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# Functional Dissection of the Promoter of the Interphotoreceptor Retinoid-Binding Protein Gene: The Cone-Rod-Homeobox Element Is Essential for Photoreceptor-Specific Expression *In Vivo*[1]

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**The essential control elements in the interphotoreceptor retinoid-binding protein gene (*IRBP*) promoter are located between −156 and +19. The −156/−109 sequence contains a retina-specific DNAse I footprint and shows a positive regulatory activity in transiently transfected retinoblastoma cells. The −105/−85 sequence is G/C rich, shows a non-tissue specific DNAse I hypersensitivity, and a negative regulatory activity in retinoblastoma cells. The −76/−42 sequence shows a retinal-specific footprint and contains a "cone-rod-homeobox element" (CRXE) and a "photoreceptor conserved element" (PCE). *IRBP* promoter fragments with mutations in either CRXE, PCE or in both were linked to reporter genes and analyzed both by transient transfection and in transgenic mice. In retinoblastoma cells, the mutated CRXE-containing promoter shows a 60% repression of the CAT activity whereas the mutated PCE-containing promoter shows a 30% repression. In HeLa cells transfected with these promoters, co-transfection of a *Crx* expression vector with wild-type, but not with CRXE mutant promoter, activates CAT activity 20-fold over the background activity. Mutation of PCE alone or conversion of CRXE to PCE reduces this Crx-activated CAT activity to only 4-fold over the background activity. In the transgenic mouse experiments, none of the 12 lines with CRXE mutant promoter show significant expression of *lacZ* in the retina. In contrast, 9 of the 17 transgenic lines with PCE mutant promoter show photoreceptor-specific *lacZ* expression. Thus the Crx interaction with CRXE is essential for the photoreceptor-specific activity of the *IRBP* promoter *in vivo*. This interaction does not appear to require PCE, but is enhanced when PCE is present.**

**Key words: *IRBP* gene regulation, photoreceptor-specific, CRXE, PCE.**

Normal development of mammalian retina is essential for the establishment of a multi-layered structure capable of conducting the visual process. The development of retina from the embryonic neuroepithelium consisting of common retinal progenitor cells involves a coordinated process of mitotic division, cell fate determination, cell mitration, and differentiation (*1, 2*), and requires a complex genetic program that determines gene expression in specific cell types at specific stages of development. To understand the molecular basis governing retinal development it is necessary to identify genes expressed during development and elucidate the regulatory mechanisms controlling their differential expression in the developing retina. Several homeo- and paired-box containing genes encoding homeodomain proteins have been found playing a critical role in retinal development (*3-5*). These genes, functioning as transcription factors, control retinal development through the regulation of their downstream specific target genes. Identification of these genes and characterization of their regulation will provide insights not only into the fundamental process of retinal development but also into the molecular pathogenesis of congenital and inherited retinal diseases (*6, 7*).

In retinal photoreceptor cells, the *IRBP* gene expression begins during the early stages of differentiation prior to outer segment formation (*8*). By comparison, proteins involved in visual transduction, such as opsin and α-trans-

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Abbreviations: IRBP, interphotoreceptor retinoid-binding protein; CRXE, cone-rod-homeobox element; PCE, photoreceptor conserved element; bp, base pair(s); CAT, chloramphenicol acetyl transferase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; β-Gal or *lacZ*, gene encoding β-galactosidase.

ducin, are expressed after outer segment formation has begun (*9, 10*). The *IRBP* gene may therefore represent a model for investigating regulation of expression of early photoreceptor genes. IRBP is a retinoid- and fatty acid-binding glycoprotein found in the interphotoreceptor matrix between photoreceptor and retinal pigment epithelial cells, in pinealocytes and in several retinoblastoma-derived cell lines (reviewed in Ref. *11*). It is believed that IRBP serves as a retinoid transporter during the bleaching and regeneration of visual pigments (*11-16*), or as a "buffer" protein to prevent the degradation of retinoids and to protect cell membranes from retinoids that are released after light bleaching (*17*). The importance of IRBP for retinal development has been demonstrated by the recent generation of mice with a targeted disruption of the *IRBP* gene, which demonstrated early-onset photoreceptor abnormalities (*18*).

As the early expression of the *IRBP* gene is critical for normal photoreceptor development and function, it is important to understand how the IRBP gene expression is regulated. During terminal differentiation tissue-specific gene expression is regulated at the transcriptional level. Gene expression is mediated by the interactions of *trans*-acting factors such as homeodomain transcription factors and their target *cis*-acting elements located in the 5′ region of the gene. Thus the first step toward understanding the regulatory mechanisms of gene expression is to identify and characterize these *cis*-elements and *trans*-acting factors. Genomic sequences upstream from the *IRBP* gene are conserved in divergent mammalian species. Two regions of homology, a distal one from about −1500 to −1200 and a proximal one from about −300 to −1, have been identified (*19*). The proximal homology domain of the human *IRBP* gene contains *cis*-acting sequences between −123 to +19 that are sufficient to provide photoreceptor-specific promoter activity (*20*). It is not clear, however, whether sequences in the homology domains or any other regions of the *IRBP* gene can modulate this promoter activity. The human *IRBP* promoter of −123/+19 contains a sequence TAATC (−50 to −54), which is present in other photoreceptor-specific genes. This sequence is recognized by a photoreceptor-specific paired-related homeobox protein, cone-rod-homeobox protein (Crx), that is capable of significant transcriptional activation of the IRBP promoter in transient transfection assays in non-photoreceptor cells (*3, 4*). The *IRBP* promoter also contains a sequence TAATT (−60 to −64), which is present in other photoreceptor-specific genes and thus has been termed the "photoreceptor conserved element" (PCE, Ref. *21*), or Ret 1 (*22*). PCE is weakly recognized by Crx and it can weakly activate the *IRBP* promoter in NIH3T3 cells (*4*). A previous study in transgenic mice showed that simultaneous mutation of both the PCE and Crx element (CRXE) can eliminate *IRBP* promoter activity in photoreceptor cells (*20*). It is not clear from these results, however, whether PCE or CRXE alone, or both, are involved in photoreceptor-specific regulation *in vivo*.

In order to address the above questions, we have first analyzed the upstream region of the *IRBP* gene from −2134 to +19 in transfected cell lines. We have localized essential control elements of the *IRBP* promoter in a region between −156 and +19. Results from the previous and current studies suggest that the sequence between −156 and −109 may be important for the early gene activation. We have mutated PCE and CRXE separately, and tested *IRBP* promoter activity in transfected cell lines and in transgenic mice. In transfected retinoblastoma cells, a mutation in CRXE decreased promoter activity more than a mutation in PCE. In transfected HeLa cells, a *Crx* expression vector transactivated a wild-type *IRBP* promoter and had minimal effect on a promoter containing a mutant CRXE. A mutation in PCE also caused a significant reduction in promoter activation. In transgenic mice, CRXE, but not PCE, is essential for promoter activity in photoreceptor cells.

## MATERIALS AND METHODS

*Cell Transfections and Chloramphenicol Acetyl Transferase (CAT) Assays*—Lipofectin (GIBCO/BRL)-mediated transfections were performed according to an established procedure (*23*) with modification. About $3.3\times10^6$ Weri-RB1 retinoblastoma cells (passage #146; ATCC) or mouse fibroblast cells (SNL; kindly provided by Dr. H. Zheng, Merck Sharp & Dohme Research Laboratories, originally from Dr. Allan Bradley, Baylor College of Medicine) were transfected with 16 μg of the *IRBP* promoter-*CAT* reporter vector (see "*Transgene Constructions*" and Fig. 1) and 4 μg of the *β-Gal* expression vector (pCH110, Pharmacia). The same number of HeLa cells (ATCC) were transfected with 10 μg of the *IRBP* promoter-*CAT* reporter vector, 2.4 μg of pCH110 and 2 μg of the *Crx* expression vector with or without the *Crx* cDNA insert (*3*). Plasmids pSV40*CAT* and pBasic*CAT*, which contain a viral promoter and no promoter, respectively, were used as positive and negative controls. CAT activity was determined by thin-layer chromatography and the autoradiographs were quantified by densitometry (*24*).

