The homeobox gene CHX10 is required for retinal progenitor cell proliferation early in retinogenesis and subsequently for bipolar neuron differentiation. To clarify the molecular mechanisms employed by CHX10 we sought to identify its target genes. In a yeast 1-hybrid assay CHX10 interacted with the Ret1 site of the photoreceptor-specific gene rhodopsin. Gel shift assays using in vitro translated protein confirmed that CHX10 binds to Ret1, but not to the similar rhodopsin sites Ret4 and BAT-1. Using retinal nuclear lysates, we observed interactions between CHX10 and additional photoreceptor-specific elements including the PCE-1 (rod arrestin/S-antigen) and the opsin locus control region (red/green cone opsin). However, chromatin immunoprecipitation assays revealed that in vivo, CHX10 bound sites upstream of the arrestin and interphotoreceptor retinoid binding protein genes but not rhodopsin or cone opsin. Thus, in a chromatin context, CHX10 associates with a specific subset of elements that it binds with comparable apparent affinity in vitro. Our data suggest that CHX10 may target these motifs to inhibit rod photoreceptor gene expression in bipolar cells.

The mammalian retina consists of three nuclear layers. The outer nuclear layer (ONL) houses the cell bodies of photoreceptors (rods and cones) while those of horizontal, bipolar and amacrine interneurons and Müller glia reside in the inner nuclear layer (INL). The innermost layer is the ganglion cell layer (GCL), which consists of a mixture of ganglion and amacrine neurons. These three cellular areas are separated by outer and inner plexiform layers that house synaptic connections. This intricate laminated structure develops through the amplification of multi-potent progenitor cells, generation of more restricted post-mitotic transition cells, and maturation of these cells into terminally differentiated neurons and glia (1). In rodents, ganglion, horizontal, cone and amacrine transition cells are born in the prenatal period, bipolar and Müller cells are born post-natally, and rods are born throughout retinal development (2). Transcription factors play critical roles in each of the stages of retinal development, but many gaps remain in our understanding of the specific target genes involved. Here, we focus on one of these factors, the homeobox gene CHX10, and its role in sculpting the characteristics of bipolar interneurons.

Homeodomain (HD) proteins regulate retinal development from the earliest stages of optic vesicle formation to the final stages of maturation in the adult (reviewed in (3,4)). Previous molecular analysis linked a naturally
occuring mutation in the homeobox of CHX10 to the ocular retardation phenotype in mice (or^r) (5). These mice display a dramatic decrease in retinal progenitor cell (RPC) proliferation and lack bipolar cells, phenotypes that reflect the expression of CHX10 in both of these cell types* (5,6). Molecular and genetic studies have begun to reveal some aspects of the mechanism by which CHX10 regulates retinal development.

The proliferation defect in the or^r mouse is partially alleviated by crossing this allele with a Mus musculus castaneous strain, thought to be due to as yet uncharacterized modifier genes (7). RPC proliferation is also partially rescued when the cyclin-dependent kinase inhibitor p27^Kip1 is deleted on an or^r background (8). Increased p27^Kip1 levels are linked to a decrease in cyclin D1 although the detailed mechanism is not yet known (8). Gene expression changes have been noted in the or^r retina including aberrant induction of MITF (9), loss of the nuclear receptor retinoid-like orphan receptor β (RORβ) (10), and up or down regulation of many other factors (11). MITF drives the formation of retinal pigment epithelium over the neural retina and RORβ promotes RPC division (9,10), so both of these changes may be linked to the perturbation of cyclin D1 and p27^Kip1 levels. The requirement for CHX10 to facilitate RPC proliferation is transient since overexpression and knockdown assays reveal that CHX10 does not alter the cell cycle in the post-natal retina*.

The mechanism by which CHX10 facilitates bipolar cell differentiation is also not clear. Indeed, until recently it was not certain that CHX10 even had a direct role in bipolar cell differentiation. These neurons are late born cell types, so their absence in the or^r retina could be explained by the severe negative effect on RPC proliferation. Indeed, RPC division drops to almost negligible levels in the post-natal mouse or^r retina*. This issue was resolved by the finding that acute CHX10 knockdown in the post-natal retina blocks bipolar cell differentiation without affecting RPC proliferation*. The decrease in bipolar neurons is accompanied by a corresponding increase in rod photoreceptors*. These data complement over-expression studies showing that CHX10 promotes bipolar cell genesis at the expense of rods* (12). CHX10 can repress transcription (13) raising the possibility that it may facilitate bipolar cell differentiation by inhibiting photoreceptor gene expression. Indeed, rod and bipolar cells express many of the same genes (14), so CHX10 could be one of the factors that defines the unique characteristics of bipolar neurons.

