Transcriptional Regulation of Cellular Retinaldehyde-binding Protein in the Retinal Pigment Epithelium

A ROLE FOR THE PHOTORECEPTOR CONSENSUS ELEMENT*" (Received for publication, October 20, 1997, and in revised form, December 16, 1997)

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Cellular retinaldehyde-binding protein (CRALBP) is abundantly expressed in the retinal pigment epithelium (RPE) and Müller cells of the retina, where it is thought to function in retinoid metabolism and visual pigment regeneration. Mutations in human CRALBP that destroy retinoid binding have been linked to autosomal recessive retinitis pigmentosa. To identify the DNA elements that regulate expression of the human CRALBP gene in the RPE, transient transfection studies were carried out with three CRALBP-expressing human RPE cell culture systems. The regions from −2089 to −1539 base pairs and from −243 to +80 base pairs demonstrated positive regulatory activity. Similar activity was not observed with cultured human breast, liver, or skin cells. Since sequence analysis of the −243 to +80 region identified the presence of two photoreceptor consensus element-1 (PCE-1) sites, elements that have been implicated in photoreceptor gene regulation, the role of these sequences in RPE expression was examined. Mutation of either PCE-1 site significantly reduced reporter activity, and mutation or deletion of both sites dramatically reduced activity. Electrophoretic mobility shift analysis with RPE nuclear extracts revealed two complexes that required intact PCE-1 sites. These studies also identified two identical sequences (GCAGGA) flanking PCE-1, termed the binding CRALBP element (BCE), that are also important for complex formation. Southern analysis with PCE-I/BCE-containing probes identified species with apparent masses near 90–100 and 31 kDa. These results begin to identify the regulatory regions required for RPE expression of CRALBP and suggest that PCE-1-binding factor(s) may play a role in regulating RPE as well as photoreceptor gene expression.

The retinal pigment epithelium (RPE) is a polarized monolayer of cells between the photoreceptors and choroid of the eye. Its primary function is maintaining and nourishing the rod and cone photoreceptor cells, including the synthesis of 11-cis-retinal for phototransduction (1). The critical importance of the RPE to the health of photoreceptors has been further illustrated by recent findings that mutations in either of two genes differentially and abundantly expressed in the RPE, namely the cellular retinaldehyde-binding protein (CRALBP) and RPE-65, can cause early onset autosomal recessive retinitis pigmentosa (2–4). The in vivo functions of CRALBP and RPE-65 have yet to be clarified; however, they are both thought to be involved in vitamin A metabolism. A retinal degeneration possibly associated with gene regulation in the RPE has been described for the vitiligo mouse; the vitiligo mouse exhibits genetic defects in transcription factor Mi and may also suffer from abnormal vitamin A metabolism (5).

CRALBP appears to serve as a substrate carrier protein in the mammalian visual cycle, modulating whether 11-cis-retinol is stored as an ester in the RPE or oxidized by 11-cis-retinol dehydrogenase to 11-cis-retinal for visual pigment regeneration (6,7). The protein carries 11-cis-retinal and/or 11-cis-retinol as an endogenous ligand in the RPE and Müller cells of the retina (7). Notably, the R150Q CRALBP mutation linked to autosomal recessive retinitis pigmentosa destroys the retinoid binding functionality of the protein (2). CRALBP is also present in ciliary body, cornea, and transiently in iris, but not in rod and cone photoreceptors. Within the ciliary epithelium, an anterior to posterior gradient of CRALBP expression exists with more abundant expression in the pars plicata region rel-


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1 The abbreviations used are: RPE, retinal pigment epithelium; CRALBP, cellular retinaldehyde-binding protein; PCE, photoreceptor consensus element; BCE, binding CRALBP element; bp, base pair(s); kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift analysis; SW, Southwestern.
ative to the pars plana region (8). Recently, CRALBP has been found in oligodendrocytes of the optic nerve and brain, but without retinoid ligands, and CRALBP is also expressed in glia cells of the pineal gland (9). During development, CRALBP is expressed in the rat RPE at E13 and in Müller cells at P1 (10), before 11-cis-retinoids or the isomerase responsible for generating 11-cis-retinoids can be found in the RPE (9). Apparently, the protein serves functions unrelated to visual pigment regeneration in brain and tissues not involved in the visual cycle and may bind ligands other than retinoids.

