



A Major Cis Activator of the IRBP Gene Contains CRX-Binding and Ret-1/PCE-I Elements

Jeffrey H. Boatright,¹ Diane E. Borst,² John W. Peoples,¹ James Bruno,¹ Carrie L. Edwards,¹ Jing-Sheng Si,¹ and John M. Nickerson¹

¹Department of Ophthalmology, Emory Eye Center, Emory University School of Medicine, Atlanta; ²Department of Anatomy and Cell Biology, Uniformed Services University of the Health Sciences, Bethesda, MD

Purpose: Interphotoreceptor retinoid binding protein (IRBP) is expressed exclusively and to high levels in photoreceptive cells. This study was an attempt to delineate the minimal regulated control region of the murine IRBP promoter involved in this expression pattern.

Methods: Fragments of the mouse IRBP 5' flanking region were tested for promoter activity in transient transfections of embryonic chick retina cells in primary culture. Electrophoretic mobility shift assays were used to identify specific cis-acting DNA elements within these fragments.

Results: Nested deletion analysis of a 1783 bp fragment of the murine IRBP 5' flanking region shows that high promoter activity is maintained with truncated fragments as short as 70 bp 5' to transcription start, but is lost with truncation to 45 bases. The 1783 bp promoter is active in cultures of retina cells but not brain cells or fibroblasts. The 70 bp fragment is active in retina and brain cells but not fibroblasts. Within retina cell cultures, the 1783 bp fragment is active in photoreceptor-like and amorphous or unidentifiable cells whereas the 70 bp is additionally active in multipolar neuron-like cells. The -70 to -45 interval contains Ret-1/PCE-I (AATTAG in the IRBP gene), a proposed retina-specific consensus sequence cis element, and a same-strand reversed copy of this sequence, GATTAA, the consensus binding element of the photoreceptor-specific trans-acting factor CRX. Mutation of either element suppresses promoter activity. Paralleling promoter tissue-specificity, the -70 to -45 fragment binds a sequence-specific protein complex found in retina and brain extracts but not fibroblasts. Mutation of both or either element inhibits this binding.

Conclusions: These data suggest that a trans-acting complex binds a cis-element in the -70 to -45 sequence. This binding fully activates transcription but confers only partial tissue-specificity to IRBP gene expression.

Interphotoreceptor retinoid binding protein (IRBP) is a large glycolipoprotein found abundantly in the interphotoreceptor matrix of many vertebrates, where it shuttles retinoids between photoreceptors and retinal pigment epithelial cells (1-4). Its gene expression is highly regulated. IRBP and its mRNA appear at an early and discrete developmental stage: just prior to the onset of outer segment formation (5-8). IRBP can be detected by postnatal day 1 (P1) in the rat (9) and the major developmental increase in IRBP protein level corresponds to the rise of mRNA between ages P1 and P9 (10). In mice, IRBP mRNA appears just after the last mitosis of precursor cells as they differentiate into photoreceptors (11). IRBP expression is limited to photoreceptors and pinealocytes (12,13). IRBP mRNA accumulates predominantly in the perikaryon (13) and the inner segment (12) of photoreceptors, but not other cells of the retina or pigment epithelium (PE). IRBP and its mRNA also are sensitive to lighting (14) and rhythmic cues (15).

Eukaryotic gene transcription often is regulated by binding between trans-acting nuclear protein complexes and cis-acting DNA elements 5' to transcription start (16,17). Recent studies have focused on identifying the cis elements that regulate IRBP

tissue specificity. Liou et al. (11) found that a 1.3-kilobase pair of human IRBP 5' flanking region drove reporter gene activity in transgenic mice and that a promoter fragment spanning -156 to +19 (relative to transcription start) conferred tissue specificity (18). Bobola et al. (19) similarly found that -123 to +18 of the human IRBP gene drove reporter activity in a transgenic mouse retina.

We recently reported that a -1783 to +101 fragment of the mouse IRBP gene drives reporter activity in transiently transfected embryonic chick retina cells, but not in brain cells or fibroblasts (20). The object of the current study was to identify a minimal regulated control region (MRCR) of the mouse IRBP gene. The data indicate that only the first 70 bp 5' to transcription start are required for promoter activity equivalent to that produced by the -1783 to +101 fragment, but that this short sequence additionally drove reporter activity in brain cells, suggesting that elements upstream of -70 are required for complete tissue-specific regulation. The -70 to -45 region contains two photoreceptor-specific consensus sequences, Ret-1/PCE-I (AATTAG in murine IRBP) and its reversed repeat, GATTAA. Ret-1/PCE-I has been identified as a functionally active cis element in several photoreceptor-specific genes (21,22) including IRBP (19,23,24). We initially characterized GATTAA as a cis-acting element of the murine IRBP gene and termed it the "GATTAA box" (23-25). Two recent reports demonstrate that this sequence binds an Otx-

*To whom correspondence should be addressed: Jeffrey H. Boatright, Ph.D., Room B-5500, Emory Eye Center, 1327 Clifton Road, Atlanta, GA, 30322, Phone: (404) 778-4113, FAX: (404) 778-2231, email: jboatright@emory.edu

like photoreceptor-specific trans-acting factor termed “CRX” (Cone rod homeobox) (26,27). Hence, the established nomenclature, “CRX-binding element,” is used in the remainder of this text in referring to this element. Mutation of either Ret-1/PCE-I or the CRX-binding element suppressed promoter activity and in vitro DNA:protein complex formation.

METHODS

Animals— These studies adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. Balb/C mice were from Jackson Labs, Bar Harbor, ME. Balb/C, a highly inbred strain of mouse, was used as “wild type” control since it is not likely to contain any appreciable incidence of polymorphisms in trans factor gene sequences.

Promoter activity assays— As previously detailed, primary cultures of embryonic chick cells can be transiently transfected with plasmid vectors containing reporter genes whose expression is regulated by retina-specific and viral promoters (20,28). Retinal cultures were prepared as previously described (20,28,29). Briefly, whole retinas from embryonic day 10 (E10) chicks were trypsinized, triturated, and plated at a density of 20 x 10⁶ cells per dish onto 60 mm plastic culture dishes pretreated with 0.1% polyornithine. Cells were cultured overnight in 5 ml of Medium 199 supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin G and 100 µg/ml streptomycin (GIBCO-BRL, Grand Island, NY).

Cultures were transfected via CaPO₄ with plasmid vectors (10 µg DNA/dish) (20) containing either a gene encoding chloramphenicol acetyltransferase (CAT) or a gene encoding an enhanced green fluorescent protein (EGFP). After incubation for 16 h, they were fed with fresh medium and incubated for an additional 48 h, fed once more, incubated another 24 h, then harvested for CAT assays or viewed through a fluorescent microscope. We previously found that co-transfection with two reporter constructs for normalization purposes can confound interpretation of results (20). To address the issue of variability due to transfection efficiency differences and other factors, the promoter activity of each construct was tested at least twice with two or more different plasmid preparations. Relative differences in activity between different promoter fragments and mutations were repeatable across experiments with little variability.

Ret-1/ PCE-I	-CBE- box
(-70) GCTTGAATTAGACAGGATTAAAGGCT (-45) Wild Type	
GCTTGccggctACAGGATTAAAGGCT	Ret-1/PCE-I Mutant
GCTTGAATTAGACAGTcggccAGGCT	CRX-binding element (CBE) Mutant
GCTTGccggctACAGTcggccAGGCT	Ret-1/PCE-I+CBE Mutant

Figure 1. Sequences tested in promoter and binding assays. Mutations of the -70 to -45 region tested in both transient transfection promoter assays and electrophoretic mobility shift assays (EMSA). Sequences are the EMSA probes (sense strands). Mutations are represented by lower case letters.