Gene targets of retinal HD proteins are largely unknown. Microarray analysis comparing mRNA from or^r versus wildtype retinas identified several potential CHX10-regulated targets (11), but whether CHX10 binds directly to these genes in vivo is not clear. In vitro, CHX10 can bind to elements found in the cone locus control region (LCR) and a Nestin regulatory element (15,16), but again, it is unclear whether these associations are recapitulated in vivo. Here, we show that CHX10 binds directly to a variety of photoreceptor gene regulatory elements in vitro, but that only a specific subset are targeted in vivo. We also show that CHX10 represses the rod arrestin promoter in a DNA-binding-dependent fashion, providing experimental support for the idea that CHX10 facilitates bipolar cell characteristics in part by silencing expression of select photoreceptor genes.

Materials and Methods

Yeast 1-hybrid screen - A yeast one-hybrid screen, using a bovine retina cDNA/GAL4AD library kindly provided by Dr. Ching-Hwa Sung (Cornell University School of Medicine), was carried out as previously described (17). The bait sequence used was a tetramer of the bovine rhodopsin promoter sequence from –148 to –126bp. Approximately 2x10^6 transformants were screened. Seventy-two colonies that grew on the His- medium were selected for further analysis.

Cell culture - NG108 cells (generated by fusion of mouse neuroblastoma and rat glioma cells) (18) and primary chicken retinal cells (E8) were maintained in culture as previously described (Dorval et al 2005). E8 cells were isolated according to the protocol described by...
Adler et al (19) and were maintained in culture for a restricted time period (~3-4 days) to prevent the overgrowth of any one cell type.

**Luciferase Assays** - Transient transfection assays were performed as previously described (Dorval et al 2005). Briefly, NG108 or freshly dissected primary chick retinal cells were plated at 70% confluency one day prior to transfection by the calcium phosphate method. As a control for transfection efficiency, 0.3 µg of CMVβGal was included in each transfection and β-galactosidase activity was determined. For luciferase assays, 20 µl of lysate was added to 50 µl or 100 µl of luciferase assay reagent (LAR) (10 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT, 270 µM co-enzyme A, 470 µM luciferin, 530 µM ATP in gly-gly buffer pH 7.8) and read immediately in a luminometer (Zylux). 100% luciferase activity is taken as that obtained in the presence of control effector plasmid. Luciferase fold activation is relative to that obtained in the presence of control effector plasmid. Luciferase activity was corrected for transfection efficiency using a β-galactosidase internal control. Individual experiments were performed in duplicate and error bars represent the standard deviation of three independent assays unless otherwise noted.

**Plasmids** - pBSKS-CHX10 (human, full-length), pECEHA-CHX10 (mouse, full-length), Chx-ABCD (human full-length), Chx-N51A (HD mutant of human CHX10) and LexCHX10 (human full-length CHX10 fused to LexA) have all been previously described (Dorval et al 2005). CHX10-VP16 (SVChxV) is a fusion protein of full-length human CHX10, the activation domain of VP16 (amino acids 410-490) and a C-terminal single FLAG tag driven by the SV40 enhancer/promoter. This plasmid was built through 4-way ligation of the following fragments: full-length human CHX10 excised from Chx-ABCD by digestion with HindIII and NheI; PCR amplified VP16 (amino acids 410-490) from GAL4-VP16 digested to yield 5’ NheI and 3’ BglII sticky ends; synthetic FLAG+TAA oligonucleotide 5’ BglII and 3’ EcoRI sticky overhangs; SV-Nmyc (20) as the backbone digested with 5’ HindIII and 3’ EcoRI. GAL4-VP16 encodes the GAL4-DNA-binding domain fused to the activation domain of VP16 (amino acids 410 to 490) (gift of Dr. Robert Kingston).

The following plasmids have been described (17): activators Nrl (mouse in a PED vector) and b-CRX or h-CRX (bovine or human in a pCDNA vector) and Luc reporter plasmids driven by the following promoters human PDEβ (PDEβ-Luc, -340 to +54), bovine IRBP (IRBP-Luc, -300 to +32), human arrestin (Arr-Luc, -316 to +112).

Deletions constructs -316Arr, -202Arr, -107Arr were generated by PCR from the human arrestin reporter (DJZ) and subcloned into pGL2Basic at the 5’ MluI and 3’ BglII sites. RSVLuc contains Luc under the control of the RSV LTR (-489 to +40; gift of HP. Elsholtz).

**CHX10 antibodies** - Polyclonal antibodies were raised by injecting sheep with GST-fusion proteins containing the human CHX10 amino terminus (1-131) or carboxy terminus (264-361) (Exalpha Biologicals, Boston, MA). These antibodies specifically recognized a protein of the correct size (~46kDa) in Western blots of retina lysates from mouse, rat, chicken and cow, and immunostained bipolar cells in the mature rodent retina*

Mouse anti-CHX10 antibodies (M1) were a gift of R. McInnes.

