A Novel Promoter Element, Photoreceptor Conserved Element II, Directs Photoreceptor-specific Expression of Nocturnin in *Xenopus laevis*

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Nocturnin is a vertebrate circadian clock-regulated gene, and in *Xenopus laevis* its mRNA is specifically expressed in retinal photoreceptor cells. We have investigated the transcriptional regulatory mechanism that drives this precise spatial expression pattern of the nocturnin gene. A deletion series of the nocturnin 5′-flanking sequence driving the green fluorescence protein (GFP) reporter was used to generate transgenic *Xenopus* tadpoles. We found that a construct containing 2.6 kilobase pairs of 5′-flanking sequence targeted high level GFP reporter expression specifically to photoreceptor cells, in a pattern identical to endogenous nocturnin. This photoreceptor-specific expression pattern was maintained with several further deletions of 5′-upstream sequence, including a short 59-base pair fragment. Within this region of 59 base pairs, three perfect repeats of a novel protein binding site were identified by electrophoretic mobility shift assay. Competitions using varying oligonucleotide sequences demonstrated that the sequence required for protein binding is CAGGCTATA, designated photoreceptor-conserved element II (PCE II). The protein complex that binds to this element is enriched in retinal extracts, and mutations of PCE II which fail to bind the protein complex also fail to direct GFP reporter expression to photoreceptors. These results indicate that the PCE II in the proximal promoter of the nocturnin gene is sufficient for driving the photoreceptor-specific expression of nocturnin.

Precise spatial patterns of gene expression are critical for the proper function of all organisms. Within most of the central nervous system of vertebrates, the study of spatial regulation of transcription is difficult because of the vast heterogeneity of this tissue. The retina is a part of the central nervous system which is more amenable to these types of study because the cells are organized in morphologically distinct layers, and the various cell types have been well characterized (1–3).

Many genes have been shown to be expressed specifically in photoreceptor cells within the retina, including those known to be involved in the phototransduction cascade or other photoreceptor processes. Biochemical studies, such as DNase I foot-
tissue-specific promoter/enhancers (22, 25, 26). To investigate the regulatory mechanism of the nocturnin gene, we analyzed the in vivo expression patterns of GFP reporters driven by various portions of the nocturnin 5′-flanking sequence in transgenic Xenopus. The in vivo experiments, coupled with in vitro binding studies, resulted in the identification of a novel element that is sufficient to drive reporter gene expression specifically in retinal photoreceptors. We have named this novel element PCE II.

**EXPERIMENTAL PROCEDURES**

**Construction of GFP Reporter Plasmids**

The transcription start site of nocturnin has been identified previously using both primer extension and 5′-rapid amplification of cDNA ends (21). Abbreviations of upstream sequences are based on the sequence position relative to the transcription start site. For example, XNP(−2.6kb/+20bp)-GFP refers to the construct of GFP driven by the Xenopus nocturnin promoter from 2.6 kb upstream to 20 bp downstream of the transcription start site.

XNP(−2.6kb/+20bp)-GFP—A Xenopus nocturnin genomic clone was cut with SacI and BsmBI to generate a fragment extending from −2.6 kb upstream of the transcription start site to 20 bp downstream. This fragment was cloned into the SacI and SmaI sites of the pGEM7 vector and named p42B2. This −2.6 kb fragment was then excised with SacI and HindIII and cloned into the SacI/HindIII sites of the pEGFP vector (CLONTECH).

XNP(−398/+20bp)-GFP—The p42B2 clone was digested with SpeI and HindIII and then cloned into the XhoI and HindIII sites of the pGL2-basic vector (Promega). XNP(−398/+20bp)-Luc. This fragment was then excised with SacI and HindIII and transferred into the pEGFP vector.

XNP(−161/+20bp)-GFP and XNP(−108/+20bp)-GFP—Specific PCR primers were designed to amplify these smaller 5′-upstream fragments from XNP(−398/+20bp)-Luc. Primer 1: 5′-GGAAAGTTGTAACTATGTGC-3′; Primer 2: 5′-GTGACGTGGCTTCTTTCT-3′; Downstream vector primer (GL primer 2; reverse): 5′-GACTGTGACGTGGCTTCTTTCT-3′.