The procedures for brain cell and fibroblast cultures were nearly identical except that tissues were minced in 0.25% trypsin in calcium- and magnesium-free Hanks’ Balanced Salt Solution (GIBCO-BRL, Grand Island, NY) to initiate trypsinization. Tissue was from E10 embryos and did not include pineal gland or cerebellum. Brain cells were cultured in medium supplemented with 20% rather than 10% FCS.

For CAT experiments, cells were harvested by scraping, centrifuged at 300 g for 2 min, medium removed, and the pellet resuspended in 100 µl of 0.1 M Tris-HCl pH 7.8. Suspensions were stored at -80° C until assayed for CAT activity. To determine CAT activity, cell suspensions were sonicated for 3 min on ice using a Vibracell cup-horn sonicator (Sonics & Materials, Danbury, CN), centrifuged, then the supernatant was transferred to fresh 1.5 ml conical tubes and heated at 65° C for 15 min. Tritiated acetyl-Co-enzyme A (New England Nuclear, Boston, MA) and chloramphenicol (Sigma Chemicals, St. Louis, MO) were added to the supernatants and this mixture added to an organic scintillation liquid (30). CAT activity, used as an index of promoter activity, was measured as the accumulation of ³H-acetylchloramphenicol in scintillation fluid over time on an LS6500 scintillation counter (Beckman Instruments, Schaumburg, IL).

The CAT plasmid vectors used were based on the

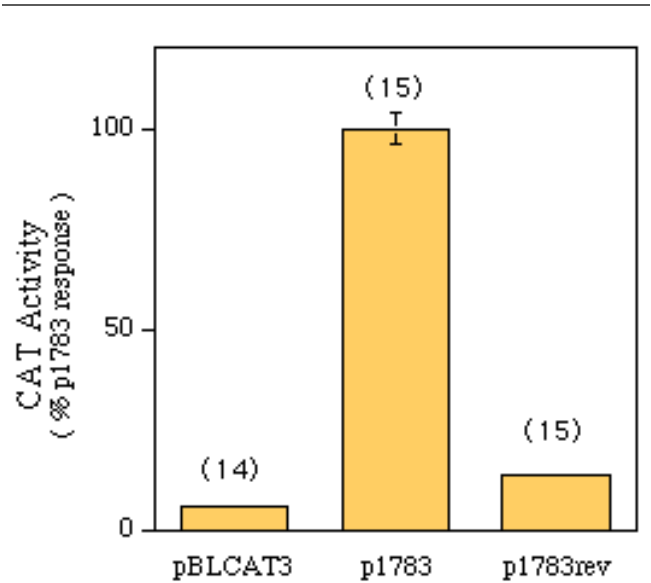


Figure 2. Orientation sensitivity of the IRBP promoter. A -1783 to +101 fragment of the murine IRBP promoter was inserted into the pBLCAT3 reporter vector either in the forward orientation (p1783) or the reverse orientation (p1783rev) and constructs transfected into primary cultures of embryonic day 10 chick retina cells as described in the text. Following four days of incubation, cells were harvested and assayed for CAT activity as a measure of promoter activity. The -1783/+101 insert stimulated CAT activity. Inverting the insert abolished most but not all of this activity. Activity with p1783 is significantly greater than that produced by transfection with the other plasmids (p < 0.05). Activity produced by p1783rev is greater than that produced by pBLCAT3 (p < 0.05). The data are from three experiments. Activities are means of percentage of p1783 ± SEM from individual experiments. Sample size (number of transfected dishes) is indicated in parentheses above error bars.

promoterless CAT vector pBLCAT3, which served as a negative control. The positive control contained the SV40 promoter and enhancer regions (pCAT Control; Promega, Madison, WI) and is referred to as pSV40. The first of the experimental plasmids tested was constructed by inserting into pBLCAT3 a BamHI fragment from -1783 to +101 (relative to transcription start) of the murine IRBP sequence (Genbank accession M32734). This plasmid is referred to as p1783. A nested set of deletions based on the -1783 to +101 fragment was prepared by the ExoIII/Mung bean nuclease method or by restriction digestion. The fragments generated and inserted into pBLCAT3 had 5' ends corresponding to -1173, -350, -204, -156, -70, and -45 of the murine IRBP gene and are referred to by these numbers.

For EGFP experiments, medium was aspirated off plated cells and glass cover slips gently laid directly onto them. Fluorescing cells were visualized through a Nikon Optiphot-2 microscope using B2-A and G1-A filters (Nikon Inc., Melville, NY) with attached camera. Photographic prints were scanned and digitized using a Bio-Rad GS-700 densitometer (Bio-Rad Laboratories, Hercules, CA) and an Apple Power Macintosh 9500/132 (Apple Computers, Inc., Cupertino, CA) running Photoshop 3.0 (Adobe Systems, Inc., San Jose, CA) and Fotolook 2.1 (Agfa, Gevaert, NV) imaging software.

The EGFP plasmid vectors were based on the promoterless pEGFP-1 reporter vector (Clontech Laboratories, Inc., Palo Alto, CA; GenBank Accession U55761), which served as negative control. The CMV promoter of pIC409 (a β -galactosidase reporter plasmid provided by Isabel Chiu and Jeremy Nathans) was PCR amplified with flanking Sall and BamHI sites and ligated into pEGFP-1. This vector served as positive control and is called pCMV-EGFP. An experimental plasmid called p1783-EGFP was constructed by inserting a

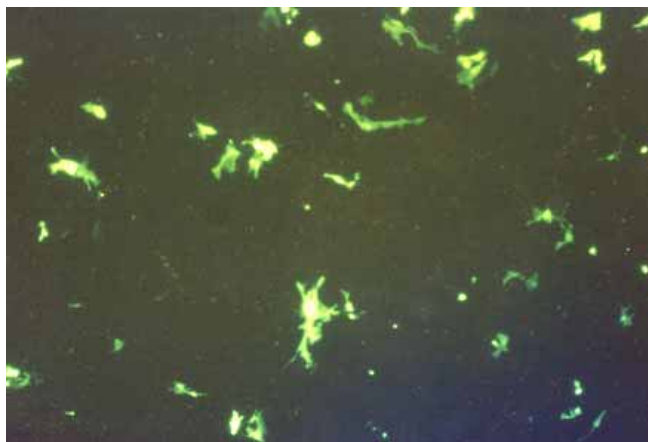


Figure 3. Transfection of E10 chick retinal cultures with the CMV promoter driving the enhanced green fluorescent protein (EGFP) gene. 10 μ g of the plasmid pCMV-EGFP, containing a PCR-amplified fragment of the CMV promoter cloned into the Sall and BamHI sites of pEGFP-1 (Clontech), was used to transfect cultures via calcium phosphate precipitation. Photo was taken two days after transfection. Hundreds to thousands of cells fluoresce brightly per 60 mm dish. Fluorescing cells exhibit a variety of morphologies, indicating that many types of cells (neurons, glia, photoreceptors, etc.) are synthesizing EGFP under the control of the CMV promoter.

BamHI fragment bases (-1783 to +101 relative to transcription start of the murine IRBP sequence) into pEGFP-1. Similarly, p70-EGFP was constructed by inserting a HindIII/BamHI fragment from the murine IRBP gene (-70 to +101 relative to transcription start) into pEGFP-1.

Electrophoretic mobility shift assays (EMSAs)—Mixtures of crude nuclear extracts and radiolabeled DNA probes were subjected to nondenaturing polyacrylamide gel electrophoresis (PAGE). Bound complexes were visualized by autoradiography. Assay conditions were optimized for each probe in terms of probe concentration (31) and competitor DNA or dye analogs (32) required to suppress nonspecific protein-DNA interactions or disperse protein aggregates.