*Preparation of Nuclear Extracts*—A recently reported procedure for the preparation of nuclear extracts from tissues was used (*25*). Briefly, bovine retinas and kidneys were homogenized and nuclear fractions were obtained by ultracentrifugation through a sucrose density cushion. The nuclear pellets were resuspended and dialyzed.

*DNAse I Footprinting*—The probes used for DNAse I footprinting analysis contained the human *IRBP* promoter region from −156 to +23. Mutations in the promoter were made by a PCR method (*26*). For the footprinting analysis, wild-type and mutant promoters were amplified by PCR using two primers, one of which was 5′ end-labeled by treatment with T4 polynucleotide kinase (New England Biolabs) in the presence of [$\gamma$-$^{32}$P]ATP (6,000 Ci/mmol, DuPont/New England Nuclear). Probes prepared in this manner had virtually identical specific activities ($2.5\times10^6$ cpm/pmol). Equal amounts of each probe ($2\times10^4$ cpm, 0.5-1 ng) were incubated with nuclear extracts or with bovine serum albumin (30 μg of protein) or Sp1 (1 fpu, Promega) and with 1 μg of poly (dI-dC) followed by partial DNAse I digestion according to the HotFoot protocol (Stratagene). DNAse I protection was analyzed by autoradiography after electrophoresis on a denaturing 8% polyacrylamide gel.

*Transgene Constructions*—The 5 plasmids used for the generation of transgenic mice were constructed by the following steps. First, the *Hin*dIII-*Bam*HI *lacZ* fragment from pCH110 (Pharmacia) was inserted into pBluescript II

KS (Strategene) to generate p$\beta$-*Gal* which contains the $\beta$-*Gal* reporter gene. This reporter gene contains SV40 polyadenylation and intron/exon splice sites and is devoid of any eukaryotic promoter sequence. Subsequently, human *IRBP* promoter fragments from −123 to +19 which contained various mutations were inserted into *Sal*I-*Hin*dIII digests of p$\beta$-*Gal* to generate the 5 plasmids. Mutations in all constructs were verified by DNA sequencing before use in the generation of transgenic mice.

*Transgenic Mice*—For each of the 5 plasmids, fragments of 3,880 bp containing the human *IRBP* promoter fused to the $\beta$-*Gal* reporter gene were excised from the plasmid backbone by *Sal*I-*Bam*HI digestion. The fragments were purified from agarose gels by Qiaex treatment (Qiagen). Mice were made transgenic by microinjection of these fragments into pronuclei of one-cell FVB/N embryos (*24*). Founder mice ($F_0$) were identified by PCR analysis of tail DNA using primers specific for the $\beta$-*Gal* sequences. Families were generated and maintained by breeding with C57BL/6. Offspring at postnatal ages 35–45 days were used for gene expression analyses unless otherwise mentioned.

*Reverse Transcriptase (RT)-PCR*—RNeasy Mini Kits (Qiagen) were used to isolate total RNA from various tissues of transgenic mice. For the RT-PCR procedure, total RNA (1 $\mu$g) was treated with DNAse and subjected to a M-MLV reverse transcriptase reaction (GIBCO/BRL) using oligo dT as the primer. The reaction product was diluted to 100 $\mu$l of which 1 $\mu$l (equivalent to 20 ng of original RNA) was used for PCR. Primer pairs for mouse *IRBP*, mouse $\beta$-*actin* and $\beta$-*Gal* transcripts were all included in each PCR. Reaction mixtures of 50 $\mu$l contained 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.16 mM of each dNTP, 0.25 $\mu$M of each of the primers and 1 unit of Taq DNA polymerase (Promega). PCR included 30 cycles of 94°C for 40 s, 58°C for 30 s, and 72°C for 60 s. At the end of PCR, reaction was extended at 72°C for 7 min. PCR products were analyzed on a 5% native polyacrylamide gel. The $\beta$-*Gal* transgene was identified by amplification of a 396 bp fragment (nucleotides 487 to 883 of pCH110). The endogenous mouse *IRBP* transcript was identified by amplification of the 639 bp fragment between +3156 and +3796 of the mouse *IRBP* gene (unpublished results). Mouse $\beta$-*actin* transcripts were identified by amplification of a 226 bp fragment of the mouse $\beta$-*actin* gene.

*RNase Protection Analysis (RPA)*—RPA was according to a previous protocol (*27*). In this procedure, 0.3 $\mu$g of total retina RNA was incubated with $^{32}$P-UTP-labeled riboprobes for $\beta$-*Gal* (from nucleotides 2929 to 3286 of pCH110), mouse opsin [from +1176 to +1279 of the mouse *opsin* cDNA (*28*)] or mouse $\beta$-*actin* (Ambion) genes. The sizes (in bases) of the probes and their protected fragments are: 432 and 357 for $\beta$-*Gal*, 163 and 108 for *opsin*, and 275 and 250 for $\beta$-*actin*, respectively.

*X-Gal Staining*—Eyes were fixed in 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, cryoprotected in 30% sucrose, embedded in OCT, frozen and sectioned at 10–15 $\mu$m thickness. The mounted sections were stained as described (*29*). Routine histological specimens were fixed 1 h in 4% glutaraldehyde in 0.1 M PBS, stored in 10% neutral buffered formalin, dehydrated in serial ethanol, embedded in JB-4, sectioned at 1.5 $\mu$m and stained with hematoxylin/eosin.

## RESULTS

*Analysis of IRBP Promoter Activity in Transiently Transfected Retinoblastoma Cells*—The retinoblastoma cell line Weri-RB1, which expresses *IRBP* as well as other photoreceptor genes (*23*), was used for transient transfections to assay the activity of *IRBP* promoter fragments linked to the *CAT* reporter gene (Fig. 1). A non-retinal mouse fibroblast cell line, SNL, was also used. Cells were transiently transfected with *IRBP-CAT* constructs containing 5′ flanking fragments of the *IRBP* gene beginning at −2134, −1311, or −156 and expression of the *CAT* gene was analyzed. The fragment beginning at −2134 contains both the distal and proximal homology domains and was used as a control. The fragments beginning at −1311 and at −156 showed retina-specific promoter activity in previous studies in transgenic mice (*24, 30*). With all of these constructs, *IRBP* promoter-directed CAT activity was observed in Weri-RB1, but not in the fibroblast cells (Fig. 1). Similar CAT activities were seen with the longer and shorter promoters, suggesting that the essential control elements are located between −156 to +19 of the *IRBP* promoter. The sequence of this region is shown in Fig. 2. Two shorter promoters were also tested: (i) −123 to +19, which contains sufficient sequence for photoreceptor-specific promoter activity (*20*); and (ii) −84 to +19, which lacks the G-rich region from −105 to −85 (Fig. 1). The truncation from −156 to −123 significantly reduced reporter gene expression, suggesting that enhancer element(s) are located in the deleted region. The removal of the G-rich region led to a modest increase in CAT activity, and point mutations in the G-rich region resulted in significant increase of CAT activity (data not shown), suggesting that this G-rich sequence has a repressor activity. A similar finding was reported previously (*20*). These results suggest that although the photoreceptor-specific regulation of *IRBP* gene transcription requires sequences within −123 and +19, the sequence further upstream within −156 may be required for a higher level of expression.

