CHX10 Targets a Subset of Photoreceptor Genes*

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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 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 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 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 *CHX10*, and its role in sculpting the characteristics of bipolar interneurons.

Homeodomain (HD)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^{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 types⁵ (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^J mouse is partially alleviated by crossing this allele with a Mus musculus castaneous 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^{*Kip1*} is deleted on an or^J 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^J retina including aberrant induction of Microphthalmia transcription factor (Mitf) (9), loss of the nuclear receptor retinoid-like orphan receptor β (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 β promotes RPC division (9, 10), so both of these changes may be linked to the perturbation of Cyclin D1 and p27Kip1 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.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^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^J retina.⁵ This issue was resolved by the finding that acute *Chx10* knockdown in the post-natal retina blocks bipolar cell differentiation without affecting RPC proliferation.⁵ The decrease in bipolar neurons is accompanied by a corresponding increase in rod photoreceptors.⁵

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⁴ The abbreviations used are: HD, homeodomain; RPC, retinal progenitor cell; LCR, locus control region; ChIP, chromatin immunoprecipitation, PDEβ, phosphodiesterase β; IRBP, interphotoreceptor retinoid binding protein; Luc, luciferase; DTT, dithiothreitol; RSV, Rous sarcoma virus; GST, glutathione S-transferase; IRBP, interphotoreceptor retinoid-binding protein; Pu, purine; Py, pyrimidine.

⁵ I. Livne-bar, M. Pacal, M. Cheung, M. Hankin, J. Trogadis, C. Chen, K. M. Dorval, and R. Bremner, submitted for publication.

These data complement overexpression studies showing that CHX10 promotes bipolar cell genesis at the expense of rods (12).⁵ 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^J *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 facilitates bipolar cell characteristics in part by silencing expression of select photoreceptor 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 -126 bp. Approximately 2×10^{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 (13). E8 cells were isolated according to the protocol described by Adler *et al.* (19) and were maintained in culture for a resticted time period (\sim 3–4 days) to prevent the overgrowth of any one cell type.

Luciferase Assays-Transient transfection assays were performed as previously described (13). Briefly, NG108 or freshly dissected primary chick retinal cells were plated at 70% confluency 1 day prior to transfection by the calcium phosphate method. As a control for transfection efficiency, 0.3 μ g of cytomegalovirus- β -galactosidase was included in each transfection and β -galactosidase activity was determined. For luciferase assays, 20 μ l of lysate was added to 50 or 100 μ l of luciferase assay reagent (10 mM MgSO₄, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 µм coenzyme A, 470 µм luciferin, 530 µм 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 (13). CHX10-VP16 (SVChxV) is a fusion protein of full-length human CHX10, the activation domain of VP16 (amino acids 410–490), and a COOH-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' BgIII sticky ends; synthetic FLAG + TAA olignucleotide 5' BgIII 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 410–490) (gift of Dr. Robert Kingston).

The following plasmids have been described (17): activators NrI (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 PDE β (PDE β -Luc, -340 to +54), bovine irbp (IRBP-Luc, -300 to +32), and human ROD ARRESTIN (Arr-Luc, -316 to +112). Deletion constructs -316Arr, -202Arr, and -107Arr were generated by PCR from the human ROD ARRESTIN reporter (DJZ) and subcloned into pGL2Basic at the 5' MluI and 3' BgIII sites. RSV-Luc contains Luc under control of the RSV long terminal repeat (-489 to +40; gift of H. P. Elsholtz).

CHX10 Antibodies—Polyclonal antibodies were raised by injecting sheep with GST fusion proteins containing the human CHX10 amino terminus (1–131) or carboxyl terminus (264–361) (Exalpha 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.⁵ 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 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 KCl, 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 mм KCl, 0.25 м sucrose, 10% glycerol, 0.5% Nonidet P-40, 0.5 mм 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 KCl, 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 KCl. Typical yield from 18 retinas is \sim 0.4 μ g of total protein, which decreased with dialysis by \sim 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 μ g of mouse P6 retinal nuclear lysate were allowed to incubate for 30 min at 30 °C. Where applicable, 1 μ l of antibody was preincubated for 15 min with GST or GST-CHX10.

Chromatin Immunoprecipitation (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 phosphate-buffered saline for 30 min, rinsed in phosphate-buffered saline, and sonicated in

TABLE 1

Oligonucleotide probes

Probe sequences used in gel shift and ChIP assays are shown. Underlined nucleotides were altered for mutated probes, 5'-A \rightarrow C, 5'-T \rightarrow G.