The oligonucleotides used (sense strands shown in Figure 1) were synthesized at the Emory University Microchemical Facility. Both strands of a complementary pair were end-labeled with 32 P-gamma ATP polynucleotide T4 DNA kinase (GIBCO-

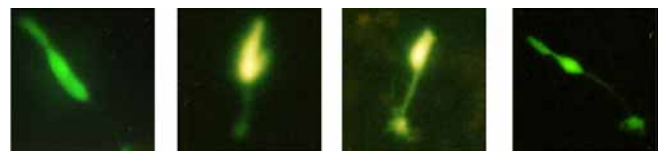


Figure 4a. Transfection of E10 chick retinal cultures with the IRBP promoter (-1783/+101 fragment) driving the green fluorescent protein (EGFP) gene. 10 μ g of the plasmid p1783-EGFP, containing the -1783 to +101 fragment of the mouse IRBP promoter cloned into the BamHI site of pEGFP-1 (Clontech), was used to transfect cultures via calcium phosphate precipitation. Photos were taken two days after transfection and are of four representative cells that fluoresce after transfection of the cultures. Only a few hundred cells fluoresce brightly per 60 mm dish, suggesting that transfection efficiency is low or that only a small fraction of cells in these cultures can express the promoter-reporter construct. Importantly, only cells with a morphology distinctly resembling photoreceptors fluoresce. Morphology suggests an axon with pedicle at the bottom, a cell body in the middle, and an inner segment with a rudimentary outer segment near the top of the photographs.

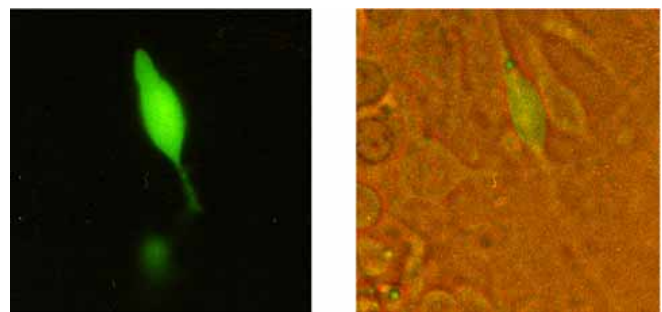


Figure 4b. Comparison of fluorescence and bright field of putative photoreceptor transfected with p1783-EGFP. The left photograph is with fluorescence lighting only. The right photograph is the same field with the transmission light turned up enough to observe an oil droplet in the same cell. The presence of an oil droplet is characteristic of photoreceptor cells in chickens.

BRL, Grand Island, NY) following the manufacturer's suggestions, then purified using a Pierce Xtreme™ oligonucleotide purification kit (Pierce, Rockford, IL). Complementary oligonucleotides were annealed by incubation together in 50 mM KCl for 5 min at 90° C followed by gradual cooling. The concentration of the DNA was determined by spectrophotometry and by fluorescence of intercalated Hoechst 33258 (Hoefer Scientific Instruments, San Francisco, CA).

Nuclear extracts from either adult mouse retina or cultured embryonic chick cells were prepared immediately before use by the method of Andrews and Faller (33). Cells were plated at E10 and incubated as above for 2-4 days before harvesting. Briefly, tissues or cells were homogenized at 4° C in 1.5 ml conical tubes using a hand-held homogenizer (Kontes, Vineland, NJ) in 10 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF and 200 µg/ml each leupeptin, pepstatin, and chymostatin (all compounds from Sigma Chemicals). Nuclei were allowed to swell on ice for 10 min then vortexed for 10 sec. Samples were spun in a microcentrifuge (14,000 g) for 10 sec and the supernatant discarded. The pellet was resuspended in 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol and protease inhibitors as above and incubated on ice for 20 min. The solution was cleared by centrifugation for 2 min at 14,000 g, 4° C. The supernatant was transferred to a fresh 1.5 ml conical tube and protein content determined by bicinchoninic acid assay (Pierce, Rockford, IL).

Each electrophoretic mobility shift assay (EMSA) reaction consisted of a double-stranded probe (~100 nM final concentration), 5 µg of nuclear protein extract, and a variable amount of nonspecific binding dispersant (poly dIdC or the protein-binding dyes R-478, T-128, or S-119 (Sigma Chemicals)) (32) incubated at room temperature for 15 min in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 2 mM DTT, 5% glycerol, and 60 mM KCl (34). Reactions were stopped with gel loading buffer (6x type III (35)). Samples were then electrophoresed on 5% nondenaturing polyacrylamide gels. The running and gel buffer was 250 mM glycine in 25 mM Tris-HCl, pH 8.3 (0.5x Tris-glycine buffer (35)). The gels were

dried overnight between sheets of cellophane and autoradiographed at -80° C for various lengths of time using Hyperfilm-MP (Amersham, Arlington Heights, IL). In some instances, radioactivity was imaged and digitized on a GS 525 Storage Phosphor Imaging system (Bio-Rad).

Statistics— CAT activity is presented as group mean ± standard error of the mean (SEM). Data were analyzed by simple analysis of variance followed by Newman-Keuls post-hoc analysis (36).

RESULTS

Activity of CMV and IRBP promoters in various cell types— We previously reported that a -1783 to +101 insert (relative to

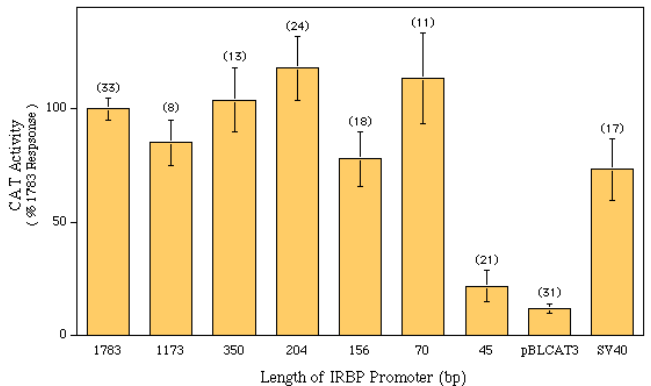


Figure 5. Reporter gene activity from a nested deletion series of the 5' flanking region of the murine IRBP promoter. Various lengths of the full-length (1783 bp) IRBP 5' flanking region were inserted into CAT plasmid constructs and transfected into primary cultures of embryonic day 10 chick retina cells as described in the text. The lengths of the promoter fragments are given below the activities. Following four days of incubation, cells were harvested and assayed for CAT activity as a measure of promoter activity. Only activities from p45 and pBLCAT3 are significantly less than that of p1783 (p < 0.05). The data are from nine experiments. Activities are means ± SEM of percentage of p1783 from individual experiments. Sample size is indicated in parentheses above error bars.

