Both PCE-1/RX and OTX/CRX Interactions Are Necessary for Photoreceptor-specific Gene Expression*

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Aira Kimura, Dhirendra Singh, Eric F. Wawrousek†, Masashi Kikuchiš, Makoto Nakamura, and Toshimichi Shinohara†

From the Center for Ophthalmic Research and §Cerebrovascular and NeuroScience Research Institute, Brigham & Women’s Hospital, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts 02115 and the ¶Laboratory of Molecular and Developmental Biology, NEI, National Institutes of Health, Bethesda, Maryland 20892

RX, a homeodomain-containing protein essential for proper eye development (Mathers, P. H. Grinberg, A., Mahon, K. A., and Jamrich, M. (1997) Nature 387, 603–607), binds to the photoreceptor conserved element-1 (PCE-1) in the photoreceptor cell-specific arrestin promoter and stimulates gene expression. RX is found in many retinal cell types including photoreceptor cells. Another homeodomain-containing protein, CRX, which binds to the OTX element to stimulate promoter activity, is found exclusively in photoreceptor cells (Chen, S., Wang, Q. L., Nie, Z., Sun, H., Lennon, G., Copeland, N. G., Gillbert, D. J., Jenkins, N. A., and Zack, D. J. (1997) Neuron 19, 1017–1030; Furukawa, T., Morrow, E. M., and Cepko, C. L. (1997) Cell 91, 531–541). Binding assay and cell culture studies indicate that RX and PCE-1 both bind to the photoreceptor cell-restricted gene elements and at least two different regulatory factors RX and CRX are necessary for high level, photoreceptor cell-restricted gene expression. Thus, photoreceptor specificity can be achieved by multiple promoter elements interacting with a combination of both photoreceptor-specific regulatory factors and factors present in closely related cell lineages.

Our goal is to understand the basic biochemical mechanisms of tissue-restricted gene expression. The vertebrate retina offers a distinct advantage in studying gene expression since it contains large numbers of well defined cell types, such as photoreceptor cells. Photoreceptor cells are highly specialized to convert light energy into neuronal impulses, a process which involves many proteins, several of which have been well characterized such as opsin, interphotoreceptor retinoid-binding protein (IRBP),1 and phosphodiesterase (4). Arrestin, also known as S-antigen, accumulates to high levels in the outer segments of retinal photoreceptor cells, where it down-regulates the phototransduction cascade (5).

The transcriptional efficiency and tissue specificity of a given gene are determined by the specific combination of regulatory factors that assemble on the gene’s regulatory elements to form the transcription initiation complex. Several homeodomain proteins such as Pax6 (6), RX (1, 7), and CRX (2, 3) which profoundly affect vertebrate eye development, bind to promoter elements of several eye-specific genes, and may aid in the activation of these genes. The RX homeobox gene has been found to be essential for normal vertebrate eye development, and its mis-expression has profound effects on eye morphology (1). Xenopus embryos injected with RX mRNA develop ectopic retinal tissue and exhibit hyperproliferation in the neural retina. Mouse embryos homozygous for a null allele of this gene fail to form optic cups and therefore do not develop eyes. It is clear that the RX gene family plays a crucial role in the establishment and/or proliferation of retinal progenitor cells. Other homeobox proteins, especially the transcriptional regulatory protein Pax6, play an important role in vertebrate and invertebrate eye formation. Mutations which affect the function of Pax6 result in severe eye malformations known as Aniridia in humans and small eye syndrome in mice (8).

Another homeodomain protein, CRX, binds to the OTX regulatory sequence found upstream of several photoreceptor cell-specific genes and can stimulate transcriptional activity of the opsin and IRBP promoters in non-retinal cells (2). Moreover, mutation of the CRX gene induces a cone-rod dystrophy in humans (9). Thus, CRX is a novel photoreceptor cell-specific factor and plays a crucial role in the differentiation of photoreceptor cells.

We have previously identified the photoreceptor conserved element 1 (PCE-1) consensus sequence which is found in the functionally important regulatory regions of all known photoreceptor cell-specific genes. The same nuclear regulatory factors which bind to the arrestin PCE-1 site also recognize PCE-1 sites in the promoter regions of other vertebrate photoreceptor-specific genes (10). A factor-binding site with the same core sequence as PCE-1 has also been identified as Ret1 in the 5′-flanking region of the rat opsin gene (11). These results suggest that the PCE-1 and Ret1 sites are structurally and perhaps functionally the same.