*Identification of Nuclear Factor Binding Sites in the IRBP Promoter*—To further identify the regulatory sequences within the *IRBP* promoter, a fragment containing the region from −156 to +23 was incubated with nuclear extracts from bovine retina or kidney then subjected to DNAse I footprint analysis. As shown in Fig. 3A, retina-specific DNAse I protection occurs between −42 to −76, similar to previous results using mouse retina extracts (from −40 to −69, Ref. *30*). These footprints are wider than those observed using nuclear proteins fromY-79 or Weri-RB1 retinoblastoma cell lines (from −45 to −58, Ref. *20*). A second retina-specific footprint is seen from −109 to −135. One of the positive regulatory elements that was detected in the transient expression analysis (Fig. 1) may be located within this footprint. It should be noted that the sequences in this footprint contain a retina-specific hypomethylation site identified previously (*27*). Hypomethylation of this site occurs during *IRBP* gene activation at the time of photoreceptor cell birth. DNAse I hypersensitive sites appear between −85 and −105 after incubation with either retinal or renal nuclear extracts (Fig. 3A). The sequences in this region are recognized by recombinant Sp1

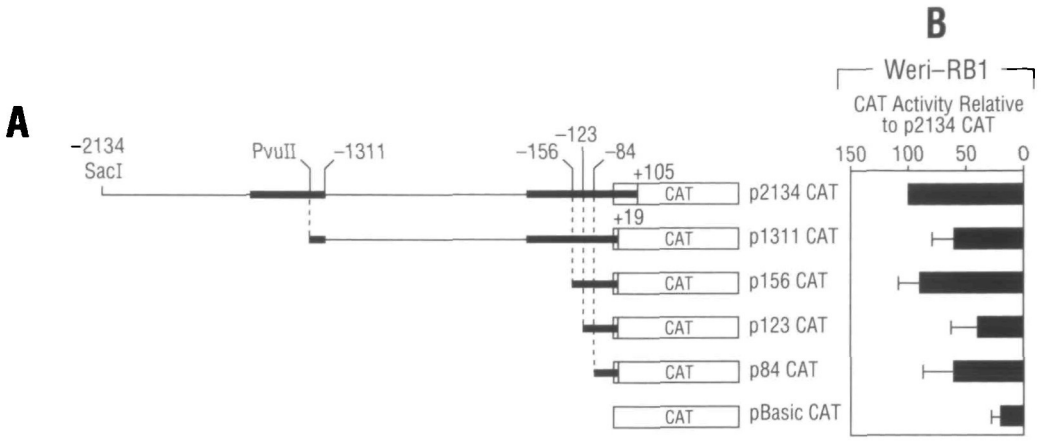


Fig. 1. **Deletional studies of the *IRBP* promoter.** A: Schematic depiction of the *IRBP-CAT* plasmids. The conserved regions are indicated by bold lines. B: Relative CAT activities (mean for 3 or 4 experiments ± SD) for the different constructs in the Weri-RB1 cells. C: CAT activities in SNL fibroblast and Weri-RB1 retinoblastoma cells transfected with the different constructs.

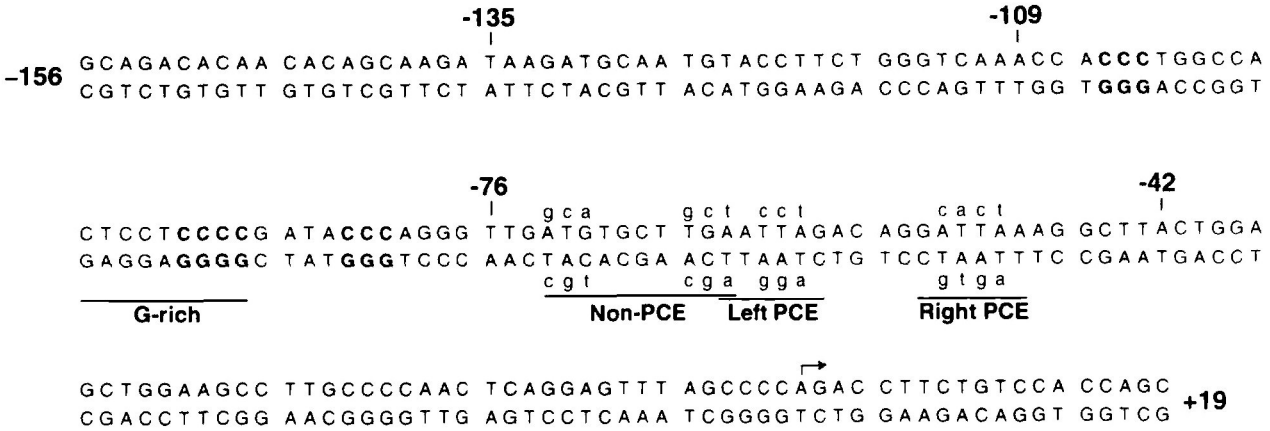


Fig. 2. **Sequence of the human *IRBP* promoter.** The underlined regions denote putative transcription factor binding sites. Bold letters denote protein-induced DNAse I hypersensitive sites (see Fig. 3). Bent arrow indicates the transcription start site.

protein (Fig. 3B). These sequences may be involved in the repressor activity detected in the retinoblastoma transfection studies (Fig. 1).

*Analysis of the Photoreceptor Conserved Element (PCE) and the Cone-Rod-Homeobox Element (CRXE)*—The sequence "AATTA" (or TAATT) which appears in the 5′ flanking region of many photoreceptor genes including *arrestin, opsin, IRBP*, and the *Drosophila* genes *trp, nina*, and *norpA* has been termed the PCE (*21*) or Ret 1 (*22*). A sequence very similar to PCE, "GATTA" (or TAATC) now termed the CRXE, is also a candidate for photoreceptor-specific regulation of many photoreceptor genes (*3, 4*). The retina-specific transcription factor, Crx, has recently been identified and shown to recognize CRXE. The human, bovine and mouse *IRBP* genes all contain two PCEs and one CRXE at similar locations. In the human *IRBP* promoter the PCEs are located at −1428 to −1433 and −64 to −60; and the CRXE is at −54 to −50. The role of the distal PCE is unclear because photoreceptor-specific regulation of the *IRBP* gene does not require the distal region (*19*). The region of the promoter containing the proximal PCE and CRXE has recently been shown in transgenic mice to be required for activity of the *IRBP* promoter (*20*). In this previous study both the proximal PCE and the CRXE were