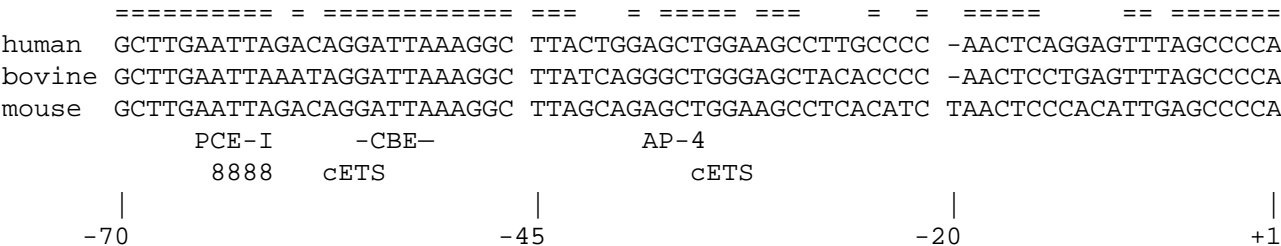


Figure 6. A multiple sequence alignment of the human, bovine, and mouse IRBP promoter regions. Shown here is the alignment from -70 to +1. The numbering shown corresponds to the mouse sequence. Identical bases are shown by the = above each nucleotide. Consensus sequence matches conserved among all three species are: the Ret-1/PCE-I element at -65 to -60, the CRX-binding element (CBE) at -55 to -50, an AP-4 consensus core sequence at -37 to -34, a cETS consensus core at -35 to -31 and another at positions -57 to -54, and an S8 homeodomain is shown by "8888" at -64 to -61. Only the core bases are marked. The criteria for searching the TRANSFAC Database by MatInspector were a match to the core sequence of at least 80% and to the entire consensus sequence of at least 85%. The Genbank entries for human, bovine, and mouse are X53044, M32733, and M32734, respectively.

transcription start) of the murine IRBP gene drives CAT reporter gene activity in primary cultures of chick retinal cells. We additionally report here that inversion of this insert causes significant loss of promoter activity in retinal cells (Figure 2), indicating that the promoter activity is unidirectional. We also reported that the SV40 but not the IRBP promoter was active in cultured brain cells and fibroblasts, suggesting that within retina cultures, the IRBP promoter may be specifically active in photoreceptor cells and not neurons or glial cells (20). To further characterize the cell types that support IRBP promoter activity within these cultures, we transfected E10 retinal cell cultures with EGFP plasmids containing the CMV promoter (pCMV-EGFP), the IRBP promoter (p1783-EGFP), or no promoter (pEGFP-1). No cells fluoresced in cultures transfected with pEGFP-1 (data not shown). Thousands of cells per plate fluoresced in cultures transfected with pCMV-EGFP (Figure 3). These cells were of varied morphology including round, multipolar, and amorphous shapes (Figure 3). Conversely, in cultures transfected with p1783-EGFP, only a few hundred cells fluoresced per plate. With the exception of a few round or undefined cells (not shown), these cells bore the morphological characteristics of photoreceptors (29,37): a synaptic pedicle attached to a single process leading to an elongated shape containing an oil droplet (Figure 4). In no instance were cells seen that were clearly neuron-like (multipolar) or glia-like (large, faintly-fluorescing, and amorphous).

Defining a minimal regulated control region of the murine IRBP promoter— Transfections with various truncations of the full-length promoter (here defined as the -1783/+101 fragment) show that promoter fragments of 70 bases (5' to the Cap site) or longer produce statistically indistinguishable high levels of activity (Figure 5). The 45 base fragment showed a slight but statistically insignificant elevation in activity over promoterless vector activity, and may be a basal promoter.

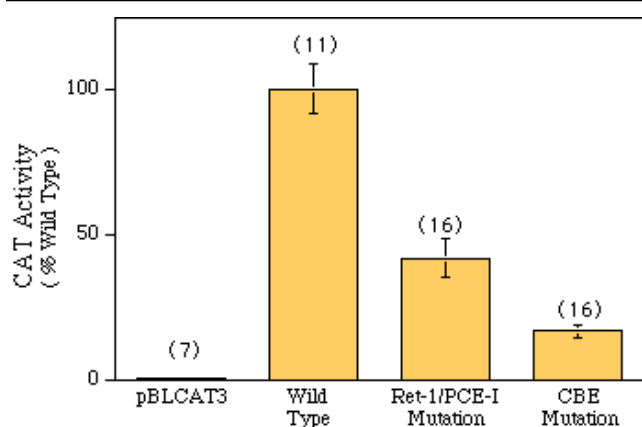


Figure 7. Effect of transversion mutations of Ret-1/PCE-I or the CRX-binding element on reporter gene activity. A -70 to +101 fragment of the IRBP gene was inserted into pBLCAT3 (p70; "Wild Type") with or without transversion mutations of the two proposed cis elements. Mutation of either sequence significantly inhibited CAT activity ($p < 0.05$), though not to background. The data are from two experiments. Activities are means \pm SEM of percentage of Wild Type from individual experiments. Sample size is indicated in parentheses above error bars.

These results suggest that the cis elements and promoter sequences required for full IRBP promoter activity are located within 70 bases of the Cap site, and that an activator resides between -70 and -45.

Figure 6 shows an alignment of three mammalian IRBP 5' flanking regions in the -70 to -45 area for the human, bovine, and mouse genes. For all three species, this area contains a Ret-1/PCE-I sequence (AATTAG), which is proposed to be a retina-specific consensus cis element (21,22). It also contains a CRX-binding element just downstream from Ret-1/PCE-I (GATTAA). Note that the CRX-binding element is a same-strand reverse copy of Ret-1/PCE-I. To determine whether either of these elements is important for the high promoter activity, we mutated them with transversion substitutions ($G \rightleftharpoons T$ and $A \rightleftharpoons C$) in vector inserts (Figure 1 and Figure 7) and transiently transfected the mutant sequences into chick retina cell cultures. Mutation of the Ret-1/PCE-I sequence reduced activity by 58% whereas mutation of the CRX-binding element reduced activity 83% (Figure 7). These results indicate that each element is required for high levels of promoter activity.

In previous work, we found that the full-length (1783 bp) promoter was active in retina cells but not brain cells or fibroblasts (20). To determine whether the truncated 70 bp promoter retained this tissue specificity, we transfected brain cells and fibroblasts in primary culture with a CAT reporter construct containing the -70 to +101 IRBP fragment (p70). Transfection with p70 produced CAT activity in brain cells, but not in fibroblasts (Figure 8), indicating that additional

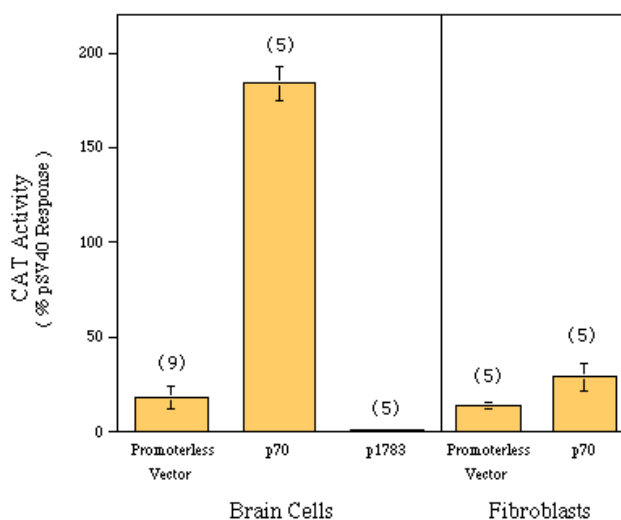


Figure 8. Reporter gene activity is driven by the 70 bp promoter in brain cells but not fibroblasts. Cells from E10 brain tissue (excluding pineal gland and cerebellum) and chick limb buds ("Fibroblasts") were transfected with pSV40, p70, or pBLCAT3 ("Promoterless Vector"). Brain cells additionally were transfected with p1783. The SV40 promoter drove activity in both cell types. In brain cells, the full-length promoter did not stimulate CAT activity, but the truncated promoter did compared to Promoterless Vector ($p < 0.05$). The truncated promoter had minimal activity in fibroblasts. Activities are means \pm SEM of percentage of the response to pSV40 from individual experiments.

regulatory elements involved in tissue-specificity reside 5' to the -70 position. To further test this, retina cell cultures were transfected with an EGFP construct containing the -70 to +101 IRBP fragment. The -70/+101 fragment drove EGFP expression in cells bearing morphological characteristics of photoreceptors (Figure 9). Additionally, cells with multiple processes characteristic of neurons fluoresced (Figure 9c). No glia-like cells fluoresced. These results parallel those of the CAT construct experiments that demonstrate that the -1783/+101 fragment acts as a promoter in primary cultures of retina cells but not brain cells or fibroblasts, whereas the -70/+101 fragment acts as a promoter in retina and brain cells but not fibroblasts.