Recently, a factor designated Erx was found to bind to the Ret 1 site (12). Here we have isolated a human homeodomain-containing protein RX, which binds to the PCE-1/Ret1 site and activates the TATA-less arrestin promoter (10, 13) and IRBP promoter. Our study established that RX is a transcriptional regulatory protein which binds to a regulatory region found in many retina-specific genes and up-regulates expression of these genes. Furthermore, our study suggested that two individual elements (PCE-1 and OTX) and two different factors (RX and CRX) are necessary for stimulating expression of photoreceptor cell-specific genes.
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TABLE I
Oligonucleotides used in EMSA

| Oligonucleotide                      | Sequence                                      |
|-------------------------------------|-----------------------------------------------|
| Mouse PCE-1 (m-PCE-1)               | CCAAAAGCCTCCAAATGACTTATTT                  |
| Mouse PCE-1 mutant (m-PCE-1 mut)    | TTCGAAAGTTATGATGATAGTT                     |
| Mouse OTX (m-OTX)                   | CCAAGCTTCTGATTAGCTATT                      |
| Bovine PCE-1 upstream (b-PCE-1 up)  | TTCGAAAGCTACTAGATGATAATTTTTGC             |
| Bovine PCE-1 intron (b-PCE-1 int)   | TGCCCGACCAATTAGCTACATGCT                 |
| Chicken Coup-TF (Coup)              | CAGGATCGATGCAATTTAGGTGAA                   |
|                                      | TAGTACCCTTATGCTAATCTTGCG                 |

EXPERIMENTAL PROCEDURES

Southwestern Screening of cDNA Library for PCE-1 Site Binding Proteins—The random-primer msg11 human retina library (CLONTECH Laboratories, Palo Alto, CA) was screened by the Southwestern method (14). Oligonucleotides were synthesized by the Life Technologies, Inc. Custom Primers Service (Grand Island, NY). The oligonucleotides containing mouse PCE-1 sites were annealed, 5’ end-labeled with γ-[^32]P]ATP (NEN Life Science Products, Boston, MA) and T4 polynucleotide kinase, and concatenated with T4 DNA ligase. The average final length of probe 1 and probe 2 was approximately 200 bp. Probe 1 was GCCGAACGCTT
TCAATTAGCATTATTCCGAAAGATTACTGATAAGTT.
Bacteriophage (2 x 10^11 per dish) were plated with Escherichia coli Y1090 on 120-cm^2 LB plates and incubated at 42 °C for 3.5 h. Nitrocellulose filters (BA-S 85 TM; Schleicher & Schuell Inc., Keene, NH) imregnated with 10 mM isopropylthio-β-D-galactopyranoside were and then overlaid onto the cultures and incubation continued at 37 °C overnight. The filters were lifted, air-dried, and submerged for 10 min in 250 ml of binding buffer (12 mM HEPES, pH 7.9, 12 mM KCl, 0.6 mM MgCl2, 1.2 mM dithiothreitol) containing 6 M guanidine hydrochloride. The filters were washed four times with gentle shaking in the same volume of the binding buffer without guanidine hydrochloride. To maximize renaturation of the proteins, we repeated this cycle (binding buffer plus 6 mM guanidine hydrochloride followed by binding buffer alone) four times. The filters were blocked with 250 ml of binding buffer containing 5% nonfat skim milk for 4 h at 4 °C with gentle shaking. They were incubated with gentle shaking at 4 °C overnight with labeled DNA probe (10^6 cpm/ml) in 250 ml of binding buffer supplemented with 0.25% skim milk. The filters were washed 3 times with 500 ml of binding buffer containing 0.25% skim milk and 50 mM KCl for 10 min at room temperature. The filters were dried on 3MM paper and exposed to Fuji X-ray Film (Fuji Film Co., Tokyo, Japan) overnight at −70 °C.
Screening of cDNA by the Plaque Hybridization Method—The 5’ fragment (479 bp) and 3’ fragment (856 bp) of the hRX cDNA were 32P-labeled with T7 QuickPrime Kit (Amersham Pharmacia Biotech) and used to screen the human retina cDNA library. Bacteriophage (5 x 10^8) were plated with E. coli strain Y1090 on 120-cm^2 plates and grown 37 °C for 8 h. Detailed procedures were described elsewhere (15).
Production and Purification of GST-RX and GST-CRX Fusion Proteins—We produced RX and CRX as GST fusion proteins in an E. coli expression system. Because there was a stop codon upstream of the translation initiation codon of hRX, a PCR primer (TCCCCGGAATTC
CCCAATGCACCCTG) was designed with an EcoRI site just upstream of the translation initiation codon of the hRX cDNA, and a second PCR primer (GGGACCTTCGTTAGGTTGACCTTG) was designed to introduce EcoNI site into the 3’ non-coding region of the hRX cDNA. These primers were used to PCR amplify 10 ng of DNA containing the entire coding region of hRX cDNA (16). The PCR amplification product (548 bp) was blunt end ligated into the HincII site of the pBluescript II SK -(Stratagene, La Jolla, CA). After confirming the fidelity of the PCR amplification by DNA sequence analysis, the fragment between the Xhol site in the pBluescript and EcoNI site in the PCR product was subcloned such that it replaced the Xhol/EcoNI fragment in the original hRX cDNA/EcoRI fragment. The EcoRI fragment of this new hRX cDNA was subcloned into the EcoRI site of pGEXKT-1 (Amersham Pharmacia Biotech), and the final ligation creates an in-frame fusion of the GST and hRX coding sequences. The cDNA for human CRX (pro-
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IgG or goat anti-mouse IgG; 1:1000; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The color was developed with 0.01% hydrogen peroxide and 0.05% 3,3-diaminobenzidine tetrahydrochloride (Bio-Rad). Protein size markers were purchased from Bio-Rad.

