A Novel, Evolutionarily Conserved Enhancer of Cone Photoreceptor-specific Expression

Vincent A. Smyth, David Di Lorenzo, and Breandán N. Kennedy

From the School of Biomolecular & Biomedical Science, UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

The α subunit of cone transducin (TαC) is expressed exclusively in cone photoreceptors of the eye and pineal. TαC is a key phototransduction protein, and inherited mutations in TαC cause total color blindness in humans. We use transgenic zebrafish to identify and characterize cone photoreceptor regulatory element 1 (CPRE-1) a novel 20-bp enhancer element in the TαC promoter (TαCP). CPRE-1 is located ~2.5 kb upstream of the translation start site and is necessary for strong cone photoreceptor-specific expression in vivo. CPRE-1 comprises of a modular arrangement of two 10-bp elements that have separate, but co-dependent transcriptional activities. In vitro, CPRE-1 specifically binds nuclear factors that are enriched in ocular tissue. Bioinformatic alignments reveal that CPRE-1 sites are evolutionarily conserved in the promoter regions of fish, rodent, and mammalian TαC orthologues and identify a 5′-CTGGAGTG(A/T)TGGA(G/A)G-GCAGGG(G/C)T-3′ consensus sequence.

The vertebrate retina contains distinct cone and rod photoreceptors that mediate scotopic and photopic vision, respectively (1). Although cones and rods originate from the same population of retinal progenitor cells, they have unique gene expression profiles that account for their differential cell fate, morphology, and signal transduction mechanisms. For example, many components of the G protein-coupled receptor phototransduction cascade are encoded by cone- or rod-specific genes (1). The molecular genetics underpinning cone photoreceptor-specific gene expression remain poorly defined.

Several transcription factors, including Crx, Nr2e3, Nrl, and Tβ2, regulate photoreceptor-specific gene expression. Cone rod homeobox is expressed in photoreceptors, and cone rod homeobox deletion leads to a developmental loss of both cone and rod photoreceptors in mice and zebrafish and to blindness (2–4). Nr2e3 and Nrl are expressed in rods where they repress expression of cone-specific genes (5–10). Mutations in Nr2e3 or Nrl lead to rods inappropriately expressing cone-specific markers and to the human retinal disease enhanced S-cone syndrome (5–12). Thyroid hormone receptor β2 is a nuclear receptor required for M-cone development (13, 14). Targeted deletion of thyroid hormone receptor β2 leads to a loss of M-cone function with an increase in functional S-cones (13, 14). Although these transcription factors are known to regulate photoreceptor genes, the molecular regulators of cone-specific gene expression are not well defined.

One method of deciphering the molecular mechanisms regulating cone-specific expression is to characterize the cis-elements in a cone-specific promoter. Several studies have identified large promoter regions sufficient to direct transgene expression in cones (15–23). However, many of these promoter regions have weak activity, exhibit ectopic, non-cone expression, or require a heterologous enhancer element (16–19, 21–23). With the exception of a cone rod homeobox-binding element, none of these studies have characterized individual cis-elements that direct cone-specific gene expression in vivo (16).

GNAT2 encodes the cone-specific α-subunit of cone transducin (TαC) that is expressed in all cone types and binds to activated cone opsin receptors (24). Missense mutations in the human TαC gene are linked with achromatopsia, a recessive disease leading to total loss of color vision (24–26). Similar mutations in the zebrafish TαC gene result in loss of visual behavior (27). The cone-specific expression of TαC makes its promoter an ideal candidate for identifying cis-elements controlling cone-specific expression. Previous studies determined that a 277-bp fragment of the human TαC promoter combined with a heterologous 214-bp interphotoreceptor retinoid binding protein enhancer element is sufficient to drive expression in murine cones (18). However, characterizing functional TαC cis-elements in mice is limited by the costs and technical expertise associated with transgenics and the limited number of cones in mice. The cost-effective and genetically tractable zebrafish is an attractive alternative model to characterize in vivo, cis-elements controlling cone-specific expression (28, 29).

Like humans, zebrafish have unique spectral cone subtypes with distinct cytology and synaptic connectivity that mediate color vision (28, 30). Zebrafish cones are divided into three morphological types; the UV-sensitive long single cones, the blue-sensitive short single cones, and the red-sensitive and green-sensitive double cones (30, 31). In vivo studies in...
zebrafish have identified large regulatory regions that direct cone photoreceptor gene expression (15, 19, 32). Transient transgenic studies identified a 105-bp cis-region of the zebrafish UV opsin promoter that can alter a rod-specific promoter to express in rod and UV cones (15). Recently a 500-bp region directing M-cone specific expression was identified that is sufficient to induce a non-retinal keratin 8 promoter to express in zebrafish M-cones (32). Previously we reported that a 3.2-kb promoter fragment of the zebrafish TαC gene is sufficient to drive strong EGFP expression in zebrafish cone photoreceptors and that the most distal 1.2-kb sequence contains an enhancer-like activity (19).

