Preliminary *In Vivo* Analysis of CRALBP Transcriptional Regulation

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Abstract

Purpose. In RPE, cellular retinaldehyde binding protein (CRALBP) serves in the visual cycle as an 11-*cis*-retinol acceptor and as a substrate carrier. CRALBP is also expressed in Müller cells, ciliary epithelium, iris, cornea, pineal, brain and optic nerve. Toward deciphering the mechanisms controlling cell-specific expression of CRALBP, transgenic mice carrying mouse CRALBP promoter constructs have been generated and analyzed.

Methods. Transgenic mice in strain B6CBA were generated in the Cleveland Clinic Transgenic Mouse Facility with EGFP reporter constructs containing either ~1.4 Kb or ~4.7 Kb of promoter from *Rlbp1*, the mouse gene encoding CRALBP. Southern blot and PCR were used for analysis of transgene expression. Founders were bred with mouse strains Balb/CbyJ or CD-1. Cryo sections of eye and brain tissues were processed for immunohistochemistry with antibodies to EGFP (BD Biosciences), GFAP (Sigma) and CRALBP. Nuclei were stained with TO-PRO-3 (Molecular Probes Inc).

Results. Three independent founder mice were obtained for the 4.7*Rlbp1*-EGFP construct and two founders were obtained for the 1.4*Rlbp1*-EGFP construct. Breeding has yielded numerous progeny carrying the 4.7 Kb construct. Immunohistochemical analysis with anti-EGFP revealed GFP positive cells in the RPE and Müller cells of mice carrying the 4.7*Rlbp1*-EGFP construct. Concomitant labeling with CRALBP antibody was observed for many cells that showed expression of GFP or GFAP.

Conclusions. The mouse CRALBP promoter region extending upstream from the transcription start site ~4.7 Kb appears to direct expression to the RPE and Müller cells of the neural retina. Progress in on going histological analyses will be presented.

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Introduction

Cellular retinaldehyde-binding protein (CRALBP) functions in the retinal pigment epithelium (RPE) as an acceptor of 11-*cis*-retinol in the isomerization step of the rod visual cycle (Saari et al., 2001) and as a substrate carrier for 11-*cis*-retinol dehydrogenase (Saari et al., 1994; Golovleva et al., 2003). In addition to RPE, the protein is expressed in Müller cells, ciliary epithelium (CE), iris, cornea, pineal, and a subset of oligodendrocytes in brain and optic nerve (Saari et al., 1997). Previous efforts toward deciphering the mechanisms controlling cell-specific expression of *RLBP1*, the human gene encoding CRALBP, were pursued in RPE, CE and Müller cell cultures using, *RLBP1* promoter constructs. Results from these *in vitro* gene regulation studies support PCE1 and PCE1-binding factor(s) as playing important roles in regulating RPE expression of CRALBP (Kennedy et al., 1998a). localize an *RLBP1* enhancer element to within –1826 to –1749 bp and a repressor element to within –702 to –635 bp and suggest that CRALBP transcriptional regulation may be similar in the CE and RPE but different in Müller cells (Kennedy et al., 2003). Previous *in vivo* gene regulation studies using human *RLBP1* promoter constructs in mice have not yielded transgene expression in the retina. In the present study, transgenic mice carrying mouse CRALBP promoter constructs were used to identify a CRALBP promoter region directing *in vivo* expression in the RPE and retina.

Methods

Mouse CRALBP promoter/EGFP fusion constructs (either ~1.4 Kb or ~4.7 Kb of promoter from Rlbp1) were generated with the mouse CRALBP gene (Kennedy et al., 1998b) and the reporter vector. Founding transgenic mice were generated in the Cleveland Clinic Foundation Transgenic Core Facility by microinjection of DNA constructs into the pronuclei of fertilized mouse eggs. Genomic DNA was prepared from mouse tail snips (Gentra DNA Kit) for analysis of transgene expression by PCR and Southern blot. PCR analysis was performed using the Advantage PCR kit (BD Biosciences) and EGFP forward primer 5579 (5'-GCC ACA AGT TCA GCG TGT CC-3') and reverse primer 5580 (5'-GAT GCC CTT CAG CTC GAT CC-3'), resulting in amplification of a 255 bp DNA fragment. Southern analysis was performed with genomic DNA digested with BamHI, NotI and ^{32}P-labeled EGFP probe, resulting in detection of ~0.72 Kb fragment. Breeding was performed with Balb/CbyJ or CD-1 mice. Cellular localization of GFP transgene expression was by immunohistochemistry using cryo tissues sections of eye, pineal, brain, liver and testis. Immunohistochemistry was performed with antibodies to EGFP (BD Biosciences, CA), GFAP (Sigma Chemical Co., St. Louis, MO.) and CRALBP (Crabb et al., 1991). Nuclei were stained with TO-PRO-3 (Molecular Probes Inc., CA). Laser scanning confocal microscopy was performed with a Leica TCS-SP2 instrument and fluoresence microscopy was performed with a Nikon EFD-3 microscope. Microscopic images were merged using AdobePhotoshop 5.5.