Due to the apparent importance of CRALBP in the visual cycle, its role in retinal disease, and its usefulness as a model system for the study of RPE gene regulation, we have begun to explore the mechanisms controlling its expression in the RPE. The structure of RLBP1, the gene encoding human CRALBP, has been reported, including the transcription start site and 3130 bases of sequence from the 5′-flanking region (11). It is composed of eight exons, with untranslated regions in exons 1, 2, and 8, and is located on human chromosome 15q26 (12). Previously, we noted the existence of two photoreceptor consensus elements between CRALBP gene positions −170 and −141 (5′-GAAGGCATTAAGGCAGGACATTAGGAC-3′) (11). The consensus sequence CAAATTAG, designated PCE-1 in vertebrates and RCS-1 in Drosophila, has been identified in a number of mammalian retina-specific genes (e.g. arrestin, interphotoreceptor retinoid-binding protein, rhodopsin, and red and green opsin) and in Drosophila rhodopsin genes and has been proposed to direct photoreceptor cell-specific expression (13). Mutation of PCE-1 sites in genes constructs from the proximal promoters of arrestin (14) and interphotoreceptor retinoid-binding protein (15) also appears to significantly reduce photoreceptor reporter gene expression in transgenic mice. The PCE-1-rich region of the RLBP1 proximal promoter exhibits significant homology to the 26-bp Ret-1 element in the rat opsin gene, which has been reported to direct opsin expression in rod photoreceptors in vivo (16). In this study, efforts have been initiated to determine the mechanisms controlling CRALBP cell-specific expression. The possibility was considered that PCE-1 might also regulate gene expression in the RPE, not just in photoreceptor cells. We report here transient transfection results, nuclear protein binding studies, and Southwestern analyses that suggest that PCE-1 influences CRALBP gene expression in the RPE.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Early passage nontransformed human RPE cell cultures (17) were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and 10% fetal bovine serum (Intergen Co.) and, for some transfections, were grown on plates coated with Matrigel (Upstate Biotechnology, Inc.) or bovine corneal endothelial cell matrix (17). The spontaneously immortalized human D407 RPE cell line (18) was grown in Dulbecco’s modified Eagle’s medium and 2% fetal bovine serum plus 210 mM Me$_2$SO (Fluka). The nontransformed human ARPE-19 RPE cell line (19) was obtained as a gift from Dr. L. Hjelmeland (University of California, Davis, CA) and grown in Dulbecco’s modified Eagle’s medium without fetal bovine serum. Human breast (MCF-7), human liver (HepG2), and human skin fibroblast transformed cell lines (available through the American Type Culture Collection) were grown in minimum Eagle’s medium (Life Technologies, Inc.) and 10% fetal bovine serum.

**Western Analysis**—Human retinal tissue was obtained from Dr. Arthur Polans and the RS Dow Neurological Sciences Institute/Good Samaritan Hospital (Portland, OR). Extracts from RPE cell cultures and human retinas were prepared with Laemmli SDS-PAGE sample buffer containing 5% 2-mercaptoethanol and 1% phenylmethylsulfonyl fluoride, and 1 mM benzamidine), boiled for 4 min, electrophoresed on 12% gels according to Laemmli (40), and electrophoretically from nitrocellulose membrane (Schleicher & Schuell). Membranes were probed with IgG-purified polyclonal anti-CRALBP peptide antibodies SI17, K235, and S31F as described previously (20), except that immunnoreactivity was detected with the commercial enhanced chemiluminescence system (Amersham Corp.). Human recombinant CRALBP (Mr = 36,343), used as a control, was produced in bacteria using the pET3a vector as described (21). The protein concentration of cell extracts was measured using the BCA protein assay (Pierce).

**Luciferase Assays**—DNA was isolated from RPE cell cultures using the RNAzol protocol (Cinna-Biotechnologies, Inc.) and 10% fetal bovine serum (Intergen Co.) and, for some transfections, were grown on plates coated with Matrigel (Upstate Biotechnology, Inc.) or bovine corneal endothelial cell matrix (17). The 3′-untranslated region of RLBP1 gene positions −165 to −2 were isolated as indicated under “Results.” Eight mutant CRALBP-luciferase reporter gene constructs were also created and are defined under “Results.” Transfections of RPE cell cultures were performed with pCMV-SEAP or pXGH5 control construct (Nichols Diagnostics, Inc.) to normalize transfection efficiency to secreted alkaline phosphatase or human growth hormone activity. For data interpretation, luciferase activity (relative luciferase units/mg of protein cell extract) was normalized using the BCA assay. For data interpretation, luciferase activity (relative luciferase units/mg of protein cell extract) was normalized using the BCA assay.