Figure 9a. Transfection of E10 chick retinal cultures with truncated IRBP promoter (-70/+101 fragment) driving the enhanced green fluorescent protein (EGFP) gene. Primary cultures of E10 chick retina cells were transiently transfected via calcium phosphate precipitation with 10 μ g of the plasmid p70-EGFP (a HindIII/BamHI -70 to +101 fragment of the mouse IRBP promoter cloned into pEGFP-1). Photos were taken three days after transfection. Photographs are of three representative cells that fluoresce after transfection of the cultures. Morphology suggests an axon with pedicle at the bottom, a cell body in the middle, and an inner segment with a rudimentary outer segment near the top of the photographs.

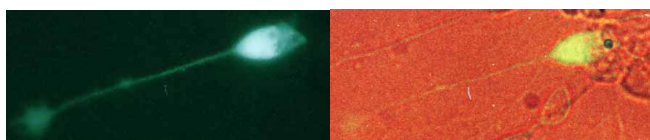


Figure 9b. Comparison of fluorescence and bright field exposure of putative photoreceptor transfected with p70-EGFP. The left photograph is with fluorescence lighting only. The right photograph is the same field with the transmission light turned up enough to observe an oil droplet in the same cell. The presence of an oil droplet is characteristic of photoreceptor cells in chickens.

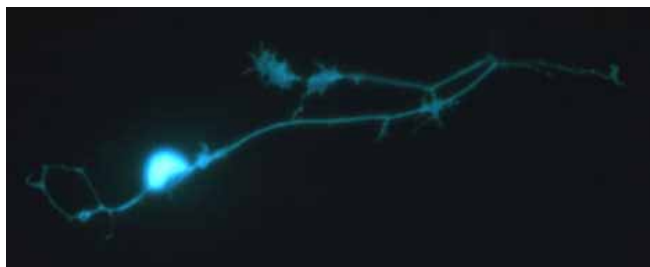


Figure 9c. Photograph of typical multipolar, neuron-like cell. Hundreds of multipolar cells with extensive arborization and lacking oil droplets fluoresced in retinal cultures transfected with p70-EGFP.

Evidence for nuclear protein binding in the activator region— Four DNA fragments were used in characterizing the ability of the -70 to -45 region to bind nuclear proteins: the wild type -70 to -45 activator sequence without mutation, a second -70 to -45 fragment in which both Ret-1/PCE-I and the CRX-binding element were mutated, a third fragment with only Ret-1/PCE-I mutated, and a final fragment with only the CRX-binding element mutated (Figure 1). Several bands appear in autoradiographs of EMSAs in which a radiolabeled wild type activator sequence was used to probe against nuclear extracts from chick retinal cells or mouse retina, indicating that the activator is a target for trans-acting complexes. However, when both Ret-1/PCE-I and the CRX-binding element were mutated by transversion, a band was lost (Figure 10). Note that a band apparently unique to the mutant corresponds to a band seen in lanes without extract, suggesting that it is artifactual. Banding patterns identical to those of Figure 10 were found regardless of the type and concentration of nonspecific competitor used to disperse protein aggregates (data not shown). Probing against mouse retina extracts produced sequence-specific banding patterns similar to those found with chick cell extracts (Figure 11). Co-incubating labeled wild type with increasing concentrations of unlabeled

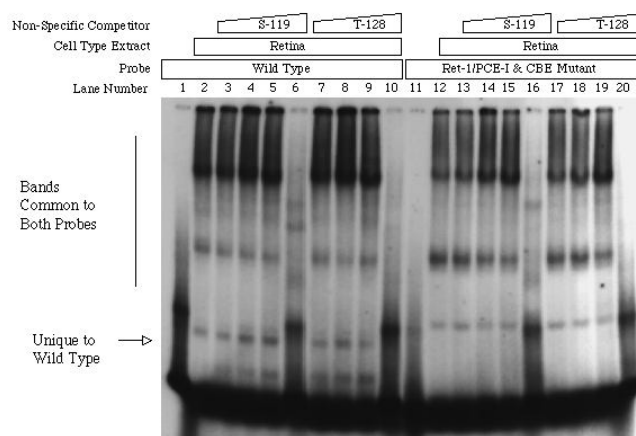


Figure 10. EMSA of wild-type and mutant activator sequence probing against extracts from cultured chick retinal cells. The -70 to -45 sequence of the murine IRBP promoter, with or without base transversions in Ret-1/PCE-I and the CRX-binding element (CBE) (see Figure 1) were radiolabeled and incubated with nuclear extracts from E10 retina cells plated four days prior to harvesting. Incubates were then electrophoresed across 5% nondenaturing polyacrylamide gels. Radioactivity was later visualized by autoradiography. Bands appeared in lanes containing both probes, however, one band (arrow) was present specifically in Wild Type lanes. A band that appears specific for the mutant is also in lanes without extract (lanes 1 and 11), suggesting that it is artifactual. Four types of nonspecific DNA-binding competitors were tested at increasing concentrations (0, 0.0625, 0.625, 6.25, and 62.5 ng/ μ l) to determine the effect of dispersing nonspecific protein aggregates. Results with all dispersants were similar; the polymeric dyes S-119 and T-128 were used in this representative experiment. The activator-specific band is seen at all concentrations of dispersant except for the highest (lanes 6, 10, 16, and 20), a condition in which probably all binding is precluded. Note that other bands that appear to be wild type-specific did not reliably appear in all experiments.

sequence containing mutations of both elements had little effect on the intensity of the activator-specific band (Figure 12), but did cause the loss of bands commonly bound by both wild type and mutant probes seen in Figure 10. These data indicate that the activator binds a sequence-specific complex in nuclear extracts from chicken retina cells and mouse retinas.

Probes with either Ret-1/PCE-I or the CRX-binding element mutated individually did not bind the activator-specific complex (lanes 13-20, Figure 13). Co-incubation of radiolabeled wild type activator sequence with either unlabeled mutant did not alter the activator-specific band (lanes 2-12, Figure 13), but did cause the loss of bands commonly bound by both wild type and mutant probes as seen in Figure 10. This suggests that both Ret-1/PCE-I and the CRX-binding element, rather than just one or the other, are required for binding of the activator-specific complex. These results are similar to those from transient transfection assays (Figure 7) in that mutation of either element causes loss of promoter activity.

To determine the tissue specificity of activator-specific binding, nuclear extracts from cultured brain cells and fibroblasts were probed with the wild type sequence. The activator-specific band appeared in lanes probing retina and brain extracts, but not fibroblasts (Figure 14). This parallels the promoter activity tissue-specificity seen with p70 transfections (Figure 8). An additional band whose migration differs from the activator-specific band appeared in lanes probing fibroblasts (Figure 14).

