in the ciliary marginal zone (CMZ) (Pan et al. 2010). To investigate the role UCE1 might play in regulating *rax* promoter activity, we amplified it from *X. tropicalis* genomic DNA and fused it to the *Tg(rax:GFP)<sup>Hme</sup>* transgene (Fig 1A). We determined the expression of this transgene in *Xenopus laevis* tadpoles, finding that it is expressed in a portion of the CMZ. We found that *Tg(UCE1,rax:GFP)<sup>Hme</sup>* is expressed in the dorsal CMZ (the CMZ in the dorsal portion of the retina; Fig 1B). We previously characterized the activity of a 3.4 kb *X. laevis* *rax.S* promoter in the developing retina (Zhang et al. 2003). We found that a *Tg(rax.S:GFP)<sup>Mjam</sup>* transgene is not expressed in the CMZ [(Zhang et al. 2003) and Fig 1C]. Addition of UCE1 also drove expression of the resulting transgene in the dorsal CMZ (tadpoles, Fig 1D).

### UCE1 contains two putative Rax binding sites

*rax* gene products can bind the photoreceptor conserved element 1 (PCE-1) site in vitro (Kimura et al. 2000). Analysis of UCE1 reveals that it contains two PCE-1-like sites. One of these was predicted to be a binding site for Nkx transcription factors by the UCSC Genome Browser (not shown). We refer to the two sites as the PCE-1 and Nkx sites (Fig 2A). To analyze binding of Rax to these sites, we performed electrophoretic mobility shift assays (EMSAs) using the UCE1 PCE-1 site as a probe. We observed the formation of two protein-DNA complexes, in the form of bands of shifted mobility, upon addition of Rax protein (S1 and S2, Fig 2B, lane 2). S1 band was essentially absent and the intensity of S2 was substantially reduced upon addition of a 100 X molar excess of unlabeled UCE1 PCE-1 double-stranded oligonucleotide but not a mutated version containing a disrupted version of the PCE-1 site (lanes 3, 4). This suggests that binding of Rax to the UCE1 PCE-1 site is specific. Formation of the complexes was similarly disrupted by addition of a UCE1 Nkx site competitor oligonucleotide (lanes 7, 8). Interestingly, addition of a competitor oligonucleotide spanning the rhodopsin gene promoter PCE-1 site only partially inhibited complex formation (lanes 5, 6), suggesting that Rax binds to this site with lower affinity than the UCE1 PCE-1 and Nkx sites. These data suggest that Rax can specifically bind the UCE1 PCE-1 and Nkx sites in vitro.

### Rax can interact with UCE-1 in vivo

We next investigated whether Rax binds UCE-1 in vivo by chromatin immunoprecipitation (ChIP). We have not identified an antibody that binds to *Xenopus* Rax, so we used an approach involving epitope-tagged Rax (Pan et al. 2010). A relatively low amount of myc-tagged Rax (MT-Rax) or myc tag alone (MT) was expressed in *X. tropicalis* embryos (see Materials and Methods) and ChIP was performed using an antibody raised against the myc tag. UCE-1 was enriched in ChIP from MT-Rax-expressing embryos as compared to embryos expressing MT alone (Fig 2C). Further, UCE1 was not precipitated by control IgG. Finally, UCE2 was not enriched in ChIP from MT-Rax-expressing embryos using the anti-

myc antibody, even though UCE2 contains a putative PCE-1 site. Taken together, these results indicate that Rax can specifically bind to UCE1 in vivo.

### The PCE-1 site is necessary for Rax-dependent regulation of UCE1-dependent reporters

We analyzed the regulation of the UCE1 by PCE-1 in the context of retinal progenitor cells. We mutated the PCE-1 and/or Nkx sites in the context of the *Tg(UCE1,rax.S:GFP)<sup>Hme</sup>* transgene and used them to produce transgenic embryos. We found that mutation of the Nkx site did not affect dorsal CMZ expression (3/3 tadpoles, Fig 3A), while mutation of the PCE-1 site abolished expression of the transgene in the dorsal CMZ (5/5 tadpoles, Fig 3B). Mutation of both the Nkx and PCE-1 sites resulted in transgene expression in apparently the same pattern as *Tg(rax.S:GFP)<sup>Mjam</sup>* lacking UCE1 entirely (3/3 tadpoles, compare to Figures 3C to 1C), suggesting that expression of *Tg(UCE1,rax.S:GFP)<sup>Hme</sup>* in the dorsal CMZ was dependent on the PCE-1 site of UCE1.