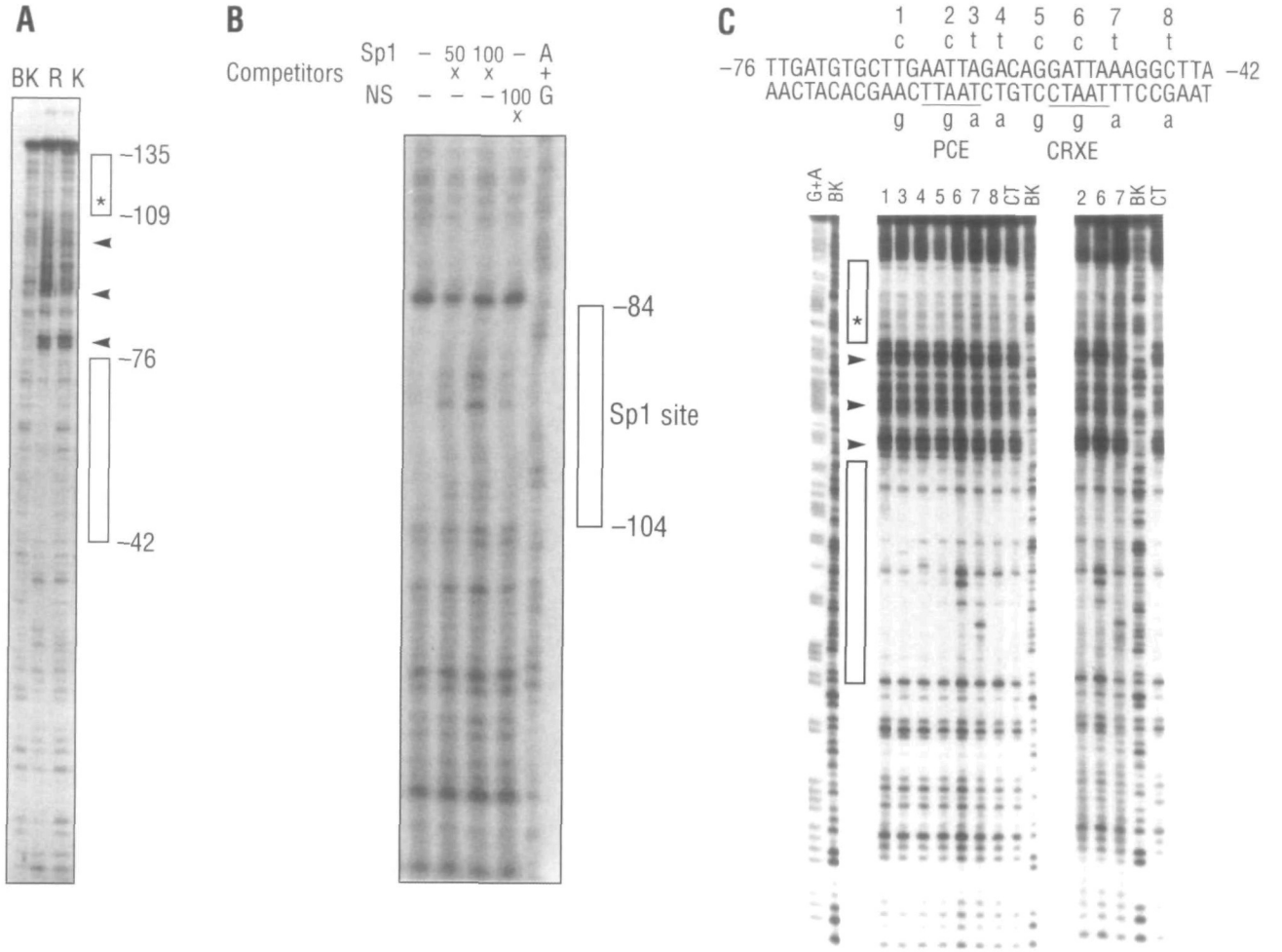


Fig. 3. **Footprinting analysis of the *IRBP* promoter.** A: DNAse I footprinting analysis of the *IRBP* promoter (−156 to +23). The *IRBP* promoter was incubated without (blank, BK) or with nuclear extracts from retinal (R) or kidney (K) cells, then subjected to partial DNAse I digestion. Protected regions of the promoter are indicated by the boxes. Arrowheads indicate protein-induced DNAse I hypersensitive sites. The asterisk is a retina-specific hypomethylation site (*27*). The lower strand was analyzed. B: DNAse I protection after incubation with recombinant Sp1. Incubations were performed in the presence of different concentrations of a consensus Sp1 oligomer or a non-specific (NS) oligomer. Upper strand was analyzed. C: DNAse I footprinting analysis of the *IRBP* promoter (−156 to +23) containing point mutations between −76 and −42. Lower strands were analyzed. Lower case letters with numbers indicate mutant probes that were incubated with retinal nuclear extracts. Lanes are identified by the same numbers that identify the mutations. BK, blank; CT, control, wild-type probe.

mutated simultaneously, so the study did not determine which element was essential for regulation of *IRBP* expression, or whether both were essential. To further analyze the PCE and CRXE in the promoter region of the *IRBP* gene, we first studied *in vitro* DNA/protein interactions by footprint analysis using probes that contained point mutations around and within each of these elements (Fig. 3C). All mutations in or around the PCE or CRXE (Fig. 3C, positions 2, 3, 4, 5, 6, and 7) altered the footprint pattern of the wild-type control probe. The mutation in the CRXE (Fig. 3C, position 6), however, altered the footprint more significantly than mutations in or around the PCE (Fig. 3C, positions 2, 3, or 4). These results and the previous results in retinoblastoma cells showing narrow footprints (from −45 to −58, Ref. *20*) suggest that although the two elements are similar in sequence, retinal nuclear factors distinguish between the two sites and appear to be more dependent on the CRXE sequence.

*Functional Analysis of PCE and CRXE in Transiently Transfected Cells*—It has been shown that PCE and CRXE sequences are functionally equivalent in *Drosophila* (*31*). In a recent study, it was shown that Crx not only binds and transactivates from the CRXE, it also weakly binds to and transactivates from the PCE (*4*). To determine whether both sequences are essential for *IRBP* promoter activity, we determined the function of the PCE and CRXE in *IRBP* gene regulation in transiently transfected cells. We used two different cells: photoreceptor-like (Weri-RB1 retinoblastoma) and non-photoreceptor (HeLa) cells. Weri-RB1 cells were transiently transfected with the following *IRBP* promoter-*CAT* constructs and expression of the *CAT* gene was analyzed: (i) the wild-type promoter, (ii) a promoterless construct, pBasic*CAT*, (iii) a PCE mutant [PCE (M)], (iv) a three-point CRXE mutant [CRXE ($M_1$)], (v) a four-point CRXE mutant [CRXE ($M_2$)], and (vi) a CRXE mutant that contains two PCEs (CRXE→PCE) (Fig. 4). The

highest CAT expression was obtained with the wild-type promoter. All mutations of the CRXE repressed CAT activity about 60%. Mutation of the PCE alone, however, repressed CAT activity about 30%. This suggests that although photoreceptor-specific regulation of the *IRBP* gene relies on Crx binding to the CRXE (*4*), PCE may also contribute to promoter activity.

HeLa cells were transiently transfected with the above *IRBP* promoter-*CAT* constructs as well as an SV40 promoter-enhancer-*CAT* construct in the presence or absence of a *Crx* expression vector (Fig. 5). In the absence of Crx, only background CAT activity was observed in HeLa cells when transfected with the *IRBP* promoters. Co-transfection of the *Crx* expression vector with the wild-type promoter increased CAT activity 20-fold over the background activity in these cells. Mutation of the CRXE [either CRXE ($M_1$) or CRXE→PCE] almost completely inhibited this Crx-activated CAT activity. Mutation of the PCE alone also significantly reduced this Crx-activated CAT activity so that only about a 4-fold increase over the background activity was observed. These results suggest that PCE can contribute to the *IRBP* promoter activity by facilitating Crx-mediated transactivation.

*Functional Analysis of PCE and CRXE in Transgenic Mice*—The function of a regulatory element of a gene analyzed in transiently transfected cells may be different from that *in vivo* due to many environmental differences in the two systems including the lack of chromatin structures in the former. To assess the *in vivo* function of the proximal PCE and CRXE individually, we used transgenic mice that carried wild-type or mutant promoters with the PCE and CRXE mutated individually. Transgenic mice were generated by microinjection of the following constructs: (i) the wild-type promoter (−123 to +19), (ii) a non-specific mutant control, in which mutations were made outside of the PCE and CRXE, (iii) a PCE mutant, (iv) a CRXE mutant, and (v) a PCE-CRXE double mutant (Fig. 6). The promoters were linked to a *lacZ* reporter gene. Four to 17 independent transgenic lines were generated for each construct and the $F_1$ or $F_2$ mice at postnatal days 35-45 were used in all analyses. Transgene expression was analyzed by RT-PCR in some lines (Fig. 6B). Endogenous

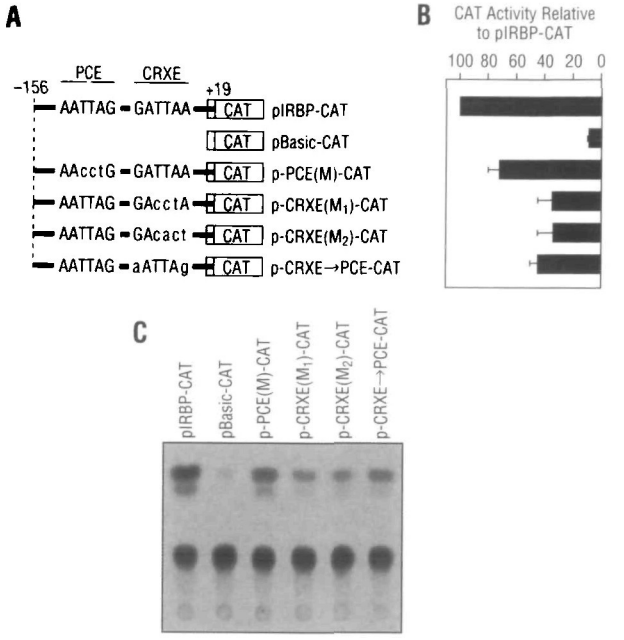


Fig. 4. **Mutational analysis of PCE and CRXE.** A: Schematic depiction of the *IRBP-CAT* plasmids. The PCE and CRXE sequences and their mutations (lower cases) are shown. B: Relative CAT activities (mean for 3 experiments ± SD) for the different constructs in the Weri-RB1 cells. C: CAT activities in Weri-RB1 cells transfected with the different constructs.