FIGURE 1. A 55-bp TαC promoter fragment enhances EGFP expression in cone photoreceptors. A, schematic of the promoter-reporter portion of the 5’ zebrafish TαCP deletion constructs and corresponding activity in vivo. The number of transgenic fish scored for each construct is indicated at the right of the histograms. Enhancer-1 activity localizes to a 55-bp region (highlighted in red) between −2521 and −2466 bp of TαCP. B, examples of the activity levels used to determine the activity of 5’ zebrafish TαCP deletion constructs. C–G, confocal micrographs of retinal sections from 5-dpf embryos injected with −2521 TαCP.

C–E, optical sections demonstrate that the EGFP-expressing cells generated co-localize with double cones (Zpr-1), UV (UV opsin), and blue cone (blue opsin) photoreceptors (highlighted by white arrows). F, EGFP-expressing cells do not co-localize with rod photoreceptors (rhodopsin). G, all EGFP-positive cells are situated in the outer nuclear layer (ONL). INL, inner nuclear layer; GCL, ganglion cell layer.
To identify this enhancer of cone-specific expression, we performed an in vivo analysis of the 3.2-kb zebrafish TaC promoter using deletion, mutant and chimeric reporter constructs, and in vitro characterization of binding to eye nuclear protein. Here we describe a novel, evolutionarily conserved 20-bp enhancer of the zebrafish TaC promoter that preferentially binds eye nuclear protein, is active upstream of a heterologous promoter, and is necessary for driving strong cone-specific expression.

**EXPERIMENTAL PROCEDURES**

**Generation of Reporter Constructs**—The −3173-bp TaCP-EGFP-1 plasmid based on the pEGFP-1 plasmid vector (Clontech) was used as the initial template for all constructs. The −3173(-397-bp ScaI-BmgBI fragment) and re-ligation. The 5ʹ deletions, internal mutants, −2510/−2501 “RR” repeat and “LR” inversion were generated by introducing PCR fragments into −3173 TaCP-EGFP-1 digested with PstI and EcoRI. The UV zebrafish opsin promoter (zUVOP) was PCR-amplified from a zebrafish genomic library clone (Stratagene) and inserted into the pEGFP-1 vector. Chimeric constructs were engineered by introducing PCR fragments upstream of the −897-bp zUVOP minimal promoter. The 3 and 6 copy repeats of “LR” were generated by cloning synthetic oligonucleotides upstream of the −897-bp zUVOP. A spacer sequence was introduced by PCR-amplifying the kanamycin cassette from the EGFP-1 vector and cloning between the −897-bp zUVOP and enhancer repeat sequence using SpeI restriction sites. The (−897-bp zUVOP)-EGFP-1 construct was generated by cloning the kanamycin cassette from the EGFP-1 vector and cloning between the −897-bp zUVOP and enhancer repeat sequence using SpeI restriction sites. The (−897-bp zUVOP)-EGFP-1 construct was generated by introducing PCR fragments upstream of the −897-bp zUVOP minimal promoter. The 3 and 6 copy repeats of “LR” were generated by cloning synthetic oligonucleotides upstream of the −897-bp zUVOP. A spacer sequence was introduced by PCR-amplifying the kanamycin cassette from the EGFP-1 vector and cloning between the −897-bp zUVOP and enhancer repeat sequence using SpeI restriction sites. The (−897-bp zUVOP)-EGFP-1 construct was generated by cloning the kanamycin cassette from the EGFP-1 vector and cloning between the −897-bp zUVOP and enhancer repeat sequence using SpeI restriction sites. The (−897-bp zUVOP)-EGFP-1 construct was generated by cloning the kanamycin cassette from the EGFP-1 vector and cloning between the −897-bp zUVOP and enhancer repeat sequence using SpeI restriction sites. The (−897-bp zUVOP)-EGFP-1 construct was generated by cloning the kanamycin cassette from the EGFP-1 vector and cloning between the −897-bp zUVOP and enhancer repeat sequence using SpeI restriction sites.

**TABLE 1**

| Number of EGFP+ cells from transient transgenic fish sections that label with rod or cone opsin antibodies |
| --- |
| Parentheses indicated number of EGFP+ cells and the number that labeled with cone opsin antibodies is highlighted in bold. |

|        | Zpr-1 | UV opsin | Blue opsin | Rhodopsin |
| --- | --- | --- | --- | --- |
| −3173 bp TaCP-EGFP-1 | 5 (22) | 5 (19) | 7 (20) | 0 (12) |
| −2521 bp TaCP-EGFP-1 | 4 (8) | 2 (9) | 2 (15) | 0 (18) |
| −2521/−890 bp TaCP/800 bp zUVOP-EGFP-1 | 0 (6) | 5 (5) | 0 (5) | 0 (6) |

**FIGURE 2.** Enhancer 1 preferentially binds eye nuclear protein. A, schematic of overlapping 42-bp probes within the 55-bp enhancer-1 sequence. B, electrophoretic mobility shift assay demonstrates that Probes 1 and 2 bind nuclear protein isolated from adult zebrafish eyes. C, electrophoretic mobility shift assay with probe 1 shows a larger quantity of radiolabeled probe 1 is bound by eye nuclear protein (ENP) than body nuclear extract (BNE) suggesting that binding of the trans-factor(s) is more highly expressed in ocular tissue. This interaction is specific, because unlabeled probe 1 at 50- or 100- fold molar excess is able to compete away binding of eye nuclear protein to labeled probe 1, whereas a non-related cold competitor for clusterin at 50- or 100-fold molar excess is not. Eye nuclear protein (ENP), body nuclear protein (BNE), cold competitor (CC).