### The PCE-1 site is not sufficient for dorsal CMZ activity of UCE1

Having determined that the PCE-1 site is necessary for transgene expression in the dorsal CMZ, we next asked if it is sufficient for transgene expression in the dorsal CMZ. We prepared the *Tg(PCE-1,rax.S:GFP)<sup>Hme</sup>* transgene, adding a single copy of the PCE-1 site to *Tg(rax.S:GFP)<sup>Mjam</sup>* and tested it in transgenic tadpoles. We found that this transgene was expressed essentially only in the photoreceptor layer and not in the CMZ, as is the *Tg(rax.S:GFP)<sup>Mjam</sup>* transgene (Figure 3D), suggesting that the PCE-1 site itself is not sufficient to drive transgene expression in the dorsal CMZ.

## DISCUSSION

In this paper, we present evidence that *rax* may regulate its own transcription by binding a conserved genomic element, UCE-1. We found that Rax can bind to a specific cis-element in UCE-1 in vitro and in vivo and that this element is necessary for UCE-1 dependent transgene expression. In transgenic *Xenopus* tadpoles, UCE-1 directed transgene expression in the dorsal CMZ. The PCE-1 site was necessary but not sufficient for this expression. These results suggest that Rax binds the PCE-1 site and cooperates with other trans-acting factors to promote transgene expression in the dorsal CMZ. It is interesting to speculate that Rax might interact with a trans-factor that binds the Nkx site. Nkx5.3 and 5.4 (SOHo) are examples of Nkx genes that are expressed in the neural retina (Deitcher et al. 1994; Stadler and Solursh 1994; Adamska et al. 2001; Kelly and El-Hodiri 2016). Although Nkx5.3 and SOHo are expressed in the mature *X. laevis* neural retina, Nkx5.3 does not exhibit differential expression between dorsal and ventral CMZ and SOHo expression is not observed in the CMZ (Kelly and El-Hodiri 2016).

The PCE-1 site is best known for its conservation among a significant subset of genes expressed specifically in photoreceptors (Kikuchi et al. 1993; Batni et al. 1996; Boatright et al. 1997; Ma et al. 2001; Mani et al. 2001; Moritz et al. 2002). It is clear that Rax binds the PCE-1 site and regulates gene expression of genes containing a PCE-1 site (Kimura et al. 2000; Pan et al. 2010). *rax* expression in photoreceptors is diminished in *rax* knockdown embryos (Pan et al. 2010), supporting the proposition that *rax* expression is, at least to some

extent, self-regulating. Even though addition of UCE1 to the *X. tropicalis rax* regulatory region seemed to decrease transgene in photoreceptors (Figure 1B), it is not clear that this effect was observed when UCE1 was added to the *X. laevis rax.s* regulatory region (compare Figure 1C and D). In any case, the knockdown results suggest that Rax is required for regulating its own expression in photoreceptors while the data presented here suggests that, in the case of UCE1-regulated transgene, Rax inhibits photoreceptor expression of the UCE1-containing transgene. Regulation of *rax* expression in the context of the endogenous genetic locus may be significantly different than in the context of the transgene, as evidenced by expression in the CMZ: in the knockdown, CMZ expression is lost, while expression of the transgene results in dorsal CMZ-specific expression.

It is interesting that UCE-1 drives transgene expression in the dorsal CMZ and represses it in the ventral CMZ. *rax* is expressed throughout the CMZ, so this represents only a portion of the *rax* expression domain. The dorsal and ventral portions of the CMZ develop with different timing. After the initial formation of the CMZ, the dorsal CMZ continues to develop rapidly whereas ventral CMZ cells exhibit decreased proliferation until somewhat later in development, when it gives rise to the post-metamorphic retina. This process is controlled by expression of *type III deiodinase* (Marsh-Armstrong et al. 1999), which is expressed exclusively in the dorsal retina as early as st 36. Other genes, such as *bmp4*, also exhibit dorsal CMZ expression (Hocking and McFarlane 2007), although their role in regulating dorsal-ventral differences in CMZ development have not been reported. In any case, it seems likely that Rax cooperates with other factors that interact with UCE1 to regulate its own differential expression in the CMZ.