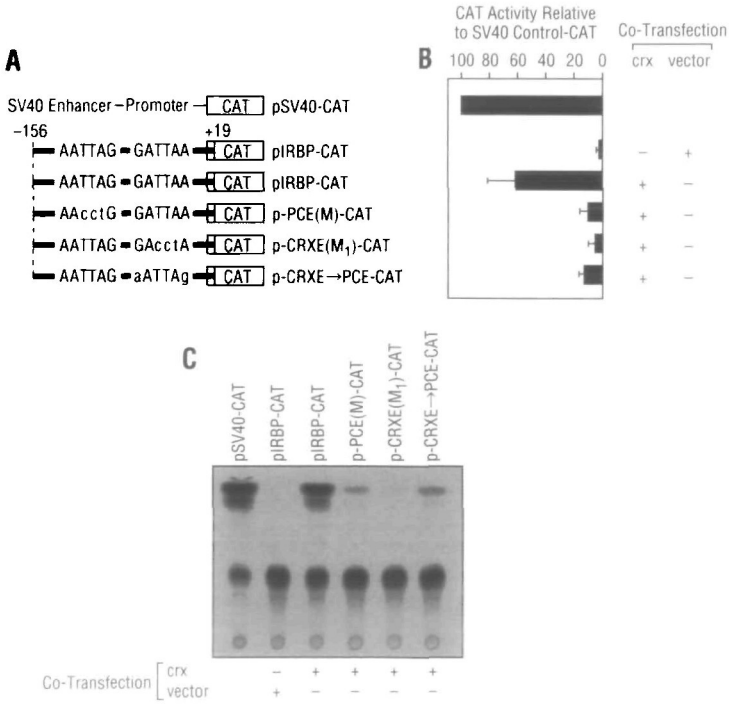


Fig. 5. **Transactivation analysis with Crx.** A: Schematic depiction of the SV40 enhancer-promoter-*CAT* (control) and *IRBP-CAT* plasmids. The PCE and CRXE sequences and their mutations (lower cases) are shown. B: Relative CAT activities (mean for 3 experiments ± SD) for the different constructs, with or without the co-transfection of a *Crx* expressing vector, in the HeLa cells. C: CAT activities in HeLa cells transfected with the different constructs, with or without the co-transfection of a *Crx* expressing vector.

*IRBP* and *β-actin* were used as controls for tissue-specificity and yield of RNA, respectively. For the wild-type promoter and for the promoters with mutations outside the CRXE, transgene expression was detected in the retinas of 7 out of 9 transgenic lines tested (*β-Gal* band, lane R; Fig. 6B). In contrast, no *β-Gal* expression was detected by RT-PCR in any of the 9 lines tested carrying the CRXE mutant or PCE-CRXE double mutant promoter. Expression in the cerebral cortex (Cr) occurred in some of these lines (for example, non-specific mutant line 1 and PCE-mutant line 5). Testes also showed occasional *β-Gal* expressions, but a similar level of *β-Gal* expression was also noted in nontransgenic controls. Sperm has been reported to contain β-Gal activity (*32*). The expression levels of the transgenes in the retina were also compared among 4 to 8 PCE mutant, CRXE mutant and PCE-CRXE

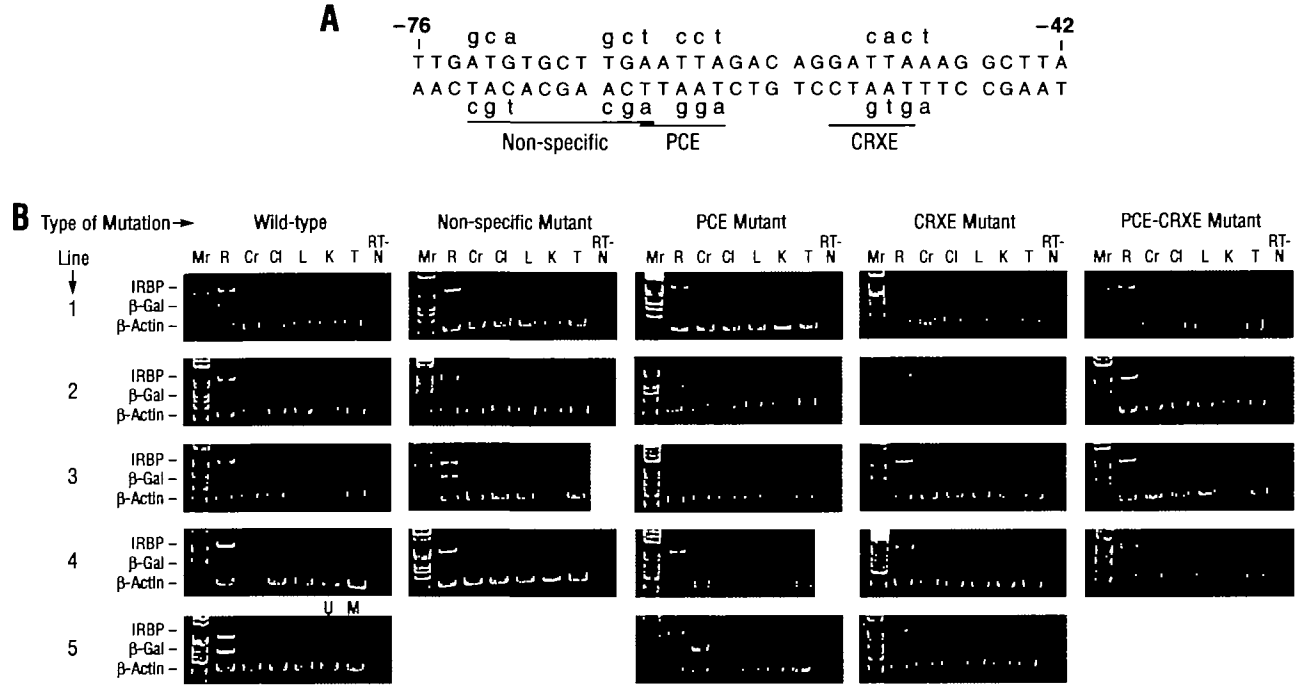


Fig. 6. **RT-PCR assays for *IRBP*, *β-Gal*, and *β-Actin* expression in transgenic mice.** A: Mutations (lower case) in the *IRBP* promoter in the transgene constructs (−123 to +19). B: RT-PCR analysis of *IRBP*, *β-Gal* and *β-Actin* expression in 4 or 5 transgenic families generated with each construct. RNA was isolated from different tissues of a transgenic mouse in each family, then amplified by RT-PCR. PCR included 30 cycles of 94°C for 40 s, 58°C for 30 s, and 72°C for 60 s, and extended at 72°C for 7 min. PCR products were analyzed on a 5% native polyacrylamide gel and assayed by ethidium bromide staining. The sizes of the amplified *IRBP*, *β-Gal*, and *β-Actin* bands are indicated. *IRBP* expression was detected only in the retinas. R, retina; Cr, Cerebrum; Cl, cerebellum; L, liver; U, uterus; M, muscle; K, kidney; T, testis. RT-N, control PCR without reverse transcriptase.