**Isolation of mouse retinal nuclear lysate** - Nuclear lysate was prepared from P6 CD-1 mouse retinas for use in gel shifts according to (21). Dissections and subsequent steps were carried out on ice. Retinas were washed in PBS then buffer A (15 mM HEPES pH 7.6, 110 mM KCl, 5mM MgCl2, 0.1 mM EDTA, 0.2 mM PMSF, 2 mM DTT). Retinas were homogenized in buffer B (10 mM Tris pH 8, 5mM MgCl2, 10 mM NaCl, 60 mM KCl, 0.25 M Sucrose, 10% Glycerol, 0.5% NP-40, 0.5 mM PMSF, 1mM DTT) and the degree of lysis monitored by nuclear staining with trypan blue over 5-10 strokes. Nuclei were spun down at 7,000 rpm at 4°C in a Sorval GSA rotor. The nuclear pellet was then washed in buffer A before nuclear lysis in buffer C (25 mM HEPES pH 7.6, 400 mM KCl, 12.5 mM MgCl2, 0.1 mM EDTA, 1.5mM DTT and 20% Glycerol). In 0.4 ml of buffer C
nuclei were lysed with 1X 2 seconds sonication burst prior to spinning down of nuclear debris for 60 minutes at 4°C at 13,000 rpm. Total protein was measured by Bradford assay before and after dialysis in 20 mM HEPES pH 7.6, 1 mM EDTA, 1 mM MgCl₂, and 50 mM KCl. Typical yield from 18 retinas is ~0.4 µg of total protein, which decreased with dialysis by approximately 3/4. Lysates were aliquoted and stored at -70°C.

Gel shifts - Oligonucleotides used as probes and primers used to amplify promoter fragments for gel shift assays, are shown in Table 1. Gel shifts were performed as previously described (Dorval et al, 2005). CHX10 or CRX plasmids were in vitro translated in the presence of 35S-labeled methionine and protein levels were adjusted for methionine content using densiometry. Gels were dried and exposed on film at room temperature. Gel shifts including 50 µg of mouse P6 retinal nuclear lysate were allowed to incubate for 30 minutes at 30°C. Where applicable, 1 µl of antibody was preincubated for 15 minutes with GST or GST-CHX10.

ChIP - The ChIP protocol used here was based on the protocol described by Pattenden and colleagues (22). P6 CD-1 mice (Charles River Canada) were decapitated and retinas dissected, frozen on dry ice and kept at –80°C until use. Retina was cross-linked with ice-cold 4% formaldehyde in PBS for 30 min, rinsed in PBS, and sonicated in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8) plus protease inhibitors (aprotinin, leupeptin and pepstatin) to an average DNA size of 1 kb (Vibra Cell, Sonics and Materials Inc., Danbury). The sonicated sample was centrifuged at 15,000 g for 10 min at 4°C, the supernatant aliquoted to 100 µl (equivalent to 1 whole retina) and diluted to 1 ml with dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl pH 8). Each diluted sample was incubated for 1 hr with 5 µl of anti-CHX10 antibody, N5, C4 or M1. Samples were centrifuged at 15,000 g for 10 min at 4°C, the supernatant mixed with 20 µl of protein G-Sepharose (Sigma), 200 µg of sonicated salmon sperm DNA (Invitrogen) and 2 mg of yeast tRNA (Invitrogen) and incubated for an additional 1 hr. Precipitates were washed sequentially for 10 min in 1X TSEI (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8, 150 mM NaCl), 4X TSEII (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8, 500 mM NaCl), 1X buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH 8) and 3X TE (10 mM Tris–HCl pH 8, 1 mM EDTA). Samples were then eluted and cross-links reversed by overnight incubation at 65°C in 100 µl of elution buffer (1% SDS, 0.1M NaHCO₃) and DNA fragments purified by GFX PCR DNA and Gel band purification kit (Amersham Biosciences) with a final elution product of 100 µl. Real time PCR (ABI PRISM 7900HT) was used to amplify 2 µl of final ChIP product, and copy number quantified by comparison to a standard curve for each primer set generated with known amounts of sonicated DNA from cross-linked mouse retina. The PCR reaction contained 2 µl each of ChIP sample or serially diluted genomic DNA, 1x SYBR Green PCR Master Mix (Applied Biosystems) and 500 nM of each primer (Table 1) in a final volume of 10 µl. Amplification involved a two-step PCR with denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min for 40 cycles. Error bars represent the standard deviation of three independent experiments.

RESULTS

CHX10 binds photoreceptor-specific elements in vitro - Since CHX10 is essential in bipolar cell development, it may accomplish this function by repressing genes required for the differentiation of other cell types. The HD proteins CRX and Rx activate photoreceptor-specific gene expression (17,23-25), thus we considered the possibility that CHX10 may repress such targets. Ret1 is a highly conserved photoreceptor gene element originally identified in the rat opsin promoter by foot-printing assays with retinal nuclear extracts (26). Four copies of this element placed upstream of a lacZ reporter gene have been reported to be sufficient to drive
photoreceptor-specific gene expression (26,27). In a one-hybrid assay using four copies of the bovine Ret1 site (−148 to −126) as bait, 58% of the identified clones encoded CHX10 (Figure 1A). This data raised the possibility that this sequence or others like it may facilitate repression of photoreceptor genes in non-photoreceptor cell types.