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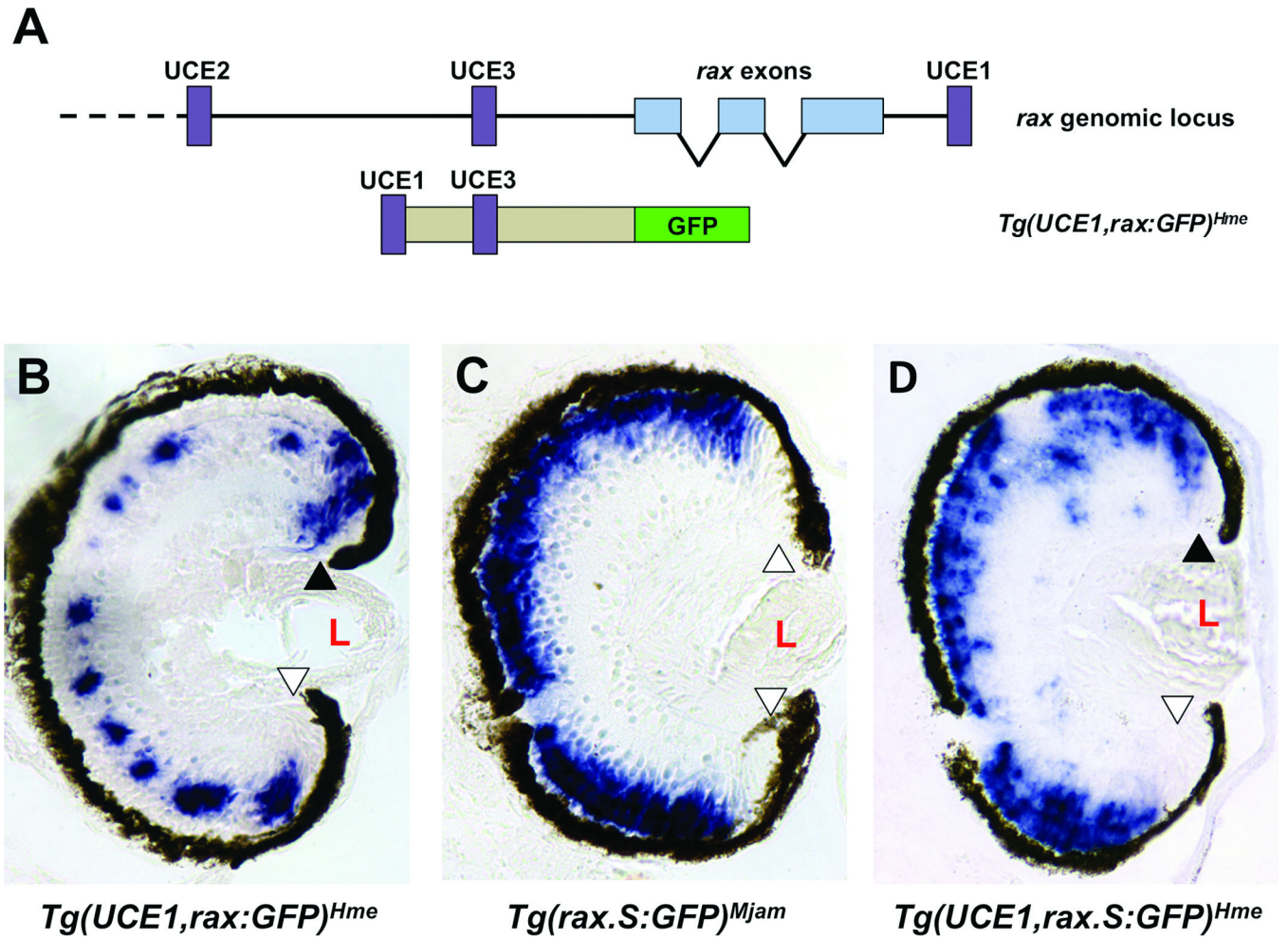
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