TABLE I. **Densitometric analysis of retinal β-Gal expression.** The β-Gal expression in the retinal of each line of transgenic mice was quantified by RPA. Total retinal RNA was incubated with riboprobes for β-Gal, opsin and β-actin genes. The densitometric measurements of the β-Gal expression as shown are arbitrary units normalized for one-tenth level of opsin (upper) or β-actin (lower). The copy number of the transgene for each line (in parentheses) was determined by quantitative dot blot analysis.

| Type of mutation | Internal standard | Lines 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Wild type (−123 | Opsin | 354 | 548 | 24 | 18 | 6 | 7 | | | | | | | | | | | |
| | | (2) | (3) | (4) | (3) | (4) | (4) | | | | | | | | | | | |
| to +19) | Actin | 189 | 313 | 16 | 13 | 5 | 0 | | | | | | | | | | | |
| Non-specific mutant | Opsin | 24 | 48 | 0 | 115 | | | | | | | | | | | | | |
| | | (2) | (2) | (5) | (2) | | | | | | | | | | | | | |
| | Actin | 15 | 26 | 0 | 103 | | | | | | | | | | | | | |
| PCE mutant | Opsin | 582 | 7 | 395 | 122 | 141 | 348 | 641 | 45 | 1 | 0 | 1 | 32 | 2 | 6 | 18 | 14 | 0 |
| | | (45) | (300) | (15) | (10) | (25) | (17) | (6) | (50) | (210) | (50) | (102) | (74) | (150) | (96) | (300) | (250) | (30) |
| | Actin | 307 | 5 | 209 | 81 | 75 | 205 | 440 | 48 | 0 | 0 | 0 | 42 | 7 | 2 | 16 | 11 | 0 |
| CRXE mutant | Opsin | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 9.5 | | | | | | | | | |
| | | (8) | (4) | (1) | (10) | (30) | (3) | (2) | (6) | | | | | | | | | |
| | Actin | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 9.7 | | | | | | | | | |
| PCE-CRXE double mutant | Opsin | 10 | 2 | 1 | 1 | | | | | | | | | | | | | |
| | | (5) | (1) | (5) | (15) | | | | | | | | | | | | | |
| | Actin | 8 | 2 | 0 | 1 | | | | | | | | | | | | | |

double mutant lines by quantitative RPA (Fig. 7). Measurable *β-Gal* expression was detected in 7 of 8 PCE mutant lines but none in CRXE mutant or PCE-CRXE double mutant lines. Table I shows the results of densitometric measurements for *β-Gal* expression in the retina from each line. Consistent with the RT-PCR results, none of the 12 transgenic lines carrying mutations in the CRXE of the *IRBP* promoter showed detectable *β-Gal* transcripts. In constrast, at least 9 of the 17 transgenic lines with PCE mutant promoter show retina-specific *β-Gal* expression. Variations in *β-Gal* expression between lines which contain the same transgenic construct presumably reflect position effects related to the transgenic integration site in the genome. The above studies in transgenic mice suggest that the CRXE is essential for the retina specificity of *IRBP* gene expression, whereas the PCE is not. To determine whether the retinal expression observed with the transgenic mice carrying the PCE-mutated constructs was photoreceptor-specific, we analyzed *lacZ* reporter gene activity by X-Gal staining. Whereas mice carrying the CRXE mutation showed no X-gal staining (data not shown), transgenic mice carrying the PCE mutation showed strong X-gal staining in the retinal outer plexiform layer, in the inner segments of the photoreceptor cells and weaker staining in the photoreceptor outer nuclear layer (Fig. 8). Thus, CRXE, but not PCE, is essential for the photoreceptor specificity of the *IRBP* gene *in vivo*.

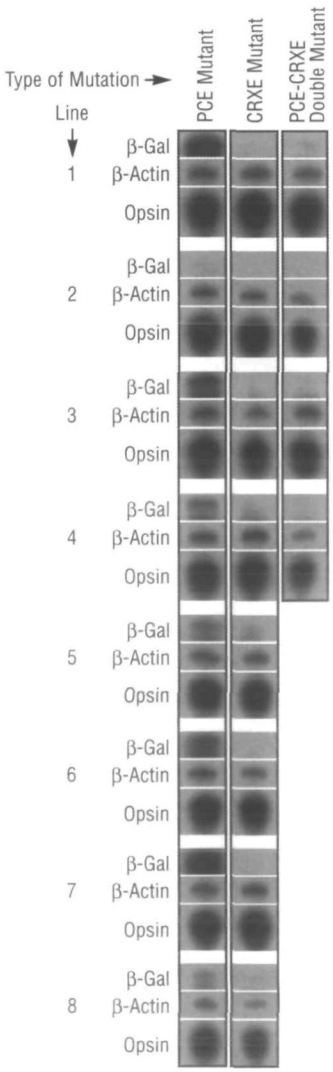


Fig. 7. **RNase protection assays for *β-Gal* expression in transgenic retinas.** Retinal *β-Gal* expression was assayed by RPA in the first 4 to 8 transgenic families for PCE, CRXE mutant or PCE-CRXE double mutant constructs. Levels of *opsin* and *actin* were also assayed.

## DISCUSSION

The results in this study strongly suggest that the early *IRBP* gene expression requires multiple elements and transcription factors in addition to the elements required for the photoreceptor-specific regulation. The region from −135 to −109 is protected from DNAse I digestion when incubated with a retinal nuclear extract. This footprinted region contains a CpG (Hpa II) site at −116 that undergoes hypomethylation during *IRBP* gene activation at the time of photoreceptor cell birth (*27*). This hypomethylation event is specifically related to *IRBP* gene activation, because another HpaII site at −725 remained methylated. Based on sequence analysis and evidence that *IRBP* gene transcription is positively modulated by cAMP (unpublished experiments, GIL; Ref. *33*), it was suggested that the region centered around −116 may contain a degenerate overlapping AP1/ATF-like element (*20*). However, additional studies still need to be done to define the relationship between this region and CRXE for *IRBP* promoter regulation. We as well as others (*20*) have demonstrated that the G-rich region between −105 and −85 of the *IRBP* gene represses promoter activity in retinoblastoma cells. It is not clear how this putative *cis*-element in the *IRBP* promoter is involved in *IRBP* gene regulation. It may modulate *IRBP* expression level through interactions with PCE/CRXE. It is likely that Sp1 or Sp1-like proteins bound to this region may help to repress non-specific promoter activity, or may be associated with the regulated activation of *IRBP* gene expression.

In this study we have demonstrated that the CRXE in the *IRBP* promoter is required to direct photoreceptor-specific promoter activity *in vivo*, and that the photoreceptor-specific transcription factor Crx can activate a transiently transfected *IRBP* promoter with an intact CRXE in a non-retinal cell type. The fact that the transfected *IRBP* promoter is active in Weri-RB1 retinoblastoma cells without the co-transfection of Crx suggests that these cells express sufficient endogenous Crx. This is not surprising since Weri-RB1 cells are derived from early photoreceptor cells and express a number of photoreceptor genes. We have also demonstrated that both CRXE and PCE contribute to *IRBP* promoter activity in transfected retinoblastoma cells. This result is similar to that of a recent study in chick retina primary cultures transfected with an *IRBP* fragment of −70/+101 (*34*). In our study we have further demonstrated that the PCE is important for maximal Crx-mediated transactivation. The cooperative function of PCE observed in transiently transfected cells may be due to its direct interaction with the Crx/CRXE complex or due to its binding to another Crx or a different homeodomain protein which then interacts with the Crx/CRXE. Both the CRXE and PCE are target sites of homeodomain proteins.