To confirm the ability of CHX10 to bind the bovine rhodopsin promoter, we performed a gel shift assay using in vitro translated CHX10 and an end-labeled Ret1 probe (Figure 1B). CHX10 bound Ret1 and was competed by excess unlabeled Ret1 probe (Figure 1B, lanes 2&3). Excess unlabeled mutated probe did not disrupt the CHX10:Ret1 complex, and CHX10 did not bind the labeled mutated Ret1 site (Figure 1B, lanes 4&7). In vitro translated luciferase did not bind either probe (Figure 1B, lanes 5&8). Therefore, CHX10 specifically interacted with the Ret1 site in vitro.

Two other developmentally important P3-like elements in the rhodopsin proximal promoter are the Ret4 and BAT-1 sites (21,28). The HD protein CRX interacts with both of these elements in vitro, (17). This is in agreement with the critical role CRX plays in photoreceptor differentiation (29,30). In previous studies, a GST-tagged version of the CRX HD bound the BAT-1 and Ret1 sites, and a His tagged form bound Ret4 in gel shift assays (17). As well, in vitro footprinting experiments showed that GST-CRX HD could protect the BAT-1, Ret1 and Ret4 sites (17). The CRX HD has a lysine at position 50 (K50) and is predicted to prefer the core TAATc binding site seen in the BAT-1 and Ret4 motifs as opposed to the TAATt present in the Ret1 motif. In contrast, CHX10 has a Q50 HD (6) which is predicted to bind to TAATt but not TAATc motifs (31-34). Thus, we reexamined the relative affinity of CRX for the three HD binding sites in the rhodopsin promoter using low amounts of in vitro translated rather than GST- or His-tagged proteins, and compared the results with those for CHX10. In vitro translated CHX10 and CRX proteins were 35S-methionine labeled and normalized using densitometry (data not shown). As before, CHX10 interacted with the Ret1 probe (Figure 1C, lane2), but at this protein level, CRX failed to bind to the Ret1 site (Figure 1C, lane4). However, CRX did bind the BAT-1 and Ret4 sequences (Figure 1C, lanes 8 and data not shown), whereas CHX10 did not (Figure 1C lane 7 and data not shown). These data illustrate the distinct binding specificities of CHX10 and CRX for TAATT and TAATC motifs, respectively.

Interaction of CHX10 with other Photoreceptor Gene Motifs - Our next goal was to examine whether CHX10 would interact with other elements found in photoreceptor genes. For instance, the PCE-1 site of the arrestin gene (Kikuchi 1993) is an attractive candidate as it is targeted by other paired-like HD proteins important for retinal development including Rx (23) and CRX (17,23) and contains a TAATt core sequence (Table 2). Retinal lysate from P6 mouse retinas mixed with an end-labeled PCE-1 probe produced a single specific band (Figure 2, lane 2). Complex formation was inhibited by addition of anti-CHX10 antibody (Figure 2, lanes 3), whereas an irrelevant anti-rhodopsin antibody had no effect (Figure 2, lane 6). Addition of the GST-CHX10 antigen used to generate the CHX10 antibody reinstated the CHX10:PCE1 complex (Figure 2, lane 5), but GST alone had no effect (Figure 2, lane 4). CHX10 binding was blocked by addition of excess unlabeled PCE1 probe, but not by an unlabeled mutated PCE-1 probe (Figure 2, c.f. lanes 7-8 with 9-10). Excess unlabeled Ret1 probe, but not a mutated version, also disrupted interaction with the labeled PCE1 probe, supporting the in vitro translated gel shifts in Fig 1 (Figure 2, c.f. lanes 11-12 with 13-14). Previously, Hayashi et al isolated CHX10 in a one- hybrid assay (15). In that case the bait was a highly conserved homeobox-binding motif in the locus control region (LCR) located upstream of the red/green cone opsin gene pair. We found that this motif but not a mutated version efficiently dislodged CHX10 from the PCE-1 site (Figure 2, c.f. lanes 15-16 with 17-18). These data indicate that CHX10 present in retinal lysate can interact, at least in vitro, with conserved elements from several photoreceptor-specific genes.
**Interaction of CHX10 with photoreceptor-specific targets in vivo** - Since chromatin structure plays a key role in mediating transcription factor binding, we next determined the DNA-binding ability of CHX10 in vivo. We performed chromatin immunoprecipitation (ChIP) analysis using P6 mouse retinal tissue and three polyclonal CHX10-antibodies (M1 rabbit, C4 sheep and N5 sheep) (Figure 3A). Normal sheep and rabbit serum as well as no antibody were run as negative controls. DNA recovered from ChIP samples was subjected to real time PCR. The primers were designed for regions upstream of arrestin, rhodopsin, red/green cone opsins and interphotoreceptor retinoid-binding protein (IRBP) (Figure 3B), all of which are known to contain homeodomain binding sites (35-38). For negative controls, we amplified the β-globin promoter and the 3′ end of all the retinal target genes we analyzed. The amount of DNA in each ChIP sample was then used to determine the relative enrichment of DNA at target areas. This analysis detected significant levels of CHX10 at the arrestin promoter, confirming that the PCE1 element is a bona fide CHX10 target. However, despite in vitro data showing that CHX10 binds the Ret1 and cone opsin LCR sites in gel shift and one-hybrid assays, low or negligible levels of CHX10 were observed at the rhodopsin promoter and the cone opsin LCR (Figure 3A). CHX10 was also absent from the immediate upstream region of the IRBP promoter, but bound a conserved region 1.4 kb upstream of the transcription start site (Figure 3A), which is known to be important for IRBP expression in vivo (39) and contains a consensus CHX10 binding site (TAATtgac). Similar results were obtained for ChIP assays performed at earlier (P0) or later (P14) time points (data not shown). CHX10 was not associated with the control β-globin promoter or 3′ regions of photoreceptor genes (Figure 3A and data not shown). These results demonstrate that CHX10 binds a subset of photoreceptor targets in vivo. Importantly, chromatin structure may have a considerable impact on the ability of CHX10 to bind different sites in vivo.