---

**Enhancer of Cone-specific Expression**

APRIL 18, 2008 • VOLUME 283 • NUMBER 16 • JOURNAL OF BIOLOGICAL CHEMISTRY
Red (Sigma) at a final concentration of 25 ng/µl. The pressure injector was regulated so that ~300 pl (~7.5 pg) of each reporter construct was injected per embryo. The covalently closed plasmids were injected into the cytoplasm of 1–2 cell stage zebrafish embryos using an air pressure-regulated Pico Pump (World Precision Instruments) attached to a Narishige micromanipulator and needle holder. Injected embryos were transferred to water containing 0.003% 1-phenyl-2-thiourea 24 h post fertilization to inhibit melanin production. Animal studies were sanctioned by the UCD Animal Research Ethics Committee (Ref: P04-18) and licensed by the Department of Health and Children, Rep. of Ireland (License Ref: B100/3358).

Screening for EGFP Expression—Injected embryos were anesthetized and analyzed by fluorescence microscopy using a Zeiss Axioplan 2 fluorescence microscope at 5 days post fertilization (dpf). Construct activity was scored on the basis of a semiquantitative assay, where one of three ordinal activity levels was assigned to an embryo based on the number of fluorescent cells within the eye. Embryos with >50 GFP + cells in the eye were assigned an activity level of +++++, fish with 5–50 GFP + cells were assigned as ++++, 1–5 fluorescent cells as ++, and fish with no GFP + cells were assigned an activity level of − (15). The sum of each ordinal level was divided by the total number of embryos to give relative activity. Statistical analysis was performed using Student’s t tests, and p values of ≤0.05 were considered significant.

Immunostaining—Whole mount immunostaining was performed on 5 dpf larvae as described previously (33) and analyzed by confocal microscopy (LSM510, Zeiss). For retinal sections, 5-dpf transgenic larvae were fixed overnight at 4 °C in 4% paraformaldehyde/5% sucrose/0.1 M phosphate buffer (pH 7.4). Following fixation fish were cryoprotected in 20% sucrose/0.1 M phosphate buffer (pH 7.4) then infiltrated with OCT (Tissue-Tek) as previously described (33). 12-µm sections were thaw-mounted to Superfrost slides (BDH Chemical Ltd.). Sections were rehydrated in TBS for 30 min, and then incubated for 60 min in blocking buffer (5% normal goat serum/1% bovine serum albumin/1% Triton X-100/TBS). Sections were incubated overnight with primary antibodies diluted in blocking buffer at the following ratios: zpr-1 (1:200, Oregon Monoclonal Bank), UV opsins (1:400), Blue opsin (1:400), and rod opsin (1:200), cone and rod opsin antibodies were generously donated by Profs. D. Hyde and T. Vih贴ic (34). Following three 5-min washes in TBS/0.1% Tween, the sections were incubated at room temperature for 1 h in secondary antibodies diluted 1:200 in blocking buffer. Prior to mounting in Anti-Fade (Molecular Probes), the sections were washed three times for 5 min in TBS. Retinal sections were analyzed by confocal microscopy (LSM510, Zeiss).

Nuclear Protein Preparation and Electrophoretic Mobility Shift Assay—Nuclear protein extracts were prepared from adult zebrafish eyes. Adult zebrafish eyes were dissected and homogenized in 4 ml of homogenization buffer (50 mM Hepes/pH 7.9, 25 mM KCl/5 mM MgCl2/200 mM sucrose/0.05 mM dithiothreitol/0.25 mM phenylmethylsulfonyl fluoride (Sigma)), centrifuged at 2000 × g for 10 min at 4 °C. The pellet was re-suspended in 600 µl of Chelser buffer (10 mM Tris, pH 7.0/10 mM NaCl/3 mM MgCl2/30 mM sucrose/1% Nonidet P-40), incubated on ice for 10 min, and centrifuged at 3000 × g for 10 min at 4 °C. Pellets were re-suspended in 600 µl of Chelser buffer plus 10 mM CaCl2, layered on sucrose cushion (50 mM Hepes, pH 7.9/25 mM KCl/5 mM MgCl2/876 mM sucrose/0.2 mM EDTA/0.5 mM dithiothreitol/0.25 mM phenylmethylsulfonyl fluoride), and centrifuged at 3000 × g for 10 min at 4 °C. Nuclei were re-suspended in Chelser buffer c-low salt (20% glycerol/20 mM Tris-CI pH 7.9/25 mM KCl/0.2 mM EDTA/0.5 mM dithiothreitol/0.25 mM phenylmethylsulfonyl fluoride), lysed by the dropwise addition of a high salt Chelser buffer c-low salt plus 1.2 mM KCl, and homogenized. Samples were incubated on ice for 30 min and centrifuged at 13,000–25,000 × g for 10 min at 4 °C. Supernatant was removed and concentrated by ultra centrifugal filter devices (Amicon ultra 4). Protein content was determined by using the BCA Protein Assay Reagent (Pierce). Oligonucleotide probes (supplemental Table S1) were synthesized with 5’ overhangs and radioactively labeled with [α-32P]dCTP using Klenow Exo- (New England Biolabs). Nuclear extracts were incubated with a 200-fold molar excess of poly(dI-dC) at 4 °C for 10 min. Labeled probe was added, and the mixture was incubated at room temperature for 30 min. Reactions were stopped and loaded on 5% non-denaturing polyacrylamide gels, followed by autoradiography. For the competition assay, cold competitor was added prior to addition of labeled probe, in 50-, 100-, and 200-fold molar excess.