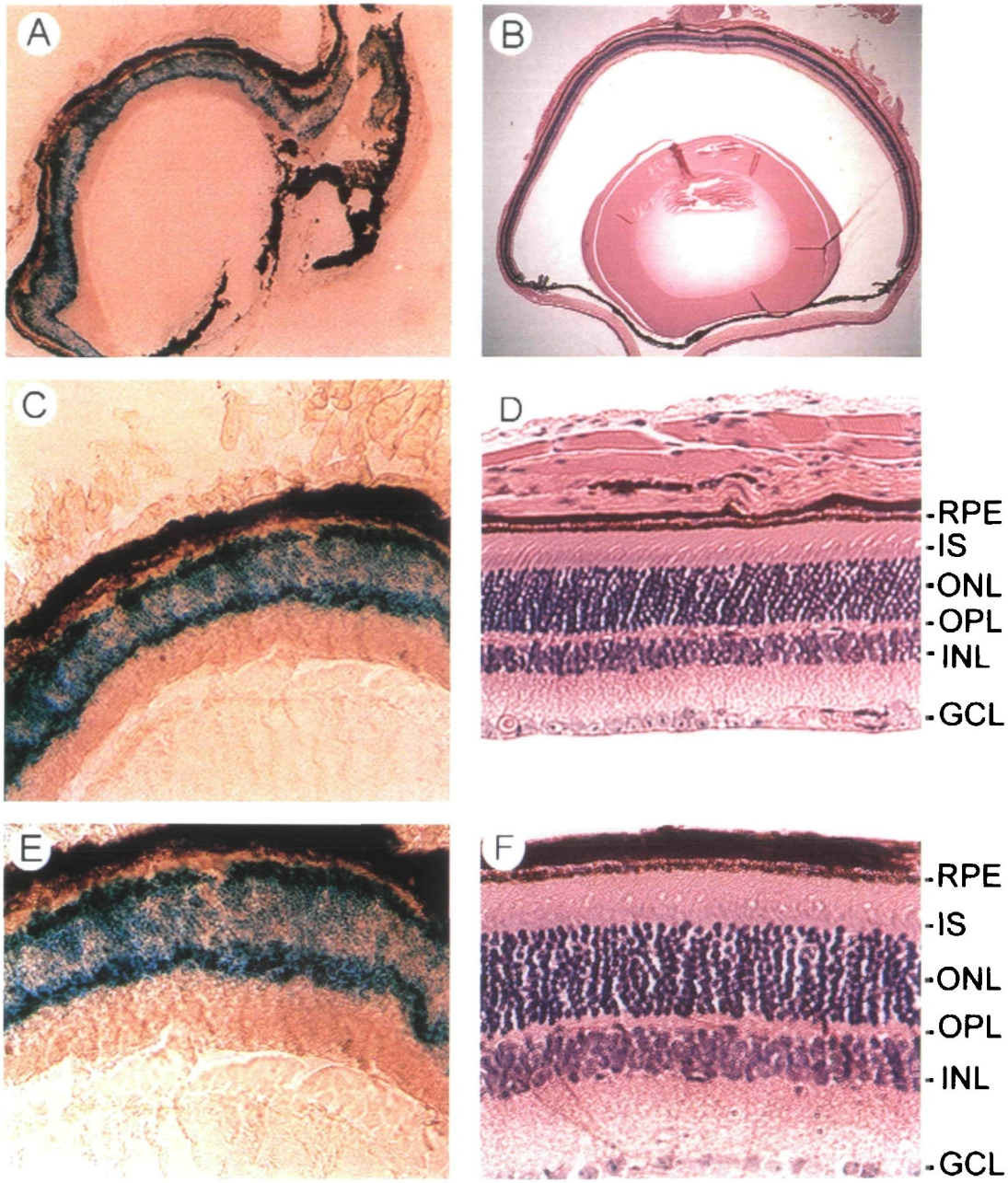


Fig. 8. **Transgene expression in retinal photoreceptors of a transgenic mouse carrying the mutant PCE.** (A, C, E) A frozen section (8 $\mu$m thick) from the eye of a 4-month-old transgenic mouse. Staining was for 2 h with the $\beta$-galactosidase substrate X-Gal. (B, D, F) JB-4 plastic-embedded sections stained with hematoxylin and eosin to demonstrate the retinal layers. GCL, ganglion cell layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments; RPE, retinal pigment epithelium. Magnifications: A and B, 25×; C and D, 200×; E and F, 400×.

Different homeodomain proteins, depending on sequences flanking the core, that bind to different target sites may be involved in the regulation of genes during different stages of development (*35*). PCE is potentially one of the target sites of the recently identified Rx (*5*), Rax (*36*), or Erx (*37*) proteins of the *paired*-type homeobox family. Rx/Rax is a member of the Antennapedia family that binds TAATT (*38*) and is expressed in proliferating cells during early retinal development. Erx is also a member of the same family but is expressed in both developing and adult retina (*37*). Crx, on the other hand, binds to a C/TTAATCC consensus (CRXE) site and is expressed during and after photoreceptor cone/rod differentiation (*3, 4*). These homeodomain proteins may therefore be involved in the regulation of the *IRBP* promoter during sequential stages of retinal development. There may also be a synergistic interaction similar to that between Crx and Nrl (*3*). In the *IRBP* promoter the PCE and CRXE are separated by 10 bases, or about one helix turn (*39*), and this spacing is conserved in all the IRBP genes studied (*19*). This specific alignment appears unique to the *IRBP* gene. In the *opsin* (*40*) and *arrestin* (*21*) genes, for example, PCE to CRXE or

CRXE to CRXE are not stereospecifically aligned. In other photoreceptor genes, for example, the monophosphate-phosphodiesterase $\beta$-subunit (*41*) and transducin $\alpha$-subunit (*42*) genes, only the CRXE is present. It is possible that the Crx/CRXE-PCE interaction observed in the current study as well as in other recent studies is due to the stereospecifically aligned positions of PCE and CRXE (*39*). Thus, although many photoreceptor genes have both PCE and CRXE, the Crx/CRXE-PCE interaction and the positive regulatory element(s) in the region between −135 and −109 may provide unique regulation of *IRBP* gene expression.

The cooperative function of the PCE, however, is only observed in transfected cells or primary cultures (*34*) but not in transgenic mice. Although gene expression patterns observed in transgenic mice are usually considered to be close to those *in vivo*, it is well known that the activity of the transgene is subject to the chromatin structure and genes surrounding the transgene. It is entirely possible that PCE as well as many other factors that contribute to the Crx-mediated transactivation *in vivo* is masked by the noise created by the chromatin structure and genes surrounding the transgene. The function of the PCE in the regulation of the *IRBP* gene *in vivo* should await future investigation using targeted disruption of this site.

