Conserved transcriptional regulation of a cone phototransduction gene in vertebrates

A.S. Viczian[a], M. Verardo[b], M.E. Zuber[a], B.E. Knox[a,*], D.B. Farber[b,*]

[a] *Departments of Ophthalmology and Biochemistry & Molecular Biology, SUNY Upstate Medical University, 750 East Adams, Syracuse, NY 13066, USA*

[b] *Brain Research Institute, Interdepartmental Neuroscience Program, and Jules Stein Eye Institute, Department of Ophthalmology, UCLA School of Medicine, Los Angeles, CA 90095, USA*

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Abstract cGMP-phosphodiesterase (PDE) is a key component in visual phototransduction. Rod and cone photoreceptors each produce their unique cGMP-PDE subunits. The α′ catalytic subunits are believed to be cone-specific. In this study, we report that transfection of the −132 to +139 sequence in the upstream region of the human α′-PDE gene fused to *luciferase* cDNA gives the highest level of reporter gene transcription in cultured retinoblastoma Y79 cells. Transgenic *Xenopus laevis* carrying this sequence fused to green fluorescent protein (GFP) expressed GFP in cones, suggesting a conserved regulatory mechanism for α′-PDE transcription in both human and frog.

Keywords: Retina; Transcription; Phosphodiesterase; Cone; Photoreceptor; *Xenopus*

1. Introduction

Phototransduction begins when the visual pigments of rods and cones absorb light. A cascade of events transforms biochemical reactions into a set of neuronal responses detected by the brain. A key step in this process is the binding of visual pigment-activated transducin to the inhibitory γ-subunit of the corresponding cyclic GMP-phosphodiesterase (PDE), which allows the catalytic subunits of the PDE to hydrolyze thousands of cyclic GMP molecules to 5′-GMP. Decreased levels of cyclic nucleotide lead to the closure of cGMP-gated cationic channels and changes in cell membrane potential and photoreceptor signaling [1]. Therefore, by hydrolyzing cyclic GMP, PDE plays a pivotal role in phototransduction.

The PDE enzyme complex has two inhibitory and two catalytic subunits. There are three separate genes encoding the catalytic subunits [2–4]. Biochemical evidence supports the fact that rod and cone photoreceptors each express a unique PDE complex [5,6]. In rods, there are two separate catalytic subunits, α and β, while in cones, the two catalytic subunits, α′, are identical [6,7]. We have previously cloned and characterized the human α′-PDE gene, which has 22 exons spanning 48 kb of genomic DNA. One of the clones isolated at that time contained approximately 1 kb of 5′ flanking region [8].

In this report, we characterized the promoter of the human α′-PDE gene using a human retinoblastoma-derived cell line (Y79) and transgenic *Xenopus laevis*. Y79 retinoblastoma cells have been shown to express cone α′-PDE mRNA [9] and its functional protein [10]. We show here that a construct containing 132 bp of the α′-PDE gene 5′ flanking region is sufficient to drive luciferase reporter gene expression in Y79 cells. In the differentiated frog retina, we find that this minimal promoter drives the expression of reporter gene specifically in cone photoreceptors. In some transgenic frogs, we also observed expression in a subset of bipolar cells. These results highlight conserved cone transcriptional regulation of cone phototransduction machinery in vertebrates and also suggest some possible common regulatory factors between cones and bipolar cells.

2. Materials and methods

2.1. Reporter gene constructs

The α′-PDE 5′ flanking region was obtained from a previously characterized human genomic DNA clone (α′3), which contains 989 bp from the translation start site [8]. Sequence specific primers, one with *Nhe*I (N48, GGGCTAGCCCTAACTAAGATACTTAGCAG) and the other with *Bgl*II (B61, GGAGATCTGGTGTGGCTTGCTTTGCTGC) restriction enzyme sites, were designed to amplify and subclone an 870 bp fragment (−731/+139) into the multicloning region of a luciferase reporter gene vector, pGL2-Basic (Promega, Madison, WI). PCRs were carried out as follows: 30 cycles of denaturation (1 min at 95 °C), annealing (1 min at 60 °C) and extension (2 min and 30 s at 72 °C) in a Robocycler 40 (Stratagene).