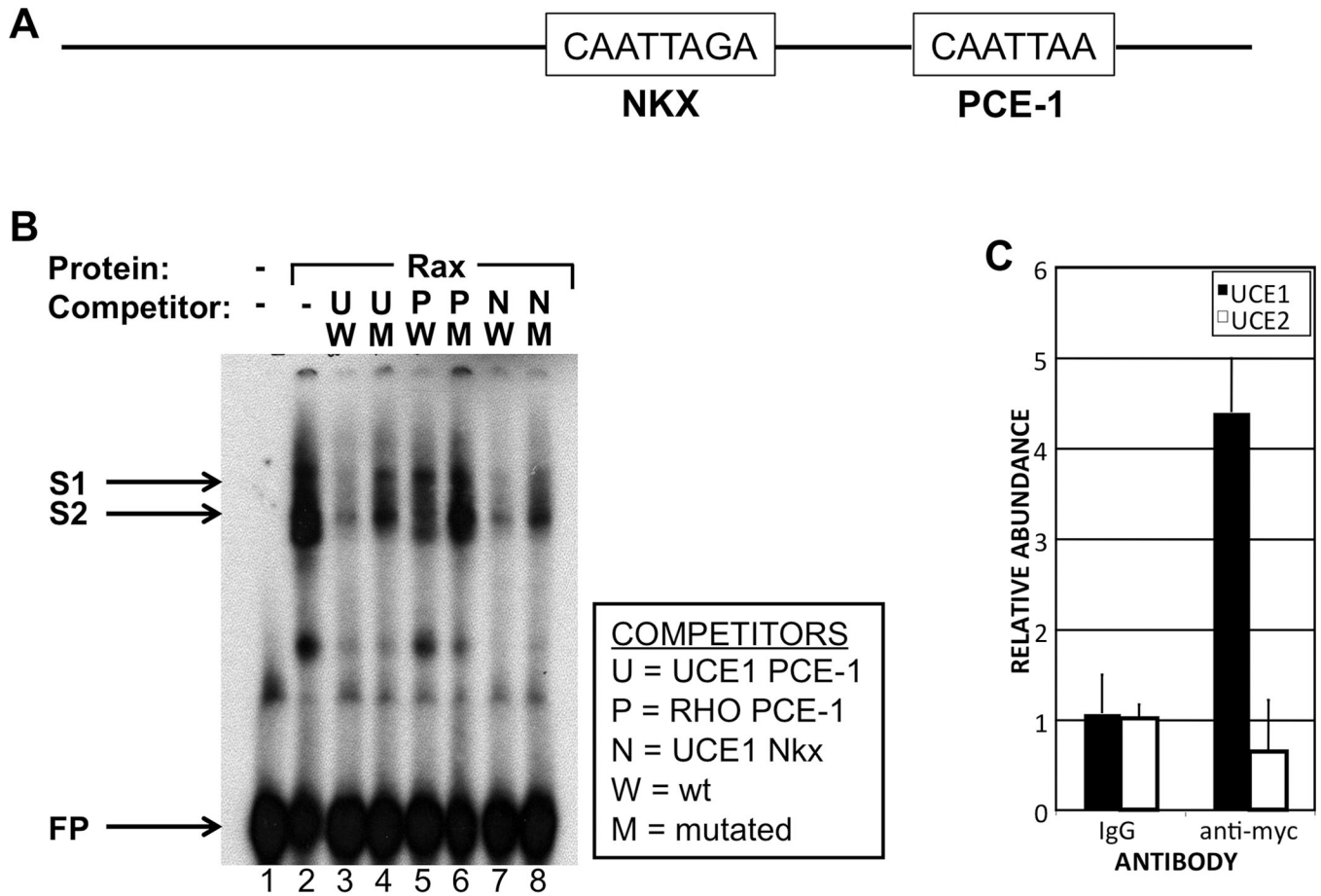
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**Figure 1.**