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## REFERENCES


1. Adler, R. and Hatlee, M. (1988) Plasticity and differentiation of embryonic retinal cells after terminal mitosis. *Science* **243**, 391-393
2. Cepko, C.L., Austin, C.P., Yang, S., Alexiades, M., and Ezzeddine, D. (1996) Cell fate determination in the vertebrate retina. *Proc. Natl. Acad. Sci. USA* **93**, 589-595
3. Chen, S., Wang, Q.-L., Nie, Z., Sun, H., Lennon, G., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and Zack, D.J. (1997) Crx, a novel otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. *Neuron* **19**, 1017-1030
4. Furukawa, T., Morrow, E.M., and Cepko, C.L. (1997) *Crx*, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. *Cell* **91**, 521-541
5. Mathers, P.H., Grinberg, A., Mahon, K.A., and Jamrich, M. (1997) The *rx* homeobox gene is essential for vertebrate eye development. *Nature* **387**, 603-607
6. Freund, C.L., Gregory-Evans, C.Y., Furukawa, T., Papaioannou, M., Looser, J., Ploder, L., Bellingham, J., Ng, D., Herbrick, J.S., Duncan, A., Scherer, S.W., Tsui, L.-C., Loutradis-Anagnostou, A., Jacobson, S.G., Cepko, C.L., Bhattacharya, S.S., and McInnes, R.R. (1997) Cone-rod dystrophy due to mutations in a novel photoreceptor-specific homeobox gene (CRX) essential for maintenance of the photoreceptor. *Cell* **91**, 543-553
7. Swain, P.K., Chen, S., Wang, Q.-L., Affatigato, L.M., Coats, C.L., Brady, K.D., Fishman, G.A., Jacobson, S.G., Swaroop, A., Stone, E., Sieving, P.A., and Zack, D.J. (1997) Mutations in the cone-rod homebox gene are associated with the cone-rod dystrophy photoreceptor degeneration. *Neuron* **19**, 1329-1336
8. Carter-Dawson, L., Alvarez, R.A., Fong, S.-L., Liou, G.I., Sperling, H.G., and Bridges, C.D.B. (1986) Rhodopsin, 11-cis vitamin A, and interstitial retinol-binding protein (IRBP) during retinal development in normal and *rd* mutant mice. *Dev. Biol.* **116**, 431-438
9. Hauswirth, W.W., Langerijt, A.V.D., Timmers, A.M., Adamus, G., and Ulshafer, R.J. (1992) Early expression and localization of rhodopsin and IRBP in the developing fetal bovine retina. *Exp. Eye Res.* **54**, 661-670
10. Timmers, A.M., Newton, R.B., and Hauswirth, W.W. (1993) Synthesis and stability of retinal photoreceptor mRNAs are coordinately regulated during bovine fetal development. *Exp. Eye Res.* **56**, 257-265
11. Pepperberg, D.R., Okajima, T.-I.L., Wiggert, B., Ripps, H., Crouch, R.K., and Chader, G.J. (1993) IRBP—Molecular biology and physiological role in the visual cycle of rhodopsin. *Mol. Neurobiol.* **7**, 61-84
12. Adler, A.J., Evans, C.D., and Stafford, W.F. (1985) Molecular properties of bovine interphotoreceptor retinoid-binding protein. *J. Biol. Chem.* **260**, 4850-4855
13. Duffy, M., Sun, Y., Wiggert, B., Duncan, T., Chader, G.J., and Ripps, H. (1993) IRBP enhances rhodopsin regeneration in the experimentally detached retina. *Exp. Eye Res.* **57**, 771-782
14. Liou, G.I., Bridges, C.D.B., Fong, S.-L., Alvarez, R.A., and Gonzalez-Fernandez, F. (1982) Vitamin A transport between retina and pigment epithelium—An interstitial protein carrying endogenous retinol (IRBP). *Vis. Res.* **22**, 1457-1467
15. Okajima, T.-I.L., Pepperberg, D.R., Ripps, H., Wiggert, B., and Chader, G.J. (1990) Interphotoreceptor retinoid-binding protein promotes rhodopsin regeneration in toad photoreceptors. *Proc. Natl. Acad. Sci. USA* **87**, 6907-6911
16. Saari, J.C., Teller, D.C., Crabb, J.W., and Bredberg, L. (1985) Properties of an interphotoreceptor retinoid-binding protein from bovine retina. *J. Biol. Chem.* **260**, 195-201
17. Ho, M.T.P., Massey, J.B., Pownall, H.J., Anderson, R.E., and Hollyfield, J.G. (1989) Mechanisms of vitamin A movement between rod outer segments, IRBP, and liposomes. *J. Biol. Chem.* **264**, 928-935
18. Liou, G.I., Fei, Y., Peachey, N.S., Matragoon, S., Wei, S., Blaner, W.S., Wang, Y., Liu, C., Gottesman, M.E., and Ripps, H. (1998) Early onset photoreceptor abnormalities induced by targeted disruption of the interphotoreceptor retinoid-binding protein gene. *J. Neurosci.* **18**, 4511-4520
19. Liou, G.I., Matragoon, S., Yang, J., Geng, L., Overbeek, P.A., and Ma, D.-P. (1991) Retina-specific expression from the IRBP promoter in transgenic mice is conferred by 212 bp of the 5′-flanking region. *Biochem. Biophys. Res. Commun.* **181**, 159-165
20. Bobola, N., Hirsch, E., Albini, A., Altruda, F., Noonan, D., and Ravazzolo, R. (1995) 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.* **270**, 1289-1294
21. Kikuchi, T., Raju, K., Breitman, M.L., and Shinohara, T. (1993) 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.* **13**, 4400-4408
22. Morabito, M.A., Yu, X., and Barnstable, C.J. (1991) 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.* **266**, 9667-9672
23. Fong, S.-L., Morris, T.A., Lee, C.-H., Donoso, L.A., and Fong, W.-B. (1993) Transfection of retinoblastoma cells: Localization of cis-acting elements for human IRBP gene in *Methods in Neurosciences* (Hargrave, P., ed.) Vol. 15, pp. 322-329, Academic Press, Orlando, FL
24. Liou, G.I., Geng, L., Al-Ubaidi, M.R., Matragoon, S., Hanten, G., Baehr, W., and Overbeek, P.A. (1990)Tissue-specific expression in transgenic mice directed by the 5′-flanking sequences of the human gene encoding interphotoreceptor retinoid-binding protein. *J. Biol. Chem.* **265**, 8373-8376
25. Fei, Y., Matragoon, S., and Liou, G.I. (1995) Simple and efficient method for the preparation of nuclear extracts. *BioTechniques* **18**, 984-986
26. Higuchi, R.B. (1990) *PCR Protocols: A Guide to Methods and Applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J., eds.) pp. 177-183, Academic Press, San Diego, CA
27. Liou, G.I., Wang, M., and Matragoon, S. (1994) Timing of interphotoreceptor retinoid-binding protein (IRBP) gene expres-

sion and hypomethylation in developing mouse retina. *Dev. Biol.* **161**, 345-356
28. Baehr, W., Falk, J.D., Bugra, K., Triantafyllos, J.T., and McGinnis, J.F. (1988) Isolation and analysis of the mouse opsin gene. *FEBS Lett.* **238**, 253-256
29. Zack, D.J., Bennett, J., Wang, Y., Davenport, C., Klaunberg, B., Gearhart, J., and Nathans, J. (1991) Unsual topography of bovine rhodopsin promoter-lacZ fusion gene expression in transgenic mouse retinas. *Neuron* **6**, 187-199
30. Liou, G.I., Matragoon, S., Overbeek, P A., and Fei, Y. (1994) Identification of a retina-specific footprint within the retina-specific regulatory region of the human interphotoreceptor retinoid-binding protein gene. *Biochem. Biophys. Res. Commun.* **203**, 1875-1881
31. Fortini, M.E. and Rubin, G.M. (1990) Analysis of *cis*-acting requirements of the Rh3 and Rh4 genes reveals a bipartite organization to rhodopsin promoters in *Drosophila melanogaster*. *Genes Dev.* **4**, 444-463
32. Droba, M. (1988) Properties of acid beta-galactosidase from fowl spermatozoa. *Br. Poult. Sci.* **29**, 555-562
33. Fassina, G., Paglialunga, G., Noonan, D.M., Chader, G.J., and Albini, A. (1993) Modulation of Y-79 retinoblastoma cell differentiation and IRBP expression by dibutyryl cyclic AMP and laminin. *Int. J. Oncol.* **2**, 745-751
34. Boatright, J.H., Borst, D.E., Peoples, J.W., Bruno, J., Edwards, C.L., Si, J.-S., and Nickerson, J.M. (1997) A major cis activator of the IRBP gene contains CRX-binding and Ret-1/PCE-1 elements. *Mol. Vis.* **3**, 15-28
35. Catron, K.M., Iler, N., and Abate, C. (1993) Nucleotides flanking a conserved TAAT core dictate the DNA binding specificity of three murine homeodomain proteins. *Mol. Cell. Biol.* **13**, 2354-2365
36. Furukawa, T., Kozak, C.A., and Cepko, C.L. (1997) *Rax*, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. *Proc. Natl. Acad. Sci. USA* **94**, 3088-3093
37. Martinez, J.A. and Barnstable, C.J. (1998) Erx, a novel retina-specific homeodomain transcription factor, can interact with Ret 1/PCE 1 sites. *Biochem. Biophys. Res. Commun.* **250**, 175-180
38. Hanes, S.D. and Brent, R. (1991) A genetic model for interaction of the homeodomain recognition helix with DNA. *Science* **251**, 426-430
39. Takahashi, K., Vigneron, M., Matthes, H., Wildeman, A., Zenke, M., and Chambon, P. (1986) Requirement of stereospecific alignments for initiation from the simian virus 40 early promoter. *Nature* **319**, 121-126
40. Chen, S. and Zack, D.J. (1996) Ret 4, a positive acting rhodopsin regulatory element identified using a bovine retina *in vitro* transcription system. *J. Biol. Chem.* **271**, 28549-28557
41. DiPolo, A., Rickman, C.B., and Farber, D.B. (1996) Isolation and initial characterization of the 5′ flanking region of the human and murine cyclic guanosine monophosphate-phosphodiesterase $\beta$-subunit genes. *Invest. Ophthal. Vis. Sci.* **37**, 551-560
42. Ahmad, I., Yu, X., and Barnstable, C.J. (1994) A cis-acting element, T$\alpha$-1, in the upstream region of rod $\alpha$-transducin gene that binds a developmentally regulated retina-specific nuclear factor. *J. Neurochem.* **62**, 396-399