| Probe | Technique | Sequence | | | |
|---|-------------------|---|--|--|--|
| Bovine Ret1 | Gel shift | 5'-GGCCCCACCTGG <u>AA</u> GCCAA <u>TT</u> AAGCC GGTGGACC <u>TT</u> CGGTT <u>AA</u> TTCGG-5' | | | |
| Bovine BAT-1 | Gel shift | 5'-GCAGCAGTGAGGATT <u>AA</u> TATGA <u>TT</u> AATAACGCCCCC CGTCGTCACTCCTAA <u>TT</u> ATACT <u>AA</u> TTATTGCGGGGG-5' | | | |
| Mouse LCR | Gel shift | 5'-GACTTGATCTTCTGTTAGCCCT <u>AA</u> TCATCAA <u>TT</u> AGCA CTGAACTAGAAGACAATCGGGA <u>TT</u> AGTAGTT <u>AA</u> TCGT-5' | | | |
| Mouse PCE-1 | Gel shift | 5'-CAAAAGCTTTCAA <u>TT</u> AGCTATTCC GTTTTCGAAAGTT <u>AA</u> TCGATAAGG-5' | | | |
| Human ROD ARRESTIN $(-202 \text{ to } +112)$ | PCR and gel shift | 5'-GCCACGCGTAGTTCCAGAGACACTGA 5'-GCGAGATCTTTCGTGCTGACAGAGTGA | | | |
| Mouse Rod arrestin promoter | ChIP | 5'-TGATGGTGAAGAGCGAAAGGA 5'-CTGGGCAAGGTGCAAAGAGA | | | |
| Mouse Rod arrestin 3'- untranslated region | ChIP | 5'-TTGTAGTTCCGGGTGCCTTG 5'-AATGCTCATGCTTTGATATCTAGCA | | | |
| Mouse Irbp | ChIP | 5'-cgtcagataatggcttccagaaa 5'-ctctgtgagctggaaacctacaag | | | |
| Mouse Irbp promoter | ChIP | 5'-AGAGTCCAGCTCATGTGCTTGA 5'-CAGCTCTGCTAAGCCTTTAATCCT | | | |
| Mouse Irbp 3'- untranslated region | ChIP | 5'-CATACTGTCTCCAAGAGCAATTCTG 5'-TCACGTGTGCCTTACTTTACATCC | | | |
| Mouse Rhodopsin promoter | ChIP | 5'-GCCTCCACCCGATGTCAC 5'-TCATACTAACGCTAATCCCACTGCT | | | |
| Mouse Red/green cone opsin LCR | ChIP | 5'-TTGAGTTCTAAGTCTTGGAGTTCCTG 5'-GGTAGTAATCCGCTTTAAGCTAAATCA | | | |
| β-Ĝlobin | ChIP | 5'-TTACTTGAGAGATCCTGACTCAACAATAA 5'-TCAATAACTGCCTTCAGAGAATCG | | | |

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, CT). The sonicated sample was centrifuged at 15,000 \times g for 10 min at 4 °C, the supernatant was aliquoted to 100 μ l (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 μ l of anti-CHX10 antibody, N5, C4, or M1. Samples were centrifuged at 15,000 \times 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 for 10 min in 1× TSEI (0.1% SDS, 1% Triton X-100, 2 mm EDTA, 20 mm Tris-HCl, pH 8, 150 mM NaCl), 4× TSEII (0.1% SDS, 1% Triton X-100, 2 mм EDTA, 20 mм Tris-HCl, pH 8, 500 mм NaCl), 1× buffer III (0.25 м LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mм EDTA, 10 mм Tris-HCl, pH 8), 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 in 100 μ l of elution buffer (1% SDS, 0.1 M NaHCO₃) and DNA fragments were purified by GFX PCR DNA and the Gel band purification kit (Amersham Biosciences) with a final elution product of 100 μ l. Real time PCR (ABI PRISM 7900HT) was used to amplify 2 μ l of the final ChIP product, and the copy number was 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 contained 2 μ l each of ChIP sample or serially diluted genomic DNA, 1× SYBR Green PCR Master Mix (Applied Biosystems), and 500 nm of each primer (Table 1) in a final volume of 10 μ l. 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—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. 1*A*). 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 (Fig. 1*B*). CHX10 bound Ret1 and was competed by excess unlabeled Ret1 probe (Fig. 1*B*, *lanes 2* and *3*). Excess unlabeled mutated probe did not disrupt the CHX10•Ret1 complex, and CHX10 did not bind the labeled mutated Ret1 site (Fig. 1*B*, *lanes 4* and 7). *In vitro* translated luciferase did not bind either probe (Fig. 1*B*, *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 Crx HD has a lysine at position 50 (Lys⁵⁰) 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 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, Crx 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.

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. 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 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 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, 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.



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 ⁵⁰ | <u>TAAT</u> PyNPuATTA | + | ND | 44 | | | |
| | Consensus | TAATtagc | + | ND | 44 | | | |
| | P3-1 | ac <u>TAAT</u> tga <u>ATTAg</u> ct | + | ND | a | | | |
| | P3-2 | gc <u>TAAT</u> taa <u>ATTAg</u> ct | + | ND | 13 | | | |
| Bovine Rhodopsin | Ret4 | cc <u>TAAG</u> ctcc | - | + | This work | | | |
| - | Ret1 | ct <u>TAAT</u> t <u>ggCTTC</u> ca | + | - | This work | | | |
| | BAT-1 | at <u>TAAT</u> cat <u>ATTA</u> at | - | + | This work | | | |
| Mouse Rod arrestin | PCE-1 | gcTAATtgaa | + | + | This work and Ref. 23 | | | |
| | OTX | ttTAATcaag | ND | + | 23 | | | |
| Mouse Red/green cone opsin | LCR | gc <u>TAAT</u> tgat | + | ND | This work and Ref. 15 | | | |
| Mouse Nestin | POU | aaTAATtagc | + | ND | 16 | | | |
| Modified CHX10 consensus: Py <u>TAAT</u> T Pu Pu | | | | | | | | |

^a K. M. Dorval and R. Bremner, unpublished data.