Unidirectional nested set deletions from the 870 bp PCR-amplified α′-PDE 5′ flanking region clone were obtained using the Erase-a-Base® System (Promega), based on a procedure developed by Henikoff [11]. To protect vector cleavage and allow sequential deletions of the 5′ end of the insert, the necessary 3′ overhang in the pGL2-Basic vector and the 5′ overhang in the insert were created by cutting *Kpn*I and *Nhe*I restriction enzyme sites, respectively. Once the DNA was prepared, deletions were made according to the protocol (Promega). Incubation was then carried out at 22 °C and samples were removed every 30 s to stop the reaction. The resulting fragments were sequenced.

The −132/+139-pGL2 construct was subcloned into a pEGFP vector using *Eco*RI and *Kpn*I. The resultant −132/+139-pEGFP construct was

* Corresponding authors. Fax: +1-315-464-8750 (B.E. Knox); +1-310-794-7904 (D.B. Farber).
E-mail addresses: vicziana@upstate.edu (A.S. Viczian), verardo@lifesci.ucsb.edu (M. Verardo), knoxb@upstate.edu (B.E. Knox), farber@jsei.ucla.edu (D.B. Farber).

Abbreviations: α′-PDE, alpha prime catalytic subunit of cyclic GMP-phosphodiesterase; GFP, green fluorescent protein; PDE, cyclic GMP-phosphodiesterase

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sequenced, linearized with *Xho*I and gel purified. This linearized vector was used for transgenic *Xenopus* generation.

2.2. Cell cultures and transffections

All cell lines described below were obtained from American Type Culture Collection (Rockville, MD). Human retinoblastoma Y79 cells (HTB18) were maintained in RPMI 1640 medium containing L-glutamine supplemented with 15% fetal bovine serum (Gibco-BRL, Gaithersburg, MD). These cells were grown in suspension and plated for transfection experiments in a monolayer as described [12]. 293 human embryonic kidney cells and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (0.25 μg/ml) [13]. SY5Y human neuroblastoma cells were cultured with the same supplements as HeLa and 293 cells, but in DMEM/F12 (50% of each v/v) instead of just DMEM. All four cell lines were transfected using the calcium phosphate precipitation method [12,14]. However, HeLa, 293 and SY5Y cells were transfected for 4.5 h instead of the 16 h used for Y79 cells, since longer incubation with calcium phosphate mixture proved toxic to these cells. Transfections were carried out using 15 μg of the experimental construct [either the pGL2-Control (an SV40 promoter and enhancer-driven luciferase reporter gene construct) or one of the human cone α′-PDE constructs], and 5 μg of an internal control construct, pSV-β-Gal (an SV40 promoter-driven lacZ gene construct) used for normalization of transfection efficiency. As a negative control, cells were either transfected with calcium phosphate and 15 μg of the pGL2-Basic vector or with water (no DNA). Cells were harvested and lysed in 100 μl of 1× Reporter Lysis Buffer (Promega) 24 h after transfection. Assays for both luciferase and β-galactosidase were performed as described [12]. Each experiment consisted of the experimental α′-PDE-luciferase constructs transfected in three separate plates alongside triplicate transfections of the control SV40-luciferase (positive control) and also, vector only (negative control) constructs, each normalized with SV40-lacZ. 'Percent of normalized SV40 driven luciferase activity' was calculated by dividing the luciferase activity of cells transfected with a given construct by their β-galactosidase activity (expressed by the co-transfected SV40-driven LacZ gene construct) and dividing this value by the average activity obtained with the normalized SV40-driven construct. We analyzed the data with the ANOVA (single factor) test (Microsoft Excel 2004) to determine statistical significance.