PCR was performed as described below. These two resulting PCR fragments were each subcloned into the pCR2.1-TOPO vector using the TOPO TA kit (Invitrogen) and then transferred into the pEGFP vector at the XhoI and HindIII sites.

XNP(−771−18bp)-GFP and Mut-PCE-GFP—Complementary oligonucleotides containing three repeats of the PCE II (−771−18bp) and mutant PCE II (Mut-PCE) elements were synthesized (Life Technologies, Inc. and MWG Biotech, Inc.) flanked with restriction enzyme cutting sites.

XNP(−771−18bp): 5′-CCAGCTGGACGGGCCGAGCATGCTTCTCATGACCATCACTCC-3′ (SacI/NotI sites are underlined).

Mut-PCE: 5′-CCCTGGAGGGAAGCTTCAGACGCACTTTCCAGCCGACAGCTTATATCGCTCAGGGCATTTAC-3′ (XhoI and BglII sites are underlined).

Products were sequenced on an ABI 377 automated sequencer (Perkin-Elmer, Foster City, CA) for confirmation. Downstream vector primer (GL primer 2; reverse): 5′-GCTTCTAGACTACTCTCTCT-3′; Downstream vector primer (GL primer 2; reverse): 5′-CCCAAGATCCATCTGCACTCATTC-3′ and (reverse) 5′-GCAATGTTCTTTCAGCTGATCAC-3′. The reaction mixture for PCR included 60 mM Tris-HCl, pH 8.5, 15 mM ammonium sulfate, 1.25 mM MgCl₂ for GFP (2.5 mM for nocturnin), 1 μM each dNTP, 5 μg/ml of each primer, and 1.5 unit of AmpliTaq-Gold (PerkinElmer Life Sciences). The PCR profile was as follows: 10 min at 95 °C followed by 30 cycles of 94 °C for 40 s, 55 °C for 1 min (for GFP, 50 °C for nocturnin), and 72 °C for 1 min, followed by a final 10 min at 72 °C. PCR results were analyzed on 1% agarose gels.

**Isolation of Retinal Nuclear Extracts**

Adult frogs were maintained on 12-h light:12-h dark cycles. Adult eyes were dissected at Zeitgeber time (ZT) 2 and ZT 11.5, in which ZT 0 is defined as the time of light onset (dawn) and ZT 12 as dark onset (dusk). Brain, muscle, liver, and heart were dissected at ZT 11.5. Nuclear extracts for EMSA were prepared from adult Xenopus retinas and other tissues using the method modified from (27). Tissues were homogenized and pelleted in phosphate-buffered saline and then quickly resuspended in hypotonic buffer (10 mM HEPEs, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) with 10% glycerol (Fisher) for 4 °C at 15 min. After centrifugation at 15,000 rpm, the pellet was dissolved in high salt buffer (20 mM HEPEs, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). The nuclei were extracted for 20–30 min on a shaking platform at 4 °C and then centrifuged at 15,000 rpm. The supernatant was quickly frozen in aliquots and then stored at −70 °C until use. Protein concentrations were tested using the standard method of D₂O, protein assay (Bio-Rad).

**EMSA**

Oligonucleotides used as probes in EMSA are listed in Fig. 8B (Life Technologies, Inc. and MWG Biotech, Inc.). Two complementary oligonucleotides were annealed in the polynucleotide kinase buffer by heating to 65 °C for 10 min and then cooling slowly to room temperature. Double-stranded oligonucleotides were end labeled with [γ-³²P]ATP (NEN Life Science Products) by T4 polynucleotide kinase (New England Biolabs) and purified by NucTrap columns (Stratagen). Binding reaction mixtures included the following: 2–4 μg nuclear extracts (for retinoic acid response element binding factor b) and 5 μg nuclear extracts (for p53).