Electrophoretic Mobility Shift Assay (EMSA)—Oligonucleotide probes listed in Table I were annealed and their 5'-ends were labeled with [γ-32P]ATP and T4 polynucleotide kinase (Promega, Madison, WI). The standard binding reaction was carried out in 20 μl of a mixture containing 12 mM HEPES (pH 7.9), 12 mM KCl, 0.6 mM MgCl2, 1.2 mM dithiothreitol, 10% glycerol, 2 μg of poly(dI-dC), 5 fmol of the 32P-labeled probe, and 8 ng to 1 μg of purified GST-RX fusion protein. In competition experiments, 50 fmol to 1 pmol (1.6-200 fold molar excess) of the competitor DNA was added to the standard mixture. After incubation on ice for 30 min, the samples were loaded onto 5% polyacrylamide gels in 0.5 × Tris borate-EDTA (TBE) buffer, and electrophoresed at 10 V/cm for 2 to 3 h at room temperature. The gel was then dried and autoradiographed at −70 °C.

For Ab supershift analysis, 1 μl of rabbit Ab to RX was added to the standard mixture halfway through the reaction (after the first 15 min of incubation). Nuclear extract from bovine retinas was prepared by the method of Gorski et al. (18).

Construction of Arrestin Promoter/Reporter Fusion Genes—A construct containing the mouse arrestin promoter (~209 to +304) chloroamphelenchus acetyltansferase (CAT) fusion gene (m-ARR-209-CAT) was prepared as described (10). To construct a CAT reporter containing the mouse arrestin promoter (~1783 to +304) fused to chloramphenicol acetyltransferase (CAT) fusion gene (m-IRBP-1983-CAT) was provided by John M. Nickerson (Emory University). CAT constructs containing PCE-1 sites or OTX sites upstream of the basal herpes simplex virus thymidine kinase (tk) promoter were generated by inserting double-stranded oligonucleotides into the pBLCAT5 (ATCC, Manassas, VA). Oligonucleotides containing either the PCE-1 or OTX element were chemically synthesized and annealed and ligated as described above. The elements were: PCE-1 BstXI-dIII and EcoNI and the fragment was replaced with a sequence of hRx-1 (2,230 bp) and hRx-2 (1,846 bp) was identical (2, 18).