Sequence Analysis—The 10-kb 5’ promoter sequence of GNA72 genes were obtained from ENSEMBL and aligned with the 20-bp CPRE-1 sequence using ClustalW (35). Only sequences with a pairwise alignment score greater than 0.61 were considered significant. The consensus CPRE-1 sequences was generated using the online Weblogo server (36).

RESULTS

A 55-bp Distal Promoter Region Is Necessary for Strong EGF Expression in Cone Photoreceptors—In transient transgenic assays, the ~3173-bp fragment of the zebrafish Toc promoter (ToCP) region drives robust EGFP expression specifically in retinal cone photoreceptors and in the pineal (Fig. 1, data not shown). The expression pattern is identical to that observed in Red.
Enhancer of Cone-specific Expression

the Tg(3.2TaCP-EGFP-1) transgenic line generated using the same promoter fragment (19). To identify cis-elements controlling this cone photoreceptor-specific expression, we tested the reporter activity of a series of 5’ deletions of the −3173-bp TaCP in vivo.

Deletion of the region between −3173 and −2749-bp of zebrafish TaCP results in a significant reduction of reporter activity, revealing a potential enhancer element, designated “enhancer region 2,” within this 424-bp region (Fig. 1A). However, deletion of the 283-bp region between −2749 and −2466-bp of the TaCP, designated “enhancer region 1,” practically abolishes EGFP expression (Fig. 1A). An internal deletion of enhancer region 1, within the −3173 bp TaCP construct, results in minimal reporter activity (Fig. 1A). These results suggest that enhancer region 1 is necessary for high level cone-specific expression and that enhancer region 2 is not sufficient to compensate for loss of enhancer region 1.

We focused on refining the enhancer element (enhancer-1) in enhancer region 1. Analyses of additional 5’ deletions initially narrowed enhancer-1 to the 55-bp region, between −2521 and −2466 bp of TaCP (Fig. 1A). The −2521-bp TaCP construct results in robust expression, with >34% of injected embryos expressing EGFP in the retina. The −2521-bp TaCP-EGFP construct confines transgene expression to all cone photoreceptor types based on expression in the outer nuclear layer and co-immunolocalization of EGFP with markers for double, short single, and long single cones, but not with a rod photoreceptor marker (Fig. 1, C–F, and Table 1). Deletion of the 55 bp between −2521 and −2466 bp of TaCP results in a significant loss of EGFP expression, with <10% of injected embryos expressing EGFP in only one to five cells per eye (Fig. 1A).

Eye Nuclear Protein Binds Specifically to Enhancer-1—The ability of enhancer-1 to bind eye nuclear protein was evaluated by testing three overlapping oligonucleotide probes that span the 55-bp sequence of −2521 to −2466 bp of TaCP (Fig. 2A). Probe 1 and Probe 2 form distinct complexes with eye nuclear protein, but Probe 1 became the focus of our studies as it generates a more intense complex (Fig. 2B).

The specificity of eye nuclear protein binding to Probe 1 was analyzed. Addition of Probe 1 as a cold competitor at 50 and 100 nM excess demonstrates a specific ability of unlabeled Probe 1 to compete for nuclear proteins binding to Probe 1 in a concentration-dependent manner (Fig. 2C). An unrelated competitor is unable to compete away binding of nuclear protein to Probe 1 (Fig. 2C). In addition, non-retinal nuclear protein isolated from zebrafish body is capable of binding to Probe 1, although at a much reduced intensity compared with eye nuclear protein (Fig. 2C). This suggests an enrichment of trans-factors in the eye that specifically bind to enhancer-1.

CPRE-1 Comprises Two 10-bp Functional cis-Elements—To narrow the minimal sequence of enhancer-1, three mutant probes containing sequential 10-bp mutations were analyzed by electrophoretic mobility shift assay (Fig. 3A). The three mutant probes fail to form a complex with eye nuclear protein (Fig. 3B). However, when used as cold competitors, Probe 1M R is unable to compete, Probe 1M L partially competes, and Probe 1M D efficiently competes away binding of eye nuclear protein to wild-type Probe 1 (Fig. 3B). Thus, the 20 bp spanning Probes 1M L and 1M R, designated “cone photoreceptor regulatory element 1” (CPRE-1) appear essential for trans-factor(s) binding to enhancer-1 (Fig. 3A). To test the significance of CPRE-1 for transcriptional activity in vivo, constructs containing complete or partial deletions of CPRE-1 were tested (Fig. 3C). The −2501-bp TaCP-EGFP construct had limited activity compared with the −2521 bp TaCP-EGFP construct demonstrating that CPRE-1 is necessary for robust cone-specific expression (Fig. 3C). 5’ deletion of the sequence from −2521 and −2510 bp of TaCP resulted in a significant loss of EGFP expression, and deletion of the sequence from −2510 and −2501 bp of TaCP results in a further statistically significant loss of EGFP expression (Fig. 3, C and D). The in vitro and in vivo assays suggest that CPRE-1 comprises two separate cis-elements, “R” located between −2521 and −2510 bp of TaCP and “L” located between −2510 and −2501 bp of TaCP. To identify the functional importance of the L and R cis-elements, they were mutated independently in the −2521-bp background and tested in vivo. Mutation of either L or R results in a significant loss of transcriptional activity compared with the −2521-bp TaCP construct, with mutation of R having a more pronounced affect (Fig. 3, C and D). However, duplication of R is insufficient to recapitulate the transgene expression levels of the L plus R arrangement, demonstrating a co-dependence of the modules for enhanced transcriptional activation (Fig. 3, C and D). The ability of CPRE-1 to increase transcription appears orientation-dependent as inverting and reversing the L and R modules results in a significant reduction in transgene expression, compared with their wild-type orientation (Fig. 3, C and D).