The two resulting PCR fragments were each subcloned into the pCR2.1-TOPO vector using the TOPO TA kit (Invitrogen) and then transferred into the pEGFP vector.
The probability of survival after transgenesis in our hands varies from 3 to 15% of total eggs injected. Our PCR results confirmed that 40–80% of the living tadpoles resulting from injections were transgenic-positive. Endogenous nocturnin bands were seen in all of the tadpoles, whereas the GFP bands were observed only in the transgenic-positive tadpoles (tadpole 2–7), and a nontransgenic tadpole (tadpole 1) is exhibited in Figs. 3 to 7. In situ hybridization demonstrated that within the Xenopus retina the expression of the nocturnin mRNA is spatially restricted to the retinal photoreceptor cells (19). A portion of a Xenopus nocturnin genomic clone containing ~2.6 kb of 5′-flanking sequence and 20 bp of transcribed sequence was isolated. A portion of the most proximal (~398/20 bp) of this sequence is shown in Fig. 1A. There are several putative protein binding sites in this region, including an E-box-like element, CRX-like elements, and three perfect repeats of a previously unidentified element (labeled PCE II). Serial deletions (constructs 1–5) and a mutant (construct 6) of the 5′-flanking sequence were made as shown in Fig. 1B. GFP reporters driven by different lengths of the nocturnin 5′-flanking sequence were used to generate transgenic tadpoles for examination of the resulting GFP expression patterns.

Analysis of Transgenic-positive Tadpoles by PCR—All resulting tadpoles were genotyped by PCR, using genomic DNA isolated from clipped tails. Endogenous nocturnin was amplified (~250-bp band) to monitor the quality of genomic DNA, and GFP coding sequence was amplified (~480-bp band) to confirm that GFP-GFP reporter constructs were analyzed by PCR. The first two lanes are PCR results using plasmids containing nocturnin (first lane) and GFP coding sequences (second lane) as templates; they serve as size controls for the amplified products. The positions of the GFP and nocturnin (Noc) bands are labeled on the left. Lanes marked 1–7 are the PCR results from transgenic tadpoles 1–7 produced by different constructs. Tadpole 1, no GFP integrated; tadpole 2, XNP−2.6kb/20bp-GFP; tadpole 3, XNP−398/20bp-GFP; tadpole 4, XNP−161/20bp-GFP; tadpole 5, XNP−108/20bp-GFP; tadpole 6, XNP−77/18bp-GFP; tadpole 7, GFP only, with no promoter. The relative positions of two of the size standards are labeled on the right. The + and − symbols across the top denote whether or not these tadpoles are carrying the transgene.

FIG. 2. Genotyping of transgenic tadpoles by PCR. DNA isolated from clipped tails of the tadpoles, produced using different XNP-GFP constructs, was analyzed by PCR. The first two lanes are PCR results using plasmids containing nocturnin (first lane) and GFP coding sequences (second lane) as templates; they serve as size controls for the amplified products. The positions of the GFP and nocturnin (Noc) bands are labeled on the left. Lanes marked 1–7 are the PCR results from transgenic tadpoles 1–7 produced by different constructs. Tadpole 1, no GFP integrated; tadpole 2, XNP−2.6kb/20bp-GFP; tadpole 3, XNP−398/20bp-GFP; tadpole 4, XNP−161/20bp-GFP; tadpole 5, XNP−108/20bp-GFP; tadpole 6, XNP−77/18bp-GFP; tadpole 7, GFP only, with no promoter. The relative positions of two of the size standards are labeled on the right. The + and − symbols across the top denote whether or not these tadpoles are carrying the transgene.

 RESULTS

In the studies presented here, our goal was to define the portion of the nocturnin promoter/enhancer responsible for correct spatial expression of the nocturnin gene. Previous analysis of the endogenous nocturnin mRNA by Northern blot showed that nocturnin mRNA can be detected only in the retina and is not evident in brain, heart, kidney, skeletal muscle, or liver (19). In situ hybridization demonstrated that within the Xenopus retina the expression of the nocturnin mRNA is spatially restricted to the retinal photoreceptor cells (19). A portion of a Xenopus nocturnin genomic clone containing ~2.6 kb of 5′-flanking sequence and 20 bp of transcribed sequence was isolated. A portion of the most proximal (~398/20 bp) of this sequence is shown in Fig. 1A. There are several putative protein binding sites in this region, including an E-box-like element, CRX-like elements, and three perfect repeats of a previously unidentified element (labeled PCE II). Serial deletions (constructs 1–5) and a mutant (construct 6) of the 5′-flanking sequence were made as shown in Fig. 1B. GFP reporters driven by different lengths of the nocturnin 5′-flanking sequence were used to generate transgenic tadpoles for examination of the resulting GFP expression patterns.