Figure 1

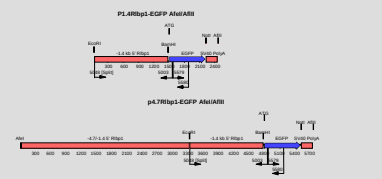

Figure 1. CRALBP promoter constructs used to generate transgenic mice. Mouse CRALBP promoter extending either ~1.4 Kb or ~4.7 Kb from the Rlbp1 start site were generated with the EGFP reporter vector. Arrows indicate the location of PCR primers used for analysis of transgene expression. Relevant restriction sites are labeled and nucleotides are numbered sequentially from 5' to 3'.

Figure 2

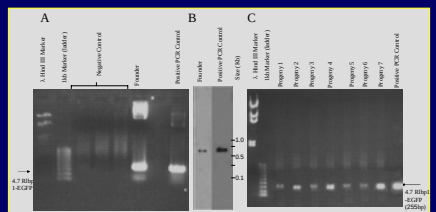

Figure 2. Analysis of Transgene Expression. Representative (A) PCR analysis and (B) Southern analysis of genomic DNA from founder mice. (C) PCR product analysis for F2 progeny. PCR was performed using EGFP primers 5579 and 5580, yielding a 255 bp fragment in EGFP positive animals. Southern analysis of BamHI/NotI digested DNA yielded a ~0.72 kb fragment in EGFP positive animals. Three independent founder mice were obtained for the 4.7*Rlbp1*-EGFP construct and two founders were obtained for the 1.4*Rlbp1*-EGFP construct using mouse strain B6CBA. From the founders carrying 4.7*Rlbp1*-EGFP, 26 F1 progeny were obtained in Balb/CbyJ (specifically 8, 8 and 10 F1 progeny) and155 F2 progeny was obtained. The founders carrying the 1.4*Rlbp1*-EGFP transgene were breed with CD-1 mice and no progeny were obtained.

Figure 3

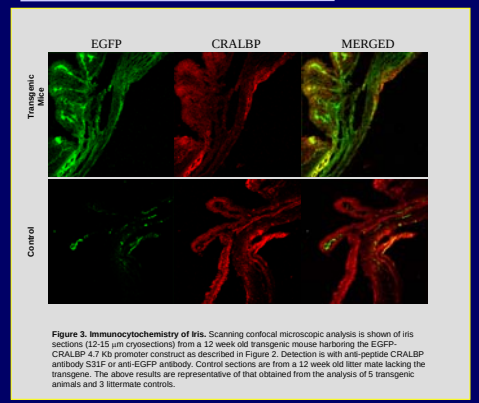

Figure 3. Immunocytochemistry of Iris. Scanning confocal microscopic analysis is shown of iris sections (12-15 µm cryosections) from a 12 week old transgenic mouse harboring the EGFP-CRALBP 4.7 Kb promoter construct as described in Figure 2. Detection is with anti-peptide CRALBP antibody S31F or anti-EGFP antibody. Control sections are from a 12 week old litter mate lacking the transgene. The above results are representative of that obtained from the analysis of 5 transgenic animals and 3 littermate controls.

Figure 4

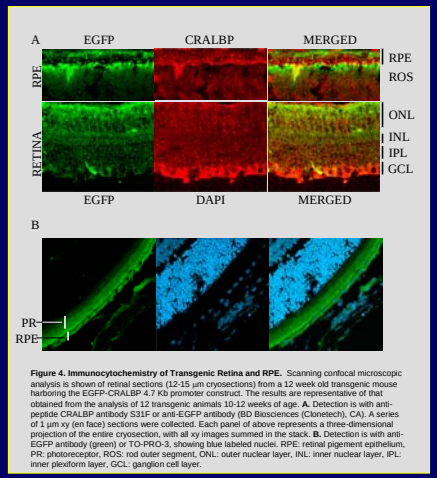

Figure 4. Immunocytochemistry of Transgenic Retina and RPE. Scanning confocal microscopic analysis is shown of retinal sections (12-15 µm cryosections) from a 12 week old transgenic mouse harboring the EGFP-CRALBP 4.7 Kb promoter construct. The results are representative of that obtained from the analysis of 12 transgenic animals 10-12 weeks of age. **A.** Detection is with anti-peptide CRALBP antibody S31F or anti-EGFP antibody (BD Biosciences (Clonetech), CA). A series of 1 µm xy (en face) sections were collected. Each panel of above represents a three-dimensional projection of the entire cryosection, with all xy images summed in the stack. **B.** Detection is with anti-EGFP antibody (green) or TO-PRO-3, showing blue labeled nuclei. RPE: retinal pigement epithelium, PR: photoreceptor, ROS: rod outer segment, ONL: outer nuclear layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