Identification of L and R Sequences Essential for trans-Factor(s) Binding—To identify the base pairs necessary for binding of trans-factor(s) to CPRE-1, the ability of L and R probes, with consecutive 3-bp mutations, to bind eye nuclear protein was tested (Fig. 4, A and B). The 6-bp sequence from −2518 to −2512 bp (5’-GAGGTG) of L is essential for binding, because mutation of these residues in Probes 1M L.2 and 1M L.3 abolishes the ability to form a complex with eye nuclear protein and to compete with binding to wild-type Probe 1 (Fig. 4C and Table 2). For R, only the 3-bp sequence from −2507 to −2504 bp (5’-GGGC), mutated in Probe 1M R.2, is essential for binding (Fig. 4D and Table 2). The sequences mutated in Probes 1M L.4 and 1M D.1, although not essential for binding, result in less intense complexes suggesting a role in stabilizing the complex. A significant role for the thymidine nucleotide at position −2511 bp is demonstrated as Probe 1M L.4 forms complexes much less efficiently than overlapping Probe 1M R.1, and this nucleotide is the distinguishing mutation (Fig. 4, C and D, and Table 2).

CPRE-1 Is Necessary but Not Sufficient to Drive Expression of a Heterologous Promoter in Vivo—The ability of CPRE-1 to drive the expression of a heterologous zebrafish UV opsin promoter (zUVOP) was characterized (Fig. 5A). We identified a −800-bp fragment of the zebrafish UV opsin proximal promoter region that directs low but specific EGFP expression in UV cones (Fig. 5A, data not shown). Chimeric constructs comprising of the heterologous UV opsin promoter with either the −3173/−890-bp or −2521/−890-bp regions of the distal TaCP upstream of the UV opsin promoter are sufficient to
enhance levels of EGFP expression (Fig. 5, A and B–F). Although the −2521/−890-bp TaCP fragment originates from a promoter expressing in all cones, in the chimera it is not sufficient to override the UV cone specificity of the heterologous promoter nor to direct expression of the ubiquitous SV40 minimal promoter to cone photoreceptors (Fig. 5, A and B). However, when CPRE-1 is removed (−2501/−890 bp) from the chimeric construct a significant loss in transgene expression was observed, confirming that CPRE-1 is necessary to drive expression from the heterologous zUVOP promoter (Fig. 5A).

CPRE-1 is not sufficient to enhance expression from the heterologous promoter, because single or multiple copies cloned upstream of the minimal UV opsin promoter do not increase expression from the minimal UV opsin promoter (Fig. 5A). To determine if this insufficiency relates to spatial context, six repeats of the 20-bp “LR” CPRE-1 sequence were placed 1721 bp upstream of the UV opsin promoter by introducing an unrelated spacer sequence. No increase in expression from the heterologous promoter was observed (Fig. 5A), suggesting that the LR CPRE-1 sequence requires interaction with specific cis-ele-
Enhancer of Cone-specific Expression

A. schematic of the promoter-reporter portion of chimeric constructs containing CPRE-1 upstream of an 800-bp minimal zebrafish UV opsin promoter (zUVOP). Blue rectangles represent the zUVOP 800-bp proximal promoter sequence, green rectangles represent EGFP coding sequences, orange rectangles represent TαC distal sequence, and the 20-bp CPRE-1 coding sequence is represented by a red rectangle. A chimeric construct containing CPRE-1 in the distal promoter (−2521/−890 TαC) significantly increases expression from the minimal zUV opsin heterologous promoter. Deletion of CPRE-1 from (−2501/−890 TαC) results in significant loss of EGFP expression. Multiple copies of the 20-bp CPRE-1 are not sufficient to enhance expression of the heterologous promoter. Chimeric constructs containing the distal zebrafish TαC (zTαC) fragment with CPRE-1 upstream of the human TαC (hTαC) or the minimal SV 40 promoter are inactive.

B–F, confocal micrographs of retinal sections from 5-dpf embryos injected with (−2521/−890 bp TαC)- (−800 bp zUVOP)-EGFP-1. Single optical section (B) and stacked z-series (C and E), Z-series projection (D) demonstrating the EGFP-expressing cells co-localize with UV cones (white arrow) and not with double cones (Zpr1), blue cones (blue opsin), or rods (rhodopsin). All EGFP cells are situated in the outer nuclear layer. ONL, outer nuclear layer; INL, inner nuclear layer; and GCL, ganglion cell layer.

