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 one-hybrid assay Chx10 interacted with the Ret1 site of the photoreceptor-specific gene Rhodopsin. Gel shift assays using \textit{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 Cone opsin locus control region (Red/green cone opsin). However, chromatin immunoprecipitation assays revealed that \textit{in vivo}, Chx10 bound sites upstream of the Rod 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 \textit{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 houses the cell bodies of photoreceptors (rods and cones), whereas those of horizontal, bipolar, and amacrine interneurons and Müller glia reside in the inner nuclear layer. The innermost layer is the ganglion cell layer, 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 multipotent 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 \textit{CHX10}, and its role in sculpting the characteristics of bipolar interneurons.

Homeodomain (HD)\textsuperscript{4} proteins regulate retinal development from the earliest stages of optic vesicle formation to the final stages of maturation in the adult (reviewed in Refs. 3 and 4)). Previous molecular analysis linked a naturally occurring mutation in the homeobox of Chx10 to the ocular retardation phenotype in mice (or\textsuperscript{J}) (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\textsuperscript{4} (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\textsuperscript{J} mouse is partially alleviated by crossing this allele with a \textit{Mus musculus castaneus} strain, thought to be because of as yet uncharacterized modifier genes (7). RPC proliferation is also partially rescued when the cyclin-dependent kinase inhibitor p27\textsuperscript{Kip1} is deleted on an or\textsuperscript{J} background (8). Increased p27\textsuperscript{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\textsuperscript{J} retina including aberrant induction of Microphthalmia transcription factor (Mitf) (9), loss of the nuclear receptor retinoid-like orphan receptor \(\beta\) (10), and up- or down-regulation of many other factors (11). Mitf drives the formation of retinal pigment epithelium over the neural retina and retinoid-like orphan receptor \(\beta\) promotes RPC division (9, 10), so both of these changes may be linked to the perturbation of Cyclin D1 and p27\textsuperscript{Kip1} levels. The requirement for Chx10 to facilitate RPC proliferation is transient because overexpression and knockdown assays reveal that Chx10 does not alter the cell cycle in the post-natal retina.\textsuperscript{5}

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\textsuperscript{J} 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\textsuperscript{J} retina.\textsuperscript{5} This issue was resolved by the finding that acute \textit{Chx10} knockout in the post-natal retina blocks bipolar cell differentiation without affecting RPC proliferation.\textsuperscript{5} The decrease in bipolar neurons is accompanied by a corresponding increase in rod photoreceptors.\textsuperscript{5}

---

\textsuperscript{4} The abbreviations used are: HD, homeodomain; RPC, retinal progenitor cell; LCR, locus control region; CHIP, chromatin immunoprecipitation, PDE5, phosphodiesterase 5; IRBP, interphotoreceptor retinoid binding protein; Luc, luciferase; DTT, dithiothreitol; RS\textsuperscript{5}, Rous sarcoma virus; GST, glutathione S-transferase; IRBP, interphotoreceptor retinoid-binding protein; Pu, purine; Py, pyrimidine.

\textsuperscript{5} L. Livne-bar, M. Palac, M. Cheung, M. Hankin, J. Trogadis, C. Chen, K. M. Dorval, and R. Bremner, submitted for publication.

---

\textsuperscript{8} This work was supported in part by the Canadian Institutes for Health Research and National Institutes of Health R01EY009769, and generous gifts from The Guerrieri Family Foundation and Robert and Clarice Smith. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1} Recipient of a Vision Science Research Program Doctoral Research Award from the University of Toronto and an E. A. Baker Foundation and The Canadian National Institute for the Blind/CIHR Partnership Doctoral Research Fellowship.

\textsuperscript{2} Guerrieri Professor of Genetic Engineering and Molecular Ophthalmology and the recipient of a Research to Prevent Blindness Senior Investigator Award.

\textsuperscript{3} To whom correspondence should be addressed: MCS – 424, Cellular and Molecular Division, 399 Bathurst St., Toronto, Ontario M5T 2S8, Canada. Tel: 416-603-5865; Fax: 416-603-5126; E-mail: rbremner@uhnres.utoronto.ca.
These data complement overexpression 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 o1 versus wild type 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 opsin 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 could be one of the factors that defines the unique characteristics of bipolar neurons.

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) (Exalba Biologicals, Boston, MA). These antibodies specifically recognized a protein of the correct size (~46 kDa) 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. McLnnes.