2.3. Electrophoretic mobility shift assay

The double stranded oligonucleotide, −132/−111, probe was made by first end-labeling the sense strand, 5′-CTAGTACTAATG-CTCAGGGATTTAGT-3′, with [γ-^{32}P]dATP and RTS T4 polynucleotide kinase (Gibco-BRL), purifying it with a Sephadex G-25 spin column and then annealing it to the antisense strand, 5′-CTA-GACTAAATCCCTGAGCATTAGTA-3′, as previously described [15]. Twenty-five μg of Y79 cell nuclear extract was prepared [15] and bound to a non-specific competitor (1 μg of poly-dIdC) for a few minutes before adding the cold competitor and radiolabeled probe ($\sim 1.5 \times 10^5$ cpm) in a total volume of 20 μl. Binding reactions were incubated at room temperature for 30 min and the products were resolved on a 8% non-denaturing polyacrylamide gel with 2.5% cross-linking. Gels were electrophoresed in 0.25× Tris-borate buffer at 15 mA for 16 h, dried and exposed to Hyperfilm (Amersham).

2.4. Transgenic frog generation

X. laevis oocytes were obtained from hormonally induced females and dejellied with 2% cysteine in 1× MMR (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM HEPES, pH 7.8, and 2.5 μg/ml gentamycin). Transgenic embryos were generated in four separate experiments using restriction enzyme-mediated integration (REMI) as previously described [16]. In each experiment, normal embryos were selected and grown to stage 39–43 at 18 °C in 0.1× MMR. At these stages, embryos that expressed green fluorescent protein (GFP) above the background auto-fluorescence detected in the yolk were selected. The embryos were then grown for another month at 18 °C in 0.1X MMR and then anesthetized, tail snips taken and frozen on dry ice. The remaining embryo was fixed in 4% paraformaldehyde for 1 h and incubated in 20% sucrose at 4 °C overnight. The next day, they were mounted in O.C.T., frozen and cryostat sectioned (10–14 μm). Immunocytochemistry was performed on the sections and these were visualized using bright field and fluorescence on the Nikon Microphot-FX microscope. The confocal images were acquired using the Nikon Eclipse E600 microscope coupled to a BioRad MRC 1024ES scanning transmission assembly. LaserSharp 2000 image acquisition software (BioRad) recorded successive *z*-axis serial sections to obtain the final images.

Genomic DNA was extracted from each tail snip using the DNeasy Tissue kit (Qiagen). To determine which embryos were carrying the transgene, PCR was performed using 1 μM kanamycin sequence-specific primers [forward, GATGGATTGCACGCAGGTTC and reverse, CGATAGAAGGCGATGCGCTGC] and 50 ng DNA in a standard 25 μl PCR (200 μM dNTPs, Promega 1× Reaction Buffer with $MgCl_2$ and 0.75 units Promega *Taq* DNA polymerase). The reactions were run on a RoboCycler® Gradient temperature cycler (Stratagene) under the following conditions: 95 °C denaturing for 3 min; 30 cycles of 95 °C denaturing for 1 min, 52 °C annealing for 1 min and 72 °C extension for 1 min; finished with one cycle at 72 °C for 5 min. The reactions were analyzed for the presence of the correct-sized PCR product using gel electrophoresis.

2.5. Immunocytochemistry

Cryostat sections were placed on Superfrost*/Plus microscope slides (Fisher Scientific) and dried at room temperature for 30 min to 2 h. Afterwards, they were stored at 4 °C for up to 2 weeks until tail snips were processed. Cryostat sections were rehydrated in 1× PBS for 3 min and fixed to slides using 100% methanol for 10 min, then rinsed in 1× PBS for 2 min and washed 3 times for 5 min in PBST (1× PBS + 0.1% Triton). The slides were blocked for 30 min with PBST + 5% heat inactivated goat serum and incubated at 37 °C for 30 min with two primary antibodies [1:3000 dilution of the mouse anti-rhodopsin antibody (4D2; [16,17]) and a 1:500 dilution of rabbit polyclonal anti-GFP IgG antibody (Molecular Probes cat#A-11122)] in PBST + 5% HIGS. Sections were washed in PBST 5 times for 3 min and incubated with 1:750 dilution of goat anti-mouse IgG-Cy3 and 1:500 dilution of donkey anti-rabbit IgG-Alexa-488 (Molecular Probes; cat#A-21206) secondary antibodies in PBST + 5% HIGS for 2 h at room temperature. The sections were washed again 3 times for 3 min in PBST and mounted in FluorSave (CalBioChem) and 2% DABCO (Sigma), drying overnight at room temperature.