Analysis of Transgenic-positive Tadpoles by PCR—All resulting tadpoles were genotyped by PCR, using genomic DNA isolated from clipped tails. Endogenous nocturnin was amplified (~250-bp band) to monitor the quality of genomic DNA, and GFP coding sequence was amplified (~480-bp band) to examine whether XNP-GFP constructs had been integrated into the tadpole genome. Fig. 2 shows the PCR results from the various samples of transgenic-positive tadpoles (tadpoles 2–7), and a nontransgenic tadpole (tadpole 1) is exhibited in Figs. 3 to 7. Endogenous nocturnin bands were seen in all of the tadpoles, whereas the GFP bands were observed only in the transgenic-positive tadpoles.

The probability of survival after transgenesis in our hands varies from 3 to 15% of total eggs injected. Our PCR results confirmed that 40–80% of the living tadpoles resulting from
these injections have GFP constructs integrated into their genome with variable copy numbers (data not shown). For each construct tested for our experiments, at least three independent PCR-positive tadpoles were examined.

XNP(2.6kb/−20bp) Directs GFP Expression to Retinal Photoreceptors—We first produced transgenic tadpoles using the construct containing 2.6 kb of 5′-flanking sequence, XNP(−2.6kb/20bp)-GFP (Fig. 3). Careful examination of the living tadpoles generally revealed no expression in any nonocular tissue. In one tadpole (out of more than 20 examined), low level GFP expression was observed in pineal gland and olfactory epithelium (data not shown), possibly because of the position effect on the inserted transgene.

We sectioned tadpoles carrying this transgene and examined retinal expression. In the phase-contrast images at low magnification (Fig. 3A), the structures of the eyes as well as some other body tissues. In the corresponding fluorescence image, GFP expression is observed only in the outer lamina of the retina, with no detectable GFP expression in other retinal cell layers, lens, or nonocular tissues. With higher magnification, it is clear that GFP is expressed in the cell bodies of the photoreceptor cells but not in outer segments of photoreceptor cells or other retinal cells (Fig. 3A). An image of the retina from a sibling tadpole that did not have the XNP(−2.6kb/20bp) construct (the PCR-negative tadpole 1, Fig. 2) exhibits only a low level of background autofluorescence at the bottom of the outer segments of the photoreceptor cells, and no detectable GFP is observed in the cell bodies (Fig. 3A). The tadpole produced using linearized GFP vector with no promoter also does not exhibit any GFP
expression in any of the examined tissues (Fig. 3B) and is identical to the PCR-negative tadpole in Fig. 3A. This same pattern of very low background autofluorescence right at the inner and outer segment junction is also observed in wild-type embryos (data not shown) and is clearly distinguishable from the GFP fluorescence observed in the transgenic retinas. It is difficult to determine whether all cells in the photoreceptor layer are expressing GFP using traditional microscopy because the cells within the photoreceptor layer are organized in a somewhat staggered pattern. Therefore, only a subset of the cells is in focus at once. To examine more closely the photoreceptor cells that express GFP, confocal microscopy was used to generate a Z-series of optical sections (see “Experimental Procedures”). Confocal images through cryosections of fixed eyes from transgenic-positive tadpoles again showed that GFP expression is limited to photoreceptor cells. Within the photoreceptor layer, the combined Z-series show that both rod and cone cells express GFP (Fig. 4). These results are consistent with the expression patterns of the endogenous nocturnin, suggesting that the XNP(–2.6kb/+20bp) is sufficient to target the GFP reporter to photoreceptor cells appropriately.

**Deletion of the Nocturnin Promoter to XNP(–108/+20bp)—** Further deletions were made from the 2.6-kb fragment to narrow the region of the nocturnin promoter which is responsible for proper spatial expression. The GFP expression pattern did not change with the deletion of the nocturnin promoter from XNP(–2.6kb/+20bp) to XNP(–398/+20bp), although the GFP signals seem to be generally weaker in the latter case (Fig. 5). Therefore, further deletions of the nocturnin promoter were performed to detect whether the spatial expression pattern of the GFP reporter would change. In tadpoles produced using both XNP(–161/+20bp)-GFP and XNP(–108/+20bp)-GFP, GFP can again only be observed in the photoreceptor layer, not in other retinal cells or other tissues (Fig. 5). These results indicate that elements sufficient for photoreceptor-specific expression must be contained within the –108/+20bp sequence.