Isolation of Mouse Retinal Nuclear Lysate—Nuclear lysate was prepared from P6 CD-1 mouse retinas for use in gel shifts according to Ref. 21. Dissections and subsequent steps were carried out on ice. Retinas were washed in phosphate-buffered saline and then buffer A (15 mM HEPES, pH 7.6, 110 mM KC1, 5 mM MgCl2, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol). Retinas were homogenized in buffer B (10 mM Tris, pH 8, 5 mM MgCl2, 10 mM NaCl, 60 mM KCl, 0.25 mM sucrose, 10% glycerol, 0.5% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol), and the degree of lysis was 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 KC1, 12.5 mM MgCl2, 0.1 mM EDTA, 1.5 mM dithiothreitol, and 20% glycerol). In 0.4 ml of buffer C nuclei were lysed with 1 × 2-s sonication burst prior to spinning down of nuclear debris for 60 min 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 KC1. Typical yield from 18 retinas is ~0.4 μg of total protein, which decreased with dialysis by ~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 (13). CHX10 or Crx plasmids were in vitro translated in the presence of 35S-labeled methionine and protein levels were adjusted for methionine content using densitometry. 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 min at 30 °C. Where applicable, 1 μg of antibody was preincubated for 15 min with GST or GST-CHX10.

CHX10 Targets Photoreceptor-specific Genes

MATERIALS AND METHODS

Yeast One-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 148-3-galactosidase internal control. Indi-
CHX10 Targets Photoreceptor-specific Genes

**TABLE 1**

| Probe | Technique | Sequence |
|-------|-----------|----------|
| Bovine Ret1 | Gel shift | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Bovine BAT-1 | Gel shift | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Mouse LCR | Gel shift | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Mouse PCE-1 | Gel shift | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Human ROD ARRESTIN (−202 to +112) | PCR and gel shift | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Mouse Rod arrestin promoter | ChIP | 5'-GACTATGCTGAGAGAAGCCA AGAGAGAAG-3' |
| Mouse Rod arrestin 3'-untranslated region | ChIP | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Mouse Irbp | ChIP | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Mouse Irbp promoter | ChIP | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Mouse Irbp 3'-untranslated region | ChIP | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Mouse Rhodopsin promoter | ChIP | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| Mouse Red/green cone opsin LCR | ChIP | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |
| B-Globin | ChIP | 5'-GACGGATCCGCATGGTACATGACCTCTGACATTCATAGTCGTAATAGGC-3' |

lysate 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, CT). The sonicated sample was centrifuged at 15,000 × g for 10 min at 4 °C, the supernatant was 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 h 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 h. Precipitates were washed sequentially with 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 1× TSEI (0.1% SDS, 0.1% Triton X-100, 2 mM EDTA, 500 mM NaCl), 1× TSEII (0.1% SDS, 0.1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 500 mM NaCl), and 3× TE (10 mM Tris-HCl, pH 8, 1 mM EDTA). Samples were then eluted and cross-links reversed by overnight incubation at 65 °C. The probe sequences are shown in Table 1. Underlined nucleotides were altered for mutated probes, 5'-A → C, 5'-T → G.

In vitro transcription and translation experiments showed that GST-Crx HD could protect the BAT-1, Ret1, and Ret4 sites (17). 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 rhodopsin promoter, we performed a gel shift assay using in vitro translated CHX10 and an end-labeled Ret1 probe (Fig. 1B). CHX10 bound Ret1 and was competed by excess unlabeled Ret1 probe (Fig. 1B, lanes 2 and 3). Excess unlabeled mutant probe did not disrupt the CHX10-Ret1 complex, and CHX10 did not bind the labeled mutated Ret1 site (Fig. 1B, lanes 4 and 7). In vitro translated luciferase did not bind either probe (Fig. 1B, lanes 5 and 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 HD proteins have a Gln50 HD (6), which is predicted to bind to TAATt but not TAATc motifs, respectively.

**RESULTS**

CHX10 Binds Photoreceptor-specific Elements in Vitro—Because 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 footprinting 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 (Fig. 1A). This data raised the possibility that this sequence or others like it may facilitate repression of photoreceptor gene expression in non-photoreceptor cell types.