3. Results and discussion

3.1. Analysis of the human α′-PDE promoter in cultured cells

We chose to transfect human Y79 retinoblastoma cells for our initial transcriptional analysis, since this immortalized cell line normally expresses the cone α′-PDE gene and its functional protein [9,10]. In order to determine the region(s) of the 5′ flanking sequence regulating the human α′-PDE gene, we cloned a fragment containing 731 bp of the 5′ flanking region and 139 bp of the 5′untranslated region upstream of luciferase. We also made a series of 5′ deletions and numbered each of the constructs based on the transcription start site (+1). The −731/+139 construct consistently drove luciferase activity above the promoter-less control and to a substantial fraction of the SV40 viral promoter (51 ± 15%) in three separate experiments (data not shown). An experiment which compared the relative luciferase activity driven by all the α′-PDE deletion constructs is shown in Fig. 1A. There was no statistically significant change in activity when nucleotides −731 to −193 were deleted. All these constructs drove high luciferase expression, although not as strong as that obtained with the SV40 promoter (Fig. 1A), demonstrating that these fragments contain sequences required for transcription in Y79 cells. Deletion analysis showed that the highest reporter gene activity was observed with the −132/+139 bp construct. This implies that the sequence between −731 and −133 may contain transcriptional repressor binding sites. Repressor *cis*-acting elements have been reported in the 5′ flanking region (−807 to −176) of another cone photoreceptor-

specific gene, transducin α-subunit (GNAT2), after transfection of corresponding constructs into WERI-Rb1 retinoblastoma cells [18]. It is possible that transcriptional repression plays a role in regulating gene expression in cone photoreceptors.

Removal of an additional 22 bp from the α′-PDE −132/+139 construct reduced expression by ~68% (Fig. 1A: −110/+139), suggesting that a strong positive regulatory factor (or factors) binds to this region. The α′-PDE gene does not contain a TATA box [8] and thus transcriptional sequences may be found in the 5′-untranslated region. Interestingly, a construct in which the 5′ flanking region was removed (construct +1/+139 bp) still retained about 40 ± 9% of the activity of the full length fragment, statistically significant from the vector alone (7 ± 1%; *P*-value = 0.01). This suggests that the 5′ untranslated region may contribute to the transcriptional regulation of cone