**CHX10 binds and regulates the arrestin promoter** - The in vitro and in vivo data described above imply that rod arrestin is an in vivo CHX10 target gene. To test whether CHX10 can regulate the rod arrestin promoter, we employed transient transfection assays. These studies utilized the human rod arrestin promoter that can be activated by CRX and Nrl (17). First, we confirmed that CHX10 binds this fragment in gel shift assays (Figure 4A). In vitro translated CHX10 interacted with an end-labeled arrestin promoter fragment (-202 to +112) (Figure 4A, lane2) and was dislodged by excess unlabeled arrestin probe but not by excess unlabeled irrelevant sequence (coding region 1-131aa of human CHX10) (Figure 4A, lanes 3&4).

Second, to recapitulate the yeast 1-hybrid assay in mammalian cells, we built a plasmid encoding CHX10 fused to the VP16 activation domain. Co-transfection of CHX10-VP16 together with the rod arrestin reporter led to dose-dependent induction of luciferase activity in NG108 cells, but had no effect on the control promoter RSV (Figure 4B). Further, a reporter containing a fragment of the photoreceptor-specific PDEβ gene promoter (-340 to +64), which lacked any homeodomain binding consensus sequences, was not regulated by CHX10-VP16 (Figure 4B). Finally, we asked whether CHX10 could repress the arrestin promoter. We found that in both NG108 and primary chick retinal cells, CHX10 inhibited CRX-induced activation of the human arrestin promoter (-316 to +112) (Figure 4C&D).

To determine whether CHX10 repressed arrestin in a DNA-binding dependent manner, we tested the effects of mutating position 51 in the HD from arginine to alanine (CHX10-N51A) on repression. We showed previously that this mutation blocks DNA binding (13). CHX10-N51A failed to repress arrestin, suggesting that CHX10-mediated repression requires DNA-binding (Figure 4E). Anti-FLAG western confirmed that the expression levels of CHX10 and CHX10-N51A were uniform (Figure 4F).
DISCUSSION

Data presented here indicate that CHX10 targets a subset of photoreceptor-specific genes. CHX10 interacted with the rhodopsin Ret1 site in one-hybrid and in vitro gel shift assays. We observed a specific interaction between CHX10 from retinal nuclear lysates and the Ret1, PCE1 (arrestin) and LCR (red/green cone opsins) sites. In vivo ChIP assays detected CHX10 upstream of the rod arrestin and IRBP genes, but not at the rhodopsin or cone opsins genes. Last, CHX10 repressed the human arrestin promoter in transient assays across different cell lines, which was dependent on DNA-binding by the HD. These data define a novel role for CHX10 in repressing photoreceptor gene targets.

Dual Role for CHX10 in Blocking Rod Photoreceptor Morphogenesis - The CHX10-deficient orJ mouse exhibits a severe defect in retinal progenitor cell proliferation and only a small portion of the thin central retina ever differentiates (5). This region contains six of the seven major retinal cell types, but lacks bipolar neurons, raising the possibility that CHX10 is critical for bipolar cell differentiation. However, it has not been clear whether the defect in bipolar cell differentiation reflects a direct requirement for CHX10 in bipolar cell development or an indirect consequence of the profound defects in the early orJ retina. The fate of cells originally destined to become bipolar neurons has also been unclear. Recently, we resolved these issues by showing that acute knockdown of CHX10 in the post-natal mouse retina does not affect cell proliferation or survival but causes a switch from bipolar cell to rod photoreceptor differentiation*. This finding, coupled with the data presented in this work, suggests that CHX10 has a dual role in promoting bipolar cell development. First, it is critical to block photoreceptor genesis through the regulation of as yet unidentified fate-determining genes. Second, CHX10 appears to block the expression of a subset of genes associated with terminal differentiation of photoreceptor differentiation, such as rod arrestin.