**Preparation of Nuclear Extracts**—Nuclear extracts for EMSA were prepared essentially according to Andrews and Fuller (24). Bovine retinal tissue was obtained from Dr. S. W. Lawrence Co. (Lincoln, NE); rat seminal vesicle extract was obtained from Dr. T. A. K. Tenen (Novartis). Bovine retinal tissue was obtained from Dr. S. W. Lawrence Co. (Lincoln, NE); rat seminal vesicle extract was obtained from Dr. T. A. K. Tenen (Novartis).
KCl, 5 mM MgCl2, 200 mM sucrose, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2 mM benzamidine. Following bench-top centrifugation (500 × g for 10 min at 4 °C), the pellet fraction was resuspended in 400 μl of cold 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, and 0.25 mM each phenylmethylsulfonyl fluoride and benzamidine. For cell cultures, confluent cells were scraped into 1.5 ml of cold phosphate-buffered saline, centrifuged at 13,000 × g for 2 min, and resuspended as described above. After a 10-min incubation on ice for lysis, cells were vortexed for 10 s, and nuclei were pelleted by centrifugation (13,000 × g). For high salt extraction of nuclear proteins, pellets were incubated for 20 min at 4 °C in 100 μl of 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.25 mM each phenylmethylsulfonyl fluoride and benzamidine and then centrifuged, and the supernatant fraction was harvested. Protein concentration in cell extracts was determined using the BCA assay.

EMSA—Oligonucleotide probes (see Table I) were synthesized with 5'-overhangs and radioactively labeled by Klenow fill-in reactions (Promega). Nuclear extracts were incubated with a 2000-fold molar excess of poly(dI-dC) (Pharmacia Biotech Inc.) at room temperature for 10–30 min in 100 mM Tris-HCl (pH 7.4), 50 mM dithiothreitol, 50 mM EDTA, and 22% glycerol (plus unlabeled probe in competition assays) before adding 1–10 ng of labeled probe. After an additional 10–30-min incubation at room temperature, the reactions were stopped and electrophoresed on 4–6% nondenaturing polyacrylamide gels, followed by autoradiography.

Southwestern Analysis—Southwestern analysis was performed according to published methods (25, 26) with modifications. Nuclear extract from human D407 RPE cells and prestained molecular mass standards (Bio-Rad) were fractionated by SDS-PAGE according to Laemmli (46–70), and S31F (C-terminal residues 286–316).

RESULTS

Expression of CRALBP in Cultured RPE Cells—Three human RPE cell culture systems were used to study regulation of RLBP1, namely early passage nontransformed RPE cell cultures (17) and RPE cell lines ARPE-19 (19) and D407 (18). Previous reports have shown by Western and Northern analyses that the early passage nontransformed RPE cultures and ARPE-19 cells express CRALBP (17, 19). We used Western analysis and reverse transcription-PCR to confirm the

CRALBP expression in D407 RPE cells previously detected by immunocytochemical methods (18). Antibodies directed against the CRALBP N-terminal 17 residues, C-terminal 31 residues, or a near N-terminal domain revealed a protein band with a slightly lower apparent mass in the D407 RPE cell extract compared with CRALBP in the human retinal extract or the purified recombinant control (Fig. 1). The strongest detection of CRALBP in the D407 RPE cell extract was obtained with the C-terminal antibody; no detection was apparent with the N-terminal antibody; and an intermediate level of detection was obtained with the third antibody. These results suggest that partial N-terminal proteolysis had occurred during protein extraction from the cultured cells as previously seen by Western analysis of CRALBP extracted from rat Müller cell cultures (28). These observations are consistent with the documented lability of the N terminus of CRALBP to proteolysis (20). In other Western analyses, the immunopositive bands could be competed away by excess purified recombinant CRALBP, but not by excess bovine serum albumin (data not shown). More intense immunodetection of CRALBP was apparent in the retinal extract than in the RPE cell extract; limited proteolysis was not apparent in the retinal extract. Reverse transcription-PCR using RNA from each of the three RPE cell culture systems followed by Southern blotting demonstrated the presence of CRALBP mRNA (data not shown). For these analyses, the PCR primers flanked intron 2 of RLBP1, and the Southern results distinguished CRALBP cDNA (241 bp) from genomic DNA (511 bp). Because the CRALBP N terminus is encoded within the reverse transcription-PCR product, these results also establish that the CRALBP mRNA in these RPE cells encodes an intact N terminus. Consistent with previous observations (17), greater apparent levels of CRALBP mRNA and protein were observed in nontransformed RPE cells grown on Matrigel or bovine corneal endothelial cell matrix than when grown on tissue culture plastic. These results, together with the previous reports, establish that CRALBP is expressed in the three RPE cell culture systems used in this study.