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 HD proteins have a Gln50 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 (Fig. 1C, lane 2), but at this protein level, Crx failed to bind the Ret1 site (Fig. 1C, lane 4). However, Chx did bind the BAT-1 and Ret4 sequences (Fig. 1C, lanes 8, and data not shown), whereas CHX10 did not (Fig. 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 ROD ARRESTIN gene (35) 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 (Fig. 2, lane 2). Complex formation was inhibited by addition of anti-CHX10 antibody (Fig. 2, lanes 3), whereas an irrelevant anti-rodopsin antibody had no effect (Fig. 2, lane 4). Chx10 binding was blocked by addition of excess unlabeled PCE1 probe, but not by an unlabeled mutated PCE-1 probe (Fig. 2, cf. 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 \textit{in vitro} translated gel shifts in Fig. 1 (Fig. 2, cf. lanes 11–12 with 13–14). Previously, Hayashi et al. (15) isolated Chx10 in a one-hybrid assay. In that case the bait was a highly conserved homeobox-binding motif in the LCR located upstream of the Red/green cone opsin gene. We found that this motif but not a mutated version efficiently dislodged Chx10 from the PCE1 site (Fig. 2, cf. lanes 15–16 with 17–18). These data indicate that Chx10 present in retinal lysate can interact, at least \textit{in vitro}, with conserved elements from several photoreceptor-specific genes.

### TABLE 2

Refined DNA-binding site for CHX10

Summary of known binding sites for CHX10 reveals a preference for a TAATtPuPu sequence.

| Locus                   | Element | Sequence         | CHX10 | CRX  | Ref. |
|-------------------------|---------|------------------|-------|------|------|
| Artificial Gln<sup>a</sup> | Consensus | TAATPyNPuATTA   | +     | ND   | 44   |
| P3-1                    |         | acTAATTgaATATAgc | +     | ND   | 44   |
| P3-2                    |         | gcTAATtaATATAgc  | +     | ND   | 13   |
| Bovine Rhodopsin Ret4   |         | gcTAATGetcc      | –     | +    | This work |
| Ret1                    |         | gcTAATTTGCTCa    | –     | +    | This work |
| BAT-1                   |         | aTAATcaATATat    | –     | +    | This work |
| Mouse Rod arrestin PCE-1 |        | gcTAATga         | +     | +    | This work and Ref. 23 |
| OTX                     | LCR     | gcTAATgat        | +     | ND   | 23   |
| Mouse Red/green cone opsin LCR |        | gcTAATgat        | +     | ND   | This work and Ref. 15 |
| Mouse Nestin POU        |         | aaTAATTagc       | +     | ND   | 16   |
| Modified CHX10 consensus: PyTAATT PuPu |       |                  |       |      |      |

<sup>a</sup> K. M. Dorval and R. Bremner, unpublished data.

\textbf{FIGURE 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 Ref. 17. Chx10 was identified in a yeast one-hybrid assay with the bait sequence \(-148\) to \(-123\) bp. B, CHX10 binds the Ret1 site. \textit{In vitro} translated pBSKS-CHX10 (lanes 2–4 and 7) or luciferase (lanes 5 and 8) were incubated with an end-labeled wild type (lanes 5–8) 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, \textit{In vitro} translated pBSKS-CHX10 (lanes 2 and 3), b-Crx (lane 4), or luciferase (lane 5) were incubated with an end-labeled Ret1 probe. 100 times excess unlabeled wild-type Ret1 probe was included in lane 3. Right panel, \textit{In vitro} translated CHX10 (lane 7), Crx (lanes 8–10 and 13), or luciferase (lanes 11 and 14) were incubated with an end-labeled wild-type (lanes 6–11) or mutated (lanes 12–14) BAT-1 probe. 100 times excess unlabeled wild type (wt) (lane 9) or mutated (lane 10) BAT-1 oligonucleotides were included in some samples. Positions of the mutations in the BAT-1 probe are shown below the gel. Asterisk indicates a nonspecific band.
Interaction of Chx10 with Photoreceptor-specific Targets in Vivo—Because chromatin structure plays a key role in mediating transcription factor binding, we next determined the DNA binding ability of Chx10 in vivo. We performed ChIP analysis using P6 mouse retinal tissue and three polyclonal CHX10 antibodies (M1 rabbit, C4 sheep, and N5 sheep) (Fig. 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 Rod arrestin, Rhodopsin, Red/green cone opsin, and Interphotoreceptor retinoid-binding protein (Irbp) (Fig. 3A). Three polyclonal CHX10 antibodies (M1 rabbit, C4 sheep, and N5 sheep) were included as a negative control. Open boxes, exons; gray boxes, homologous to human; ball/stick, CHX10 consensus site. B, schematic diagram illustrating organization of murine target genes analyzed in A. β-Globin was included as a negative control. Arrows denote CHX10 consensus site.