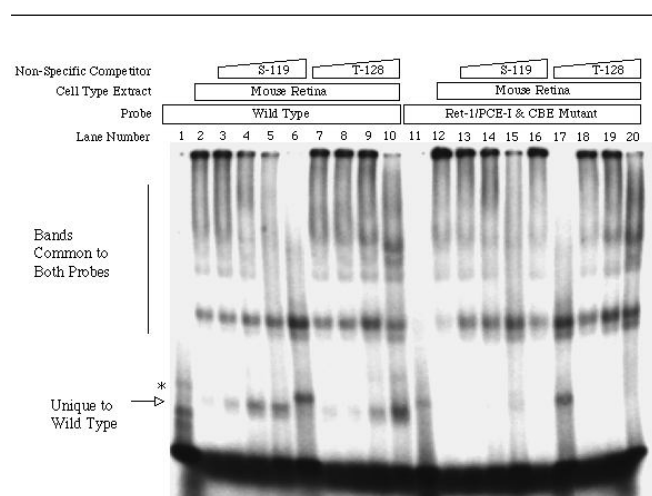


Figure 11. EMSA of wild-type and mutant activator sequence probing against extracts from whole mouse retina. This experiment was conducted identically to the previous experiment (Figure 10), except extracts probed were from mouse retina tissue rather than chick retinal cell cultures; results are similar. Both probes (the -70 to -45 sequence of the murine IRBP promoter, with or without base transversions in Ret-1/PCE-I and the CRX-binding element [CBE]) bind many complexes with similar migration patterns. The wild type probe binds a complex (arrow) that appears just below the artifactual band (asterisk) seen in lanes with probe alone (lanes 1 and 11) and in lanes where protein binding is precluded by high concentrations of dispersant.

DISCUSSION

The regulation of photoreceptor gene expression is being intensely pursued through several strategies, including the use of transgenic mice, transfection of immortalized cells, and in vitro binding assays. We recently reported the usefulness of transient transfection of primary chicken retinal cell cultures with DNA plasmid reporter constructs in this pursuit (20). The current data further establish the validity of this protocol. In retina, IRBP is expressed only in photoreceptors (12,13). In the primary chick retina cell cultures, the CMV promoter drove EGFP expression in many morphologically-distinct cell types, whereas the mouse IRBP promoter drove expression in cells bearing the morphological characteristics of photoreceptors and occasionally in small, round cells. This indicates that the transfected mouse IRBP promoter is active selectively in the photoreceptor cells of the primary chicken retinal cell cultures, paralleling the in vivo expression of IRBP.

Recent studies from our laboratories and others have demonstrated that 123 to 156 bases of the 5' flanking region is sufficient for sporadically regulated promoter activity for either the human (18,19) or murine (38,39) IRBP gene. The present data advance our understanding of the IRBP promoter by demonstrating a minimal regulated control region of only 70 bases. Further, not all of this region is required for full activity. The sequence between -70 and -45 is critical for high activity, while the -45 to Cap site region may be a basal promoter. The -70 to -45 activator contains a Ret-1/PCE-I element and a CRX-binding element, both proposed to be photoreceptor-specific cis elements (19,21-27). Mutation of either of these sites causes loss of promoter activity in transient transfection assays and binding activity in EMSAs. This suggests that these sequences are cis-acting elements required for MRCR activity.

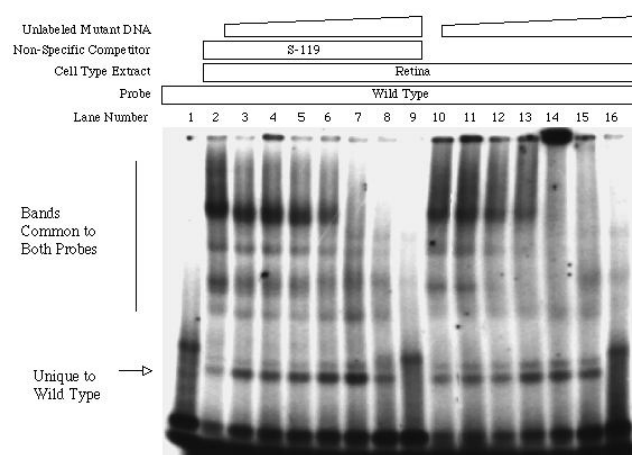


Figure 12. EMSA of the effects of co-incubation of wild type probe with increasing concentrations of mutant. Radiolabeled wild type probe was co-incubated with increasing concentrations (0, 0.1, 1, 10, 100, 1000 fold excess) of unlabeled probe that had both Ret-1/PCE-I and the CRX-binding element mutated. The upper bands apparent with both probes in Figure 10 disappear with increasing concentration of competitor. The lower, activator-specific band did not diminish until competitor was at 1000 fold. Co-incubation with the dispersant S-119 had little effect on this response.

Although the MRCR provides activity equivalent to the full-length promoter, it is not fully regulated. The -1783/+101 fragment provides high activity in cultured retina cells, but not in brain cells or fibroblasts (20). The -70/+101 fragment provides high activity in both retina (Figure 5) and brain cells but not fibroblasts (Figure 8). Additionally, the -70/+101 fragment drives EGFP expression in cells bearing morphological characteristics of photoreceptors and cells with multiple processes characteristic of neurons (Figure 9), but not in glia-like cells. These results indicate that truncation down to 70 bases causes loss of regulatory factors that suppress expression in neurons. This suggests the existence of an inhibitory regulatory mechanism common to many non-retinal cell types. As we and others have found evidence for tissue specificity regulation within the first 123 to 156 bases of the Cap site (18,19,38,39), it may be that this lost regulatory mechanism requires a cis element within the -123 to -70 sequence. A CLUSTAL W (40) multiple sequence alignment of the human, bovine, and murine IRBP 5' flanking regions shows that there are many sequences between -164 and +1

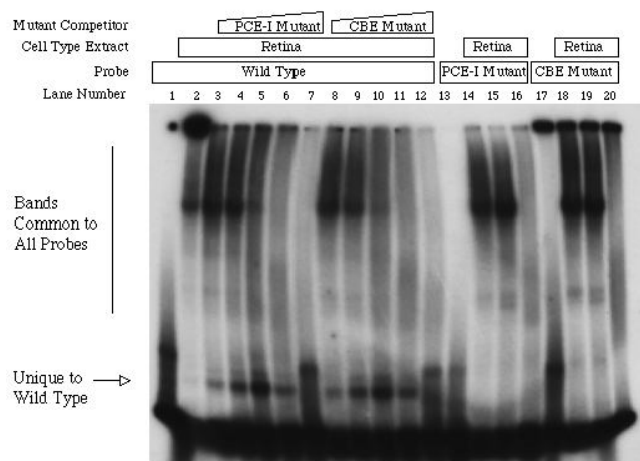


Figure 13. EMSA of the effects of transversion mutation of either Ret-1/PCE-I or the CRX-binding element. Radiolabeled wild type probe was co-incubated with increasing concentrations of unlabeled activator sequence with either Ret-1/PCE-I mutated or the CRX-binding element (CBE) mutated. As with the experiment in Figure 12, complexes common to wild type and mutant sequence diminished with an increasing excess of unlabeled mutant probe. The intensity of the wild type-specific band lessened only at the highest concentration of either mutant. These data indicate that both putative elements must be intact for the activator-specific complex to bind. The last eight lanes (13-20) have one or the other of the mutants as radiolabeled probe. Lane 13 is the probe with Ret-1/PCE-I mutated (intact CRX-binding element) without extract. Lane 14 is this probe incubated with extract. Lane 15 is lane 14 conditions with 6.25 µg/µl S-119. Lane 16 is lane 15 conditions co-incubated with a 100 fold excess of unlabeled activator sequences with the CRX-binding element mutated (Ret-1/PCE-I intact). Lanes 17-20 are the same as lanes 13-16 except the roles are reversed for the two mutant sequences. Lanes 14, 15, 18, and 19 have upper bands common to all probes, but lack the activator-specific probe. These data indicate that both putative elements must be intact for the activator-specific complex to bind.

that are highly conserved. Using MatInspector (41) to search the TRANSFAC database (42) for matches with these conserved sequences reveals the existence of an estrogen receptor consensus sequence at -112 to -109 and a RORA1 retinoic acid receptor site at -118 to -109 (with core at -113 to -110). These sequences are completely conserved across species, and their appearance by chance is but every 2250 nucleotides. Additionally, DNA probes containing these sites in human, murine, and bovine IRBP promoters bind nuclear factors from several tissues in *in vitro* binding assays (19,25; unpublished data). These considerations and data suggest that the sites are cis-acting elements, raising the possibility that they contribute to the tissue specificity that is lost with their truncation.