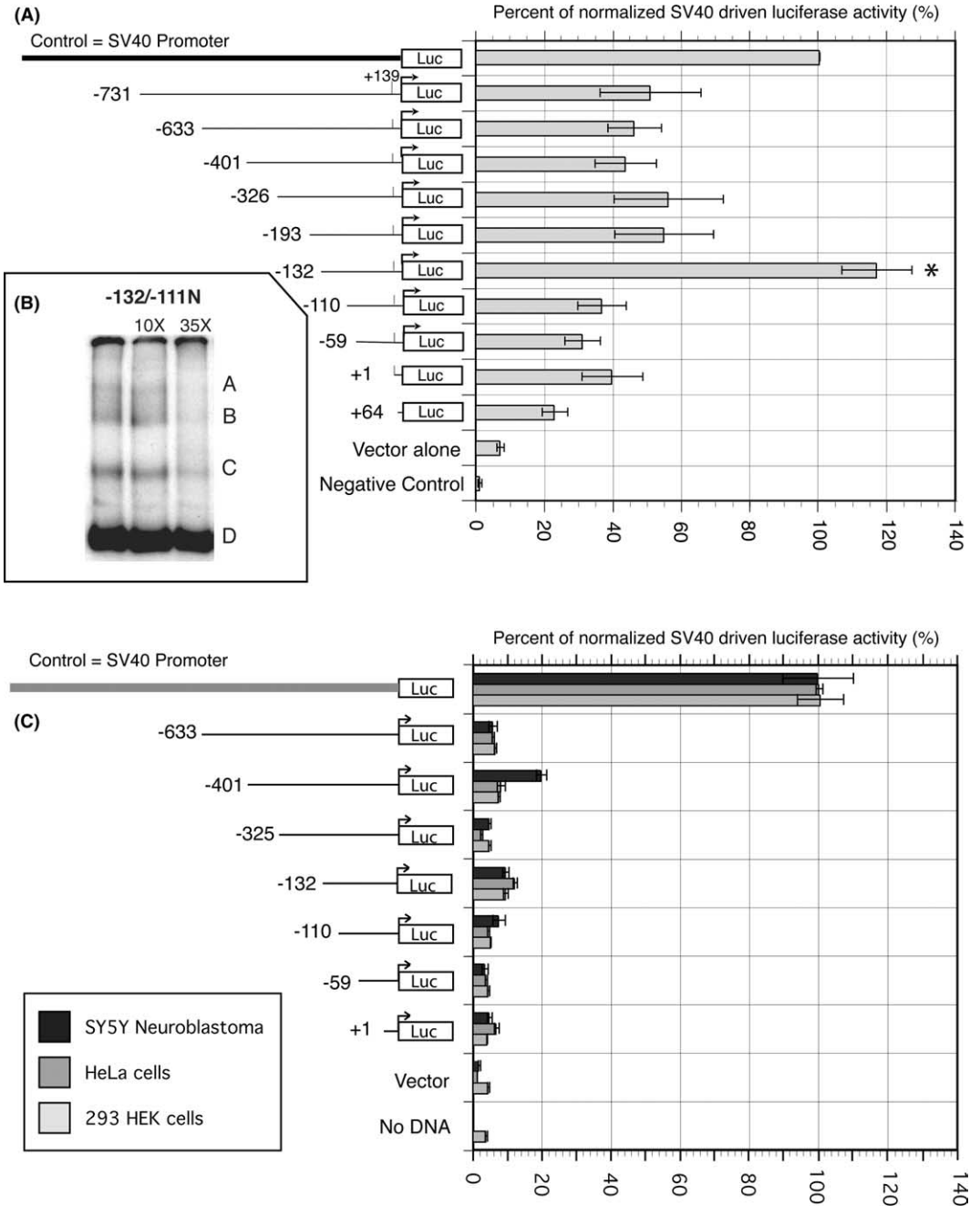

Fig. 1. Relative luciferase activity expressed by constructs containing different lengths of the human cone α′-PDE 5′ flanking region upon transient transfection into (A) human retinoblastoma Y79 cells, (C) human neuroblastoma (SY5Y), cervical cancer (HeLa) and 293 embryonic kidney (HEK) cells. On the left are schematic representations of the experimental constructs used. The SV40 promoter served as a positive control. The value bars graph the average percent relative luciferase activity of at least three separate transfection experiments (A) and one transfection experiment in each of the non-retinal cell lines (C) with error bars indicating standard error. Asterisk indicates construct that is statistically significant (*P*-value < 0.00001) within the experimental group. (B) Electrophoretic gel shift assay with Y79 cell nuclear extract and radiolabeled −132/−111 probe competed away with 10 times (lane 2) and 35 times (lane 3) unlabeled −132/−111 double stranded DNA.

α′-PDE, which has also been shown for a number of genes, including the α- and β-PDE genes [19].

To identify whether there were proteins in Y79 cell nuclear extracts that could bind the 22 bp region, we performed gel shift assays. Three protein–DNA complexes were observed (Fig. 1B) that were effectively competed with unlabeled probe. A much larger concentration of the cold oligonucleotide (100–500X) was necessary to compete with the protein–DNA complex D (data not shown). Sequence analysis of *cis*-acting elements within this 22 bp region using MatInspector (Matrix family library version 4.2; http//:www.genomatix.de/cgi-bin/matinspector/matinspector.pl) showed one potential binding site for an Otx family member (*core consensus binding site:* ATTA at −129 to −126). An additional Otx binding site was also found at −94 to −91. Both Otx family members, Otx2 and Crx, are expressed in the retina and are important in photoreceptor formation. Our results suggest a role for Crx in activating the α′-PDE promoter as we have shown previously that Crx can activate the −132/+139 construct in 293 human embryonic kidney cells [20].