RESULTS

Cloning of the Human RX Homeodomain Protein—We isolated cDNAs for seven individual factors from a human retinal library by Southwestern screening with the PCE-1 probes (probe 1 and probe 2; see “Experimental Procedures”), and a clone which bound strongly to both probes was selected for further study. Sequencing revealed that the clone contained a region homologous to the highly conserved DNA binding consensus (homeobox) in homeodomain proteins. The full size of this clone was 1790 bp long and encoded a 346-amino acid protein, which we have named human RX (GenBank accession number AF115392). This protein shares 85% identity (1, 7). The homeodomain regions are in bold letters, and the three putative helical regions are underlined. An arrow with int-1 or int-2 indicates an insertion sequence. B, three different types of human RX cDNA clones probably arose by alternative RNA splicing, hRx can be translated into the 346-amino acid RX protein shown as hRx-1 has a 303-bp insertion (Int-1) between 484 and 485 bp of hRx. The hRx-2 has the same Int-1 insertion and also has an additional 159-bp insertion (Int-2) between 742 and 743 bp of hRx. The Int-1 and Int-2 insertions each contain two in-frame stop codons. AA indicates the poly(A) tail.
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Fig. 2. Immunoblot analysis. Proteins from bovine retina, lens, iris, and mouse brain and liver, GST purified RX (100 ng) and CRX (100 ng) immunostained with RX-specific Abs (1/1000 dilution) (A). Immunoblot analysis of duplicate blots stained with RX-specific Abs (1/1000 dilution) which have been preincubated with purified GST-RX (B).

FIG. 2.

Fig. 3. Immunohistochemistry. A, rat retinas were immunostained with RX-specific antibodies (1/500 dilution). B, retinas were immunostained with RX-specific antibodies (1/500 dilution) which had been preincubated with purified GST-RX (200 μg). The bar indicates 20 μm.

bp 484/485 and bp 742/743, respectively, in hRx-2 (Fig. 1B). We believe that these cDNAs might be products of alternative RNA splicing. A functional role of these two clones is presently unknown. It does, however, seem unlikely that these cDNA clones could encode a homeodomain-containing protein, since the first inserted sequence (Int-1) contains two in-frame stop codons upstream of the homeodomain, and the second insertion (Int-2) also contains two stop codons.

Expression Pattern of RX in Various Tissues—We studied the expression pattern of RX in various tissues by immunoblot analysis. Specificity of the this procedure was demonstrated on duplicate immunoblots probed with the same RX-specific Abs (Fig. 2A) which had been preincubated with purified GST-RX (Fig. 2B) or with preimmune serum (data not shown). Two predominant bands in the 37-kDa molecular mass region were detected in retina (Fig. 2A), but only one of these was RX (Fig. 2A, shown by an arrowhead) since this band could be eliminated by addition of GST-RX to anti-RX Ab (Fig. 2B). This RX-specific band was also detected in iris (Fig. 2A). The specificity of the RX Abs for GST-RX but not the related GST-CRX is shown in Fig. 2A. These results suggest that RX is present in neural retina and iris, but not detectable lens, brain, and liver. The expression pattern of RX in the retina was further investigated by immunohistochemistry using the affinity purified rabbit anti-RX Ab (1/500 dilution). This analysis revealed that RX was present in the outer nuclear layer (nucleus of photoreceptor cells), in addition, it was present in neuroretinal cells including ganglion cells and the inner nuclear layer (Fig. 3A).

Control experiments, RX Abs which had been preincubated with purified GST-RX failed to stain any of these cell types (Fig. 3B).

RX Binds to the PCE-1 Element—We investigated the binding properties of RX to the PCE-1 element using an EMSA. Double-stranded oligonucleotides containing the PCE-1 element of the mouse arrestin gene (10) and the mouse OTX element (m-OTX) (see Refs. 2 and 3 and Table I) were 5′-end labeled with [32P]. Fusion proteins of GST with RX (GST-RX) or with the human CRX (GST-CRX) were produced in an E. coli expression system and purified by glutathione column chromatography.

Strong binding was observed when the m-PCE-1 element was incubated with the GST-RX protein at higher concentrations (Fig. 4A). In contrast, less strong binding of GST-CRX to m-PCE-1 was observed. Conversely, binding was observed between m-OTX and GST-RX only at higher concentrations, but stronger binding was observed between m-OTX and GST-CRX at those concentrations. In the control experiments, GST bound to neither the m-PCE-1 site nor the m-OTX site.