**GFP Reporter Is Only Expressed in Fully Differentiated Photoreceptor Cells**—The ciliary marginal zone (CMZ) is the region at the peripheral edge of the retina and contains undifferentiated retinal progenitor cells. The youngest cells and stem cells are closest to the periphery, the proliferative retinoblasts in the middle, and the cells that have stopped dividing at the central stage (28). In amphibians, the retina grows throughout life by adding new cells of all types from the CMZ. Fig. 6 shows that the GFP reporter directed by the XNP(–108/+20bp) is targeted only to the mature photoreceptor cells. Those cells in the CMZ which have not differentiated to photoreceptors do not exhibit any detectable GFP (Fig. 6). The same pattern has been observed in the other GFP constructs directed by longer nocturnin promoters (data not shown). These results indicate that the transcription of nocturnin is a feature of the fully differentiated photoreceptor cells (see “Discussion”).

**The GFP Expression Pattern Is Maintained in the Transgenic Tadpoles Produced by XNP(–77/–18bp)—** Within the –108/+20bp sequence, we noticed several elements of interest. One was a sequence with high similarity to an E-box, the other was three nearly perfect repeats of a novel sequence (Fig. 1A). To determine whether the E-box-like element was involved in directing spatial expression of nocturnin, we deleted this element to generate a XNP(–77/–18bp) fragment which only contains the three repeated elements. The GFP expression levels from this shorter promoter fragment are low in some of the photoreceptor cells (Fig. 7A), but from the confocal image it appears that the GFP is still expressed in both rod and cone cells (Fig. 7B). No cells were found within the photoreceptor layer that did not express GFP, although the levels of expression were variable.

**PCE II Was Identified by EMSA**—To determine the protein binding sequences within XNP(–77/–18bp), EMSAs were performed using nuclear extracts isolated from *Xenopus* retina (Fig. 8). As mentioned previously, we had observed three perfect repeats of 5′-CAGACAGCTTTA-3′ within the proximal promoter of the nocturnin gene (Fig. 8A), suggesting that the repeated sequence may be the protein binding site. One prom-
Fig. 7. **XNP (−77/−18bp) is sufficient to direct GFP to photoreceptor cells.** A diagram of the construct is shown on top of the images. Panel A, phase-contrast (left), fluorescent (center), and merged images (right) are shown. Panel B, confocal image (Z-series composite over 20 μm) shows that GFP is expressed in all cells of the retinal photoreceptor layer. OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer.

Fig. 8. **EMSAs using double-stranded oligonucleotides containing PCE II as a probe.** Panel A, alignment of the three nearly perfect repeats of PCE II (underlined) in the proximal nocturnin promoter region, XNP (−77/−18bp). Panel B, first two gels (lanes 1–13 and lanes 14–28) were run using the same conditions. Lane 1 is the free probe (FP) without retinal nuclear extracts. Each group of three lanes contains 0, 50, and 200 × cold competitors along with the radiolabeled PCE II probe. Identities of the competitors are labeled on top of each group of three lanes. Poly(dI-dC) was used as nonspecific competitor in the last group (lanes 26–28). The sequences of the competitors are shown at the bottom of the figure. Nuclear extracts isolated from retina (R), brain (B), muscle (M), liver (L), and heart (H) were used to test the tissue specificity of the complex in lanes 29–34. The arrowhead marks the position of specific DNA-protein complex.

The specific shifted band was detected when this element with flanking sequences was used as a probe (lane 2, Fig. 8B). The addition of excess unlabeled oligonucleotides identical to the radiolabeled probe resulted in a dosage-dependent inhibition of the DNA binding activity (lanes 2–4), whereas the addition of the nonspecific competitor poly(dI-dC) (lanes 26–28) had no effect, suggesting that the binding activity is specific. The binding was not inhibited by competition with oligonucleotides that were mutated in any part of the sequence of 5′-CAGACAGGCTTATA-3′ (lanes 11–22), suggesting that this sequence is involved in the DNA-protein interaction. However, the competitors with mutations outside of the recognition site still resulted in a dosage-dependent inhibition (lanes 5–10, 23–25), which is similar to that seen when using competitor identical to the probe.