Regulation of RPE-specific Expression of CRALBP—As a first step toward identifying the elements in the CRALBP gene responsible for its expression in the RPE, we transfected re-
porter gene constructs containing 0.3–2.3 kb of the human CRALBP gene promoter region into three human RPE cell model systems and into several human non-ocular cell systems (Fig. 2). The results with all three RPE cells were similar and showed that the 2.1- and 2.3-kb CRALBP-reporter gene constructs direct high level luciferase expression in the RPE cells, but not in the non-ocular cells. Also evident, the 0.3-kb CRALBP-reporter gene construct, but not the reverse orientation (0.3R) construct, directs above background level luciferase expression in the RPE (Fig. 2, C–E). This suggests that basal promoter elements in RLBP1 exist between −243 and +80 bp. The results also suggest that element(s) that enhance CRALBP expression in RPE cells exist between −2089 and −1539 bp. None of the CRALBP-reporter constructs directed significant levels of luciferase expression in cultures of human liver or skin cells, although low levels of nonspecific leaky expression were observed in cultures of human breast cells (Fig. 2, F–H).

Identification of PCE-1 as a Possible Positive Regulator in the RPE—The presence of two PCE-1 sequences in the −243 to +80 region was intriguing given that similar sequences have been implicated in the regulation of photoreceptor-specific gene expression (13, 15, 16). To investigate the possible role of CRALBP PCE-1, we prepared mutant 0.3- and 2.1-kb reporter constructs with altered PCE-1 sites (Fig. 3A) and performed transient transfections with human D407 RPE cells. The transfection results (Fig. 3B) show that altering the PCE-1 sites in the 2.1-kb construct decreased the luciferase reporter activity relative to the 2.1-kb wild-type constructs. Reporter activity for the 2.1-kb mutant constructs was reduced −35% with either PCE-1 site altered (pce-dM or pce-pM), −60% with both PCE-1 sites mutated (pce-2M), and >70% when PCE-1 and adjacent sequence were deleted and mutated (pee-2M). Reporter activity for the 0.3-kb mutant constructs approximated that observed for the 0.2-kb wild-type construct lacking PCE sites. These results support a role for PCE-1 in regulating CRALBP expression in the RPE.

PCE in the CRALBP Proximal Promoter Interacts with Proteins in the Retina and RPE—To follow up on the transient transfection results, EMSAs were performed to investigate possible DNA-protein interactions involving the CRALBP PCE region. EMSA with a 42-bp probe, designated PCE42 (Table I), demonstrated four shifted protein complexes with a bovine retinal nuclear extract (indicated by arrows in Fig. 4A). These complexes were not apparent in nuclear extract from rat seminiferous vesicle or human breast cells (Fig. 4A). The bovine retinal complexes were reduced in number and diminished in intensity by competition with excess unlabeled PCE42 probe (Fig. 4B). These complexes could be competed away with excess amounts of a structurally related probe (arrestin-42; Table I), but not with the unrelated clusterin probe (data not shown). These results suggest that the observed DNA-protein complexes are tissue- and sequence-specific.