We also recently found that a CpG dinucleotide at -114 is hypomethylated in photoreceptor DNA compared to DNA from other tissues (unpublished data). DNA probes containing this site bind nuclear extracts from many tissues, and this is suppressed by DNA methylation. Exogenous methylation of this site and another at -725 suppresses promoter activity (unpublished data). It may be that methylation at this site in non-photoreceptor cells suppresses promoter activity, suggesting an alternative regulatory role for the -123 to -70 region.

It has been proposed that Ret-1/PCE-I and the CRX-binding element together confer tissue-specific expression upon the IRBP gene (19). The data of that study and the results presented here are not contradictory, but the conclusion of that study is not supported entirely by our results. In the previous report, data from one transgenic mouse indicated that an IRBP fragment out to -123 bases, which contains the two putative cis elements, confers absolute photoreceptor specificity within

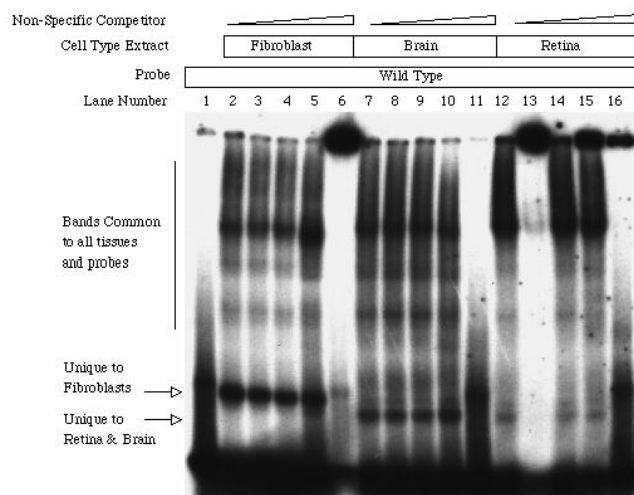


Figure 14. EMSA showing tissue specificity of activator-specific binding. Radiolabeled wild type activator sequence was incubated with nuclear extracts from cultured fibroblast, brain, and retina cells. The activator-specific band appeared in lanes with retina and brain extract. A band whose mobility differed from the activator-specific band appeared in fibroblast lanes. Lanes 6 and 13 ran anomalously and were discounted from analysis.

the retina (19), a result not contradicted by the present data. However, our 70 bp IRBP promoter fragment, which also contains these elements, drives reporter gene activity in brain cells. The tissue specificity ascribed to the two elements in the earlier study was based upon in vitro DNA:protein binding assays probing against extracts from immortalized cell lines of retinoblastomas, which produce IRBP, versus HeLa cells and lymphocytes, which do not (19). Our results parallel and extend these data: DNA probes containing the two elements bind specific nuclear factors from both retina and brain cells, but not fibroblasts. Extracts from cells or tissue more similar to photoreceptors, such as neurons or neuroblastomas, were not probed in the earlier study (19), precluding detection of possible neuron-specific DNA-binding complexes. Both sets of data are consistent with a mechanism in which the two-element activator region binds permissive or coordinating factors that, in conjunction with elements in the -123 to -70 region, regulate absolute tissue specificity.

In addition to tissue specificity, the activator may regulate temporal specificity. The CRX-binding element clearly binds an Otx-like homeodomain protein (26,27). This binding appears to allow IRBP transcription in non-expressing cells (26,27) and the data presented here indicate that an intact CRX-binding element is required for promoter activity in expressing cells. Additionally, the Ret-1/PCE-I consensus sequence is remarkably similar to the S8 homeodomain consensus, ANYYAATTAARC (43), which also resembles the Antp-homeodomain consensus sequence. The S8 protein is specific to mesodermal cells, but the binding activity also is found in neuroblastoma cells. Most homeodomain proteins bind the ATTA core (44). The IRBP Ret-1/PCE-I and CRX-binding element exhibit the same ATTA core in each motif. The two sites are spaced 10 nucleotides apart, or one DNA helix turn, so that they are perfectly aligned on one side, which would be advantageous for concurrent protein binding. It may be that two homeodomain proteins (or one large one) bind to the two ATTA sites simultaneously. Our EMSA data suggest that both sites bind the same protein complex (Figure 7 and Figure 13). Conversely, EMSA experiments using purified GST-CRX fusion protein show that Ret-1/PCE-I binding is much weaker than binding with the CRX-binding element (26). Our promoter assays indicate that mutating the CRX-binding element profoundly suppresses transcription, whereas mutating Ret-1/PCE-I has a less dramatic effect. Though purely speculative, it may be that the Ret-1/PCE-I and CRX-binding elements of the MRCR bind distinct homeodomain proteins that comprise a heterodimeric trans-acting complex.

ACKNOWLEDGEMENTS

This work was supported by Research to Prevent Blindness, Inc.; NIH RO1 EY09378 and P30 06360 to JMN; NIH F32 EY06409 to JHB; a Fight for Sight Grant-in-Aid to JHB; and a Fight for Sight Student Fellowship to CLE.

The authors gratefully acknowledge discussions of cell culture techniques with Dr. P. M. Iuvone and the help of Ms. Joy Wu in preparing some of the plasmids.

Preliminary reports of some of these data were presented at the annual meeting of the Association for Research in Vision and Ophthalmology in 1993, 1994, and 1996 (23-25).