The flanking sequences of the different competitors of the PCE II probe are similar but not identical, and the results of the competition assays (lanes 5–10 and 23–25) demonstrate that they are probably not critical for the DNA-protein interactions. In addition, when wild-type short oligonucleotides (like M3–M6, only with the wild-type 14-bp core sequence) were used as probes, we observed the same prominent shift as that using the longer PCE II oligonucleotides as probes (data not shown). In fact, no changes in the flanking sequence, outside the 14-bp core, had any detectable effect on protein binding. Therefore, the protein-binding element is defined as CAGGCTTATA. Because this sequence appears to be a novel protein binding site involved in photoreceptor-specific expression, we have named it PCE II.

Tissue specificity of PCE II was also tested using nuclear extracts isolated from Xenopus brain, muscle, liver, and heart (lanes 29–34). The results show that, as before, there is a strong and specific shift with nuclear extracts from retinas. A similar complex is seen with nuclear extracts from brains, but it is much weaker than seen in retinal extracts. There also may be low levels of complex formation in extracts from other tissues, but again the levels are much weaker than seen in retinal extracts. These results confirm that the protein complex that binds to PCE II is enriched in retina.

**Mutants of PCE II Failed to Target the GFP Reporter to the Photoreceptor Cells**—A mutant PCE II was designed based on the results from the competition assays of EMSAs: CAGACA was mutated to TGAGTC (M3 and M4 in Fig. 8). These base changes should disrupt complex formation. Three repeats of mutant PCE II were subcloned upstream of the GFP reporter to generate the Mut-PCE-GFP construct and was used to produce transgenic tadpoles. Six individual PCR-positive tadpoles were examined, and results from three of them are shown in Fig. 9. One of the six tadpoles exhibited a very low level of GFP in all tissues (data not shown). Within the retina, GFP was seen in a few cells, but there was no evidence of photoreceptor specificity (Fig. 9, tadpole 2). The other five...
PCR-positive tadpoles did not show any GFP expression in any tissue, including photoreceptor cells. Note the autofluorescence in the inner and outer segment junctions (Fig. 9) as seen before in nontransgenic tadpoles (Fig. 3). Altogether, these results demonstrate that XNP(−77/−18bp) containing sufficient information to direct GFP expression to photoreceptor cells in vivo.

DISCUSSION

We have isolated and characterized a *Xenopus* nocturnin promoter element capable of directing correct spatial expression. Transgenic experiments showed that a small portion of the nocturnin promoter was capable of driving reporter gene expression specifically to photoreceptors, a pattern identical to endogenous nocturnin expression. This small piece of nocturnin promoter contains three perfectly repeated sequences, and EMSAs defined the protein binding site as CAGACAGGC-TATA. Photoreceptor specificity was abolished when mutations of PCE II were used to direct the GFP reporter in the transgenic experiments. Comparison of this sequence with elements that have been shown to regulate photoreceptor-specific expression of other genes revealed that this was a novel element, and we therefore designated it as PCE II.

Several promoter elements have been reported to be involved in the rod-specific (opsin and rod arrestin) or photoreceptor-specific (IRBP and nocturnin) control of transcription (Table I). These protein binding motifs were identified by EMSAs and DNase I footprinting assays from a number of different laboratories. Comparing these sequences with each other makes it obvious that even the binding motifs with the same name found from different genes or from different species are quite variable. However, PCE II, the novel element described here, has a unique sequence that is not similar to any of the elements described previously.

Despite the work done investigating photoreceptor-specific transcriptional regulation in a number of species, no clear general mechanism for photoreceptor-specific gene expression has yet emerged. It has been shown that Ret1 can direct gene expression in rod photoreceptors in transgenic rats (29). A 221-bp fragment of the mouse opsin promoter which contains Ret1 and BAT-1 also directs expression specifically to the rod photoreceptors of transgenic mice (4). A reporter gene driven by the 70-bp promoter of the murine IRBP which contains Ret1/PCE I and CRX binding sites is active in cultures of retinal cells and brain cells (30). A portion of the rod arrestin promotor, including CRX and API binding sites, directs expression of a reporter gene only in rod cells in the transgenic *X. laevis* (7).