FIGURE 5. CPRE-1 is necessary but not sufficient to drive expression of a heterologous promoter. A, schematic of the promoter-reporter portion of chimeric constructs containing CPRE-1 upstream of an 800-bp minimal zebrafish UV opsin promoter (zUVOP). Blue rectangles represent the zUVOP 800-bp proximal promoter sequence, green rectangles represent EGFP coding sequences, orange rectangles represent TαC distal sequence, and the 20-bp CPRE-1 coding sequence is represented by a red rectangle. A chimeric construct containing CPRE-1 in the distal promoter (−2521/−890 TαC) significantly increases expression from the minimal zUV opsin heterologous promoter. Deletion of CPRE-1 from (−2501/−890 TαC) results in significant loss of EGFP expression. Multiple copies of the 20-bp CPRE-1 are not sufficient to enhance expression of the heterologous promoter. Chimeric constructs containing the distal zebrafish TαC (zTαC) fragment with CPRE-1 upstream of the human TαC (hTαC) or the minimal SV 40 promoter are inactive.

B–F, confocal micrographs of retinal sections from 5-dpf embryos injected with (−2521/−890 bp TαC)- (−800 bp zUVOP)-EGFP-1. Single optical section (B) and stacked z-series (C and E), Z-series projection (D) demonstrating the EGFP-expressing cells co-localize with UV cones (white arrow) and not with double cones (Zpr1), blue cones (blue opsin), or rods (rhodopsin). All EGFP cells are situated in the outer nuclear layer. ONL, outer nuclear layer; INL, inner nuclear layer; and GCL, ganglion cell layer.

Thus, CPRE-1 is necessary, in the context of the −2521/−890 distal zebrafish TαC-UV opsin chimeric construct, to enhance transgene expression from a heterologous promoter. However, it is not sufficient to enhance transcription from the heterologous promoter nor to overwrite the UV cone specificity of the minimal promoter by itself.

CPRE-1 Is Conserved in TαC Orthologues—Alignment of the CPRE-1 sequence with orthologous TαC promoters reveals a conserved copy within divergent vertebrate species (Fig. 6A). The location of the CPRE-1 site is also conserved among more closely related species, e.g. primates and rodents. Bioinformatics reveal a CPRE-1 consensus sequence of 5′-CTGGAGTG(A/
T)TGGA(G/A)GCAGGG(G/C)T-3'
/H11032
(Fig. 6B), which does not match any known transcription factor site in the Transfac data base (37). We searched for CPRE-1 consensus sites in the promoter regions of other zebrafish cone-specific (arr3, cnga3, gngt2, pde6C, and pde6h), rod-specific (rho, pde6a, and gnat1), and housekeeping genes (tubulin and β-actin). A significantly matching consensus site was identified in the cone-specific arrestin (arr3) and cone cyclic-gated nucleotide channel α3 subunit (cnga3) genes. Thus, CPRE-1 is an evolutionarily conserved enhancer element in a cohort of cone-specific genes.

**DISCUSSION**

Cone photoreceptors are highly specialized sensory neurons that enable color vision and visual acuity. Severe forms of human blindness associated with cone defects include rare genetic diseases, e.g. achromatopsia, and prevalent complex diseases, e.g. age-related macular degeneration. To develop our understanding of the molecular genetics of cones we focused on identifying cis-elements in the zebrafish T/H9251C promoter region. This study identifies and characterizes cone photoreceptor regulatory element-1 (CPRE-1) as a 20-bp enhancer of cone-specific expression. CPRE-1 is located in the distal zebrafish T/H9251C promoter region, ~2.5 kb upstream of the coding sequence. CPRE-1 is necessary for robust, cone-specific expression, and regulates expression in all cone photoreceptor types. CPRE-1 comprises a modular arrangement of two 10-bp cis-elements. These L and R elements have separate but co-dependent transcriptional activities that are required to drive high levels of cone-specific expression. The activity of CPRE-1 appears orientation-dependent as inversion and reversion of LR result in significantly less activity than the wild-type orientation. CPRE-1 is not sufficient to drive expression from heterologous promoters. However,
Enhancer of Cone-specific Expression

a larger distal fragment of the zebrafish ToCP is sufficient to enhance expression from a heterologous UV cone-specific promoter, and CPRE-1 is required for this enhanced activity. Notably, CPRE-1 does not override the UV specificity of the heterologous promoter. Thus, CPRE-1 is an enhancer module that increases the rate of transcriptional activity, but not the tissue specificity, of cone-specific promoters in vivo.

Enhancement of transcriptional activity by CPRE-1 may result from direct binding of transcriptional activators on site or by modification of the chromatin architecture to enable access of transcription factors to the promoter region. However, in the transient transgenesis assay, the reporter constructs are microinjected as closed-circular DNA, which does not integrate into the zebrafish genome, but remains as episomal DNA (38). Chromatin assembly on episomal DNA is a “time-dependent” process with the majority of episomal DNA not bound by chromatin until 5 to 10-fold less episomal DNA bound by chromatin than at 5 weeks (39). Because our reporter constructs are assayed for activity at 5 days post microinjection, it is unlikely that regulation of chromatin accessibility is a significant determinant of reporter activity. Thus, we conclude that CPRE-1 most likely enhances transcriptional activity by assembly of transcriptional activators.