REFERENCES

1. Bunt-Milam AH, Saari JC. Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. *J Cell Bio* 1983; 97:703-712.
2. Carlson A, Bok D. Promotion of the release of 11-cis-retinal from cultured retinal pigment epithelium by interphotoreceptor retinoid-binding protein. *Biochemistry* 1992; 31:9056-9062.
3. Okajima TI, Pepperberg DR, Ripps H, Wiggert B, Chader GJ. Interphotoreceptor retinoid-binding protein: role in delivery of retinol to the pigment epithelium. *Exp Eye Res* 1989; 49:629-644.
4. Okajima TI, Pepperberg DR, Ripps H, Wiggert B, Chader GJ. Interphotoreceptor retinoid-binding protein promotes rhodopsin regeneration in toad photoreceptors. *Proc Natl Acad Sci U S A* 1990; 87:6907-6911.
5. Carter-Dawson L, Alvarez RA, Fong SL, Liou GI, Sperling HG, Bridges CD. Rhodopsin, 11-cis vitamin A, and interstitial retinol-binding protein (IRBP) during retinal development in normal and rd mutant mice. *Dev Biol.* 1986; 116:431-438.
6. Timmers AM, Newton BR, Hauswirth WW. Synthesis and stability of retinal photoreceptor mRNAs are coordinately regulated during bovine fetal development. *Exp Eye Res* 1993; 56:257-265.
7. van Veen T, Ekstrom P, Wiggert B, Lee L, Hirose Y, Sanyal S, Chader GJ A developmental study of interphotoreceptor retinoid-binding protein (IRBP) in single and double homozygous rd and rds mutant mouse retinæ. *Exp Eye Res* 1988; 47:291-305.
8. Wiggert B, Mizukawa A, Kuwabara T, Chader GJ. Vitamin A receptors: multiple species in retina and brain and possible compartmentalization in retinal photoreceptors. *J Neurochem* 1978; 30:653-659.
9. Eisenfeld AJ, Bunt-Milam AH, Saari JC. Immunocytochemical localization of retinoid-binding proteins in developing normal and RCS rats. *Prog Clin Biol Res* 1985; 190:231-240.
10. Gonzalez-Fernandez F, Van Niel E, Edmonds C, Beaver H, Nickerson JM, Garcia-Fernandez JM, Campohiaro PA, Foster RG. Differential expression of interphotoreceptor retinoid-binding protein, opsin, cellular retinaldehyde-binding protein, and basic fibroblastic growth factor. *Exp Eye Res* 1993; 56:411-427.
11. Liou GI, Wang M, Matragoon S. Timing of interphotoreceptor retinoid-binding protein (IRBP) gene expression and hypomethylation in developing mouse retina. *Dev Biol* 1994; 161:345-356.
12. Porrello K, Bhat SP, Bok D. Detection of interphotoreceptor retinoid binding protein (IRBP) mRNA in human and cone-dominant squirrel retinas by in situ hybridization. *J Histochem Cytochem* 1991; 39:171-176.
13. van Veen T, Katial A, Shinohara T, Barrett DJ, Wiggert B, Chader GJ, Nickerson JM. Retinal photoreceptor neurons and pinealocytes accumulate mRNA for interphotoreceptor retinoid-binding protein (IRBP). *FEBS Lett* 1986; 208:133-137.
14. Kutty G, Duncan T, Nickerson JM, Si JS, Van Veen T, Chader GJ, Wiggert B. Light deprivation profoundly affects gene

- expression of interphotoreceptor retinoid-binding protein in the mouse eye. *Exp Eye Res* 1994; 58:65-75.
15. Rajendran R, Niel E, Stenkamp D, Cunningham L, Raymond P, Gonzalez-Fernandez F. Zebrafish interphotoreceptor retinoid-binding protein: differential circadian expression among cone subtypes. *J Exp Biol* 1996; 199:2775-2787.
 16. Buratowski S. The basics of basal transcription by RNA polymerase II. *Cell* 1994; 77:1-3.
 17. Goodrich JA, Cutler G, Tjian R. Contacts in context: Promoter specificity and macromolecular interactions in transcription. *Cell* 1996; 84:825-830.
 18. Liou GI, Matragoon S, Overbeek PA, Fei Y. Identification of a retina-specific footprint within the retina-specific regulatory region of the human interphotoreceptor retinoid-binding protein gene. *Biophys Res Comm* 1994; 203:1875-1881.
 19. Bobola N, Hirsch E, Albin A, Altruda F, Noonan D, Ravazzolo R. A single cis-acting element in a short promoter segment of the gene encoding the interphotoreceptor retinoid-binding protein confers tissue-specific expression. *J Biol Chem* 1995; 270:1289-1294.
 20. Boatright JH, Buono R, Bruno J, Lang RK, Si JS, Shinohara T, Peoples JW, Nickerson JM. The 5' flanking regions of IRBP and arrestin have promoter activity in primary embryonic chicken retina cell cultures. *Exp Eye Res* 1997; 64:269-277.
 21. Kikuchi T, Raju K, Breitman ML, Shinohara T. The proximal promoter of the mouse arrestin gene directs gene expression in photoreceptor cells and contains an evolutionarily conserved retinal factor-binding site. *Mol Cell Biol* 1993; 13:4400-4408.
 22. Morabito MA, Yu X, Barnstable CJ. Characterization of developmentally regulated and retina-specific nuclear protein binding to a site in the upstream region of the rat opsin gene. *J Biol Chem* 1991; 266:9667-9672.
 23. Boatright JH, Borst DE, Nagpal S, Ram H, Nickerson JM. Characterization of trans-acting factors and cis-acting elements in the IRBP gene. *Invest Ophthalmol Vis Sci* 1994; 35:1578.
 24. Boatright JH, Si JS, Patel D, Peoples J, Nickerson JM. Functional Characterization of a photoreceptor cis-acting element. *Invest Ophthalmol Vis Sci* 1996; 37:S464.
 25. Boatright JH, Peoples JW, Edwards CL, Borst DE, Si JS, Nickerson JM. The minimal regulated control region of the IRBP gene. *Invest Ophthalmol Vis Sci* 1997; 38:S241.
 26. Furukawa T, Morrow EM, Cepko CL. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* 1997; 91:531-541.
 27. Chen S, Wang QL, Nie Z, Sun H, Lennon G, Copeland NG, Gilbert DJ, Jenkins NA, Zack DJ. Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* 1997; 19:1017-1030.
 28. Werner M, Madreperla S, Lieberman P, Adler R. Expression of transfected genes by differentiated, postmitotic neurons and photoreceptors in primary cell cultures. *J Neurosci Res* 1990; 25:50-57.
 29. Adler R, Magistretti PJ, Hyndman AG, Shoemaker WJ. Purification and cytochemical identification of neuronal and non-neuronal cells in chick embryo retina cultures. *Dev Neurosci* 1982; 5:27-39.
 30. Neumann JR, Morency CA, Russian KO. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* 1987; 5:444-448.
 31. Hennighausen L, Lubon H. Interaction of protein with DNA in vitro. *Methods Enzymol* 1987; 152:721-735.
 32. Lee TC, Schwartz RJ. Novel use of a polymeric chromophore in detecting sequence-specific DNA-binding proteins. *Biotechniques* 1993; 15:390-393.
 33. Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 1991; 19:2499.
 34. Lee TC, Schwartz RJ. Using proteases to avoid false identification of DNA-protein complexes in gel shift assays. *Biotechniques* 1992; 12:486-490.
 35. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Press; 1989.
 36. Glantz SA. *Primer of Biostatistics*. New York: McGraw-Hill; 1997.
 37. Politi LE, Adler R. Generation of enriched populations of cultured photoreceptor cells. *Invest Ophthalmol Vis Sci* 1986; 27:656-665.
 38. Borst DE, Si JS, Nickerson JM. Transient Expression of IRBP promoter/CAT constructs in retinoblastoma cells. *Invest Ophthalmol Vis Sci* 1990; 31[Suppl]:585.
 39. Borst DE, Si JS, Nickerson JM. The mouse IRBP promoter: functional assays for activity. *Invest Ophthalmol Vis Sci* 1991; 32:1011.
 40. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; 22:4673-4680.
 41. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995; 23:4878-4884.
 42. Wingender E, Dietze P, Karas H, Knuppel R. TRANSFAC: a database on transcription factors and their DNA binding sites. *Nucleic Acids Res* 1996; 24:238-241.
 43. de Jong R, van der Heijden J, Meijlink F. DNA-binding specificity of the S8 homeodomain. *Nucleic Acids Res* 1993; 21:4711-4720.
 44. Laughon A. DNA binding specificity of homeodomains. *Biochemistry* 1991; 30:11357-11367.

Erratum— 30 Dec 1997: In **REFERENCES**, the original reference 25 “Boatright JH, Ram H, Nickerson JM. Protein and DNA complexes in the distal 5' flanking region of the IRBP gene. *Invest Ophthalmol Vis Sci* 1993; 34:830.” was corrected to “Boatright JH, Peoples JW, Edwards CL, Borst DE, Si JS, Nickerson JM. The minimal regulated control region of the IRBP gene. *Invest Ophthalmol Vis Sci* 1997; 38:S241.”