RPE nuclear extracts also demonstrated binding activity for the PCE-1-containing sequences. EMSAs using RPE extracts

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** RPE cell-specific expression of human CRALBP promoter constructs. Wild-type RLBP1-luciferase reporter gene constructs are shown in A, with the exon indicated as a black box. Results from transient transfection of human RPE cells are shown in B–E. B, nontransformed RPE cells cultured on Matrigel; C, nontransformed RPE cells cultured on plastic; D, D407 RPE cells cultured on plastic; E, ARPE-19 cells cultured on plastic. Results from transient transfection of non-RPE control cells on plastic are shown in F–H. F, MCF-7 human breast cells; G, HepG2 human liver cells; H, human skin fibroblasts (HSF). Transient transfections were performed with the indicated CRALBP promoter constructs as described under “Experimental Procedures.” Data in B and D–G are from the LipofectAMINE transfection protocol, and data in C and H are from the calcium phosphate protocol. All data are from n ≥ 3 experiments, except for the 2.1-kb construct in B, where n = 2. Each construct was assayed in triplicate per experiment; S.D. is shown by error bars. huCRALBP, human CRALBP; RLU, relative luciferase units; Basic, the pGL2-luciferase vector lacking a promoter; CMV, cytomegalovirus enhancer and promoter control vector (indicates transfection efficiency).
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and the shorter 29-bp wild-type probe, designated PCE29, demonstrated two shifted bands (Fig. 4C). EMSAs with RPE nuclear extracts and altered PCE29 probes (Table I) are shown in Fig. 4C. Mutations of the PCE-1 sites prevented the formation of the two complexes observed with the RPE extracts and the wild-type probe (Fig. 4C). Mutations in the identical sequence adjacent to each PCE-1 also prevented formation of the two complexes (Fig. 4C). We have designated this 6-bp sequence (GCAGGA) as the binding CRALBP element (BCE). These results support a role for PCE-1 and the BCE in forming DNA-protein interactions in the RPE.

The specificity of the DNA-protein interactions with the RPE extract was further assessed by examining the ability of unlabeled probes to compete with the 32P-labeled PCE29 probe for complex formation. The results in Fig. 4D show the following: (i) unlabeled wild-type PCE29 and PCE42 probes are effective competitors and block formation of the two complexes; (ii) the mutant probe with both PCE-1 sites altered (mutant pce-2M) competes away the upper but not the lower complex; (iii) the mutant probe with both BCE sites altered (mutant bce-2M) competes away both complexes; and (iv) the unlabeled probe with mutations in both the BCE and BCE sites (p&b-2M) and the unrelated clusterin control probe do not block formation of either complex. Overall, the results support the specificity of the DNA-protein interactions and are consistent with a specific role for PCE-1 and the BCE in forming RPE nuclear protein complexes.

Southwestern Analysis of RPE Proteins Interacting with the CRALBP PCE—Southwestern analysis was used to investigate the size and complexity of proteins in the D407 RPE nuclear extract that bind to the wild-type CRALBP 32P-labeled PCE42 probe. The results (Fig. 5) reveal major doublet bands with apparent molecular masses near 90–100 kDa and a less predominant lower band at ~31 kDa. Unlabeled probes that contain intact PCE sequences (i.e. PCE42, PCE29, bce-2M and arrestin-42) were found to be the most effective competitors of binding. Unlabeled probes with both PCE-1 sites altered (pce-2M) or with both PCE-1 sites and both BCE sites altered (p&b-2M) could not block binding of the wild-type PCE42 probe with RPE nuclear protein. These results are consistent with the EMSA results in Fig. 4 and further demonstrate a specific interaction between PCE and RPE nuclear proteins.

DISCUSSION

CRALBP was first detected and purified from bovine retina ~20 years ago and subsequently found to be abundant in the RPE and to carry 11-cis-retinal and 11-cis-retinol as endogenous ligands in the RPE and retina (7). Structure-function studies have defined ligand stereoselectivity and photosensitivity and a topological and epitope map and established in vitro evidence for a substrate carrier function in the RPE (6, 7, 20). Human and bovine CRALBP cDNAs have been cloned, and human recombinant CRALBP has been produced and used in functional domain analyses (21, 29, 30). This study has explored the molecular mechanisms controlling expression of the human CRALBP gene (RLBP1) in the RPE.