Binding of RX to the m-PCE-1 element was decreased significantly by a 40-fold molar excesses of unlabeled m-PCE-1 competitor, but was only slightly diminished by a 200-fold molar excesses of unlabeled m-OTX competitor (Fig. 4B). Complex formation between RX and PCE-1 sites was unaffected by a large excess of an unlabeled, unrelated oligonucleotide containing the chicken ovalbumin upstream promoter transcription factor (COUP-TF) site. Conversely, binding of CRX to m-OTX was inhibited slightly by a large molar excess of unlabeled m-PCE-1, and strongly by a large molar excess of unlabeled of m-OTX, but was unaffected by the COUP competitor. These results indicated that the stronger binding was between m-PCE-1 and RX and between m-OTX and CRX, and that the affinity of CRX for m-PCE-1 and the affinity of RX for m-OTX are much weaker. We have therefore concluded that PCE-1 and OTX are the binding sites for RX and CRX, respectively.

We used an antibody (Ab) supershifting technique in combination with EMSA to confirm that the PCE-1 elements bind to RX contained in retina extracts. In a previous publication (10), when m-PCE-1 was incubated with bovine retinal nuclear extract, EMSA revealed a complex designated Bp1 (see also Fig. 5, lane 1, indicated by an arrow). Initially we generated complexes between m-PCE-1 and bovine nuclear extract followed by addition of Abs (Fig. 5, lanes 1 and 3, an arrow and an open arrowhead, respectively). Similarly, we generated complexes between m-PCE-1 and purified RX, then added RX-specific Abs (Fig. 5, lanes 4 and 5, an arrowhead and an open arrowhead, respectively). The RX-specific Abs recognized both purified GST-RX and RX from bovine nuclear extracts bound in PCE-1 complexes. The complexes formed between m-PCE-1 and purified RX were smaller than those formed with the nuclear extract, but both of these complexes were supershifted by RX-specific Abs (Fig. 5, lanes 3 and 5, open arrowheads, respectively). We therefore concluded that the Bp1 complexes formed between bovine retina nuclear extract and the m-PCE-1 element contained RX factor. As reported previously (10), m-PCE-1 and a bovine nuclear factor generated a second complex, Bp2 (Fig. 5, arrow). Interestingly, the Bp2 complex failed to supershift upon addition of RX-specific Abs suggesting that it does not contain RX. We speculated that the Bp2 may be composed of m-PCE-1 element and other homeodomain proteins such as CRX (2, 3), Pax6 (6, 8), or Erx (12), since bovine retinal extract is derived from various cell types.
Two Nucleotide Differences in the PCE-1 Sequence Determine RX versus CRX Binding Specificity—We next investigated whether the RX factor binds to the PCE-1 element in the murine and bovine arrestin promoters. One PCE-1 element is present in the mouse arrestin gene at position 2115 to 221 (CAATTAG). The bovine arrestin gene, however, has three PCE-1 sites associated with it; one in the 5'-flanking region (21830 CAATTAG 21824) and two in the first intron (1561 GTAATTG 1567 and 1656 TTAATTG 1663) (see below). Complete sequences of all oligonucleotides used in the following EMSA experiments are shown in Table I. We conducted EMSA using purified GST-RX and oligonucleotide probes containing the identical mouse (221 CAATTAG 215) and bovine up-stream (21830 CAATTAG 21824) PCE-1 site, and one bovine first intron PCE-1 site (1663 CAATTAA (rev) +656). These oligonucleotides bound strongly to GST-RX to form complexes (Fig. 6, lanes 1–10) suggesting that the PCE-1 core consensus (CAATTA(G/A)) was an essential binding element and the flanking sequence contributed minimally to the observed binding activity.

Although the consensus PCE-1 and OTX sequences are similar, the difference is in two nucleotides at their 5'-ends: the

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**Fig. 4.** A, EMSA with mouse arrestin PCE-1 (m-PCE-1) (lanes 1–9) and mouse arrestin OTX (m-OTX) (lanes 10–18) oligonucleotide probes. The EMSA assays were conducted with 8, 40, 200, or 1000 ng of GST-RX (lanes 2–5 and 11–14) or GST-CRX (lanes 6–9 and 15–18). Lanes 1 and 10 are control experiments with 1000 ng of GST protein. B, competition EMSA with m-PCE-1 32P-labeled oligonucleotide probe and GST-RX protein (lanes 1–18), and m-OTX 32P-labeled oligonucleotide probe with GST-CRX protein (lanes 14–26). Each lane contained 400 ng of GST-RX or GST-CRX. Unlabeled competitor oligonucleotides, m-PCE-1 (lanes 2–5 and 15–18), m-OTX (lanes 6–9 and 19–22), and COUP (lanes 10–13 and 23–26) were added in 1.6-, 8-, 40-, and 200-fold molar excess. No unlabeled competitor was added in lanes 1 and 14.