UCE1 drives transgene expression in the dorsal CMZ. A. Schematic diagram of the *rax* genomic locus (top) and the *Tg(UCE1,rax:GFP)<sup>Hme</sup>* transgene. B. Addition of UCE1 to the *Tg(rax:GFP)<sup>Hme</sup>* transgene results in expression in the dorsal CMZ but not the ventral CMZ. C, D. Addition of UCE1 to the *X. laevis rax.S* promoter drives expression in the dorsal CMZ. C. The *X. laevis Tg(rax.S:GFP)<sup>Mjam</sup>* transgene is not expressed in the CMZ (open arrowheads). D. Addition of UCE1 to the *Tg(rax.S:GFP)<sup>Mjam</sup>* transgene results in expression in the dorsal CMZ (filled arrowhead). Transgene expression was visualized by *in situ* hybridization using a GFP antisense riboprobe (blue color). L – lens. Sections are oriented so that the dorsal aspect of the embryo is toward the top of the figure.

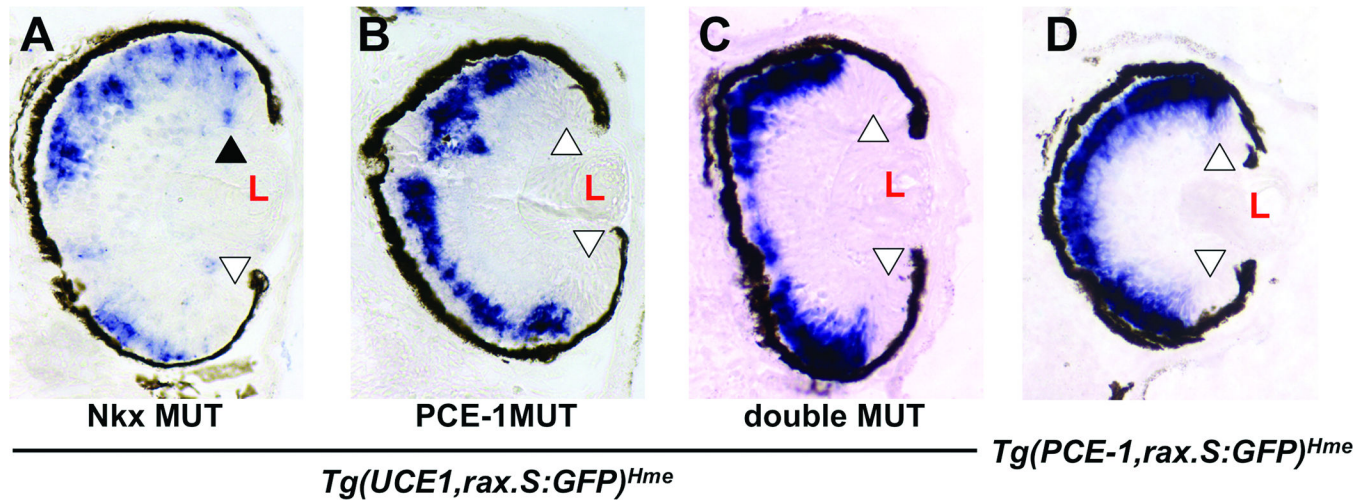


**Figure 2.**

Rax can bind to UCE1 in vitro and in vivo. A. Schematic diagram of UCE1 showing the putative PCE-1 and Nkx sites. B. Rax can bind the PCE-1 and Nkx sites in vitro.

Electrophoretic mobility shift assay (EMSA) using an oligonucleotide spanning the UCE1 PCE-1 site as probe and competitor oligonucleotides spanning the same site (lanes 3, 4), the mouse rhodopsin promoter PCE-1 site (lanes 5, 6) or the UCE1 Nkx site (lanes 7, 8). For each competitor we used a wild type version (lanes 3, 5, 7) or a version in which the PCE-1 site was mutated (lanes 4, 6, 8). Addition of in vitro translated Rax protein to the probe resulted in two bands of shifted mobility (S1, S2). C. Rax can bind UCE1 in vivo.

Chromatin immunoprecipitation (ChIP) performed using embryos injected with RNA encoding myc-tagged Rax (MT-Rax) and immunoprecipitated using an antibody raised against the myc tag. UCE1 is specifically precipitated by anti-myc antibody and not control IgG. UCE1 is specifically precipitated but UCE2 is not.



**Figure 3.** *rax*-dependent UCE1 activity is mediated through the PCE-1 site. A – C. Expression of the  $Tg(UCE1, rax.S:GFP)^{Hme}$  transgene containing mutations in the Nkx site (A), PCE-1 site (B), or both (C). D. Expression of  $Tg(PCE-1, rax.S:GFP)^{Hme}$  transgene. Expression is visualized by in situ hybridization of sectioned retinal tissue using a GFP antisense riboprobe. Open arrowheads indicate lack of expression in the CMZ; black arrowhead indicates expression in the CMZ. L – lens. Sections are oriented so that the dorsal aspect of the embryo is toward the top of the figure.