The presence of photoreceptor consensus elements in genes other than those expressed in photoreceptors suggests a broader tissue spectrum of potential regulatory activity for PCE-1. We investigated the potential role of PCE-1 in human CRALBP gene expression in three human RPE cell culture systems. The 0.3-kb CRALBP-reporter construct, which contains two PCE-1 sites, consistently generated above background level reporter gene expression. None of the CRALBP

![Image](http://www.jbc.org/)

**Table I**

Oligonucleotide probes

<table>
<thead>
<tr>
<th>Oligonucleotide Probes</th>
<th>5'-Overhang Sequences as Shown in Fig. 3</th>
<th>5'-Overhang Sequences as Shown in Fig. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type CRALBP PCE42</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
</tr>
<tr>
<td>Wild-type CRALBP PCE29</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
</tr>
<tr>
<td>PCE29-pce-2M</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
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<tr>
<td>PCE29-pce-2M</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
</tr>
<tr>
<td>Arrestin-42</td>
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</tr>
<tr>
<td>Clusterin</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
<td>5'-TCTTGAACGACTTAGCATTTAGGCCAGGAGA</td>
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**Fig. 3.** Identification of the PCE as a positive regulator of CRALBP expression in the RPE. Mutant human CRALBP-reporter gene constructs were prepared with the 0.3- and 2.1-kb pGL2-CRALBP-luciferase vectors as shown in A. pce-dM is the distal and proximal PCE site; pce-pM is the proximal PCE mutant; pce-2M is the distal and proximal PCE site; and pce-3M is the mutant with a deletion of the sequence adjacent to each PCE-1 also prevented formation of the two complexes (Fig. 4C).

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Wild-type CRALBP PCE29

Wild-type CRALBP PCE42

PCE29-pce-2M

pce-2M

pce-dM

pce-3M

Arrestin-42

Clusterin

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Table I

The oligonucleotide probes indicated below were designed from the indicated upstream sequences from the human CRALBP gene (11), the mouse arrestin gene (13), and the rat TRPM-2 clusterin gene (GenBank™ accession number M64733). The wild-type CRALBP probes contain a 13-bp wild-type CRALBP PCE29, dem-
gene constructs provided significant reporter activity in three different non-ocular cell lines. These results demonstrate that basal promoter function lies in the sequences contained in the 0.3-kb construct (RLBP1 positions 2243 to 180). The increase in reporter activity observed with the 2.1-kb construct compared with the 1.6-kb construct suggests the presence of an enhancer element between 22089 and 21539 bp. Notably, Alu repetitive sequence exists in the 1.6-kb construct between 21438 and 21157 bp; whether this region influences CRALBP gene expression remains to be determined. Given the consistent low level reporter activity of the 0.8-kb construct, a repressor element may exist within the 702 to 243 region. Reporter activity in RPE cells for the 2.1-kb construct with either of the two PCE-1 sites altered was significantly reduced relative to wild-type constructs and even more dramatically reduced with both PCE-1 sites mutated or deleted (Fig. 3). These data suggest a positive role for PCE-1 in regulating CRALBP expression in the RPE. The apparent enhancer activity in RLBP1 between 2089 and 1539 bp appears to be modulated in part by DNA-binding protein(s) interacting with PCE-1 between -165 and -140 bp.

The nature of the RLBP1-protein interactions were probed by EMSA using a 42-bp wild-type probe (PCE42) from RLBP1 designed to mimic an arrestin probe used in other binding studies. The binding pattern obtained with PCE42 resembled that obtained with bovine retinal extract in a previous study (13). Two of the PCE42-protein interactions in the retinal extract appear to be specific based on competition results and the fact that similar complexes were not observed with nuclear extracts from seminal vesicle and breast cells (Fig. 4, A and B). The apparent low molecular mass complexes in extracts from seminal vesicle and breast cells are not present in retinal extracts. These complexes do not appear to be relevant to CRALBP gene expression in visual tissues; however, their sig-
nuclear proteins. Notably, a PCE-1-containing probe designed
ing the specificity of the interaction between PCE-1 and RPE
plexes observed by Southwestern analysis, further emphasiz-
PCE-1 sequences were required to block formation of the com-
RPE nuclear complexes observed in the present analysis. Intact
different proteins, the apparent masses of their respective nu-
PCE42 over the labeled probe is indicated. These results were repro-
labeled probe; the approximate molar excess concentration of unlabeled
Ret-1 element in the opsin gene appears to bind a 40-kDa
bind to the 31-kDa component, suggesting that the smaller
ative to the two 90–100-kDa complexes, less probe appears to
frayed or post-translationally modified versions of one polypep-
study is needed to determine whether these components are
Fig. 5. Southwestern analysis of CRALBP PCE binding activ-
human RPE cells. Nuclear proteins from human D407 RPE
cells were separated by SDS-PAGE, transferred to nitrocellulose, rena-
tured, and probed with wild-type (wt) human CRALBP 32P-labeled
PCE42 (Table I) in the presence or absence of excess unlabeled compe-
tor. PCE29 probes were used at a 290-fold molar excess over the
labeled probe; the approximate molar excess concentration of unlabeled
Fig. 5. Southwestern analysis of CRALBP PCE binding activ-
ity in human RPE cells. Nuclear proteins from human D407 RPE
cells were separated by SDS-PAGE, transferred to nitrocellulose, rena-
tured, and probed with wild-type (wt) human CRALBP 32P-labeled
PCE42 (Table I) in the presence or absence of excess unlabeled competitor. PCE29 probes were used at a 290-fold molar excess over the labeled probe; the approximate molar excess concentration of unlabeled PCE42 over the labeled probe is indicated. These results were reproducible over two to three Southwestern analyses. Arr42; arrestin-42.