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**Fig. 5.** RX-specific antibodies supershift EMSA bands. EMSAs were performed with 32P-labeled m-PCE-1 probe in combination with either bovine retina nuclear extract (lanes 1–3) or purified GST-RX fusion protein (lanes 4 and 5). When RX-specific antibodies were added to the EMSA reactions (lanes 3 and 5) the Bp1 band shifted to a higher molecular weight. Addition of unlabeled competitor m-PCE-1 at 200-fold molar excess (lane 2) eliminated the specific EMSA band. The arrow in the left margin indicates the Bp2 complex (10) which did not supershift with addition of RX-specific antibodies. The arrowhead indicates the DNA-RX-Ab complex.

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**Fig. 6.** EMSA with various naturally occurring and mutant PCE elements and GST-RX or GST-CRX proteins. The 32P-labeled oligonucleotide probes used were: bovine arrestin upstream PCE-1 element (lanes 1–5), bovine arrestin intron PCE-1 element (lanes 6–10), mouse arrestin PCE-1 mutant (lanes 11–20). Assays were conducted with 8, 40, 200, or 1000 ng of GST-RX (lanes 2–5, 7–10, and 12–15) or GST-CRX (lanes 6–9 and 19–22). In control experiments, 1000 ng of GST protein was used (lanes 1, 6, 11, and 16).

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PCE-1 consensus sequence is CAATTA and OTX is TGAT-Tag. Oligonucleotide pair m-PCE-1-mut was synthesized in which the two 5' nucleotides of the mouse PCE-1 core sequence were altered (CAATTA → TGATTA) but the sequences within the core and adjacent remained unchanged. The RX bound much more weakly to m-PCE-1-mut than to wild type...
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The present study established that in addition to the expression of RX factor, known to be essential for eye morphogenesis, binds to, and can act through, the PCE-1 element fused to the CAT reporter gene, mouse IRBP. The mouse IRBP promoter at least one PCE-1 site in all known retina-specific genes, to determine RX binding to the PCE-1 site activates an arrestin promoter, we generated two heterogeneous promoter constructs (Fig. 9). One construct had two tandem repeats of the PCE-1 site and the other had two tandem repeats of the OTX site upstream of the weak HSVtk promoter which drives CAT reporter gene expression. Each of these reporter constructs was co-transfected into 293 cells with constructs expressing either RX or CRX, and promoter activation was quantified by CAT assay. Promoter activity of the tk promoter alone was not affected by RX or CRX (1.4- and 1.2-fold induction, respectively, Fig. 9). Promoter activity of the construct containing two tandem repeats of PCE-1 was stimulated by up to 3.9-fold by RX, but unaffected by CRX. Conversely, the promoter activity of the construct containing two tandem repeats of OTX was stimulated up to 2.5-fold by CRX, but it was unaffected by RX. This result clearly demonstrates that RX acts through PCE-1 site to activate transcription, and CRX acts through the OTX site to activate transcription.

DISCUSSION

The PCE-1 Element Interacts with the Product of an Eye Morphogenesis Gene—We have isolated a novel homeodomain protein, RX, from a human retinal cDNA library. The RX factor bound to the PCE-1 element (Figs. 4 and 6) and activated the promoter constructs of arrestin and IRBP genes in embryonic chick retina cells and kidney 293 cells (Figs. 7 and 8). RX is expressed in most cell types in the adult retina including photoreceptor cells, and other neuroretinal cell types (Figs. 2 and 3). It is an essential factor for normal eye development and its misexpression has profound effects on eye morphogenesis (1). The present study established that in addition to the expression seen during development, a low level of RX, comparable to that of other known homeodomain proteins, was present in cells of the adult retina. Double shift assays indicated that there is sufficient RX in adult retina to bind to PCE-1 element (see Fig. 5). Interestingly, expression of most photoreceptor-specific genes is initiated after RX was dropped at lower levels (1). Our results showed that the RX factor, known to be essential for eye morphogenesis, binds to, and can act through, the PCE-1 site.