Bioinformatic analyses suggest that CPRE-1 sequences are evolutionarily conserved in the promoter regions of ToC orthologues from zebrafish to humans and identify a novel consensus sequence of 5′-CTGGAGTG(A/T)TGGA(G/A)GCAGGG(G/C)T-3′. In vitro experiments confirm that DNA probes encompassing the CPRE-1 element preferentially bind eye nuclear protein in a dose-dependent manner, and CPRE-1 is required for these interactions. Within the CPRE-1 of zebrafish ToCP we identify nine central nucleotides essential for binding eye nuclear factors (−2518/−2512 and −2507/−2504 bp), and four nucleotides regulating the efficacy of binding (−2511 and −2501/−2499 bp). These sites include four of the eight most conserved bases identified in the CPRE-1 consensus sequence.

This study advances our understanding of the transcriptional regulation of the cone-specific GNA72 gene family. Previous studies of the human promotor reported several silencer sequences that bind nuclear protein in vitro, but their functional importance in vivo has not been reported (40). A transgenic line consisting of a 277-bp proximal region of the human ToC promoter directs expression in murine cones when a heterologous enhancer from the human interphotoreceptor retinoid-binding protein gene is placed upstream (18), but a native human ToC enhancer has not been identified. Chimeric reporter constructs with a distal fragment of the zebrafish ToCP promoter region containing CPRE-1 cannot regulate a proximal human ToC promoter, and vice versa, suggesting species-specific differences in promoter organization or transcription factor binding.

Our proposed model of transcriptional regulation of the zebrafish ToC gene is depicted in Fig. 7. The proximal 0.7 kb of promoter has weak transcriptional activity but controls the “all” cone specificity of expression (19). In the distal promoter, enhancer activities are located between −3.2/−2.8 kb (enhancer region 2) and −2.8/−2.5 kb (enhancer region 1). A novel 20-bp enhancer, CPRE-1 is located within enhancer region 1. CPRE-1 comprises of separate but co-dependent L and R modules necessary for robust, cone-specific expression. CPRE-1 enhances the rate of transcriptional activity, but not the tissue specificity. The region from −2.5/−0.9 kb, bridging the tissue specificity and enhancer domains, is essential for the enhancer activity of CPRE-1. This dependence is not a trivial spatial dependence, because neither a generic spacer nor an equivalent region of the human ToC distal promotor can substitute in chimeric reporter assays. We speculate that transcription factors binding to these regulatory DNA regions are brought into contact in a complex resulting in high level cone-specific expression (Fig. 7B). Our future studies are focused on identifying these factors and characterizing their expression and function.

Acknowledgments—We thank Prof. Tom Vihtelic and David Hyde for the generous gift of zebrafish opsin antibodies; Prof. Finian Martin, Dr. Yolanda Alvarez, Dr. Victor Vendrell, Maria Morrissey, Sarah Mc Loughlin, and Theresa Heffernan for helpful technical assistance and comments on the manuscript; and Beata Sapetto- Rebob for management of our zebrafish facility.

REFERENCES

1. Forrester, J. V., Dick, A. D., McMenamin, P. G., and Lee, W. R. (2002) The Eye: Basic Sciences in Practice, Elsevier Health Sciences, New York
2. Kimble, T. D., and Williams, R. W. (2000) Anat. Embryol. (Berl.) 201, 305–316
3. Silva, E., Yang, J. M., Li, Y., Dharmaraj, S., Sundin, O. H., and Maumenee, I. H. (2000) Invest. Ophthalmol. Vis. Sci. 41, 2076–2079
4. Furukawa, T., Morrow, E. M., and Cepko, C. L. (1997) Cell 91, 531–541
5. Cheng, H., Khanna, H., Oh, E. C., Hicks, D., Mitton, K. P., and Swaroop, A. (2004) Hum. Mol. Genet. 13, 1563–1575
6. Chen, J., Rattner, A., and Nathans, J. (2005) J. Neurosci. 25, 118–129
7. Peng, G. H., Ahmad, O., Ahmad, F., Liu, J., and Chen, S. (2005) Hum. Mol. Genet. 14, 747–764
8. Mears, A. J., Kondo, M., Swain, P. K., Takada, Y., Bush, R. A., Saunders, T. L., Sieving, P. A., and Swaroop, A. (2001) Nat. Genet. 29, 447–452
9. Nikonor, S. S., Daniele, L. L., Zhu, X., Craft, C. M., Swaroop, A., and Pugh, E. N., Jr. (2005) J. Gen. Physiol. 125, 287–304
10. Oh, E. C., Khan, N., Novelli, E., Khanna, H., Strettoi, E., and Swaroop, A. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 1679–1684
11. Haider, N. B., Jacobson, S. G., Cideciyan, A. V., Swiderski, R., Streb, I. M., Searby, C., Beck, G., Hockey, R., Hanna, D. B., Gorman, S., Duhl, D., Carmi, R., Bennett, J., Weleber, R. G., Fishman, G. A., Wright, A. F., Stone, E. M., and Sheffield, V. C. (2000) Nat. Genet. 24, 127–131
12. Haider, N. B., Demarco, P., Nyustuen, A. M., Huang, X., Smith, R. S., McCall, M. A., Naggett, J. K., and Nishina, P. M. (2006) Vis. Neurosci. 23, 917–929
13. Ng, L., Hurley, J. B., Dierks, B., Srinivas, M., Salto, C., Vennstrom, B., Reb, T. A., and Forrest, D. (2001) Nat. Genet. 27, 94–98
14. Roberts, M. R., Srinivas, M., Forrest, D., Murrel de Escobar, G., and Reb, T. A. (2006) in Proc. Natl. Acad. Sci. U. S. A. 103, 6218–6223
15. Luo, W., Williams, I., Smallwood, P. M., Touchman, J. W., Roman, L. M., and Nathans, J. (2004) J. Biol. Chem. 279, 19286–19293
16. Pickrell, S. W., Zhu, X., Wang, X., and Craft, C. M. (2004) Invest. Ophthalmol. Vis. Sci. 45, 3877–3884
17. Glushakova, L. G., Timmers, A. M., Pang, J., Teusner, J. T., and Hauswirth, W. W. (2006) Invest. Ophthalmol. Vis. Sci. 47, 3505–3513
18. Ying, S., Fong, S. L., Fong, W. B., Kao, C. W., Converse, R. L., and Kao, W. W. (1998) Curr. Eye Res. 17, 777–782
19. Kennedy, B. N., Alvarez, Y., Brockerhoff, S. E., Stearns, G. W., Sapetto-Reboc, B., Taylor, M. R., and Hurley, J. B. (2007) Invest. Ophthalmol. Vis.
Enhancer of Cone-specific Expression