significance was not explored. Shorter oligonucleotide probes
(PCE29) were designed to more specifically evaluate RPE pro-
tein interactions with PCE-1 and the 6-bp BCE site adjacent to
each PCE-1 site. Two RPE nuclear protein complexes were
observed with the PCE29 probes, which require intact PCE-1 and BCE sequences (Fig. 4C). The results from competitive
EMSA with mutant probes pce-2M and bce-2M suggest that
PCE-1 mediates formation of both complexes and that PCE-1 and the BCE are involved in formation of the more retarded
complex (Fig. 4D). Complex formation with PCE-1 appears to
be required for the more retarded complex to form, encompass-
thesing the BCE (Fig. 4D). Overall, the results from the reporter
gen assay and EMSA strongly support a role for PCE-1 in
controlling CRALBP expression in the RPE. It is not yet clear
whether PCE-1 and the BCE comprise a single cis-element or
two separate elements.

Up to three RPE nuclear complexes were detected by South-
western analysis using the PCE42 probe from

PCE-1 in the RLPB1 proximal promoter appears to contain
essential nucleotides for CRALBP expression in the RPE. As
determined for the photoreceptor-specific genes opsin (16, 37,
38) and interphotoreceptor retinoid-binding protein (15) and as
suggested for arrestin (14), the RLPB1 proximal promoter may
determine the cell specificity of CRALBP expression, whereas
upstream elements may govern other aspects such as the quan-
ty and timing of expression. Regulation of opsin and inter-
photoreceptor retinoid-binding protein genes involves more dis-
tal upstream elements, including enhancer elements (34, 37).
Indeed, our in vitro results suggest that enhancer elements exist in the RLPB1 distal region between −2089 and −1539 bp.
The current work extends the spectrum of gene regulatory
activity for PCE-1 from rod photoreceptor cells to the RPE.
However, the spectrum may be even broader since CRALBP is
expressed in several ocular cell types and in brain oligodendro-
cytes. Furthermore, a recent transgenic mouse study suggested
that the related Ret-1 element may influence gene expression in brain (16). A growing number of retinal transcription factors appear to be coexpressed in the central nervous system (39), suggesting that a common PCE-1-binding protein for RLPB1 may function in the retina, RPE, brain, and perhaps other tissues. Future in vivo studies will be needed to more directly test this hypothesis.

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REFERENCES
2. Maw, M. A., Kennedy, B. N., Knight, A., Bridges, R., Roth, K., Mani, E.,
Genet. 17, 198–200
Nicotelli, A., Murphy, K. R., Rathmann, M., Kumarananickavel, G.,
Liu, S.-Y., Harris, E., Redmond, T. M., Arnaud, B., Claustres, M., and
Visual Sci. 37, 1097–1111
7. Saari, J. C. (1994) in “The Retinoids” (Sporn, M. A., Roberts, A. B., and
Transcriptional Regulation of Cellular Retinaldehyde-binding Protein in the Retinal Pigment Epithelium: A ROLE FOR THE PHOTORECEPTOR CONSENSUS ELEMENT
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