RX Binds to PCE-1 Sites and May Stimulate Retina-specific Promoters—The finding that RX is expressed in most cell types in the retina suggested that the promoters of a variety of retina-specific genes might contain PCE-1 elements. Computer searches of genes expressed in the retina revealed the presence of at least one PCE-1 site in all known retina-specific genes, and multiple PCE-1 sites in the 5'-flanking region of several of these genes (10). Since RX binds tightly to the PCE-1 element and regulates promoter activity in our assay systems, it is reasonable to speculate that RX binds to the PCE-1 sites of retina-restricted genes and enhances retinal specific promoter activity in vivo.

RX has been shown by protein immunoblot analysis and immunocytochemistry to be expressed in most retinal cell types (predominantly in the photoreceptor cells). Our results are also in agreement with those of Yu et al. (21), who showed that in transgenic mice carrying four tandem copies of the PCE-1/Ret1 element fused to the lacZ reporter gene, β-gal was expressed...
not only in photoreceptor cells, but also in the ganglion cell layer and ciliary epithelium. The PCE-1 element in the 5'-flanking region of cellular retinaldehyde-binding protein may play an important role in the expression of cellular retinaldehyde-binding protein in RPE cells (22). In addition, present supershift analysis suggested that the PCE-1 site bound to RX as well as some other factor(s). One such factor, Erx, was recently isolated and found to bind to the PCE-1/Ret1 site and activate transcription (12).

RX and CRX Bind to the PCE-1 and OTX Sites, respectively—In our present study, EMSA and competition EMSA assays confirmed that the PCE-1 and OTX sites bound tightly to RX and CRX. 293 cells were co-transfected with 10 μg of mouse arrestin promoter (−209 to +304 bpi/CAT (m-ARR-209-CAT) fusion gene, together with expression vector(s) encoding RX or CRX, or RX and CRX together. RX and CRX (0.2 μg) appear to act synergistically in activating the arrestin promoter. B, similarly, 293 cells were co-transfected with 10 μg of mouse arrestin mutant promoter (m-ARR-209-PCE-1-mut-CAT) fusion gene, together with expression vector(s) encoding RX or CRX, or RX and CRX. CRX, but not RX, activated the mutant mouse arrestin promoter. All CAT activity values were normalized to β-galactosidase activity to control for transfection efficiency.
RX factor and CRX factor, respectively. We also demonstrated the functional transformation of a PCE-1 element (CAATTAG) into an OTX element (TGATTAG) by a simple two-nucleotide substitution (CA3TG) adjacent to the core sequence of the m-PCE-1 element. The ATTA core was known to be essential for DNA binding activity of many homeodomain proteins, while nucleotides immediately flanking this core determine the specificity of binding (23). In addition, the S8 factor (24), a mesoderm-specific homeodomain protein, was shown to bind to a sequence element identical to that of PCE-1, and it bound strongly to the mouse and bovine PCE-1 element.2

The predicted amino acid sequences of human and mouse RX show high degree of homology to S8 (25), aristless (26), Chx10 (27), pax6 (6), and paired (28). The DNA-binding domain of the homeobox is comprised of three regions which presumably me-

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2 A. Kimura D. Singh, E. F. Wawrousek, M. Kikuchi, M. Nakamura, and T. Shinohara, unpublished data.
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RX binds to PCE-1 to activate photoreceptor-specific genes, indicating that RX binds to the PCE-1 promoter to promote high level expression of photoreceptor-specific genes. Furthermore, RX and CRX (another photoreceptor-specific transcription factor) can bind to the same DNA sequences, suggesting that they interact with the same DNA-binding sites.

Our transgenic studies indicated that both PCE-1 and OTX elements are necessary for high levels of photoreceptor-specific gene expression. However, neither element alone could stimulate high level photoreceptor-restricted gene expression, but when the two elements were present, they could induce high level photoreceptor-restricted gene expression. The transgenic and present in vitro studies with Rx and CRX factors suggest that tissue specificity can be achieved by multiple promoter elements interacting with a combination of both highly cell type-specific regulatory factors and factors present in closely related cell lineages.

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