20. Gouras, P., Kjeldbye, H., and Zack, D. J. (1994) Vis. Neurosci. 11, 1227–1231
21. Chen, J., Tucker, C. L., Woodford, B., Szel, A., Lem, J., Gianella-Borradori, A., Simon, M. I., and Bogennmann, E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2611–2615
22. Boatright, J. H., Borst, D. E., Peoples, J. W., Bruno, J., Edwards, C. L., Si, J. S., and Nickerson, J. M. (1997) Mol. Vis. 3, 15
23. Babu, S., McIlvain, V., Whitaker, S. L., and Knox, B. E. (2006) FEBS Lett. 580, 1479–1484
24. Kohl, S., Baumann, B., Rosenberg, T., Kellner, U., Lorenz, B., Vadala, M., Jacobson, S. G., and Wissinger, B. (2002) Am. J. Hum. Genet. 71, 422–425
25. Weinstein, L. S., Chen, M., Xie, T., and Liu, J. (2006) Trends Pharmacol. Sci. 27, 260–266
26. Michaelides, M., Aligianis, I. A., Holder, G. E., Simunovic, M., Mollon, J. D., Maher, E. R., Hunt, D. M., and Moore, A. T. (2003) Br. J. Ophthalmol. 87, 1317–1320
27. Brockerhoff, S. E., Rieke, F., Matthews, H. R., Taylor, M. R., Kennedy, B., Ankoudinova, I., Niemi, G. A., Tucker, C. L., Xiao, M., Cilliuffo, M. C., Fain, G. L., and Hurley, I. B. (2003) J. Neurosci. 23, 470–480
28. Goldsmith, P., and Harris, W. A. (2003) Semin. Cell Dev. Biol. 14, 11–18
29. Collery, R. F., Cederlund, M. L., Smyth, V. A., and Kennedy, B. N. (2006) Adv. Exp. Med. Biol. 572, 201–207
30. Raymond, P. A., and Barthel, L. K. (2004) Int. J. Dev. Biol. 48, 935–945
31. Branchek, T., and Bremiller, R. (1984) J. Comp. Neurol. 224, 107–115
32. Tsujimura, T., Chinen, A., and Kawamura, S. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 12813–12818
33. Barthel, L. K., and Raymond, P. A. (1990) J. Histochem. Cytochem. 38, 1383–1388
34. Vihtelic, T. S., Doro, C. J., and Hyde, D. R. (1999) Vis. Neurosci. 16, 571–585
35. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, I. D., Gibson, T. J., and Higgins, D. G. (2007) Bioinformatics 23, 2947–2948
36. Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004) Genome Res. 14, 1188–1190
37. Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A. E., Kel, O. V., Ignatieva, E. V., Ananko, E. A., Podkolodnaya, O. A., Kolpakov, F. A., Podkolodny, N. L., and Kolchanov, N. A. (1998) Nucleic Acids Res. 26, 362–367
38. Chen, Z. Y., Yant, S. R., He, C. Y., Meuse, L., Shen, S., and Kay, M. A. (2001) Mol. Ther. 3, 403–410
39. Riu, E., Chen, Z. Y., Xu, H., He, C. Y., and Kay, M. A. (2007) Mol. Ther. 15, 1348–1355
40. Morris, T. A., Fong, W. B., Ward, M. J., Hu, H., and Fong, S. L. (1997) Invest. Ophthalmol. Vis. Sci. 38, 196–206