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-rhodopsin antibody had no effect (Fig. 2, *lane 6*). Addition of the GST-CHX10 antigen used to generate the CHX10 antibody reinstated the Chx10·PCE1 complex (Fig. 2, *lane 5*), but GST alone 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 *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 *in vitro*, with conserved elements from several photoreceptor-specific genes.



FIGURE 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* and 8) or mutated (*lanes 9* and 10) PCE-1 probe; wild-type (*lanes 11* and 12) or mutated (*lanes 13* and 14) Ret1 probe; or wild-type (*lanes 15* and 16) or mutated (*lanes 17* and 18) LCR probe was included in some samples. LCR, Red/green cone opsin. *Asterisk* denotes nonspecific band. *Arrowhead* denotes Chx10 complex.

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. 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 Rod arrestin promoter, confirming that the PCE1 element is a bona fide Chx10 target (Fig. 3A). However, despite in vitro data showing that Chx10 binds the Ret1 and Red/green 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 Red/green cone opsin LCR (Fig. 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 (Fig. 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 (Fig. 3A and data not shown). These results demonstrate that Chx10 binds a subset of photoreceptor targets in vivo. Importantly, the chromatin structure may have a considerable impact on the ability of Chx10 to bind different sites in vivo.



FIGURE 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 (*M*1, rabbit; *C*4 or *N5*, both sheep) and subjected to real time PCR for targets shown in *B*. Normal rabbit (*R5*) or sheep serum (*S5*) or no antibody (*Ab*(-)) were 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; gray boxes, homologous to human; ball/stick, CHX10 consensus site. Bglo, β -globin; Arr pro, Rod arrestin promoter; IRB pro, IRBP promoter; Rho pro, Rhodopsin promoter; LCR, Red/green cone opsin LCR; UTR, untranslated region.

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 endlabeled 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 dosedependent 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 PDE β 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.

FIGURE 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) were incubated with an end-labeled probe containing -202 to +112 of the ROD ARRESTIN promoter. 200 times excess unlabeled wild-type ROD ARRES-TIN (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 (0.2-2.5 µg), 2.0 µg of pECEHA-CHX10, or 1.9 µg of GAL4-VP16, together with 1 μ g of the reporter plasmid PDE β -Luc, Arr-Luc, or RSV-Luc. C, CHX10 represses Crx-mediated activation of ROD ARRESTIN. NG108 cells were cotransfected with increasing amounts of the expression vector pECEHA-CHX10 together with 3 μ g of the activator b-Crx and 1 μ g 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 5 μ g of the expression vector Lex-CHX10 or Lex together with 3 μ g of the activator b-Crx and 1 μ g 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 μ g), together with 3 μ g of the activator h-Crx and 1 μ g 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.



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. 4*E*). Anti-FLAG Western blot confirmed that the expression levels of CHX10 and CHX10-N51A were uniform (Fig. 4*F*).

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 opsin) sites. *In vivo* ChIP assays detected Chx10 upstream of the Rod arrestin and Irbp genes, but not at the Rhodopsin or Cone opsin 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^J 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^J 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.⁵ 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 or¹ Retina—Recently Rowan *et al.* (11) performed microarray analysis using embryonic retinal mRNA isolated from wild type or Chx10-deficient (or¹) retinas (11). Intriguingly, up-

regulation 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). Because the Rowan *et al.* (11) 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.* (11) 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 opsin gene, respectively, supporting data from one-hybrid assays that used multiple copies of the Ret1 or cone LCR motifs as bait (this work and Ref. 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 opsin gene 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 Pdx1 (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 PCRbased binding selection method, Ferda Percin et al. (44) identified TAATtagc 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 Nestin gene containing a TAATtagc core (16). The Ret1 (tTAATtggc), PCE1 (cTAATtgaa), and cone LCR (cTAATtgat) 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 because modifying this base to a C, as seen in the BAT-1 or Ret4 sites, disrupts association with CHX10 and favors binding to Lys^{50} HD proteins, such as Crx (Table 2). Similarly, the Bicoid HD is a Lys⁵⁰ that strictly prefers a C nucleotide 3' of the TAAT core (TAATc) (32). Switching Bicoid's Lys⁵⁰ to Gln⁵⁰, as found in the Antennapedia HD, alters its specificity to a Gln⁵⁰ binding site of TAATt (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. Acknowledgments—We thank Drs. Samantha Pattenden and Izzy Livne-Bar for critical reading of this manuscript.

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