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 (Fig. 4A). In vitro translated CHX10 interacted with an end-labeled ARRESTIN promoter fragment (−202 to +112) (Fig. 4A, lane 2) and was dislodged by excess unlabeled ARRESTIN probe but not by excess unlabeled irrelevant sequence (coding region 1–131 amino acids of human CHX10) (Fig. 4A, lanes 3 and 4). Second, to recapitulate the yeast one-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 (Fig. 4B). Furthermore, 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 (Fig. 4B). Finally, we asked whether CHX10 could repress the ROD 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) (Fig. 4, C and D).

To determine whether CHX10 repressed ROD ARRESTIN in a DNA-binding dependent manner, we tested the effects of mutating position 51 in the HD from asparagine 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 (Fig. 4E). Anti-FLAG Western blot confirmed that the expression levels of CHX10 and CHX10-N51A were uniform (Fig. 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 (Rod 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 ROD 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 or’ 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 genesis or whether it is an indirect consequence of the profound defects in the early or’ 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 or’ Retina**—Recently Rowan et al. (11) performed microarray analysis using embryonic retinal mRNA isolated from wild type or Chx10-deficient (or’) retinas (11). Intriguingly,
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 2–3 nucleotides (43). Using a PCR-based binding selection method, Ferda Percin et al. (44) identified TAATgcg as a CHX10 target sequence, suggesting that a palindromic motif is not absolutely critical. Recently, CHX10 was shown to interact with an enhancer upstream of the Nstn gene containing a TAATgcg core (16). The Ret1 (TAATgtcg), PCE1 (cTAATtgaa), and cone LCR (cTAATtggg) 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′ of this motif is likely the most critical determinant because modifying this base to a C, as seen in the BAT-1 or Ret4 sites, disrupts association with CHX10 and favors binding to Lys48-HD proteins, such as Crx (Table 2). Similarly, the Bicoid HD is a Lys48-binding protein, but this is uninformative for CHX10 and CRX (Table 2). 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 that 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 vitro analyses, will improve our understanding of the fundamental mechanisms involved in retinogenesis and eye development.

Acknowledgments—We thank Drs. Samantha Pattenden and Izzy Livne-Bar for critical reading of this manuscript.