Figure 5

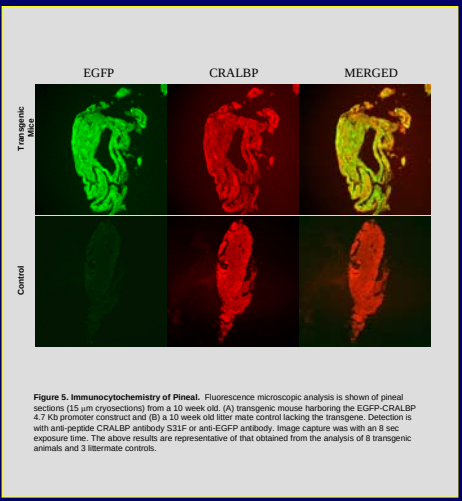

Figure 5. Immunocytochemistry of Pineal. Fluorescence microscopic analysis is shown of pineal sections (15 µm cryosections) from a 10 week old. (A) transgenic mouse harboring the EGFP-CRALBP 4.7 Kb promoter construct and (B) a 10 week old litter mate control lacking the transgene. Detection is with anti-peptide CRALBP antibody S31F or anti-EGFP antibody. Image capture was with an 8 sec exposure time. The above results are representative of that obtained from the analysis of 8 transgenic animals and 3 littermate controls.

Figure 6

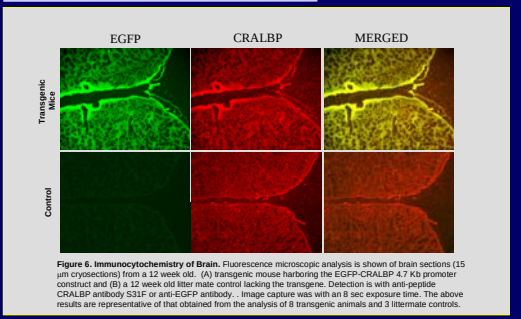

Figure 6. Immunocytochemistry of Brain. Fluorescence microscopic analysis is shown of brain sections (15 µm cryosections) from a 12 week old. (A) transgenic mouse harboring the EGFP-CRALBP 4.7 Kb promoter construct and (B) a 12 week old litter mate control lacking the transgene. Detection is with anti-peptide CRALBP antibody S31F or anti-EGFP antibody. Image capture was with an 8 sec exposure time. The above results are representative of that obtained from the analysis of 8 transgenic animals and 3 littermate controls.

Figure 7

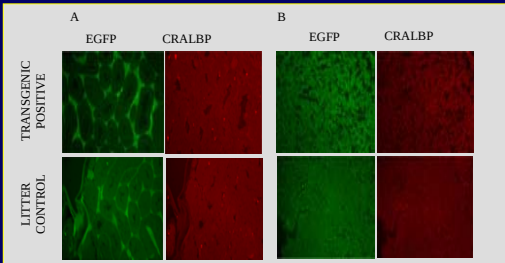

Figure 7. Immunocytochemistry of Liver and Testis. Fluorescence microscopic analysis is shown of (A) liver and (B) testis (15 µm cryosections) from either a 10 week old transgenic mouse harboring the EGFP-CRALBP 4.7 Kb promoter construct or a litter mate control lacking the transgene. Detection is with anti-peptide CRALBP antibody S31F or anti-EGFP antibody. Image capture was with an 15 sec exposure time. Approximately equal intensity background staining was apparent in liver and testis from transgenic and littermate controls, with staining intensity significantly weaker than in retina and RPE. The above results are representative of that obtained from the analysis of 8 transgenic animals and 3 littermate controls.

Conclusion

- These preliminary results suggest that the mouse CRALBP promoter region extending upstream from the transcription start site ~4.7 Kb directs expression to RPE, retina, iris, pineal and brain but not to the liver and testis. Previous studies have demonstrated CRALBP is expressed in retinal Müller cells and a subset of oligodendrocytes in the brain.
- Efforts continue toward the production and analysis of transgenic mice carrying the EGFP-CRALBP 1.4 Kb promoter construct

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