In this paper we showed that the XNP(−77/-18bp) containing three repeated PCE II is sufficient to target the GFP reporter to the photoreceptors in *X. laevis*.

In several cases the transcriptional factors that bind to the retina-specific elements have been characterized (Table I) even though their interactions and in vivo functions are not yet well defined. Maf and Nrl can bind in vitro to AP-1 sites and form heterodimers with Fos and Jun (15, 31–33). The homeodomain protein RX has been shown to bind to the Ret1/PCE I site and activates the TATA-less arrestin and IRBP promoters (29, 34, 35). Because the nocturnin PCE II is novel, the photoreceptor protein(s) that bind to this sequence are unknown.

Several transcriptional factors are expressed in the developmental process in *Xenopus* which produce rod and cone photoreceptors, including genes that encode paired-type homeodomain proteins, such as PAX6, CHX10, RX, and NeuroD (36–42). PAX6 and CHX10 are expressed in the retinal progenitors and maintained in inner nuclear layer cells, but their expression is excluded from developing photoreceptor cells in zebrafish, mouse, and *X. laevis* (36, 38, 43). RX is also expressed in retinal progenitors in *Xenopus* and subsequently down-regulated in all cells upon differentiation (44). NeuroD is a member of the basic helix-loop-helix family usually expressed during and/or after the terminal mitosis of neuronal precursors in rodent (45). Its expression is maintained in a subset of mature photoreceptors (46). The hierarchical pathway of these transcriptional factors has been examined using double in situ hybridizations on cross-sections of the CMZ in *Xenopus* (28). However, the targets of these transcriptional factors during development are largely unknown, and most of their known roles are limited to early development. Therefore, it seems unlikely that these proteins control the PCE II-mediated photoreceptor-specific expression pattern because nocturnin expression is a feature of fully differentiated photoreceptor cells.

Another transcription factor that is known to be present in both developing and mature photoreceptor cells is CRX (5, 6). This *otd/otx* gene family encodes paired-like homeodomain proteins that are involved in the regulation of anterior head structure and sensory organ development (47). CRX expression is restricted to developing and mature photoreceptor cells (6). It has been shown that CRX binds the sequence TAATCA/A, resulting in transactivation of transcription. This element is found in the upstream sequence of several photoreceptor-specific genes (5, 6). The nocturnin 5′-flanking region has several sequences that are similar to CRX binding sites, but deletion of these elements does not alter spatial expression (Figs. 1A and
5). The \textit{Xenopus} homolog of CRX has not been cloned. Therefore, the role of CRX in the regulation of nocturnin is unclear.

The transgenic technique in frogs is a powerful tool to investigate the spatial expression pattern and regulation of genes of interest (7, 22, 25, 26). GFP has been commonly used as the reporter because of its stability and ease of detectability for in vivo studies. Compared with making transgenic mice, it is relatively easy and inexpensive to generate these animals, and several hundreds of transgenic embryos can be produced within 1 day. One limitation of the transgenic technique in \textit{Xenopus} is the difficulty in controlling the levels of GFP because the copy numbers and positions of the construct are randomly determined in the individual tadpoles. Among those PCR-positive transgenic tadpoles in this study, there was variable expression. This variability makes it difficult to make quantitative assessments of gene expression levels. Although we observed generally homogeneous copy numbers and integration sites (24). These more quantitative methods will also be important in the temporal analyses of nocturnin expression because it will be necessary to compare expression levels at different times of day.

\textit{Xenopus} offers a unique advantage of combining the rapid and powerful transgenic approach with \textit{in vitro} methods for the study of cis-elements in controlling cell-specific expression in vertebrates. Using these methods, we have demonstrated that the nocturnin promoter PCE II is sufficient to drive photoreceptor-specific expression in \textit{Xenopus}. The novel sequence of PCE II suggests that an as yet undescribed transcriptional mechanism is driving photoreceptor-specific expression of nocturnin.

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