The same constructs were also transiently transfected into a variety of non-retina-derived cells to test for retinal specificity (Fig. 1C). In each cell-line, the SV40 control construct drove luciferase expression to levels equal to those in Y79 cells. However, when neuroblastoma, HeLa and 293 human embryonic kidney cells were transfected with the human cone α′-PDE constructs, luciferase expression was similar to that produced by the vector alone – only the −401/+139 construct caused a small increase in luciferase activity in neuroblastoma cells, comparable to that driven by the +64/+139 construct in Y79 cells. The rest of the α′-PDE constructs drove luciferase expression at consistently low levels among the triplicate plates. Therefore, it appears that a short construct, −132/+139, is sufficient to support reporter gene expression in a retinal cell line but not in a neural, ovarian or kidney cell line. This suggests that the −132/+139 construct has the elements necessary for strong expression of cone α′-PDE and may include elements required for retina-specific expression.

Several other genes expressed in cone photoreceptor cells have also been tested in retinoblastoma cell lines. Cone photoreceptor-specific GNAT2 upstream sequence is able to drive reporter gene expression with equal intensity in WERI-Rb1 retinoblastoma cells and human 293 cells [18], while cone arrestin upstream sequences direct expression of a reporter gene in WERI-Rb1 and Y79 cells but have not been tested in other cell lines [21]. Human cone red and green visual pigment genes are expressed in WERI but not Y79 cells. In WERI cells, reporter gene expression can be activated 2–4 times more effectively using the green visual pigment regulatory region than the red [22]. Human interphotoreceptor retinoid binding protein (IRBP) is expressed in rods, cones and pinealocytes; its upstream sequence drives expression of a reporter gene in WERI-Rb1 but not in HeLa cells [23]. In order to test whether cone α′-PDE could direct retina-specific expression in vivo, we generated transgenic *X. laevis* with the −132/+139 construct.

3.2. *Human α′-PDE promoter drives retina-specific expression in transgenic Xenopus*

We produced transgenic *Xenopus* using the REMI approach with a DNA construct containing −132/+139 bp α′-PDE fused to GFP (α′-PDE prom → GFP). Under a fluorescent dissecting microscope, GFP was observed only in the eye beginning at stage 39–40 and fluorescence intensity increased slightly at later stages (Fig. 2A–F). We cannot exclude the possibility that there were very low levels of expression (below our detection level) in other areas of the tadpole. GFP expression level was