Altered gene expression in the orJ retina – Recently Rowan et al performed microarray analysis using embryonic retinal mRNA isolated from wild-type or Chx10-deficient (orJ) retinas (11). Intriguingly, upregulation of several peripheral- and/or retinal pigment epithelial-specific genes was observed in concordance with the transdifferentiation of neuroretina into pigmented epithelium (11). In particular, data supported Mitf and Tfec as strong Chx10 candidate target genes (11). Since the Rowan et al study utilized embryonic retinas at a time point prior to expression of genes analyzed in our work, a direct comparison of the results is not possible. One exception is that of IRBP (40), however, this gene was not reported by the Rowan et al study.

In vitro versus in vivo DNA binding by CHX10 - In vitro assays reported here show that CHX10 can interact with several motifs known to be important for the regulation of photoreceptor specific genes. Gel shifts revealed that CHX10 could bind the Ret1, PCE1 and LCR motifs found in the rhodopsin promoter, rod arrestin promoter and cone LCR, respectively, supporting data from one-hybrid assays that used multiple copies of the Ret1 or cone LCR motifs as bait (this work and (15). In stark contrast to these findings, however, ChIP analysis showed, within the limits of the assay, that CHX10 does not bind either the rhodopsin or cone LCR elements in vivo, but does target the rod arrestin promoter. We also discovered that CHX10 is associated with a conserved region upstream of the IRBP locus in vivo, but not with the immediate upstream IRBP promoter. Thus, in vivo CHX10 binds to a specific subset of targets from a larger group of motifs that exhibit binding in vitro. Inconsistencies between in vitro and in vivo data have been documented for other HD proteins, such as engrailed and Pbx1 (41,42). It will be important to utilize ChIP to determine whether CHX10 binds to other putative targets identified through expression analyses or in vitro binding assays (9-11,16).

A Refined CHX10 Consensus site; CHX10 and CRX target distinct motifs - The identification of novel target sequences for
CHX10 refines the consensus binding site for this HD protein. Paired-like HD proteins such as CHX10 and CRX are predicted to bind palindromic TAAT core motifs separated by 2-3 nucleotides (43). Previously we showed that CHX10 binds to so-called “P3” sites that contain two HD TAAT core motifs separated by the consensus pyNpu (13)(Table 2). Using a PCR-based binding selection method, Ferda-Percin et al identified TAATtagc as a CHX10 target sequence (44), suggesting that a palindromic motif is not absolutely critical. Recently, CHX10 was shown to interact with an enhancer upstream of the Nestin gene containing a TAATtagc core (16). The Ret1 (tTAATtgc), PCE1 (cTAATtgaa), and cone LCR (cTAATtgcgat) elements are variations of this motif. A summary of the known binding sites suggests the consensus PyTAATtPuPu as the optimal target for CHX10 (Table 2). Other than the TAAT core, the immediate 3’ T is likely the most critical determinant since modifying this base to a C, as seen in the BAT-1 or Ret4 sites, disrupts association with CHX10 and favours binding to K50 HD proteins, such as CRX (Table 2). Similarly, the Bicoid HD is a K50 that strictly prefers a C nucleotide 3’ of the TAAT core (TAATc) (32). Switching Bicoid’s K50 to Q50, as found in the Antennapedia HD, alters its specificity to a Q50 binding site of TAATq (31,33,34). As CHX10 and CRX are coexpressed in bipolar cells (45), our data raise the possibility that CHX10 may repress CRX activity by binding to distinct motifs in the same promoters, rather than by competing for CRX binding sites.

Refinement of the consensus binding site will aid in the identification of putative CHX10 target genes. To fully understand the role of CHX10 in retinal development, it is necessary not only to identify such sites, but also to confirm they are bona fide targets, and to elucidate whether CHX10 activates or represses expression of the associated genes. Continued studies to explore these issues, with an emphasis on in vivo analyses, will improve our understanding of the fundamental mechanisms involved in retinogenesis and eye development.

REFERENCES

FOOTNOTES


1The abbreviations used are: HD, homeodomain; RPC, retinal progenitor cell; ONL, outer nuclear layer; INL, inner nuclear layer; LCR, locus control region; ChIP, chromatin immunoprecipitation, PDEβ, phosphodiesterase beta, IRBP, interphotoreceptor retinoid binding protein; Luc, luciferase