REFERENCES

1. Dyer, M. A., and Brenner, R. (2005) Nat. Rev. Cancer 5, 91–101
2. Young, R. W. (1985) Anot. Rec. 212, 199–205
3. Jean, D., Ewan, K., and Gruss, P. (1998) Mech. Dev. 76, 3–18
4. Freund, C., Horsford, D. J., and McInnes, R. R. (1996) Hum. Mol. Genet. 5, 1471–1488
5. Burmeister, M., Novak, J., Liang, M. Y., Basu, S., Ploider, L., Hawes, N. L., Vidgen, D., Hoover, F., Goldmann, D., Kalnins, V. I., Roderick, T. H., Taylor, B. A., Hankin, M. H., and McInnes, R. R. (1996) Nat. Genet. 12, 376–384
6. Liu, I. S., Chen, J., Ploider, L., Vidgen, D., van der Kooy, D., Kalnins, V. I., and McInnes, R. R. (1994) Neuron 13, 377–393
7. Bone-Larson, C., Basu, S., Radel, D. J., Liang, M., Perozetz, T., Kapoutsa-Brnuee, N., Green, D. G., Burmeister, M., and Hankin, M. H. (2000) J. Neurobiol. 42, 232–247
8. Green, E. S., Stubbs, J. L., and Levine, E. M. (2002) Development 130, 539–552
9. Horsford, D. J., Nguyen, M. T., Sellar, G. K., Kothy, R., Arberhite, H., and McInnes, R. R. (2005) Development 132, 177–187
10. Chow, L., Levine, E. M., and Reh, T. A. (1998) Mech. Dev. 77, 149–164
11. Rowan, S., Chen, M. C., Young, T. L., Fisher, D. E., and Cepko, C. L. (2004) Development 131, 5139–5152
12. Hatakeyama, J., Tomita, K., Inoue, T., and Kageyama, R. (2001) Development 128, 1313–1322
13. Dorval, K. M., Bobechock, B. P., Ahmad, K. F., and Brenner, R. (2005) J. Biol. Chem. 280, 10100–10108
14. Blackshaw, S., Fraioli, R. E., Furukawa, T., and Cepko, C. L. (2001) Cell 107, 579–589
15. Hayashi, T., Huang, J., and Deeb, S. S. (2000) Genomics 67, 128–139
16. Rowan, S., and Cepko, C. L. (2005) Dev. Biol. 281, 240–255
17. 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) Neuron 19, 1017–1030
18. Daniels, M. P., and Hamprecht, B. (1974) J. Cell Biol. 63, 691–699
19. Adler, R. (1990) Methods Neurosci. 2, 134–149
20. Bremner, R., Cohen, B. L., Sopta, M., Hamel, P. A., Ingles, C. J., Gallie, B. L., and Phillips, R. A. (1995) Mol. Cell. Biol. 15, 3256–3265
21. Deslaurds, L. E., and Hauswirth, W. W. (1996) Invest. Ophthalmol. Vis. Sci. 37, 154–165
22. Pattenden, S. G., Klose, R., Karavas, E., and Brenner, R. (2002) EMBO J. 21, 1978–1986
23. Kimura, A., Singh, D., Wawrousek, E. F., Kikuchi, M., Nakamura, M., and Shinozara, T. (2000) J. Biol. Chem. 275, 1152–1160
24. Freund, C. L., Gregory-Evans, C. Y., Furukawa, T., Papaioannou, M., Looser, J., Ploder, L., Beltingham, J., Ng D., Herbrick, J. A., Duncan, A., Scharer, I. S., Wu, C. L., Loutredis-Anagnostou, A., Jacobson, S. G., Cepko, C. L., Bhattacharya, S. S., and McInnes, R. R. (1997) Cell 91, 543–553
25. Furukawa, T., Morrow, E. M., and Cepko, C. L. (1997) Cell 91, 531–541
26. Morabito, M. A., Yu, X., and Barnstable, C. J. (1991) J. Biol. Chem. 266, 9667–9672
27. Yu, X., Leconte, L., Martinez, J. A., and Barnstable, C. J. (1996) J. Neurochem. 67, 2494–2504
28. Chen, and Zack, D. J. (1996) J. Biol. Chem. 271, 28549–28557
29. Freund, C. L., Wang, Q. L., Chen, S., Muskat, B. L., Wiles, C. D., Sheffield, V. C., Jacobson, S. G., McInnes, R. R., Zack, D. I., and Stone, E. M. (1998) Nat. Genet. 18, 311–312
30. 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) Neuron 19, 1329–1336
31. Hanes, S. D., and Brent, R. (1991) Science 251, 426–430
32. Driever, W., and Nusslein-Volhard, C. (1989) Nature 337, 138–143
33. Hanes, S. D., and Brent, R. (1989) Cell 57, 1275–1283
34. Treisman, J., Gonczy, P., Vashishtta, M., Harris, E., and Desplan, C. (1989) Cell 59, 553–562
35. Kikuchi, T., Raju, K., Brittain, M. L., and Shinozara, T. (1993) Mol. Cell. Biol. 13, 4400–4408
36. Liou, G. I., Matragoos, S., Yang, J., Geng, L., Overbeek, P. A., and Ma, D. P. (1991) Biochem. Biophys. Res. Commun. 181, 159–165
37. Zack, D. J., Bennett, J., Wang, Y., Davenport, C., Klaunberg, B., Geardhart, J., and Nathans, J. (1991) Neuron 7, 161–167
38. Wang, Y., Macke, J. P., Merbs, S. L., Zack, D. J., Klaunberg, B., Bennett, J., Geardhart, J., and Nathans, J. (1992) Neuron 9, 429–440
39. Liou, G. I., Geng, L., al-Ubaidi, M. R., Matragoos, S., Hanten, G., Baehr, W., and Overbeek, P. A. (1990) J. Biol. Chem. 265, 8373–8376
40. Liou, G. I., Wang, M., and Matragoos, S. (1994) Dev. Biol. 161, 345–356
41. Tolkunova, E. N., Fujioka, M., Kobayashi, M., Deka, D., and Jaynes, J. B. (1998) Mol. Cell. Biol. 18, 2804–2814
42. Chakrabarti, S. K., James, J. C., and Mirmira, R. G. (2002) J. Biol. Chem. 277, 13286–13293
43. Wilson, D., Sheng, G., Lecuit, T., Dostatni, N., and Desplan, C. (1993) Genes Dev. 7, 2120–2134
44. Ferda Percin, E., Ploder, L. A., Yu, J. J., Arici, K., Horsford, D. I., Rutherford, A., Bapat, B., Cox, D. W., Duncan, A. M., Kalnins, V. I., Kokak-Altintas, A., Sowden, J. C., Traboulsi, E., Sarfarazi, M., and McInnes, R. R. (2000) Nat. Genet. 25, 397–401
45. Bibb, L. C., Holt, J. K., Tarttelin, E. E., Hodges, M. D., Gregory-Evans, K., Rutherford, A., Lucas, R. J., Sowden, J. C., and Gregory-Evans, C. Y. (2001) Hum. Mol. Genet. 10, 1571–1579