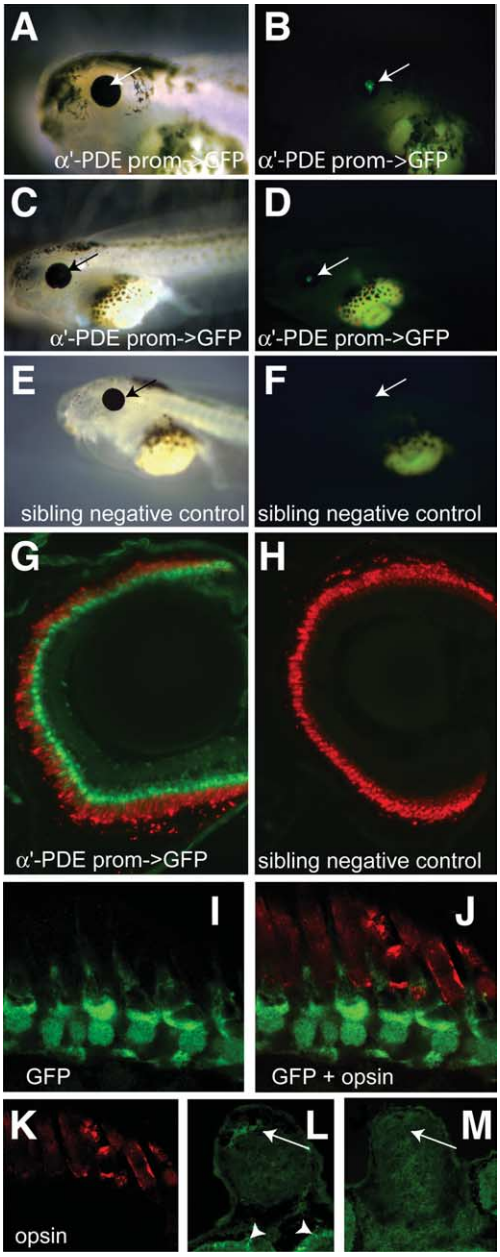

Fig. 2. The α′-PDE promoter directs expression in *Xenopus* cones. (A–F) *Xenopus* embryos (stage 43) carrying the −132/+139 α′-PDE + GFP transgene (A–D) express GFP in the eye (arrow) while a sibling, non-transgenic embryo (E, F) does not. Note the autofluorescence in the yolk of all embryos. (G–K) Retinal cryosections (12 μm) of one month old embryos stained with GFP and rhodopsin antibodies show that GFP-positive cells are only photoreceptors. (I–K) Confocal images of section in panel G at higher magnification. (K), staining of rod photoreceptors by antibodies against rhodopsin. (I) Staining of cone photoreceptors by anti-GFP antibodies. (J) Overlay of both the anti-rhodopsin and anti-GFP antibody stainings shows that GFP-positive cones are distinct from rhodopsin containing rods (K). (L, M) Cross-sections of transgenic positive (L) and negative (M) tadpole brain at stage 39, stained with GFP antibody. Arrow points to pineal in both sections, while arrowheads in L point to GFP-positive cone cells.

less than that observed using the rod-specific β-PDE promoter [24]. At approximately one month, expression was observed only in the outer portion of the retina (Fig. 2G). Anti-GFP staining was found in cone cells and was distinct from the anti-rhodopsin staining (Fig. 2I–K). Thus, a small fragment of the α′-PDE promoter (−132/+139) is capable of selectively directing gene expression to cone photoreceptors in transgenic *Xenopus*.

Interestingly, in some young embryos (stage 39/40), we did detect weak GFP expression in developing pinealocytes (Fig. 2L). Ectopic pineal expression has also been observed in transgenic frogs harboring the *Xenopus* rhodopsin promoter driving GFP, yet rhodopsin is undetectable in the pineal [25]. Further experiments will be required to interpret the pineal expression.

In other animals, we also observed expression of GFP in cells at the outer edge of the inner nuclear layer that appeared to be bipolar cells, with processes that spanned the INL from the pedicles of the photoreceptor layer to the tips of the ganglion cell dendrites (inlay Fig. 3C). A quantitative analysis was performed on a total of 21 tadpoles, 16 transgene positive (animals 1–16) and 5 negative (animals C1–C5, Fig. 3A). We counted the number of GFP-positive retinal cells within the space covered by 25–30 rhodopsin-positive cells in the center of a lens-containing retinal section (Fig. 3A). Animals not carrying the transgene did not express GFP (Fig. 2E–F, H; 3A and E). We found animals with only GFP-positive cones (e.g. Fig. 2). The retinas of these animals exhibited fairly uniform expression in every cone photoreceptor. A few animals exhibited mostly bipolar cell expression in a highly mosaic pattern (Fig. 3B and C). Most animals exhibited at least some cone expression. These results suggest that transgene insertion site may influence the precise expression pattern in individual transgenic animals.

Previous studies on mammalian retina have only detected α′-PDE expression in photoreceptors, although low-level expression in some bipolar cells cannot be excluded [6]. A search of the *Xenopus tropicalis* genome site (http://genome.jgi-psf.org/xenopus0/xenopus0.info.html, v.2.0) identified a putative gene with homology to the human α′-PDE (*data not shown*). However, antibodies to the mammalian α′-PDE did not cross-react with the *Xenopus* protein (*data not shown*). The possible expression of this gene in bipolar cells remains to be elucidated. Previous studies by others and us have suggested a relationship between cones and a subset of bipolar cells. For example, we have shown that *XOtx5b*, the *Xenopus* ortholog of mammalian *Crx*, is also expressed in photoreceptors and a subset of bipolar cells [26]. This also appears to be the case for zebrafish *Crx* [27]. Futhermore, transgenic mice carrying the blue cone opsin promoter expressed a reporter gene in both blue cones and bipolar cells [28,29]. Taken together, the results presented here suggest the possibility that cones and some bipolar cells may share a common regulatory mechanism that can activate the minimal α′-PDE (−132/+139) promoter. The native α′-PDE promoter may contain additional sequences that function as targets for repressor(s) to prevent expression outside of cones. Results from studies on the opsin promoter provide support for this type of mechanism. We found that a closely related family member, Otx2, which is expressed in bipolar cells, blocks the ability of Otx5b to bind to the opsin promoter and activate transcription [26]. It is possible that the α′-PDE upstream region may contain such *cis*-acting sequences.