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Figure Legends

Fig. 1. CHX10 and CRX bind distinct sites. A, Schematic diagram illustrating the organization of the bovine rhodopsin proximal promoter. For a detailed description of all the sites shown see (17). CHX10 was identified in a yeast-1 hybrid assay with the bait sequence –148 to –123bp. B, CHX10 binds the Ret1 site. In vitro translated pBSKS-CHX10 (lanes 2-4, 7) or luciferase (lanes 5&8) were incubated with an end-labeled wild-type (lanes 2-5) or mutated (lanes 6-8) Ret1 probe. 100 times excess unlabeled wild-type (lane 3) or mutated (lane 4) Ret1 oligonucleotides were included in some samples. Positions of the mutations in the Ret1 probe are shown below the gel. C, CHX10 and CRX bind different sites. (Left panel) In vitro translated pBSKS-CHX10 (lanes 2&3), b-CRX (lane 4) or luciferase (lane 5) were incubated with an end-labeled wild-type (lanes 2-5) or mutated (lanes 6-8) Ret1 probe. 100 times excess unlabeled wild-type (lane 3) or mutated (lane 4) Ret1 oligonucleotides were included in some samples. Positions of the mutations in the Ret1 probe are shown below the gel. Asterisk indicates a non-specific band.

Fig. 2. Endogenous CHX10 in retinal nuclear lysates binds multiple photoreceptor elements. P6 mouse retinal nuclear lysates were incubated with an end-labeled wild-type PCE-1 probe (lanes 2-18). Anti-CHX10 antibody (lanes 3-5) or anti-rhodopsin antibody (lane 6) was included in some samples. GST protein was added to lane 4 and CHX10 antigen was added to lane 5. 30 or 100 times excess unlabeled wild-type (lanes 7&8) or mutated (lanes 9&10) PCE-1 probe; wild-type (lanes 11&12) or mutated (lanes 13&14) Ret1 probe; or wild-type (lanes 15&16) or mutated (lanes 17&18) LCR probe was included in some samples. LCR=red/green mouse cone opsin. * denotes non-specific band. Arrowhead denotes CHX10 complex.

Fig. 3. CHX10 associates with the rod arrestin and IRBP loci in vivo. A, ChIP assays. Chromatin from cross-linked/sonicated P6 mouse retinas was immunoprecipitated with polyclonal CHX10 antibodies (M1-rabbit, C4 or N5- both sheep) and subjected to real-time PCR for targets shown in (B). Normal rabbit (RS) or sheep serum (SS) or no antibody (Ab(-)) was included as a negative control. B, Schematic diagram illustrating organization of murine target genes analyzed in (A). β-globin was included as a negative control. Open boxes: exons, grey boxes: homologous to human, ball/stick: CHX10 consensus site. Bglo=β-globin, Arr pro=rod arrestin promoter, IRBP=interphotoreceptor retinoid binding protein, IRB pro=IRBP promoter, Rho pro=rhodopsin promoter, LCR=red/green cone opsin LCR.