The most significant finding of this report is the identification of a short 271 bp fragment of human DNA that can direct reporter gene expression to cone but not rod photoreceptors in *Xenopus* tadpoles. Previously, we had obtained similar results using the mouse cone arrestin promoter, but there was also expression in many other *Xenopus* tissues [30]. Mouse IRBP 5′

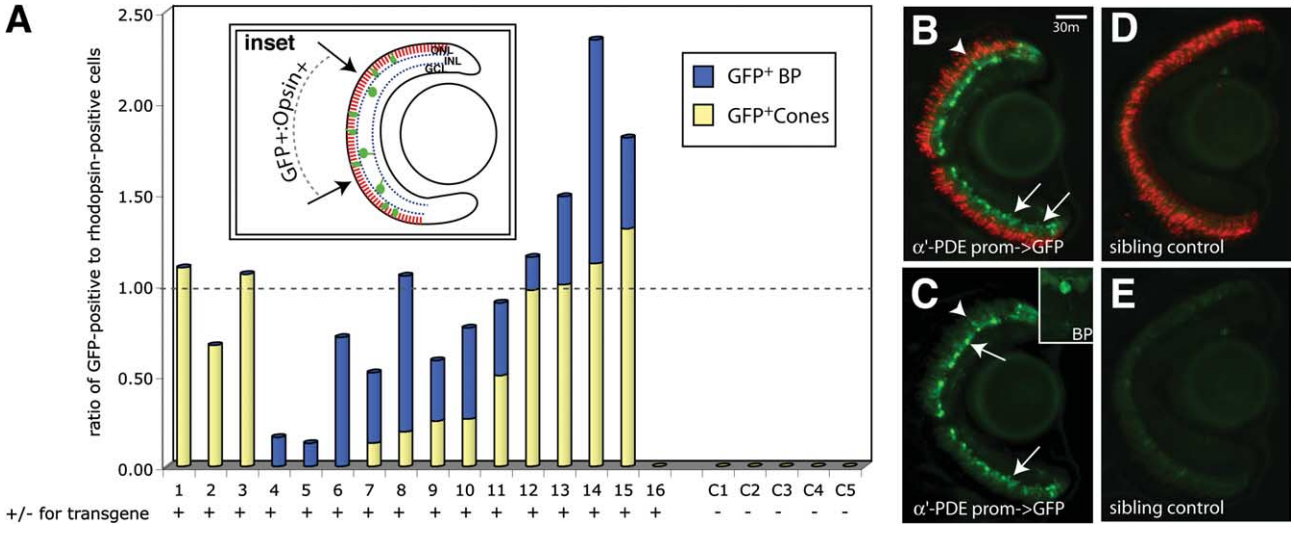

Fig. 3. Transgenic α′-PDE prom→GFP *X. laevis* embryos also express GFP in a subset of bipolar cells. (A) A graph representing the ratio of GFP-positive bipolar (BP) or cones cells to rhodopsin-positive rod cells. (A, inset) A schematic of a retinal section showing the region where cell counts were made. The number of rods and GFP-positive cone, BP were determined. Arrows point to the boundaries of the approximate region where cells were counted. (B) An example of a transgenic *X. laevis* (sample 6) animal that expresses GFP mostly in inner nuclear layer cells (arrows) and a few cones (arrowhead). (C) Inlay shows a GFP bipolar cell (BP) spanning the INL. (C and E) Rhodopsin-positive cells are red. (D, E) Sibling not carrying the transgene (negative control) does not express GFP. (F) Weak background auto-fluorescence can be seen in the sibling negative control. The bar in panel C represents 30 μm. Outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layers (GCL).

upstream regulatory sequences directed reporter gene expression in a more restrictive pattern in transgenic frogs, but both rods and cones expressed the transgene [31]. This is the first report of a mammalian cone promoter directing expression to frog cone photoreceptors. Our study emphasizes the importance of using transgenic frogs and highlights the value of the human α′-PDE regulatory region for studying cone transcriptional gene regulation.

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