Fig. 4. CHX10 regulates the human rod arrestin promoter. A, CHX10 binds the rod arrestin promoter in vitro. In vitro translated CHX10-ABCD (lanes 2-4) or luciferase (lane 5) was incubated with an end-labeled probe containing –202 to +112 of the rod arrestin promoter. 200 times excess unlabeled wild-type rod arrestin (lane 3) or irrelevant (lane 4) oligonucleotides were added to some samples. B, CHX10-VP16 activates rod arrestin. NG108 cells were transfected with increasing amounts of the expression vector CHX10-VP16 (0.2-2.5µg), 2.0ug pECEHA-CHX10 or 1.9µg GAL4-VP16, together with 1µg of the reporter plasmid PDEβ-Luc, Arr-Luc or RSV-Luc. C, CHX10 represses CRX-mediated activation of rod arrestin. NG108 cells were co-transfected with increasing amounts of the expression vector pECEHA-CHX10 together with 3µg of the activator b-CRX and 1µg of the reporter plasmid Arr-Luc or RSV-Luc. Error bars represent the range across two independent experiments. D, CHX10 represses rod arrestin in chick retinal cultures. E8 primary chick retinal cells were co-transfected with 5µg of the expression vector LexCHX10 or Lex together with 3µg of the activator b-CRX and 1µg of the reporter plasmid Arr-Luc. E, CHX10 requires the
HD to repress rod arrestin. NG108 cells were co-transfected with increasing amounts of the expression plasmid CHX10-ABCD or CHX10-N51A (1, 2 or 5 µg), together with 3 µg of the activator h-CRX and 1 µg of the reporter plasmid Arr-Luc. F, Anti-FLAG western analysis on transfected lysates from (D). The amount of lysate loaded was normalized for transfection efficiency. For transfections, equimolar amounts of effector plasmid were achieved by adding appropriate amounts of empty effector plasmid. 100% luciferase activity is taken as that obtained in the presence of control effector plasmid. Luciferase fold activation is relative to that obtained in the presence of control effector plasmid. Luciferase activity was corrected for transfection efficiency using a β-galactosidase internal control.
<table>
<thead>
<tr>
<th>Probe</th>
<th>Technique</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Bovine Ret1</td>
<td>Gel shift</td>
<td>5’ GGCCCCACCTGGAAAGCCAATTAAGGC GGTGCCACCTGGTTAAATCCGG 5’</td>
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<tr>
<td>Bovine BAT-1</td>
<td>Gel shift</td>
<td>5’ GCAGCAGTGGAGGATATATATAATGTTAAATACCGCCCTCGG 5’</td>
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<tr>
<td>Mouse LCR</td>
<td>Gel shift</td>
<td>5’ GACCTGGATCTTCTGTAGCCCCCTATCCTACAAATAGCA CTGAACTAGAAGACAATCGGGATT 5’</td>
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<tr>
<td>Mouse PCE-1</td>
<td>Gel shift</td>
<td>5’ CAAAAGCTTTCAATTAGCTATCCC GTTTCCAAAGTTAACGATAAGG 5’</td>
</tr>
<tr>
<td>Human arrestin (-202 to +112)</td>
<td>PCR &amp; Gel shift</td>
<td>5’ GCCACGCGTATCCTCAGAGACACTGA 5’ GCCAGATCTTCTGCTGACAGAGTGA</td>
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<tr>
<td>Mouse arrestin promoter</td>
<td>ChIP</td>
<td>5’ TGATGGTGAAAGACAGAAAAGGA 5’ CTTGGCAAGGTTGCAAAGAGA</td>
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<tr>
<td>Mouse arrestin 3’ UTR</td>
<td>ChIP</td>
<td>5’ TGTAGTTCCCGGTGCCCTTG 5’ AACGCTCATGCTTGTATCTAGCA</td>
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<tr>
<td>Mouse IRBP</td>
<td>ChIP</td>
<td>5’ CTCAGATTAATGGCTCCAGAAAA 5’ CTCTGTAGCTGGAAACCTACAAG</td>
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<tr>
<td>Mouse IRBP promoter</td>
<td>ChIP</td>
<td>5’ AGAGTCCAGCTGCTGCTTGA 5’ CAGCTCTGCTAAGCCTTTAATCCT</td>
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<tr>
<td>Mouse IRBP 3’ UTR</td>
<td>ChIP</td>
<td>5’ CTAAGTTTCAGAGAAGAATTCTG 5’ TCAGCTGCTGCTTACTTTACATCC</td>
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<td>Mouse rhodopsin promoter</td>
<td>ChIP</td>
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<td>5’ TGAGTTGCAGAAGATACCTAGCTG 5’ GGTAGTAAATCCGCTGCTAAGCTAATCA</td>
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<tr>
<td>β-globin</td>
<td>ChIP</td>
<td>5’ TACCTGGAGAGATACCTGACTCAACAATAA 5’ CAAATACAGCTCCAGAGAATCG</td>
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Table 1. Oligonucleotide probes. Probe sequences used in gel shift and ChIP assays are shown above. Underlined nucleotides were altered for mutated probes, 5’:A -> C, 5’:T-> G.
<table>
<thead>
<tr>
<th>Locus</th>
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<th>Sequence</th>
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<th>CRX</th>
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<tr>
<td>(P3G2CAT)</td>
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<td>acTAATcbaATTAgc</td>
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<td>ND</td>
<td>**</td>
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<tr>
<td>Bovine Rhodopsin</td>
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<td>Ret1</td>
<td>ctTAATtagcCTTca</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>gcTAATTgaa</td>
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<td>+</td>
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<tr>
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<td>OTX</td>
<td>ttTAATcaag</td>
<td>ND</td>
<td>+</td>
<td>(23)</td>
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<tr>
<td>Mouse red/green</td>
<td>LCR</td>
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<tr>
<td>Mouse Nestin POU</td>
<td></td>
<td>aaTAATTgac</td>
<td>+</td>
<td>ND</td>
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**Modified CHX10 Consensus: PyTAATT Pu Pu**

Table 2: Refined DNA-binding site for CHX10. Summary of known binding sites for CHX10 reveals a preference for a TAATtPuPu sequence.
Figure 2

anti-Chx10: + + +
anti-rho: +
GST: +
GST-Chx: +
Comp: PCE1 PCE1 PCE1 PCE1 PCE1 PCE1 PCE1

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Figure 3

A

B

Arrestin
IRBP
Rhodopsin
Cone opsin
Beta-globin

Arbitrary units

Arr pro  Arr 3'UTR  IRBP  IRBP pro  IRBP 3'UTR  Rho pro  LCR  Bglo

1 Kb
Figure 4

A
Probe: Arr
Protein: Chx luc
Competitor: Arr irrel

B
Fold Activation

C
% Luc activity
μg Chx10:

D
% Luc activity
Lex
LexChx10

E
% Luc activity
μg transfected:
Chx10 Chx10-N51A

F
α-FLAG
μg transfected:
Chx10 Chx10-N51A
Rel: 1.0 1.3 5.6 2.9 3.1 5.4
Chx10 targets a subset of photoreceptor genes
Kimberley M. Dorval, Brian P. Bobechko, Hiroki Fujieda, Shiming Chen, Don J. Zack and Rod Bremner

J. Biol. Chem. published online October 18, 2005

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